DEVELOPING BRAIN IN DANGER : CRITICAL PERIODS OF VULNERABILITY FROM IN-UTERO TO ADOLESCENCE

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DEVELOPING BRAIN IN DANGER : CRITICAL PERIODS OF VULNERABILITY FROM IN-UTERO TO ADOLESCENCE

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Table of Contents

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Exposure of Developing Male Rats to One or Multiple Noise Sessions and Different Housing Conditions: Hippocampal Thioredoxin Changes and Behavioral Alterations

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Exposure of developing rats to noise has shown to induce hippocampal-related behavioral alterations that were prevented after a week of housing in an enriched environment. However, neither the effect of repeated exposures nor its impact on key endogenous antioxidants had been studied yet. Thus, the aim of the present work was to reveal novel data about hippocampal oxidative state through the measurement of possible age-related differences in the levels of hippocampal thioredoxins in rats exposed to noise at different developmental ages and subjected to different schemes and housing conditions. In addition, the possibility that oxidative changes could underlie hippocampal-related behavioral changes was also analyzed. Developing male Wistar rats were exposed to noise for 2 h, either once or for 5 days. Upon weaning, some animals were transferred to an enriched cage for 1 week, whereas others were kept in standard cages. One week later, auditory and behavioral assessments, as well as measurement of hippocampal thioredoxin, were performed. Whereas no changes in the auditory function were observed, significant behavioral differences were found, that varied according to the age, scheme of exposure and housing condition. In addition, a significant increase in Trx-1 levels was found in all noise-exposed groups housed in standard cages. Housing animals in an enriched environment for 1 week was effective in preventing most of these changes. These findings suggest that animals become less susceptible to undergo behavioral alterations after repeated exposure to an environmental challenge, probably due to the ability of adaptation to an unfavorable condition. Moreover, it could be hypothesized that damage to younger individuals could be more easily prevented by a housing manipulation.

Keywords: noise, thioredoxin, behavior, hippocampus, enriched environment

Abbreviations: HC, Hippocampus; PND7, Rats exposed to noise at 7 days of age; PND15, Rats exposed to noise at 15 days of age; N1/N5, Exposure schemes: 1 day and 5 days, respectively; St, Standard cage; EE, Enriched environment; Trx-1, Trx-2, Thioredoxin-1 and Thioredoxin-2, respectively; CNS, Central Nervous System.

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INTRODUCTION

Data from the literature have shown that exposure to noise could be capable to induce damage to the auditory system (Frenzilli et al., 2004; Gourévitch et al., 2014) as well as to structures of different extra-auditory tissues, such as brain structures (prefrontal cortex and hippocampus), cardiac tissues or adrenal and thyroid glands (Trapanotto et al., 2004; Manikandan et al., 2006; Uran et al., 2010; Gannouni et al., 2013; Molina et al., 2016a; Miceli et al., 2018). However, whereas exposure to occupational noise seems to be one of the main causes of disabling hearing loss, limited data are available concerning the effects of noise exposure on everyday lives of the ordinary population (Kopke et al., 2007). Actually, people living in big cities should be aware that they might be involuntarily exposed to high levels of noise coming from different sources. The urban traffic, the use of noisy household appliances or the attendance to concerts venues and discotheques might be examples of some of the many health-threatening environments.

It is well known that several environmental challenges increase the production of reactive oxygen species (ROS) in different tissues, which may overwhelm the endogenous antioxidant defenses and trigger a disturbance in the redox homeostasis (Erkal et al., 2006; Halliwell, 2006). In particular, it has been reported that exposure to noise was able to induce changes in the cochlear oxidative state (Yamane et al., 1995; Yamasoba et al., 1998; Dehne et al., 2000; Yamashita et al., 2004; Fetoni et al., 2015). In addition, Ohlemiller et al. (1999) reported a significant increase in ROS cochlear levels 1 h after exposure to noise, even when the acoustic stimulus is no longer present and Tamura et al. (2012) found that oxidative stress might be induced in the Corti organ of the inner ear after noise exposure in a rodent animal model. Finally, Kurioka et al. (2014) reported an increase in mitochondrial ROS production and excitotoxicity in the cochlea of rats exposed to noise.

ROS are unstable molecular species that contain one or more unpaired electrons that make them highly reactive (Halliwell, 1992). Hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet -}$) or hydroxyl radicals ($OH^{\bullet}$) are ROS that have the ability to damage cellular lipids, proteins and to mitochondrial and nuclear genome through oxidative mechanisms, leading to mutations and cellular death (Halliwell and Gutteridge, 1991; Halliwell, 1992, 2006; Harman, 1992; Uttara et al., 2009; Massaad and Klann, 2011; Hanschmann et al., 2013). Although these species are persistently generated during aerobic respiration as derivatives of redox reactions and considering that even low amounts are required to regulate certain signaling pathways, an imbalance between the production of ROS and the system of endogenous antioxidants (i.e., a disproportionate increase in ROS levels and/or excessive decrease in antioxidant enzymes activities) might lead to cell damage (Jones, 2006). In fact, although the classic definition of oxidative stress focuses on an imbalance between pro- and anti-oxidative molecules in a given structure, at present this definition has been approached to a new concept in which oxidative stress is defined as the disruption of normally occurring redox signaling events (Jones, 2006).

It should be highlighted that brain is more susceptible to oxidative damage when compared with other tissues for different reasons. First, it consumes higher oxygen amounts; second, it has more iron content; third, it has high levels of unsaturated fatty acids and finally, it has lower activities of antioxidant enzymes such as superoxide dismutase and catalase. The high vulnerability can be observed after hypoxia (Romero et al., 2015; Ten and Starkov, 2012), ionizing radiation exposure (Caceres et al., 2009, 2010) and different nervous system disorders (Chen et al., 2010; Ma et al., 2012). Of importance, it has been reported that an environmental threat such as noise was able to induce an oxidative imbalance in different tissues (Cassarino and Bennett, 1999; Sathyasaikumar et al., 2007; Samson et al., 2008; Uran et al., 2010, 2012; Massaad and Klann, 2011; Molina et al., 2016b). A study of Zheng and Ariizumi (2007) showed an increase in oxidative stress and a suppression of the immune function after noise exposure during 28 days, whereas Cheng et al. (2011) found that only 1 week of moderate noise was capable to induce oxidative stress in different structures of mice brain. Cui and Li (2013) reported an increase in brain oxidative stress, as well as alterations of spatial memory in adult animals exposed to noise. Finally, several behavioral and biochemical changes were found in extra-auditory tissues of noise-exposed animals, including impairment of hippocampal-dependent reference and working spatial memory as well as changes in hippocampal antioxidant enzymes activities (Manikandan et al., 2006; Rabat et al., 2006) and a decrease in the number of hippocampal neurons (Jáuregui-Huerta et al., 2011).

Thioredoxins (Trx) are part of an endogenous family of oxido-reductases, recognized as the major reductant among a variety of antioxidant enzymes (Lillig and Holmgren, 2007; Lillig et al., 2008; Romero et al., 2015). Even though the Trx family includes various proteins, the main Trx isoforms are the cytosolic Trx-1 and the mitochondrial Trx-2 (Lillig et al., 2008; Aon-Bertolino et al., 2011; Godoy et al., 2011). Trx-1 is a regulator of cellular functions that take place in response to redox signals and modulates various signaling pathways. Different literature data show an increase in Trx-1 when an oxidative imbalance is induced in different nervous areas of animals subjected to neonatal hypoxia (Romero et al., 2015), intended to maintain a reduced environment to protect cells and tissues from oxidative damage (Silva-Adaya et al., 2014). In addition, Cunningham et al. (2015) showed that Trx-1 overexpression extended lifespan of transgenic mice by protecting against oxidative stress and Wu et al. (2015) found that a treatment with Trx-1 siRNA induced behavioral deficits. Therefore, it could be hypothesized that under physiological conditions the balance between ROS generation and antioxidant activity is highly controlled. However, when an injury is going on, an activation of the endogenous antioxidant defense systems can primarily occur as an attempt to counteract the oxidative process. Nevertheless, the endogenous antioxidant system often can fail in restoring redox homeostasis and the defense activity might result insufficient to prevent damage.

Last, a non-pharmacological neuroprotective strategy, the enriched environment (Laviola et al., 2008) has shown to be an effective tool that could be protective against different central

nervous system (CNS) injuries (Lores-Arnaiz et al., 2006). It consists of a cage larger than the standard, which contains different toys, ramps and wheels. Although we have reported that EE was able to prevent noise-induced behavioral alterations in PND28 animals exposed at PND7 and PND15 to noise for 2 h (Molina et al., 2016a), data in animals exposed for 5 days have not been obtained yet.

Unfortunately, data obtained from developing animals exposed to noise are very scarce in the literature. The results from our laboratory showed different behavioral, biochemical and histological alterations when immature rats were exposed to noise. In addition, housing in an enriched environment has demonstrated to be an effective neuroprotective tool when rats were exposed to noise for a single day (Molina et al., 2016a). However, a comparison between the effects of single or repeated exposures to noise, at different developmental ages and/or housing conditions, as well as a possible relationship with the hippocampal oxidative state, has not been made yet.

Thus, the main hypothesis was that hippocampal thioredoxins might be responsible, at least in part, of the behavioral changes induced in developing rats after exposure to noise. Therefore, the aim of the present work was to reveal novel data about hippocampal oxidative state through the measurement of possible age-related differences in the levels of hippocampal Trx-1 and Trx-2, the major members of the thioredoxin family of endogenous antioxidants, in animals exposed to noise at 7 and 15 days according to different schemes. In addition, the possibility that oxidative changes could underlie hippocampal-related behavioral changes was also analyzed. Finally, the impact of housing conditions on noise-induced changes was additionally evaluated. To discard hearing alterations, the auditory pathway function was assessed.

MATERIALS AND METHODS

Animals

Healthy male and female albino Wistar rats were obtained from the animal facilities of the Biochemistry and Pharmacy School, University of Buenos Aires, Argentina. A total of 30 multiparous females and 10 males were used for mating procedures. Pregnant rats were isolated and left undisturbed until delivery. The day of birth was designated as postnatal day (PND) 0. In average, 10 pups per litter were delivered and only male rats (usually 4–6 per litter) were used for the different experimental procedures.

To prevent from litter effects, no more than one animal from each litter was used to measure each parameter.

After behavioral and auditory experiments at PND28, animals were euthanized under a CO_2 chamber for final disposal. Those animals assigned to western blot experiments were sacrificed through guillotine decapitation, the brain was exposed and the hippocampus was subsequently dissected.

PND7 and PND15 littermates were randomly assigned to four experimental groups: sham (control) at PND7, sham (control) at PND15, noise-exposed at PND7 and noise-exposed at PND15 ($n = 84$ each group). In turn, within each group, animals received one of the following exposure schemes: single (N1) or five consecutive daily sessions (N5; $n = 42$ each group). Finally, each subgroup was divided into standard (St) or enriched (EE) cages housing, conforming 16 experimental groups ($n = 21$ each group). To reduce confounding factors, animals within each group were randomly assigned to the different measurements, being different those animals used for behavioral experiments (with some rats performing two behavioral tests, usually seven for OF and elevated plus maze (EPM) and other seven animals for IA) western blot experiments (four rats for each group) and auditory assessment (three rats for each group). **Figure 1** depicts the experimental groups used.

All littermates were kept with their dams until weaning, at 21 days of age. Then, rats were separated and were put in groups of 2–3 in standard and 3–4 in enriched cage for 1 week with food and water *ad libitum*, on 12 h light-dark cycles (lights on at 7 A.M.) at 21 ± 2°C and mashed cornflower for bedding.

Animals were handled and sacrificed according to the Institutional Committee for the Use and Care of Laboratory Animal rules (CICUAL, School of Medicine, University of Buenos Aires, Argentina). The present experimental protocol was approved by this Committee and registered with the number 53679/16. The CICUAL adheres to the rules of the "Guide for the Care and Use of Laboratory Animals" (NIH; 2011 revision) and to the EC Directive 86/609/EEC (2010 revision) for animal experiments.

To avoid circadian rhythm alterations, noise exposures were performed in the intermediate phase of the light cycle, between

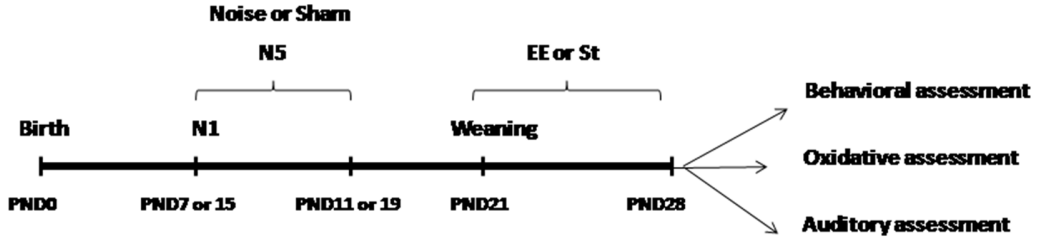

FIGURE 1 | Experimental design. Sham: non-exposed animals; N1: single noise exposure; N5: five-daily noise exposure. St: standard housing. EE: enriched environment.

10 A.M. and 2 P.M. All experiments were performed in PND28 animals.

Noise Exposure

For this procedure, the computer software TrueRTA was chosen to produce white noise, using a bandwidth from 20 Hz to 20,000 Hz in octave bands. For sound amplification, an active 2 way monitor (SKP, SK150A, 40 W RMS per channel) was used, located 30 cm above the animal cage, placed in an "*ad hoc*" wooden sound chamber of 1 m × 1 m × 1 m fitted with a ventilated top as reported by Cui et al. (2009). Before exposure, noise intensity was measured with an omnidirectional measurement condenser microphone (Behringer ECM 8000) by positioning the microphone in the sound chamber at several locations and taking an average of the different readings. Animals were kept in their home cages and the entire litter was assigned to the same group so that they were not handled throughout the exposure period. Sham animals were placed in the same chamber, but without being exposed to noise. Given that experimental animals were still being breastfed and mothers had to be transiently removed for the period in which the pups were being exposed to noise, this action was carried out also in non-exposed sham animals in order to discard possible changes that could be attributed to mother separation.

Based on previous publications of our laboratory (Uran et al., 2010) with further modifications (Molina et al., 2016a), PND7 and PND15 animals were exposed for 2 h to white noise at 95–97 dB SPL (20–20,000 Hz), either a single day (N1) or for five consecutive days (N5). Background noise level ranged between 50 and 55 dB SPL, being within the harmless interval suggested by the WHO guidelines (NIOSH, 1998) and by others (Campeau et al., 2002; Sasse et al., 2008). Lighting was provided by a 20 W lamp located in the upper left corner of the sound chamber. In addition, the chamber had a sound attenuation system made with Celotex™.

The intensity and duration of noise used in the present work were chosen considering its potential translational value, as it could be comparable to the intensity and duration perceived in various workplaces, mainly induced by different machines, data that can be found even in the earliest WHO report (WHO, 1999).

Enriched Environment (EE)

At weaning (PND21), a subset of animals was housed in an EE with 3–4 animals residing together whereas a subset of 2–3 was accommodated in standard cages (St). In contrast to St, conventional top-wired, stainless steel rectangular cages of 40 cm × 25 cm × 16 cm, EE consisted of 54 cm × 40 cm × 41 cm plastic cages with two levels, containing two connecting ramps. Different plastic toys and tunnels, as well as a running wheel, were placed in the cage. A palatable food, such as Froot Loops®, was added regularly in small quantities in addition to the conventional balanced food. It should be highlighted that the minimal sugar and fat amounts of the Froot Loops® offered are much below those contained in a "cafeteria diet," known to induce *per se* metabolic and behavioral changes (Zeeni et al., 2015). The objects were changed every 2 days to ensure continued novelty. Rats were maintained in their housing condition (St or EE) for 1 week, prior to behavioral studies.

Auditory Pathway Assessment (ABR)

The auditory brainstem responses (ABRs) are sound-evoked potentials generated by neuronal circuits in the ascending auditory pathways and consequently require functional integrity of hair cells, as well as their afferent neurons.

PND28 animals were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.) and placed in an acoustically electrically shielded chamber maintained at 30°C. Methods for measuring ABRs were essentially as described (Kujawa and Liberman, 2009; Maison et al., 2013). Briefly, acoustic stimuli were delivered through an acoustic system consisting of two miniature dynamic earphones used as sound sources and an electret condenser microphone coupled to a probe tube to measure sound pressure near the eardrum. Digital stimulus generation and response processing were handled by digital I-O boards from National Instruments driven by custom software written in LabVIEW. ABRs were recorded with needle electrodes inserted at vertex and pinna with a ground reference near the tail. Auditory responses were evoked with 5 ms tone pips, amplified (10,000×), filtered to six different frequencies (0.1–3 kHz), and acquired on a computer. The sound level was raised in 10 dB steps and "threshold" was defined as the lowest SPL level at which a wave is detected.

To avoid potential data misinterpretation, animals assigned to ABR assessments were not subjected to further behavioral or biochemical evaluations and were euthanized in a CO_2 chamber for final disposal.

Behavioral Assessment

PND28 animals were used for all behavioral experiments. To control for variables that could significantly alter physiological and behavioral indicators of stress (Walf and Frye, 2007), animals remained in their home cage and placed in a separate area of the main housing room for 30 min prior to the behavioral assessments. Thereafter, they were individually housed for 5 min in the same area and finally were taken to the adjacent testing room, which had identical environmental conditions, for additional 3 min to complete the acclimation period, prior to the behavioral assessments.

Open Field Task (OF)

An open field device was used to analyze habituation memory and exploratory activity, behaviors known to depend on the hippocampus (Vianna et al., 2000; Barros et al., 2006). In this task, the reduction of locomotor activity triggered by a repeated exposure to the same environment can be taken as a measure of preservation of habituation memory (Vianna et al., 2000; Pereira et al., 2011). In addition, the activity in the first session of the OF can be used to assess changes in emotionality induced by exposure to a novel environment. In consequence, vertical exploratory activity can be quantified by recording the number of rearing and climbing, holding on the hind legs. The activity was recorded using a camcorder (Handycam DCR-DVD810, Sony).

- Apparatus: OF device consists of a 50 cm × 50 cm × 50 cm wooden box, with a floor divided into 25 equal squares by black lines.
- First session: rats were withdrawn from the cage, placed on the center rear quadrant of the OF box and allowed to freely explore the box for 5 min. The number of crossed lines as well as the number of rearing and climbing, were recorded over the session.
- Second session: after 1 h inter-trial in their home cages, animals were acclimatized to the behavioral room and allowed to explore the apparatus for another 5 min. The number of crossed lines was recorded and compared with the number crossed in the first session to evaluate habituation to the device (Barros et al., 2000).

Elevated Plus Maze (EPM)

This task was used to evaluate anxiety-related behaviors that depend on the integrity of the hippocampus (Montgomery, 1955; Brenes et al., 2009; Violle et al., 2009).

Anxiety-related behaviors are calculated as the number of entries to the open arms as well as the latency required to access the open arms. When an increase in the first and a decrease in the latter are observed, it could be stated that a decrease in anxiety-like behaviors could have occurred.

Additionally, some ethological parameters can be evaluated using this task (Carobrez and Bertoglio, 2005), designated as risk assessment behavior because they have been associated to detection and analysis of threats or threatening situations (Rodgers and Cole, 1993). One of these parameters is called head dipping (HD). As closed arms and center platform were designated as "protected" areas (i.e., offering relative security), the percentage of head-dipping in closed arms (%HD in closed arms) was calculated as the percentage of these behaviors displayed in or from the protected areas. Therefore, this parameter describes the action of the animal when it is positioned on a closed arm and, at the junction with the open arm, stretches the head over the ledge of an open arm and bends down.

- Apparatus: the wooden apparatus consists of four arms of equal dimensions (50 cm × 10 cm) and raises 50 cm above the floor. Two arms, enclosed by walls 40 cm high, are perpendicular to the two other opposed open arms.
- Session: rats were placed in one of the closed arms, facing the center of the maze, and were recorded for 5 min using a camcorder (Handycam DCR-DVD810, Sony). The number of entries to open arms, the latency to reach the open arms, as well as the percent of HD in closed arms, were calculated. Only few rats randomly distributed across experimental groups fell when they walked along the open arms; these animals were excluded from the study.

Inhibitory Avoidance Task (IA)

Inhibitory avoidance task was used to measure the memory of an aversive experience through the simple avoidance of a location in which the unpleasant experience occurred. This task is thought to depend heavily on the dorsal hippocampus and is a reliable index of associative memory (Ennaceur and Delacour, 1988; Izquierdo and Medina, 1997).

- Apparatus: the apparatus consists of a box (60 cm × 60 cm × 40 cm), divided into two compartments: one is illuminated while the other is equipped with a removable cover to allow it to be dark, as described by Roozendaal (2002). A removable partition divides the two compartments. The floor of the dark compartment consists of a stainless steel grid at the bottom, through which a continuous current could be delivered.
- Habituation session: the rat was placed into the lit box and allowed to freely explore the apparatus. Either after passing three times to the dark side or after 3 min staying in the dark side, the rat was removed from the apparatus. After 10 min, the rat was placed again in the lit side and when it entered the dark side, the doors were closed and the rat was retained for 10 s on this side.
- Training session (T1): after 1 h, each rat was placed in the lit compartment, facing away from the dark compartment; the latency to move into the dark compartment was recorded. When the rat stepped with all four paws in the dark compartment, a foot shock (1.2 mA, 2 s) was delivered. The rat was quickly removed from the apparatus and returned to its home cage.
- Retention session (T2): retention test was performed 1 h after the training session by following a similar procedure, except for the fact that no footshock was delivered. The ratio between the latency to move into the dark compartment in the *retention* and the *training* sessions (T2 and T1, respectively) was taken as a measure of associative memory retention (T2/T1).

Western Blot Experiments

The levels of the Trx-1 and Trx-2 were determined in hippocampal homogenates of rats from all experimental groups through Western blot experiments. To prevent from confounding influences, those animals destined to western blot experiments were not previously used for behavioral or auditory measurements. Animals were euthanized through guillotine decapitation, brain was exposed, and hippocampus dissected. Briefly, tissues were homogenized in ice-cold lysis buffer (25 mM Hepes, 6 mM MgCl, 1 mM EDTA, mix of protease inhibitors) and centrifuged at 10,000 *g*. The supernatants were analyzed for total protein concentration using Bradford solution, with bovine serum albumin (BSA) as standard. According to the determined protein concentration, the samples were diluted with sample buffer solution (6×: 0.346 M SDS, 30% glycerol, 6% 2-mercaptoethanol, 0.179 mM bromophenol blue, 0.998 M Tris–HCl, pH 6.8) in order to have 10 μg of tissue/ml. Therefore, homogenates were preincubated with 1 μl DTT 1 M per 10 μl of sample for 30 min at room temperature and then heated to 94°C for 10 min. Then, samples were run on 14% polyacrylamide gels under denaturing conditions. The samples were electro-transferred to PVDF membranes which were blocked with 5% non-fat milk and 1% BSA and incubated overnight with the primary antibody at 4°C [Trx-1 and Trx-2 rabbit antibodies, used in a dilution of 1:1,000, were a generous gift of Dr. Lillig from

University of Greifswald, Germany; sc-32233 GAPDH (load control) rabbit antibody from Santa Cruz Biotech. was used in a dilution of 1:5,000]. After that, samples were incubated at room temperature with the secondary anti-rabbit HRP-conjugated antibody (sc-2768 Santa Cruz Biotech., diluted 1:5,000) for 2 h under shaking, scanned densitometrically by the Image Quant analyzer and quantified using ImageJ software.

Statistical Analysis

Normality test was performed for each group (KS-test). Significant differences between groups were analyzed through one-, two- or three-way analysis of variance (ANOVA) tests with LSD *post hoc* comparisons using the Infostat/L software. When the normality tests failed, a non-parametric analysis was made, using the Kruskal–Wallis test. Different letters (a, b, c, d) were used to depict significant differences between the means, being significantly different one bar from another when they have no common letters. For example, if a bar received an "a" score and another a "b" score, it means that they differ statistically with $p < 0.05$. Considering the large number of groups to be compared, with the consequent difficulties in data interpretation, the differences between the results of PND7 and PND15-exposed animals were analyzed separately. A probability < 0.05 was accepted as significant.

When interactions were significant, a simple effect analysis was performed, through which one-way ANOVA analyses were performed. The results were expressed as mean values ± standard error of the mean (SEM) and graphs were performed with Prism Graphpad software v5.

RESULTS

Auditory Function

No significant changes in ABRs thresholds in any of the frequencies tested were observed in PND28 animals exposed to noise at PND7 and PND15 [non-parametric Kruskall–Wallis test, $H < 4$ and $p > 0.05$ (NS) for all frequencies, **Figures 2A,B**].

Open Field (OF) Task

(i) The number of lines crossed in two sessions of 5 min in an OF, separated by an interval of 1 h, was taken as an index of short-term habituation to a new environment.

Data show that exposure to noise at PND7, according to N1 and N5 schemes, induced a decrease in the number of lines crossed in the second session of the OF when compared with the first session, both in standard or in enriched conditions, that resulted similar to what was observed in sham animals, when evaluated at PND28 [**Figure 3A**, *N1*: Three-way ANOVA, $F_{(7,65)} = 7.77$, $p < 0.01$. Between factors: exposure (sham or noise), $F_{(1,65)} = 0.22$, NS; housing (St or EE), $F_{(1,65)} = 5.4$, $p < 0.05$; within factor: session (first or second), $F_{(1,65)} = 43.88$, $p < 0.01$. *post hoc* comparisons: first vs. second session: all groups, $p < 0.01$. **Figure 3B**, *N5*: Three-way ANOVA, $F_{(7,67)} = 6.29$, $p < 0.01$. Between factors: exposure (sham or noise), $F_{(1,67)} = 0.04$, NS; housing (St or EE), $F_{(1.67)} = 0.23$, NS; within factor: session (first or second) $F_{(1,67)} = 41.79$, $p < 0.01$. *Post hoc* comparisons: first vs. second session: all groups, $p < 0.05$].

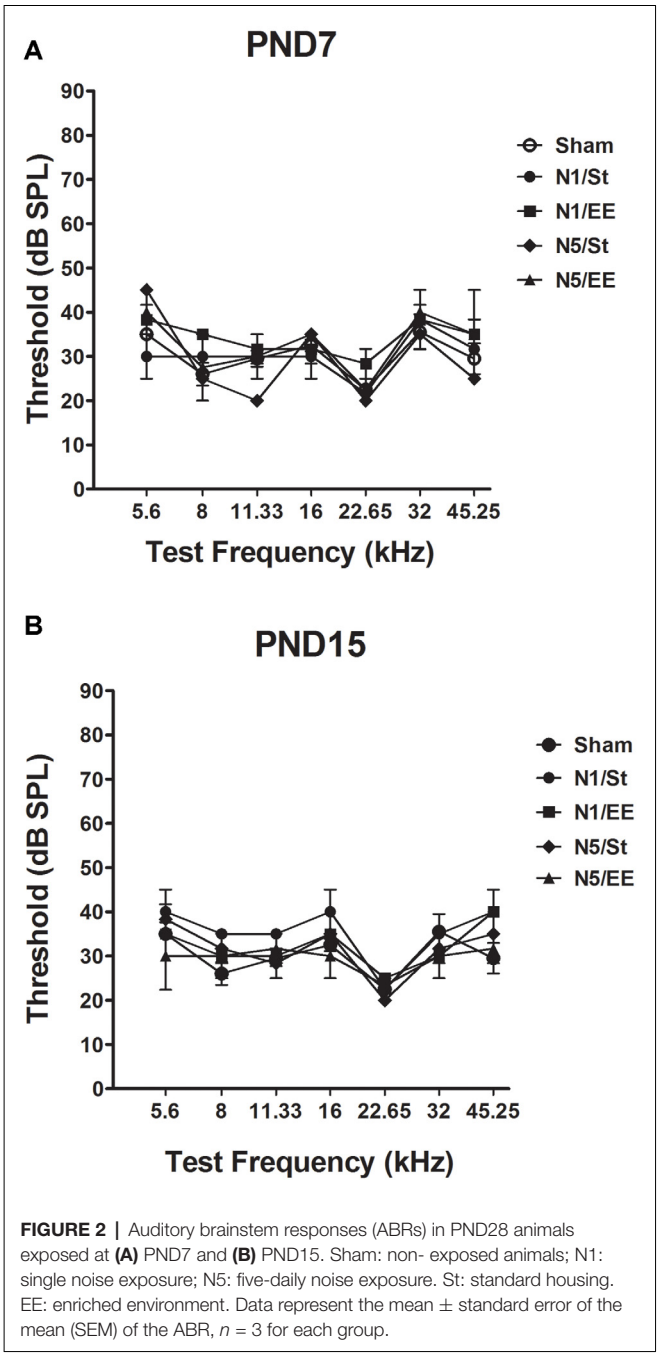

FIGURE 2 | Auditory brainstem responses (ABRs) in PND28 animals exposed at **(A)** PND7 and **(B)** PND15. Sham: non- exposed animals; N1: single noise exposure; N5: five-daily noise exposure. St: standard housing. EE: enriched environment. Data represent the mean ± standard error of the mean (SEM) of the ABR, $n = 3$ for each group.

In addition, most groups showed a decrease in the lines crossed in the second session of the OF when the animals were exposed at PND15 according to N1 scheme [Three-way ANOVA, $F_{(7,47)} = 9.65$, $p < 0.01$. Between factors: exposure (sham or noise), $F_{(1,47)} = 9.49$, $p < 0.01$; housing (St or EE), $F_{(1,47)} = 3.67$, NS; within factor: session (first or second), $F_{(1,47)} = 38.91$, $p < 0.01$]. However, given that a significant interaction between exposure and session was found ($F_{(1,47)} = 10.14$, $p < 0.01$), a simple effect analysis was performed. Data show that whereas

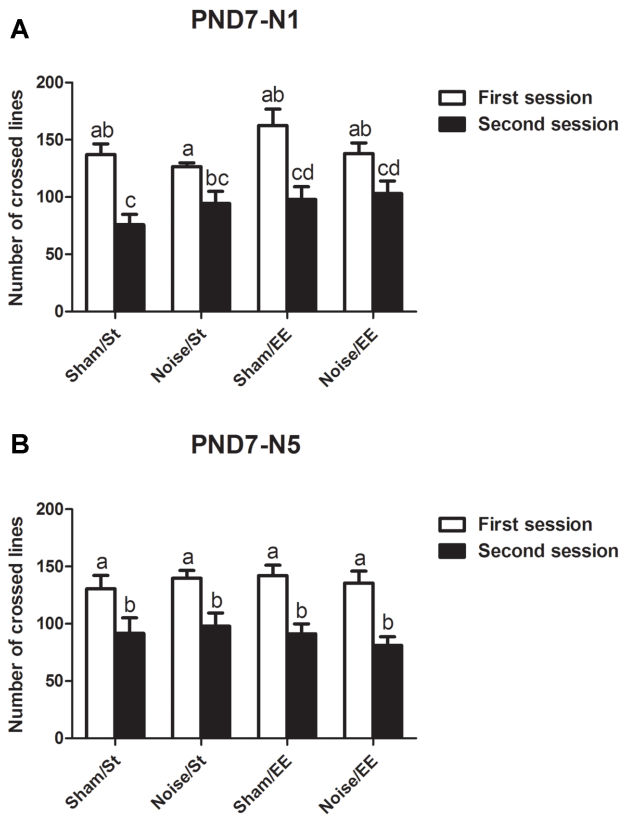

FIGURE 3 | Number of lines crossed in the first and second session of the OF by PND28 animals exposed to noise at PND7. **(A)** PND7 animals exposed according to N1; **(B)** PND7 animals exposed according to N5. Sham: non-exposed animals; N1: single noise exposure; N5: five-daily noise exposure. St: standard housing. EE: enriched environment. Different letters (a, b, c, d) symbolize significant differences with $p < 0.05$. Data represent the mean ± SEM of the number of lines crossed in the first and second session of the OF, $n = 7$ for each group.

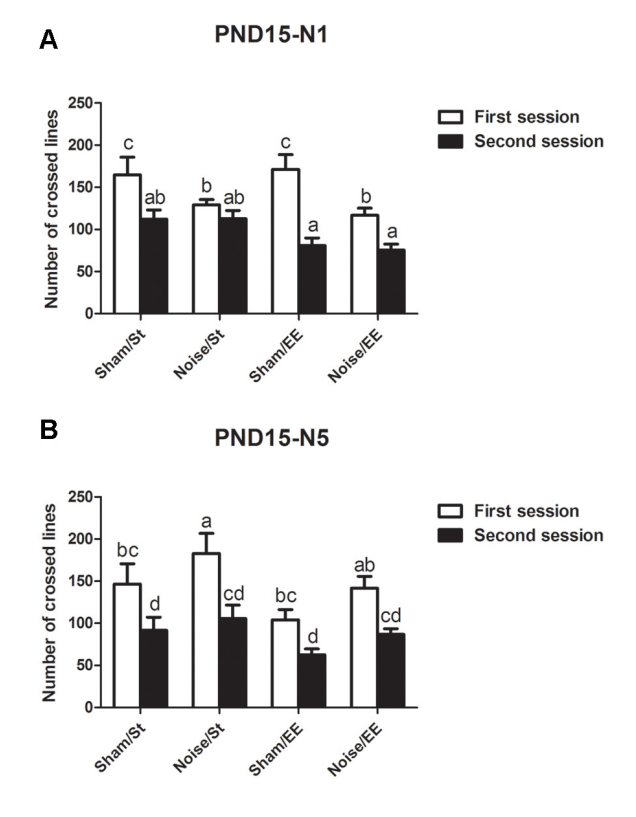

FIGURE 4 | Number of lines crossed in the first and second session of the OF by PND28 animals exposed to noise at PND15. **(A)** PND15 animals exposed according to N1; **(B)** PND15 animals exposed according to N5. Sham: non-exposed animals; N1: single noise exposure; N5: five-daily noise exposure. St: standard housing. EE: enriched environment. Different letters (a, b, c, d) symbolize significant differences with $p < 0.05$. Data represent the mean ± SEM of the number of lines crossed in the first and second session of the OF, $n = 7$ for each group.

significant differences were observed between the first and second session in most groups, non-significant differences were observed in animals exposed to noise according to N1 scheme and housed in St conditions (**Figure 4A**, *Sham*: Two-way ANOVA, $F_{(3,21)} = 9.34$, $p < 0.01$, *post hoc* comparisons: first session vs. second session, St and EE, $p < 0.05$. *Noise*: Two-way ANOVA, $F_{(3,25)} = 8.21$, $p < 0.01$. *Post hoc* comparisons: first session vs. second session, St, NS; EE, $p < 0.05$). Finally, when animals exposed at PND15 according to N5 scheme were evaluated, significant differences were observed between the lines crossed in the first and second session of the OF in all groups [**Figure 4B**, *N5*: Three-way ANOVA, $F_{(7,59)} = 5.81$, $p < 0.01$. Between factors: exposure (sham or noise), $F_{(1,59)} = 5.68$, $p < 0.05$; housing (St or EE), $F_{(1,59)} = 5.97$, $p < 0.05$; within factor: session (first or second) $F_{(1,59)} = 26.81$, $p < 0.01$. *Post hoc* comparisons: first vs. second session: St (sham and noise), $p < 0.01$; EE (sham and noise), $p < 0.05$].

In summary, results show a significant decrease in the number of lines crossed in the second session of the OF when compared with the first session in most groups, both exposed at PND7 and PND15, except for animals exposed to noise at PND15 according to N1 scheme and housed in St conditions.

(ii) The number of forelimb elevations (i.e., rearing and climbing) made in the first session of the OF task was taken as an index of exploratory activity.

Data show a significant main effect in this parameter [**Figure 5A**, Three-way ANOVA, $F_{(7,85)} = 3.42$, $p < 0.01$. Between factors: exposure (sham or noise), $F_{(1,85)} = 0.57$, NS; housing (St or EE), $F = 13.08$, $p < 0.01$; within factors: scheme of exposure (N1 or N5) $F_{(1,85)} = 0.55$, NS]. As the interaction between exposure and housing was significant ($F_{(1,85)} = 8.43$, $p < 0.01$), a simple effect analysis was performed [*St*: Two-way ANOVA, $F_{(3,46)} = 2.09$, NS. Between factor: scheme (N1 or N5), NS; within factor: exposure (sham or noise), $p < 0.05$. *EE*: Two-way ANOVA, $F_{(3,38)} = 1.53$, NS]. The results show a significant increase in animals exposed at PND7 according to N1 and housed in St conditions when compared to their respective controls. In contrast, no changes were observed after EE housing of N1-exposed rats. Finally, exploration activity of N5-exposed animals (both after St and EE housing) remained unaltered [*post*

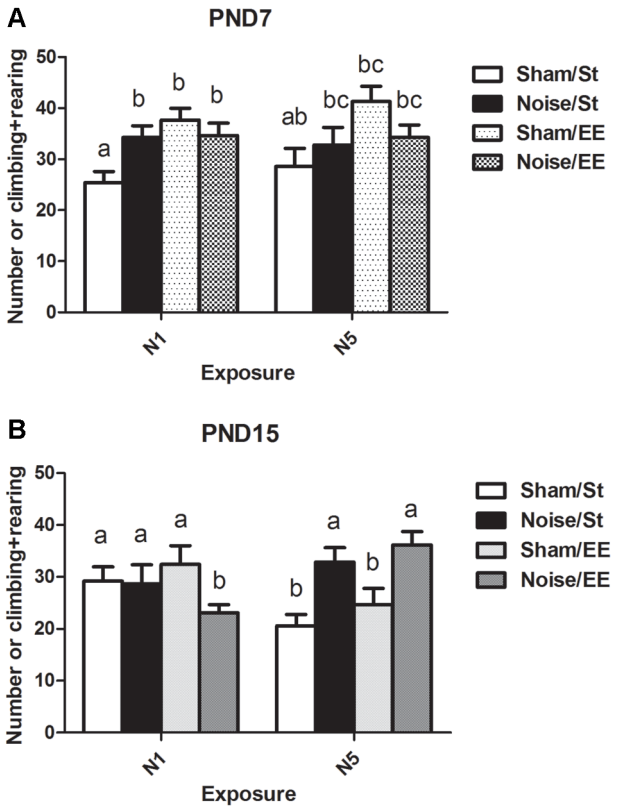

FIGURE 5 | Number of elevations (climbing and rearing) made by PND28 animals exposed to noise at PND7 and PND15 in the OF task. **(A)** PND28 animals exposed at PND7; **(B)** PND28 animals exposed at PND15. Sham: non- exposed animals; N1: single noise exposure; N5: five-daily noise exposure. St: standard housing. EE: enriched environment. Different letters (a, b, c) symbolize significant differences with $p < 0.05$. Data represent the mean ± SEM of the number of elevations (climbing and rearing) made in the OF task, $n = 7$ for each group.

hoc comparisons: sham vs. noise: N1: St, $p < 0.05$; EE, NS; N5 (St and EE), NS].

On the other hand, a significant main effect was found in animals exposed at PND15 (**Figure 5B**, Three-way ANOVA, $F_{(7,77)} = 2.69$, $p < 0.05$). As the interaction between exposure and scheme was significant ($F_{(1,77)} = 12.65$, $p < 0.01$), a simple effect analysis was performed [*N1*: Two-way ANOVA, $F_{(3,38)} = 1.29$, NS. *N5*: Two-way ANOVA, $F_{(3,37)} = 6.35$, $p < 0.01$. Between factor: exposure (sham or noise), $F_{(1,37)} = 17.29$, $p < 0.01$; within factor: scheme (St or EE), $F_{(1,37)} = 1.74$, NS, *post hoc* comparisons: sham vs. noise: N1: St, NS; EE, $p < 0.05$; N5 (St and EE), $p < 0.05$].

In summary, results show an increase in the number of forelimb elevations in animals exposed at PND7 according to N1 scheme housed in St when compared to the sham group. In contrast, no changes were observed when these animals were housed in EE or in groups exposed to N5 scheme (both after St and EE housing). On the other hand, results show that whereas no changes were observed in this parameter after exposure of PND15 animals to noise according to N1 scheme and St housing, a significant decrease was observed when exposed animals were housed in EE. In contrast, a significant increase was observed in animals repeatedly exposed to noise, both after St or EE housing.

Elevated Plus Maze (EPM) Task

Open arms-related parameters measured in the EPM, such as the decrease in the latency to enter and an increase in the number of entries, are thought to be associated with a reduction of anxiety-like behaviors. HD in an open arm might be related with risk assessment behaviors.

Latency to Enter to the Open Arms in the Elevated Plus Maze (EPM) Task

Figure 6A shows a significant main effect on the latency to enter the open arms of the EPM when animals exposed at PND7 were evaluated [Three-way ANOVA, $F_{(7,52)} = 10.69$, $p < 0.01$; between factors: exposure (sham or noise), $F_{(1,52)} = 23.35$, $p < 0.01$; housing (St or EE), $F_{(1,52)} = 15.60$, $p < 0.01$; within factors: scheme of exposure (N1 or N5), $F_{(1,52)} = 30.63$, $p < 0.01$]. The results show a significant decrease in animals exposed to noise according to N1 scheme, both in St and EE housing conditions, without changes when exposure was done according to N5 (*post hoc* comparisons: sham vs. noise, St: N1, $p < 0.05$; N5, NS. EE: N1, $p < 0.05$; N5, NS).

When PND15 animals were exposed, a significant main effect was observed [**Figure 6B**, Three-way ANOVA, $F_{(7,55)} = 5.85$, $p < 0.01$; between factors: exposure (sham or noise), $F_{(1,55)} = 5.59$, $p < 0.01$; housing (St or EE), $F_{(1,55)} = 0.45$, NS; within factor: scheme (N1 or N5), $F_{(1,55)} = 3.25$, NS]. As a significant interaction was observed between exposure and scheme ($F_{(1,55)} = 28.12$, $p < 0.01$), a simple effect analysis was performed. A significant increase in the latency to open arms was found in noise-exposed animals according to N1, both in St and EE housing [Two-way ANOVA, $F_{(3,28)} = 7.07$, $p < 0.01$; between factor: exposure (sham or noise), $F_{(1,28)} = 19.61$, $p < 0.01$; within factor: housing (St or EE), $F_{(1,28)} = 1.40$, NS. *Post hoc* comparisons: sham vs. noise: St and EE, $p < 0.05$]. As a significant main effect was observed after N5 scheme [Two-way ANOVA, $F_{(3,26)} = 4.67$, $p < 0.01$; between factor: exposure (sham or noise), $F_{(1,26)} = 8.18$, $p < 0.01$; within factor: housing (St or EE), $F_{(1,26)} = 0.03$, NS] and an interaction was observed ($F_{(1,26)} = 5.79$, $p < 0.05$), a simple effect analysis was performed, which showed a significant decrease in noise-exposed animals housed in St cages when compared with their respective controls ($p < 0.05$).

In summary, results show a decrease in the latency to enter to the open arms in noise-exposed animals according to N1 at PND7 and an increase in this parameter when animals were exposed to N1 at PND15, after St and EE housing conditions. On the other hand, no changes were found when exposure was done according to N5 at PND7 whereas a significant decrease was observed when animals were exposed to N5 at PND15 and housed in St, without changes when animals were housed in EE.

Number of Entries to the Open Arms in the Elevated Plus Maze (EPM) Task

Figure 6C shows a significant main effect on the number of entries to the open arms of the EPM in animals exposed at PND7 (Three-way ANOVA, $F_{(7,55)} = 4.19$, $p < 0.01$). As some

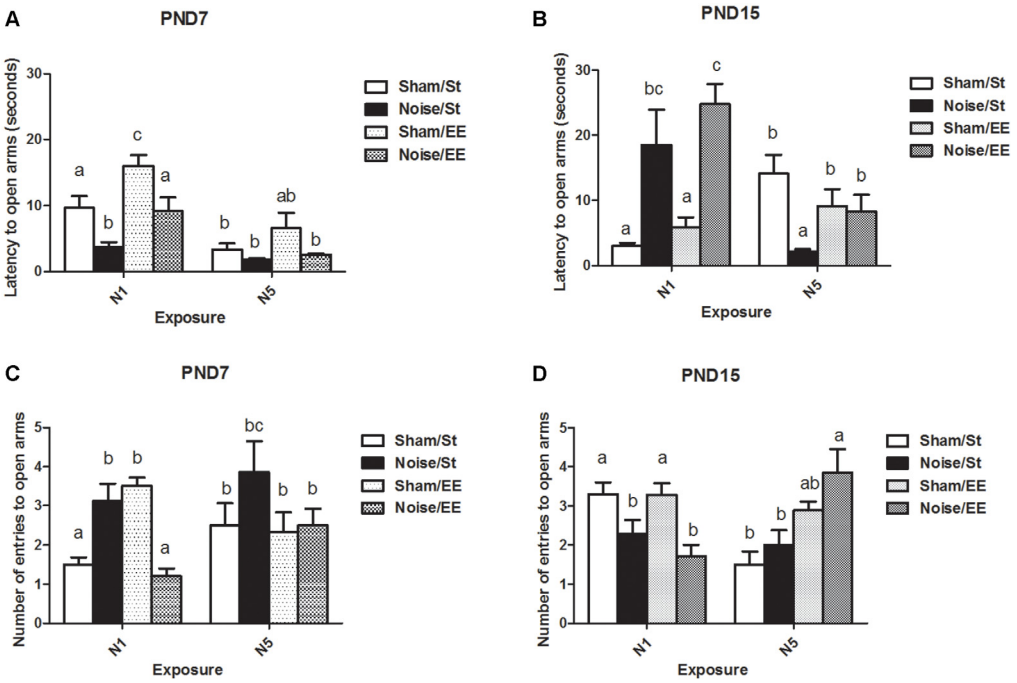

FIGURE 6 | Anxiety-related behaviors measured in the elevated plus maze (EPM; latency and number of entries to open arms) of PND28 animals exposed to noise at PND7 and PND15 in the EPM task. Latency to open arms (in seconds): **(A)** PND28 animals exposed at PND7; **(B)** PND28 animals exposed at PND15. Entries to open arms: **(C)** PND28 animals exposed at PND7; **(D)** PND28 animals exposed at PND15. Sham: non- exposed animals; N1: single noise exposure; N5: five-daily noise exposure. St: standard housing. EE: enriched environment. Different letters (a, b, c) symbolize significant differences with $p < 0.05$. Data represent the mean ± SEM of latency (seconds) or entries to open arms made in the EPM task, $n = 7$ for each group.

interactions were significant (between exposure and housing: $F_{(1,55)} = 18.31$, $p < 0.01$; between exposure, housing and scheme: $F_{(1,55)} = 4.47$, $p < 0.05$) a simple effect analysis was performed [*St*: Two-way ANOVA, $F_{(3,28)} = 3.82$, $p < 0.05$. Between factor: exposure (sham or noise), $p < 0.01$; within factor: scheme (N1 or N5), NS. *Post hoc* comparisons: sham vs. noise: St, $p < 0.05$; EE, $p < 0.01$. *EE*: Two-way ANOVA, $F_{(3,26)} = 8.61$, $p < 0.01$. Between factor: exposure (sham or noise), $p < 0.01$; within factor: scheme (N1 or N5), NS].

Data show a significant increase in St-housed animals exposed according to N1 scheme and a decrease when exposed animals were housed in EE (*post hoc* comparisons: sham vs. noise: St, $p < 0.05$; EE, $p < 0.01$). No changes were observed in rats exposed for 5 days [between factors: exposure (sham or noise) or housing (St or EE), NS; within factors: scheme of exposure (N1 or N5), NS]. However, as the interaction between exposure and scheme in rats housed within EE group was significant ($F_{(1,26)} = 13.27$, $p < 0.01$), a simple effect analysis was performed. In summary, results show a significant increase in noise-exposed animals according to N1 ($p < 0.05$) and no changes in rats exposed according to N5.

Figure 6D shows a significant main effect on the number of entries to the open arms of the EPM in animals exposed at PND15 (Three-way ANOVA, $F_{(7,60)} = 4.77$, $p < 0.01$). A decrease in this parameter was observed in animals housed in St and EE conditions and exposed according to N1 scheme. In contrast, no changes were observed in animals exposed according to N5 scheme [Between factors: exposure (sham or noise) or housing (St or EE), NS; within factors: scheme of exposure (N1 or N5), NS]. As some interactions were significant (between exposure and scheme: $F_{(1,60)} = 12.19$, $p < 0.01$; between exposure, scheme and housing: $F_{(1,60)} = 15.21$, $p < 0.01$), a simple effect analysis was performed [*St*: Two-way ANOVA, $F_{(3,30)} = 5.15$, $p < 0.01$. Between factor: exposure (sham or noise), NS; within factor: scheme (N1 or N5), $F_{(1,30)} = 8.81$, $p < 0.01$. *EE*: Two-way ANOVA, $F_{(3,29)} = 4.85$, $p < 0.01$. Between factor: exposure (sham or noise), NS. Within factor: scheme (N1 or N5), $F_{(1,29)} = 5.83$, $p < 0.05$. *Post hoc* comparisons: sham or noise: St: N1, $p < 0.05$; N5, NS. EE: N1, $p < 0.05$; N5, NS]. As a significant interaction was found between exposure and scheme, both within St and EE-housed animals (*St*: $F_{(1,30)} = 4.63$, $p < 0.05$; *EE*: $F_{(1,29)} = 8.56$, $p < 0.01$), simple effect analysis were performed. Data show a significant decrease in noise-exposed animals according to N1 scheme, both in St and EE conditions ($p < 0.05$).

In summary, results show significant differences in the number of entries to the open arms in noise-exposed animals when compared to their controls according to N1 scheme, without changes after N5 noise-exposure scheme. When animals were exposed to N1 at PND7, an increase in this parameter in St-housed animals and a decrease when animals were housed in EE were observed. Moreover, when animals were exposed at PND15 a decrease was observed, both for St and EE housing.

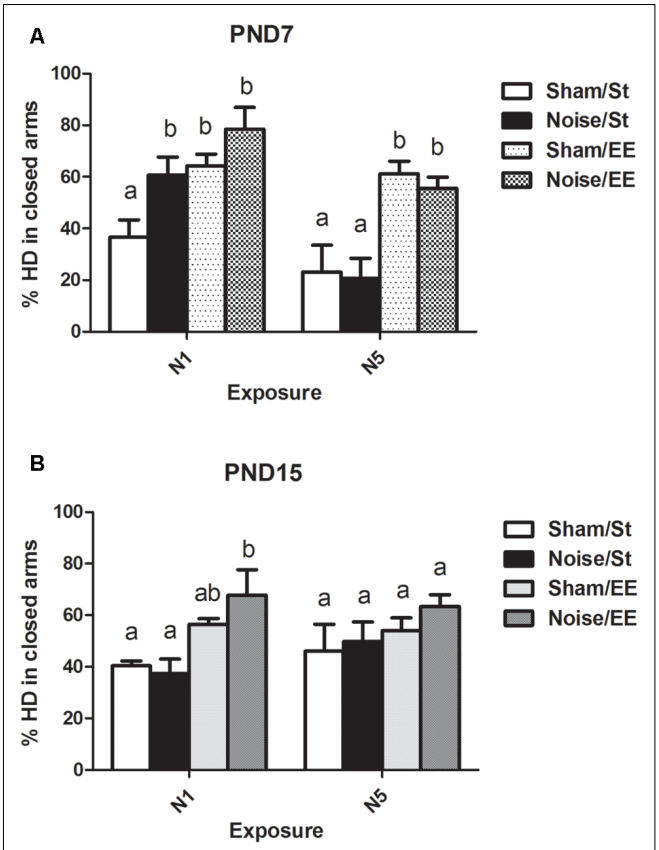

FIGURE 7 | Percentage of head dipping (HD) in closed arms in the EPM task made by PND28 animals exposed to noise at PND7 and PND15. **(A)** PND28 animals exposed at PND7; **(B)** PND28 animals exposed at PND15. Sham: non- exposed animals; N1: single noise exposure; N5: five-daily noise exposure. St: standard housing. EE: enriched environment. Different letters (a, b) symbolize significant differences with $p < 0.05$. Data represent the mean ± SEM of the % of HD in closed arms made in the EPM task, $n = 7$ for each group.

Head Dipping (HD)

When HD was analyzed, a significant main effect was observed when animals were exposed at PND7 (**Figure 7A**, Three-way ANOVA, $F_{(7,56)} = 8.38$, $p < 0.01$). Data show a significant increase in percentage of HD in closed (protected) arms (%HD in closed arms) between animals exposed to noise at PND7 according to N1 scheme and housed in standard conditions and sham animals, without changes when animals were housed in EE. No changes were observed when animals were exposed according to N5 scheme in comparison with the corresponding sham group [Between factors: exposure (sham or noise), NS; housing (St or EE), $F_{(1,56)} = 36.86$, $p < 0.01$; within factors: scheme of exposure (N1 or N5), $F_{(1,56)} = 13$, $p < 0.01$]. As the interaction between exposure and scheme was significant ($F_{(1,56)} = 5.29$, $p < 0.05$), a simple effect analysis was performed [*N1*: Two-way ANOVA, $F_{(3,29)} = 6.62$, $p < 0.01$. Between factor: exposure (sham or noise), $p < 0.05$. Within factor: housing (St or EE), $p < 0.01$. *N5*: Two-way ANOVA, $F_{(3,26)} = 7.26$, $p < 0.01$. Between factor: exposure (sham or noise), NS; within factor: housing (St or EE), $p < 0.01$. *Post hoc* comparisons, sham vs. noise, N1: St, $p < 0.05$; EE, NS. N5: St and EE, NS].

Finally, non-significant differences were observed between N1 and N5 noise-exposed and the corresponding sham group in PND15 animals, both in standard and EE conditions (**Figure 7B**, Three-way ANOVA, $F_{(7,63)} = 1.08$, NS).

In summary, results show no significant changes in %HD in closed arms in most groups, both exposed at PND7 and PND15, except from animals exposed to noise at PND7 according to N1 scheme and housed in St conditions, which showed an increase in this parameter when compared to their sham group.

Inhibitory Avoidance (IA) Task: Ratio Between the Latency to Enter the Dark Compartment in the Retention and the Training Sessions

In the IA task, T1 is defined as the time required to enter the dark compartment (i.e., the side in which an electric shock, an aversive stimulus, was delivered) in the training session and T2 is the time required to enter the same compartment in the retention session, after an interval of 1 h. The ratio T2/T1 is the relationship between the seconds measured in the retention and the training sessions and might be taken as an index of associative memory. **Figure 8A** shows a significant main effect in the T2/T1 ratio in rats exposed at PND7 (Three-way ANOVA, $F_{(7,50)} = 5.49$, $p < 0.01$).Whereas non-significant differences were induced after exposure to noise under standard conditions according to N1 scheme when compared with sham animals, a significant increase was observed under EE housing [between factors: exposure (sham or noise), $F_{(1,50)} = 9.92$, $p < 0.01$; housing (St or EE), NS; within factors: scheme of exposure (N1 or N5), NS]. In contrast, an increase in this ratio was observed after repeated exposures to noise of animals housed in standard cages, without changes observed after housing in EE when compared with the corresponding sham rats. As some interactions were significant (between housing and scheme: $F_{(1,50)} = 5.50$, $p < 0.05$; between exposure, scheme and housing: $F_{(1,50)} = 20.32$, $p < 0.01$), a simple effect analysis was performed [*St*: Two-way ANOVA, $F_{(3,24)} = 5.36$ $p < 0.01$. Between factor: exposure (sham or noise), NS; within factor: scheme (N1 or N5), NS. *EE*: Two-way ANOVA, $F_{(3,25)} = 6.85$, $p < 0.01$. Between factor: exposure (sham or noise), $F_{(1,25)} = 7.71$, $p < 0.01$; within factor: scheme (N1 or N5), NS]. As the interaction between exposure and scheme was significant both in St and EE animals (*St*: $F_{(1,24)} = 10.43$, $p < 0.01$; *EE*: $F_{(1,25)} = 10.36$, $p < 0.01$), simple effect analyses were performed. *Post hoc* comparisons show a significant increase in the T2/T1 ratio of N5, St-housed, noise-exposed animals ($p < 0.05$) and in N1, EE-housed, noise-exposed animals ($p < 0.05$).

Finally, noise exposure at PND15 induced a significant main effect in the T2/T1 ratio (**Figure 8B**, Three-way ANOVA, $F_{(7,53)} = 2.79$, $p < 0.01$). Although a significant increase was observed in St housed animals exposed according to N1 scheme, five consecutive daily exposures did not produce changes in this parameter. Housing in an EE induced a significant increase only when rats were exposed once daily, for five consecutive days,

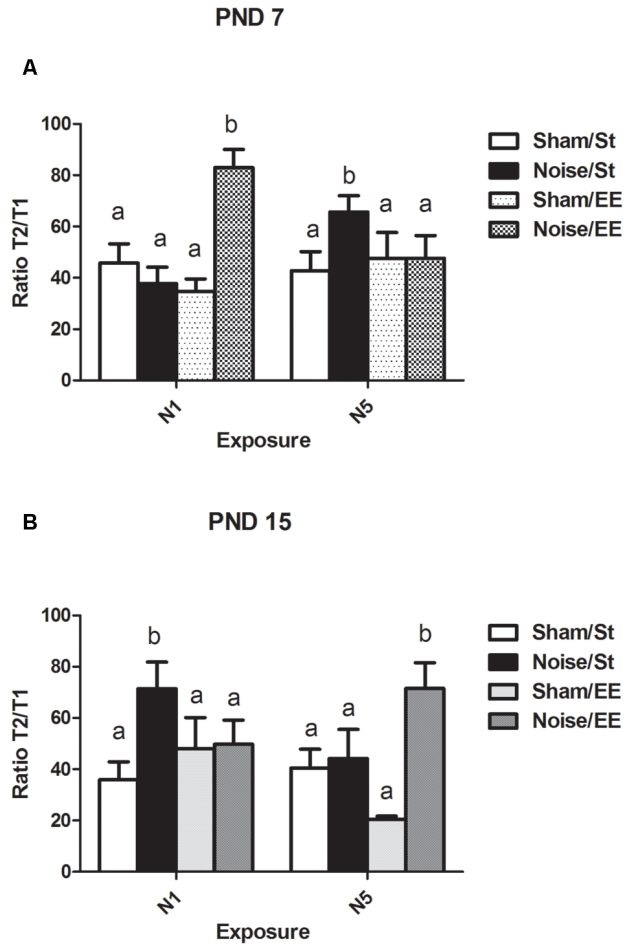

FIGURE 8 | Ratio between the latency to enter the dark compartment (in seconds) in the retention session and the training session (T2/T1) in the inhibitory avoidance (IA) task in PND28 animals exposed to noise at PND7 and PND15. **(A)** PND28 animals exposed at PND7; **(B)** PND28 animals exposed at PND15. Sham: non-exposed animals; N1: single noise exposure; N5: five-daily noise exposure. St: standard housing. EE: enriched environment. Different letters (a, b) symbolize significant differences with $p < 0.05$. Data represent the mean ± SEM of T2/T1in the IA task, $n = 7$ for each group.

without changes observed after N1 scheme [between factors: exposure (sham or noise), $F_{(1,53)} = 7.84$, $p < 0.01$; housing (St or EE), NS; within factors: scheme (N1 or N5), NS]. As the interaction between exposure, housing and scheme was significant ($F_{(1,53)} = 7.89$, $p < 0.01$), a simple effect analysis was performed [*St*: Two-way ANOVA, $F_{(3,26)} = 3.30$, $p < 0.05$. Between factors: exposure (sham or noise), $F_{(1,26)} = 2.42$, NS; within factor: scheme (N1 or N5), NS. *EE*: Two-way ANOVA, $F_{(3,26)} = 3.20$, $p < 0.05$. Between factors: exposure (sham or noise), $F_{(1,26)} = 5.62$, $p < 0.05$; within factor: scheme (N1 or N5), NS. *Post hoc* comparisons: sham vs. noise, EE: N1, NS; N5, $p < 0.05$]. As the interaction between exposure and scheme was significant in St animals ($F_{(1,26)} = 4.16$, $p < 0.05$), a simple effect analysis was performed. Data show a significant increase in the T2/T1 ratio of N1 St-housed, noise-exposed animals ($p < 0.05$), without changes observed in animals exposed according to N5 scheme.

In summary, data showed an increase in the ratio between the latency to enter the dark compartment in the retention and the training sessions in noise-exposed animals housed in St conditions according to N5 at PND7 and N1 at PND15, when compared with the respective controls, without changes in these groups after housing in an EE. On the other hand, an increase in noise-exposed animals was observed when compared to their sham groups, only when animals were housed in EE, according to N1 at PND7 and N5 at PND15, without changes when housed in standard cages.

Hippocampal Trx1 and Trx2 Levels

Figure 9A shows that noise exposure at PND7 induced a significant increase in hippocampal Trx-1 levels, when exposed according to both N1 or N5 schemes [Three-way ANOVA, $F_{(7,42)} = 2.82$, $p < 0.05$. Between factors: exposure (sham or noise), $F_{(1,42)} = 6.08$, $p < 0.05$; housing (St or EE), $F_{(1,42)} = 4.17$, $p < 0.05$; within factors: scheme (N1 or N5), NS], that remained similar to the corresponding sham levels when animals were housed in an EE. As interaction between exposure and housing was significant ($F_{(1,42)} = 8.32$, $p < 0.01$), a simple effect analysis was performed [*St*: Two-way ANOVA, $F_{(3,20)} = 5.47$, $p < 0.01$. Between factor: exposure (sham or noise), $F_{(1,20)} = 16.05$, $p < 0.01$; within factor: scheme (N1 or N5), NS. *EE*: Two-way ANOVA, $F_{(3,21)} = 0.27$, NS. Between factor: exposure (sham or noise), NS; within factor: scheme (N1 or N5), NS. *Post hoc* comparisons: St: sham vs. noise: N1 and N5, $p < 0.05$. EE: N1 and N5, NS].

Similarly, animals exposed to noise at PND15 showed a significant increase, according to N1 or N5 schemes and housed in St conditions, that remained similar to the corresponding sham values when housed in EE [**Figure 9B**, Three-way ANOVA, $F_{(7,36)} = 2.67$, $p < 0.05$. Between factors: exposure (sham or noise), $F_{(1,36)} = 4.18$, $p < 0.05$; housing (St or EE), $F_{(1,36)} = 4.76$, $p < 0.05$; within factors: scheme (N1 or N5), NS]. As interaction between exposure and housing was significant ($F_{(1,36)} = 9.72$, $p < 0.01$), a simple effect analysis was performed [*St*: Two-way ANOVA, $F_{(3,19)} = 3.47$, $p < 0.05$. Between factor: exposure (sham or noise), $F_{(1,19)} = 10.41$, $p < 0.01$; within factor: scheme (N1 or N5), NS. *EE*: Two-way ANOVA, $F_{(3,16)} = 0.40$, NS. Between factor: exposure (sham or noise), NS; within factor: scheme (N1 or N5), NS. *Post hoc* comparisons: sham vs. noise: St: N1 and N5, $p < 0.05$. EE: N1 and N5, NS].

Figure 9C shows a significant main effect on Trx-2 in animals exposed at PND7, although significant differences between sham and noise-exposed animals were observed only after five repeated exposures in standard housing [Three-way ANOVA, $F_{(7,36)} = 6.05$, $p < 0.01$. Between factors: exposure (sham or noise), $F_{(1,36)} = 3.17$, NS; housing (St or EE), $F_{(1,36)} = 1.80$, NS; within factors: scheme (N1 or N5), $F_{(1,36)} = 28.17$, $p < 0.01$. *Post hoc* comparisons: sham vs. noise: *N1*: St and EE, NS; *N5*: St, $p < 0.05$; EE, NS]. In contrast, non-significant main effects were observed in animals exposed at PND15 (**Figure 9D**, Three-way ANOVA, $F_{(7,34)} = 1.34$, NS). However, as a significant interaction was found between exposure, housing and scheme ($F_{(1,34)} = 4.43$,

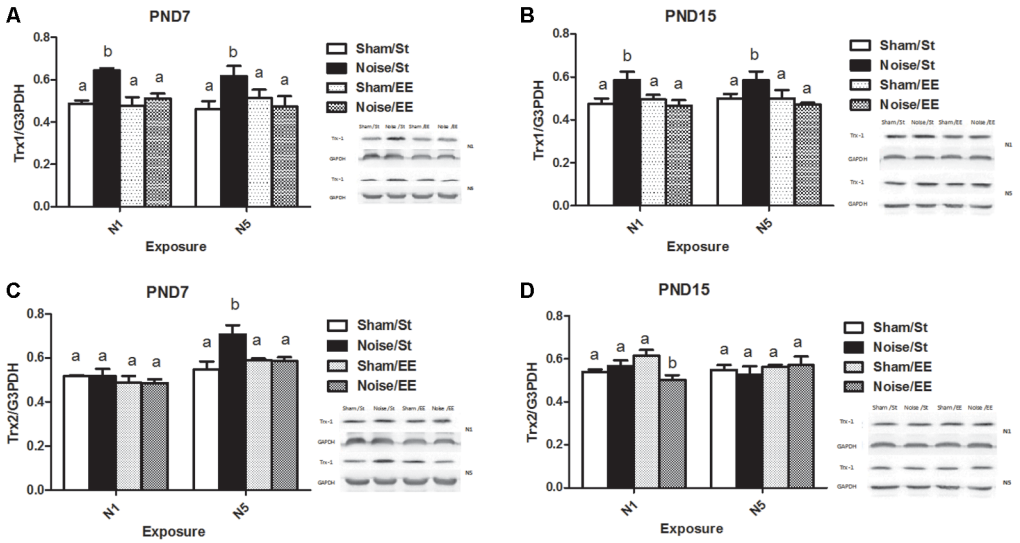

FIGURE 9 | Hippocampal Thioredoxin (Trx) levels.Trx-1: **(A)** PND28 animals exposed at PND7; **(B)** PND28 animals exposed at PND15. Trx-2: **(C)** PND28 animals exposed at PND7; **(D)** PND28 animals exposed at PND15. Sham: non- exposed animals; N1: single noise exposure; N5: five-daily noise exposure. St: standard housing. EE: enriched environment. Different letters (a, b) symbolize significant differences with $p < 0.05$. Data represent the mean ± SEM of the levels of hippocampal Trx-1 or Trx-2, $n = 4$ for each group.

$p < 0.05$), a simple effect analysis was performed. In addition, although no changes were induced in rats housed both in St and EE conditions (Two-way ANOVA, St: $F_{(3,17)} = 0.40$, NS; EE: $F_{(3,16)} = 2.47$, NS), a significant interaction was found between exposure and scheme in rats housed in EE ($F_{(1,16)} = 4.42$, $p < 0.05$), with a significant decrease only in rats exposed according to N1 scheme ($p < 0.05$).

In summary, results show an increase in hippocampal Trx1 levels in all noise-exposed animals when compared to their respective sham groups, according to both schemes (N1 and N5) and ages of exposure (PND7 and PND15), when animals were housed in St condition, without changes after EE housing. Moreover, data show an increase in hippocampal Trx2 levels in PND7 noise-exposed animals only after five repeated exposures in standard housing when compared to their sham group, without changes after EE housing. Finally, although no changes in Hippocampal Trx2 levels were induced in rats exposed to noise according to N1 scheme and housed both in St and EE conditions at PND15, a significant decrease was observed in animals exposed to noise according to N1 and housed in EE when compared to the sham group.

DISCUSSION

Present results show that exposure of 7 and 15-days-old animals to moderate levels of white noise (95–97 dB SPL, 2 h), using single or repeated session's exposures, was capable to trigger hippocampal-related behavioral alterations as well as oxidative-related molecular changes when evaluated after several days, that differed according to the scheme used. In addition, animals were not uniformly affected when different ages of exposure were compared. The housing in an enriched environment, a non-pharmacological strategy of neuroprotection, was effective in preventing some of these changes that differed between the different groups. Finally, non-significant changes in auditory function were found in neither group.

Auditory Pathway Evaluation

No changes in the auditory thresholds were induced, neither when the rats were exposed at PND7 nor at PND15, supporting results in other animal models (Pienkowski and Eggermont, 2012; Gourévitch et al., 2014). The fact that auditory system become mature at approximately PND12, could explain why there were no significant changes in the auditory threshold of animals exposed at PND7, considering that auditory pathway was not functional at the age of exposure. For this reason, the observation of damage when exposure was done at PND7, an age at which the auditory pathway is still immature, might suggest that moderate noise exposure can produce the behavioral and biochemical effects through a direct rather than an indirect mechanism, as hypothesized by Säljö et al. (2011). Otherwise, it is possible that in the case of rats exposed at PND15, which already had a functional auditory pathway, the intensity of noise used was not high enough to generate an effect on the auditory thresholds at PND28. However, it should not be discarded that animals' auditory system could be affected after PND 12 and prior to PND28, age at which animals were evaluated.

Behavioral Assessment

The behavioral alterations found in PND15 animals exposed according to N5 scheme differed from those observed in PND7 rats subjected to the same noise scheme, as was previously found for animals exposed at PND7 and PND15 according

to N1 scheme (Uran et al., 2010, 2012, 2014; Molina et al., 2016a). Even more, when exposed animals were housed in an EE, prevention of most behavioral alterations was observed in all groups. These data suggest that a prompt housing intervention, soon after single or multiple exposures to an environmental potentially hazardous agent, could be effective to avoid unfavorable effects, mainly if it is implemented in early stages of development (Smith et al., 2018; Gong et al., 2018).

It is important to highlight that habituation memory refers to behavioral changes that could be triggered in response to repeated exposure to novelty (Leussis and Bolivar, 2006). In addition, fear conditioning (i.e., inhibitory avoidance) implies a predictive relationship between a stimulus and an event (Ennaceur and Delacour, 1988). Interestingly, both depend on the hippocampal integrity (Vianna et al., 2000; Leussis and Bolivar, 2006). Finally, exploration is a behavior that can be measured in the OF and is triggered by novel stimuli: consists of behavioral acts and postures that permit an animal to collect information about new aspects of the environment (Barros et al., 2006). However, there are some debate in the literature (Ennaceur, 2014), as several authors suggested that as the anxiety-like behavior decreases, the animals increase the exploration of the environment (Escorihuela et al., 1999; Prut and Belzung, 2003; Lever et al., 2006; Kalouda and Pitsikas, 2015), whereas others postulated that it may be interpreted as an anxiogenic-like behavior (Barnett and Cowan, 1976; Lamprea et al., 2008).

Habituation Memory

When a rodent is placed in a novel environment, it begins to form an internal representation of the surrounding spatial information. Once this hippocampal-dependent map is "complete," the animal decreases the exploration of the environment because it would be considered habituated to the new context (O'Keefe and Nadel, 1979; Leussis and Andersen, 2008). Given that impairment in this parameter was observed only in PND15N1 animals and considering that the deficit was not evident when younger animals were exposed to noise, habituation memory might be used as a marker of vulnerability. Therefore, as the auditory system becomes active between PND7 and PND15 (de Villers-Sidani et al., 2008; Säljö et al., 2011), it could be postulated that more immature animals could be refractory to the damaging effects of noise on this type of memory, probably due to the impossibility of noise to affect CNS by means of a functional auditory system. As no effect was observed when PND15 animals were exposed to noise for 5 days, it could be suggested that repeated exposures might trigger adaptive mechanisms intended to counteract potential damage (Febbraro et al., 2017; Scott et al., 2017). The ability of EE to prevent noise-induced changes in PND15N1 animals might depend on the same adaptive mechanisms.

Exploratory Activity

Significant differences among groups were observed in exploratory activity, with an increase in those exposed to noise at PND7N1 and PND15N5 and without changes in the other groups. As a decrease in the latency and/or an increase in the number of entrances to open arms of the EPM was also observed in both groups, it could be suggested that greater exploration might be associated with decreased anxiety-like behavior, supporting Kalouda and Pitsikas (2015) and Wright et al.'s (2011) results. In addition, it could be claimed that an increase in exploratory activity with the consequent collection of information from the environment can favor the habituation and adaptability of these animals. Furthermore, an increase in novelty anxiety triggered by the new environment might affect exploration and habituation (Leussis and Bolivar, 2006), because shared mechanisms might be involved (Izquierdo and Medina, 1997; Salomons et al., 2012).

Conversely, animals with impairment in habituation memory (i.e., those exposed at PND15N1) did not exhibit changes exploratory activity. Even more, the increase in anxiety-related observed in animals exposed at PND15N1 might be related to a deficit in habituation memory (Venero et al., 2005; Barzegar et al., 2015). Furthermore, rats exposed at PND15N1 could have an increased fear response, which would imply that these animals would have greater emotional reactivity.

However, whereas housing in an EE prevented the changes in exploration, as observed in PND7N1 rats, no prevention was observed when animals exposed at PND15N5 were evaluated. These data suggest that there would seem to be a window of opportunity to intervene using a neuroprotection strategy that depends on the developmental stage at which the injury took place (Smith et al., 2018; Gong et al., 2018).

Emotional Reactivity: Anxiety-Like Behavior and Risk Assessment Behavior

It should be considered that decreased anxiety-like behavior could be interpreted as a behavioral improvement. However, it could not be true in the wild, because certain minimal anxiety levels might be required to cope with eventual dangerous situations. In contrast, although low or moderate levels of anxiety may be positive for learning and memory processes, it has been shown that high levels could lead to a cognitive deficit (Silva and Brandão, 2000).

A decrease in the entries to open arms of the EPM might be taken as a sign of an increase in anxiety-like behavior, as observed in animals exposed at PND15N1 and supported by Angrini and Leslie (2012). Conversely, an increase in the entries might imply a decrease in anxiety-like behavior, as observed in PND7N1 and supported by Eraslan et al. (2015). Therefore, it could be suggested that not only the developmental stage at which the animals are exposed to the environmental agent but also the scheme of exposure come into play to determine the development of emotional alterations. The lack of change of anxiety-like behavior in animals subjected to five daily noise sessions (PND7N5 and PND15N5) could be explained by a possible compensation that could be triggered as a consequence of the repeated exposure to the environmental challenge.

%HD in closed arms is a significant behavioral dimension whose biological function is to inform behavioral strategies in potentially dangerous situations (Carobrez and Bertoglio, 2005). Noise was capable to increase this parameter only when animals were exposed at PND7N1. Actually, animals with decreased anxiety-like behaviors would be less cautious and

could be more exposed to potential hazards. As a decrease in anxiety-like behaviors was observed in the group exposed at PND7N1, the finding of an increase in risk assessment behavior might not support this hypothesis. This result implies that at an early developmental age noise exposure increased the consciousness against potential dangers, such as the open environment of the OF task (Rodgers and Cole, 1993). Neither repeated exposure sessions nor maturation was able to induce changes, suggesting that this unique defensive behavior in mammals that reduces the chances of the animal to being harmed might be more important in helpless animals and tend to disappear with the advancement of CNS maturation. In contrast, the increases in this behavior observed in PND7N1 animals can be effectively prevented by housing in EE, suggesting that animals exposed to noise at earlier ages could be handled through the modification of rearing conditions when subjected to a threatening situation.

Interestingly, EE has shown "*per se*" to increase %HD when non-exposed PND7N1 rats were tested, when compared with the respective groups housed in standard conditions, indicating that these ethological readings might be altered through an environmental intervention, supporting results of Pietropaolo et al. (2004a) using a mice model of housing in an enriched environment. Usually, an increase in this risk assessment behavior is correlated with a decrease in anxiety-like behaviors (Cole and Rodgers, 1995). In contrast, non-exposed PND15 animals housed in EE cages showed unchanged %HD when compared with those animals housed under standard conditions, suggesting that the age of exposure is critical to driving this emotional output.

Associative Memory

Associative memory can be evaluated through the IA task by means of the ratio between the seconds taken to enter the dark compartment in the retention and the training sessions (T2/T1, Roozendaal, 2002). Although all animals retained associative memory in this task, the performance in the associative memory task was increased in rats exposed at PND7N5 and PND15N1, suggesting that these animals would have a more detailed representation of the traumatic event, as reported by Atucha and Roozendaal (2015). Again, the lack of change in the other groups might be related to either immature associative mechanisms (PND7N1) or to adaptive mechanisms (PND15N5) that could be triggered by repeated exposures, intended to counteract potential damage, as observed in different stress models (Febbraro et al., 2017; Scott et al., 2017). However, as memory retention has been observed in both groups, it should not be discarded that PND7N5 and PND15N1 rats experimented an increase in fear sense instead of an improvement in associative memory (i.e., there seems not to be a memory acquisition trouble). It must be underlined that fear can be distinguished from anxiety as it occurs in response to threats perceived as imminent, while anxiety could occur in response to potential or sustained threats (Izquierdo et al., 2016). In other words, a greater fear sense that could explain the increase in T2 in the IA task could be distinguished from the anxiety in response to a potential danger that occurs in the EPM test. There is also evidence supporting this statement, demonstrating that anxiety and fear response could depend on different CNS structures (Kjelstrup et al., 2002; Pentkowski et al., 2006).

In consequence, it could be suggested that the longer latency to enter into the dark compartment might be related to an increased emotional reactivity, a non-adaptive response, as suggested by Costanzi et al. (2011) that might be also related to the increase in anxiety-related behavior, as observed in humans (Michael et al., 2007; Ponomarev et al., 2010). Therefore, although fear is essential for survival, destined to learn about a potential danger, the lack of behavioral flexibility might expose individuals to environmental changes that might affect not only hippocampus but also other structures-related behaviors (Barros et al., 2000; Izquierdo et al., 2016).

In addition, whereas EE was able to prevent the noise-induced changes in the associative memory of PND7N5 and PND15N1 groups, this housing condition induced an improvement in the performance of noise-exposed PND7N1 and PND15N5 groups, suggesting that differences in environmental stimulation could favor different behavioral phenotypes in the presence of an unfavorable previous condition, such as exposure to noise.

EE as a Neuroprotective Strategy

The EE has shown to be an effective tool to protect against CNS injury (Lores-Arnaiz et al., 2006), obtaining benefits on learning and memory (Schrijver et al., 2002; Baraldi et al., 2013) as well as on anxiety-like behaviors (Friske and Gammie, 2005; Lima et al., 2014).

It should be highlighted that short periods of housing in an enriched environment appeared to be enough to produce brain changes in young, but not in adults rats, suggesting that in rodent species adolescence is a highly sensitive period likely to be modified by environmental challenges (Spear, 2000). Actually, only 1 week of EE used in the present experimental model as a neuroprotective strategy contrasts with the long periods required to be protective when adult animals are the experimental subjects, supporting this hypothesis.

In addition, housing in EE generated changes on its own in some behavioral parameters. For example, behavioral differences were observed between control groups depending on the type of housing in parameters such as anxiety-like behavior, %HD and exploratory activity. In addition, in some cases, exposed animals presented changes in their behavior when compared with their respective sham group only when they were housed in an EE, whereas no differences were observed when housed under standard conditions. Supporting these observations, several authors found behavioral changes in untreated animals after housing in EE, even during short periods (van Praag et al., 1999; Nithianantharajah and Hannan, 2006; Mitra and Sapolsky, 2012; Sampedro-Piquero and Begega, 2017). It has been postulated that beneficial effects of EE could be due to the novelty and increased social contact and exercise, which are rewarding for animals as well as efficacious in supplying for their ethological needs (Pietropaolo et al., 2004b; Crofton et al., 2015).

Therefore, it could be suggested that housing for a week in an EE was able to generate behavioral changes by itself, as well as to

unmask differences between exposed animals and their controls, which highlight the importance of the interaction with the environment that surrounds the animal, given that differences in environmental stimulation may favor the development of certain behavioral phenotypes (Nithianantharajah and Hannan, 2006; Mychasiuk et al., 2012).

Is Hippocampal Oxidative State Involved?

Finally, it is known that the balance of cellular oxidative status might be affected after several insults (Bendix et al., 2012; Sies, 2015). A significant increase in the antioxidant enzymes activities might indicate that a prior increase in ROS production could have been triggered, suggesting that the brain endogenous antioxidant defense system is capable of being activated in response to excessive ROS generation. As some changes in hippocampal oxidative status were observed after noise exposure of PND15N1 animals (Uran et al., 2010, 2014), the measurement of Trx, an endogenous antioxidant often involved in brain injuries, could be taken as a marker of damage in the present model that could underlie behavioral changes. However, although a similar increase in hippocampal Trx-1 levels was found in all groups, dissimilar changes in the behavioral parameters in each group were observed. This lack of correlation suggests that this endogenous antioxidant could not be the main responsible for the behavioral changes. Although, Trxs are a part of the vast antioxidant machinery, these key enzymes are frequently altered in oxidative-related pathologies. Nevertheless, other markers should be measured to confirm these findings.

CONCLUSION

In conclusion, noise exposure using single or repeated session's schemes was capable to trigger hippocampal-related behavioral alterations as well as oxidative-related molecular changes in animals exposed at PND 7 and PND15 and evaluated after several days that differed according to the scheme used and the age of exposure. Housing in an enriched environment, a non-pharmacological strategy of neuroprotection, was effective in preventing some of these changes. In addition, an oxidative imbalance might be triggered in the hippocampus of rats from all groups, without changes in the auditory function.

The different ages of exposure, as well as the different schemes applied, might predispose animals to undergo different alterations: more behavioral alterations were observed in younger animals, exposed for a single day. Therefore, it could be suggested that immature animals might be more vulnerable to noise impact and that the alterations induced by repeated exposures might be more effectively compensated in younger animals.

Therefore, these findings suggest that after repeated exposure to an environmental challenge animals become less susceptible to noise-induced behavioral changes, probably due to the ability of adaptation to an unfavorable condition. Moreover, it could be hypothesized that damage to younger individuals could be more easily prevented by an environmental manipulation.

The knowledge of the mechanisms involved in the damage, as well as the strategies aimed to prevent them, is of clinical relevance considering noise exposure as a public health problem that is increasing in urbanized societies.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the Institutional Committee for the Use and Care of Laboratory Animal rules (CICUAL, School of Medicine, University of Buenos Aires, Argentina). The present experimental protocol was approved by this Committee and registered with the number 53679/16. The CICUAL adheres to the rules of the "Guide for the Care and Use of Laboratory Animals" (NIH; 2011 revision) and to the EC Directive 86/609/EEC (2010 revision) for animal experiments.

AUTHOR CONTRIBUTIONS

SM performed the experiments, with the collaboration of GB and MR. MG-C performed auditory measurements. LG wrote the manuscript, with the collaboration of FC.

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REFERENCES

Angrini, M. A., and Leslie, J. C. (2012). Vitamin C attenuates the physiological and behavioural changes induced by long-term exposure to noise. *Behav. Pharmacol.* 23, 119–125. doi: 10.1097/fbp.0b013e32834f9f68

Aon-Bertolino, M. L., Romero, J. I., Galeano, P., Holubiec, M., Badorrey, M. S., Saraceno, G. E., et al. (2011). Thioredoxin and glutaredoxin system proteins-immunolocalization in the rat central nervous system. *Biochim. Biophys. Acta* 1810, 93–110. doi: 10.1016/j.bbagen.2010.06.011

Atucha, E., and Roozendaal, B. (2015). The inhibitory avoidance discrimination task to investigate accuracy of memory. *Front. Behav. Neurosci.* 9:60. doi: 10.3389/fnbeh.2015.00060

Baraldi, T., Schöwe, N. M., Balthazar, J., Monteiro-Silva, K. C., Albuquerque, M. S., Buck, H. S., et al. (2013). Cognitive stimulation during lifetime and in the aged phase improved spatial memory and altered neuroplasticity and cholinergic

markers of mice. *Exp. Gerontol.* 48, 831–838. doi: 10.1016/j.exger.2013.05.055

Barnett, S. A., and Cowan, P. E. (1976). Activity, exploration, curiosity and fear: an ethological study. *Interdiscip. Sci. Rev.* 1, 43–62. doi: 10.1179/030801876789768534

Barros, D., Amaral, O. B., Izquierdo, I., Geracitano, L., do Carmo Bassols Raseira, M., Henriques, A. T., et al. (2006). Behavioral and genoprotective effects of Vaccinium berries intake in mice. *Pharmacol. Biochem. Behav.* 84, 229–234. doi: 10.1016/j.pbb.2006.05.001

Barros, D. M., Izquierdo, L. A., Mello e Souza, T., Ardenghi, P. G., Pereira, P., Medina, J. H., et al. (2000). Molecular signalling pathways in the cerebral cortex are required for retrieval of one-trial avoidance learning in rats. *Behav. Brain Res.* 114, 183–192. doi: 10.1016/s0166-4328(00)00226-6

Barzegar, M., Sajjadi, F. S., Talaei, S. A., Hamidi, G., and Salami, M. (2015). Prenatal exposure to noise stress: anxiety, impaired spatial memory, and deteriorated hippocampal plasticity in postnatal life. *Hippocampus* 25, 187–196. doi: 10.1002/hipo.22363

Bendix, I., Weichelt, U., Strasser, K., Serdar, M., Endesfelder, S., Von Haefen, C., et al. (2012). Hyperoxia changes the balance of the thioredoxin/peroxiredoxin system in the neonatal rat brain. *Brain Res.* 1484, 68–75. doi: 10.1016/j.brainres.2012.09.024

Brenes, J. C., Padilla, M., and Fornaguera, J. (2009). A detailed analysis of open-field habituation and behavioral and neurochemical antidepressant-like effects in postweaning enriched rats. *Behav. Brain Res.* 197, 125–137. doi: 10.1016/j.bbr.2008.08.014

Caceres, L. G., Aon Bertolino, L., Saraceno, G. E., Zorrilla Zubilete, M. A., Uran, S. L., Capani, F., et al. (2010). Hippocampal-related memory deficits and histological damage induced by neonatal ionizing radiation exposure. Role of oxidative status. *Brain Res.* 1312, 67–78. doi: 10.1016/j.brainres.2009.11.053

Caceres, L. G., Rios, H., and Guelman, L. R. (2009). Long-lasting effects of neonatal ionizing radiation exposure on spatial memory and anxiety-like behavior. *Ecotoxicol. Environ. Saf.* 72, 895–904. doi: 10.1016/j.ecoenv.2008.09.009

Campeau, S., Dolan, D., Akil, H., and Watson, S. J. (2002). c-fos mRNA induction in acute and chronic audiogenic stress: possible role of the orbitofrontal cortex in habituation. *Stress* 5, 121–130. doi: 10.1080/10253890290027895

Carobrez, A. P., and Bertoglio, L. J. (2005). Ethological and temporal analyses of anxiety-like behavior: the elevated plus-maze model 20 years on. *Neurosci. Biobehav. Rev.* 29, 1193–1205. doi: 10.1016/j.neubiorev.2005.04.017

Cassarino, D. S., and Bennett, J. P. (1999). An evaluation of the role of mitochondria in neurodegenerative diseases: mitochondrial mutations and oxidative pathology, protective nuclear responses and cell death in neurodegeneration. *Brain Res. Rev.* 29, 1–25. doi: 10.1016/s0165-0173(98)00046-0

Chen, T. Y., Tsai, K. L., Lee, T. Y., Chiueh, C. C., Lee, W. S., and Hsu, C. (2010). Sex-specific role of thioredoxin in neuroprotection against iron-induced brain injury conferred by estradiol. *Stroke* 41, 160–165. doi: 10.1161/strokeaha.109.562850

Cheng, L., Wang, S. H., Chen, Q. C., and Liao, X. M. (2011). Moderate noise induced cognition impairment of mice and its underlying mechanisms. *Physiol. Behav.* 104, 981–988. doi: 10.1016/j.physbeh.2011.06.018

Cole, J. C., and Rodgers, R. J. (1995). Ethological comparison of the effects of diazepam and acute/chronic imipramine on the behaviour of mice in the elevated plus-maze. *Pharmacol. Biochem. Behav.* 52, 473–478. doi: 10.1016/0091-3057(95)00163-q

Costanzi, M., Cannas, S., Saraulli, D., Rossi-Arnaud, C., and Cestari, V. (2011). Extinction after retrieval: effects on the associative and nonassociative components of remote contextual fear memory. *Learn. Mem.* 18, 508–518. doi: 10.1101/lm.2175811

Crofton, E. J., Zhang, Y., and Green, T. A. (2015). Inoculation stress hypothesis of environmental enrichment. *Neurosci. Biobehav. Rev.* 49, 19–31. doi: 10.1016/j.neubiorev.2014.11.017

Cui, B., and Li, K. (2013). Chronic noise exposure and Alzheimer disease: is there an etiological association? *Med. Hypotheses* 81, 623–626. doi: 10.1016/j.mehy.2013.07.017

Cui, B., Wu, M., and She, X. (2009). Effects of chronic noise exposure on spatial learning and memory of rats in relation to neurotransmitters and NMDAR2B alteration in the hippocampus. *J. Occup. Health* 51, 152–158. doi: 10.1539/joh.l8084

Cunningham, G. M., Roman, M. G., Flores, L. C., Hubbard, G. B., Salmon, A. B., Zhang, Y., et al. (2015). The paradoxical role of thioredoxin on oxidative stress and aging. *Arch. Biochem. Biophys.* 576, 32–38. doi: 10.1016/j.abb.2015.02.025

de Villers-Sidani, E., Simpson, K. L., Lu, Y. F., Lin, R. C. S., and Merzenich, M. M. (2008). Manipulating critical period closure across different sectors of the primary auditory cortex. *Nat. Neurosci.* 11, 957–965. doi: 10.1038/nn.2144

Dehne, N., Lautermann, J., Ten Cate, W. J. F., Rauen, U., and De Groot, H. (2000). *In vitro* effects of hydrogen peroxide on the cochlear neurosensory epithelium of the guinea pig. *Hear. Res.* 143, 162–170. doi: 10.1016/s0378-5955(00)00036-8

Ennaceur, A. (2014). Tests of unconditioned anxiety—pitfalls and disappointments. *Physiol. Behav.* 135, 55–71. doi: 10.1016/j.physbeh.2014.05.032

Ennaceur, A., and Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. 1: behavioral data. *Behav. Brain Res.* 31, 47–59. doi: 10.1016/0166-4328(88)90157-x

Eraslan, E., Akyazi, I., Ergül-Ekiz, E., and Matur, E. (2015). Noise stress changes mRNA expressions of corticotropin-releasing hormone, its receptors in amygdala, and anxiety-related behaviors. *Noise Health* 17, 141–147. doi: 10.4103/1463-1741.155838

Erkal, H. S., Batçioglu, K., Serin, M., Uyumlu, B., and Yücel, N. (2006). The evaluation of the oxidant injury as a function of time following brain irradiation in a rat model. *Neurochem. Res.* 31, 1271–1277. doi: 10.1007/s11064-006-9159-y

Escorihuela, R. M., Fernández-Teruel, A., Gil, L., Aguilar, R., Tobeña, A., and Driscoll, P. (1999). Inbred roman high- and low-avoidance rats: differences in anxiety, novelty-seeking, and shuttlebox behaviors. *Physiol. Behav.* 67, 19–26. doi: 10.1016/s0031-9384(99)00064-5

Febbraro, F., Svenningsen, K., Tran, T. P., and Wiborg, O. (2017). Neuronal substrates underlying stress resilience and susceptibility in rats. *PLoS One* 12:e0179434. doi: 10.1371/journal.pone.0179434

Fetoni, A. R., Paciello, F., Rolesi, R., Eramo, S. L. M., Mancuso, C., Troiani, D., et al. (2015). Rosmarinic acid up-regulates the noise-activated Nrf2/HO-1 pathway and protects against noise-induced injury in rat cochlea. *Free Radic. Biol. Med.* 85, 269–281. doi: 10.1016/j.freeradbiomed.2015.04.021

Frenzilli, G., Lenzi, P., Scarcelli, V., Fornai, F., Pellegrini, A., Soldani, P., et al. (2004). Effects of loud noise exposure on DNA integrity in rat adrenal gland. *Environ. Health Perspect.* 112, 1671–1672. doi: 10.1289/ehp.7249

Friske, J. E., and Gammie, S. C. (2005). Environmental enrichment alters plus maze, but not maternal defense performance in mice. *Physiol. Behav.* 85, 187–194. doi: 10.1016/j.physbeh.2005.03.022

Gannouni, N., Mhamdi, A., Tebourbi, O., El May, M., Sakly, M., and Rhouma, K. B. (2013). Qualitative and quantitative assessment of noise at moderate intensities on extra-auditory system in adult rats. *Noise Health* 15, 406–411. doi: 10.4103/1463-1741.121236

Godoy, J. R., Funke, M., Ackermann, W., Haunhorst, P., Oesteritz, S., Capani, F., et al. (2011). Redox atlas of the mouse. Immunohistochemical detection of glutaredoxin-, peroxiredoxin- and thioredoxin-family proteins in various tissues of the laboratory mouse. *Biochim. Biophys. Acta* 1810, 2–92. doi: 10.1016/j.bbagen.2010.05.006

Gong, X., Chen, Y., Chang, J., Huang, Y., Cai, M., and Zhang, M. (2018). Environmental enrichment reduces adolescent anxiety- and depression-like behaviors of rats subjected to infant nerve injury. *J. Neuroinflammation* 15:262. doi: 10.1186/s12974-018-1301-7

Gourévitch, B., Edeline, J. M., Occelli, F., and Eggermont, J. J. (2014). Is the din really harmless? Long-term effects of non-traumatic noise on the adult auditory system. *Nat. Rev. Neurosci.* 15, 483–491. doi: 10.1038/nrn3744

Halliwell, B. (1992). Reactive oxygen species and the central nervous system. *J. Neurochem.* 59, 1609–1623. doi: 10.1111/j.1471-4159.1992.tb10990.x

Halliwell, B. (2006). Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiol.* 141, 312–322. doi: 10.1104/pp.106.077073

Halliwell, B., and Gutteridge, J. M. C. (1991). Free radicals in biology and medicine, second edition. *Free Radic. Biol. Med.* 10, 449–450. doi: 10.1016/0891-5849(91)90055-8

Hanschmann, E.-M., Godoy, J. R., Berndt, C., Hudemann, C., and Lillig, C. H. (2013). Thioredoxins, glutaredoxins, and peroxiredoxins—molecular mechanisms and health significance: from cofactors to antioxidants to redox signaling. *Antioxid. Redox Signal.* 19, 1539–1605. doi: 10.1089/ars.2012.4599

Harman, D. (1992). Role of free radicals in aging and disease. *Ann. N Y Acad. Sci.* 673, 126–141. doi: 10.1111/j.1749-6632.1992.tb27444.x

Izquierdo, I., and Medina, J. H. (1997). Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. *Neurobiol. Learn. Mem.* 68, 285–316. doi: 10.1006/nlme.1997.3799

Izquierdo, I., Furini, C. R. G., and Myskiw, J. C. (2016). Fear memory. *Physiol. Rev.* 96, 695–750. doi: 10.1152/physrev.00018.2015

Jáuregui-Huerta, F., Garcia-Estrada, J., Ruvalcaba-Delgadillo, Y., Trujillo, X., Huerta, M., Feria-Velasco, A., et al. (2011). Chronic exposure of juvenile rats to environmental noise impairs hippocampal cell proliferation in adulthood. *Noise Health* 13, 286–291. doi: 10.4103/1463-1741.82961

Jones, D. P. (2006). Redefining oxidative stress. *Antioxid. Redox Signal.* 8, 1865–1879. doi: 10.1089/ars.2006.8.1865

Kalouda, T., and Pitsikas, N. (2015). The nitric oxide donor molsidomine induces anxiolytic-like behaviour in two different rat models of anxiety. *Pharmacol. Biochem. Behav.* 138, 111–116. doi: 10.1016/j.pbb.2015.09.004

Kjelstrup, K. G., Tuvnes, F. A., Steffenach, H.-A., Murison, R., Moser, E. I., and Moser, M.-B. (2002). Reduced fear expression after lesions of the ventral hippocampus. *Proc. Natl. Acad. Sci. U S A* 99, 10825–10830. doi: 10.1073/pnas.152112399

Kopke, R. D., Jackson, R. L., Coleman, J. K. M., Liu, J., Bielefeld, E. C., and Balough, B. J. (2007). NAC for noise: from the bench top to the clinic. *Hear. Res.* 226, 114–125. doi: 10.1016/j.heares.2006.10.008

Kujawa, S. G., and Liberman, M. C. (2009). Adding insult to injury: cochlear nerve degeneration after "temporary" noise-induced hearing loss. *J. Neurosci.* 29, 14077–14085. doi: 10.1523/JNEUROSCI.2845-09.2009

Kurioka, T., Matsunobu, T., Satoh, Y., Niwa, K., and Shiotani, A. (2014). Inhaled hydrogen gas therapy for prevention of noise-induced hearing loss through reducing reactive oxygen species. *Neurosci. Res.* 89, 69–74. doi: 10.1016/j.neures.2014.08.009

Lamprea, M. R., Cardenas, F. P., Setem, J., and Morato, S. (2008). Thigmotactic responses in an open-field. *Braz. J. Med. Biol. Res.* 41, 135–140. doi: 10.1590/s0100-879x2008000200010

Laviola, G., Hannan, A. J., Macrì, S., Solinas, M., and Jaber, M. (2008). Effects of enriched environment on animal models of neurodegenerative diseases and psychiatric disorders. *Neurobiol. Dis.* 31, 159–168. doi: 10.1016/j.nbd.2008.05.001

Leussis, M. P., and Andersen, S. L. (2008). Is adolescence a sensitive period for depression? Behavioral and neuroanatomical findings from a social stress model. *Synapse* 62, 22–30. doi: 10.1002/syn.20462

Leussis, M. P., and Bolivar, V. J. (2006). Habituation in rodents: a review of behavior, neurobiology, and genetics. *Neurosci. Biobehav. Rev.* 30, 1045–1064. doi: 10.1016/j.neubiorev.2006.03.006

Lever, C., Burton, S., and O'Keefe, J. (2006). Rearing on hind legs, environmental novelty and the hippocampal formation. *Rev. Neurosci.* 17, 111–133. doi: 10.1515/REVNEURO.2006.17.1-2.111

Lillig, C. H., Berndt, C., and Holmgren, A. (2008). Glutaredoxin systems. *Biochim. Biophys. Acta* 1780, 1304–1317. doi: 10.1016/j.bbagen.2008.06.003

Lillig, C. H., and Holmgren, A. (2007). Thioredoxin and related molecules-from biology to health and disease. *Antioxid. Redox Signal.* 9, 25–47. doi: 10.1089/ars.2007.9.25

Lima, A. P., Silva, K., Padovan, C. M., Almeida, S. S., and Fukuda, M. H. (2014). Memory, learning and participation of the cholinergic system in young rats exposed to environmental enrichment. *Behav. Brain Res.* 259, 247–252. doi: 10.1016/j.bbr.2013.10.046

Lores-Arnaiz, S., Bustamante, J., Arismendi, M., Vilas, S., Paglia, N., Basso, N., et al. (2006). Extensive enriched environments protect old rats from the aging dependent impairment of spatial cognition, synaptic plasticity and nitric oxide production. *Behav. Brain Res.* 169, 294–302. doi: 10.1016/j.bbr.2006.01.016

Ma, Y. H., Su, N., Chao, X. D., Zhang, Y. Q., Zhang, L., Han, F., et al. (2012). Thioredoxin-1 attenuates post-ischemic neuronal apoptosis *via* reducing oxidative/nitrative stress. *Neurochem. Int.* 60, 475–483. doi: 10.1016/j.neuint.2012.01.029

Maison, S. F., Usubuchi, H., and Liberman, M. C. (2013). Efferent feedback minimizes cochlear neuropathy from moderate noise exposure. *J. Neurosci.* 33, 5542–5552. doi: 10.1523/jneurosci.5027-12.2013

Manikandan, S., Padma, M. K., Srikumar, R., Jeya Parthasarathy, N., Muthuvel, A., and Devi, R. S. (2006). Effects of chronic noise stress on spatial memory of rats in relation to neuronal dendritic alteration and free radical-imbalance in hippocampus and medial prefrontal cortex. *Neurosci. Lett.* 399, 17–22. doi: 10.1016/j.neulet.2006.01.037

Massaad, C. A., and Klann, E. (2011). Reactive oxygen species in the regulation of synaptic plasticity and memory. *Antioxid. Redox Signal.* 14, 2013–2054. doi: 10.1089/ars.2010.3208

Miceli, M., Molina, S. J., Forcada, A., Acosta, G. B., and Guelman, L. R. (2018). Voluntary alcohol intake after noise exposure in adolescent rats: hippocampal-related behavioral alterations. *Brain Res.* 1679, 10–18. doi: 10.1016/j.brainres.2017.11.001

Michael, T., Blechert, J., Vriends, N., Margraf, J., and Wilhelm, F. H. (2007). Fear conditioning in panic disorder: enhanced resistance to extinction. *J. Abnorm. Psychol.* 116, 612–617. doi: 10.1037/0021-843x.116.3.612

Mitra, R., and Sapolsky, R. M. (2012). Short-term enrichment makes male rats more attractive, more defensive and alters hypothalamic neurons. *PLoS One* 7:e36092. doi: 10.1371/journal.pone.0036092

Molina, S. J., Capani, F., and Guelman, L. R. (2016a). Noise exposure of immature rats can induce different age-dependent extra-auditory alterations that can be partially restored by rearing animals in an enriched environment. *Brain Res.* 1636, 52–61. doi: 10.1016/j.brainres.2016.01.050

Molina, S. J., Miceli, M., and Guelman, L. R. (2016b). Noise exposure and oxidative balance in auditory and extra-auditory structures in adult and developing animals. Pharmacological approaches aimed to minimize its effects. *Pharmacol. Res.* 109, 86–91. doi: 10.1016/j.phrs.2015.11.022

Montgomery, K. C. (1955). The relation between fear induced by novel stimulation and exploratory drive. *J. Comp. Physiol. Psychol.* 48, 254–260. doi: 10.1037/h0043788

Mychasiuk, R., Zahir, S., Schmold, N., Ilnytskyy, S., Kovalchuk, O., and Gibb, R. (2012). Parental enrichment and offspring development: modifications to brain, behavior and the epigenome. *Behav. Brain Res.* 228, 294–298. doi: 10.1016/j.bbr.2011.11.036

NIOSH. (1998). "Occupational noise exposure," in *National Institute for Occupational Safety and Health, DHHS (NIOSH), (Cincinnati, OH)*, 98–126.

Nithianantharajah, J., and Hannan, A. J. (2006). Enriched environments, experience-dependent plasticity and disorders of the nervous system. *Nat. Rev. Neurosci.* 7, 697–709. doi: 10.1038/nrn1970

O'Keefe, J., and Nadel, L. (1979). Précis of O'Keefe and Nadel's The hippocampus as a cognitive map. *Behav. Brain Sci.* 2, 487–533.

Ohlemiller, K. K., McFadden, S. L., Ding, D. L., Flood, D. G., Reaume, A. G., Hoffman, E. K., et al. (1999). Targeted deletion of the cytosolic Cu/Zn-superoxide dismutase gene (Sod1) increases susceptibility to noise-induced hearing loss. *Audiol. Neurootol.* 4, 237–246. doi: 10.1159/000013847

Pentkowski, N. S., Blanchard, D. C., Lever, C., Litvin, Y., and Blanchard, R. J. (2006). Effects of lesions to the dorsal and ventral hippocampus on defensive behaviors in rats. *Eur. J. Neurosci.* 23, 2185–2196. doi: 10.1111/j.1460-9568.2006.04754.x

Pereira, M., Dombrowski, P. A., Losso, E. M., Chioca, L. R., Da Cunha, C., and Andreatini, R. (2011). Memory impairment induced by sodium fluoride is associated with changes in brain monoamine levels. *Neurotox. Res.* 19, 55–62. doi: 10.1007/s12640-009-9139-5

Pienkowski, M., and Eggermont, J. J. (2012). Reversible long-term changes in auditory processing in mature auditory cortex in the absence of hearing loss induced by passive, moderate-level sound exposure. *Ear Hear.* 33, 305–314. doi: 10.1097/AUD.0b013e318241e880

Pietropaolo, S., Branchi, I., Chiarotti, F., and Alleva, E. (2004a). Utilisation of a physically-enriched environment by laboratory mice: age and gender differences. *Appl. Anim. Behav. Sci.* 88, 149–162. doi: 10.1016/j.applanim.2004.02.015

Pietropaolo, S., Branchi, I., Cirulli, F., Chiarotti, F., Aloe, L., and Alleva, E. (2004b). Long-term effects of the periadolescent environment on exploratory activity and aggressive behaviour in mice: social versus physical enrichment. *Physiol. Behav.* 81, 443–453. doi: 10.1016/j.physbeh.2004.02.022

Ponomarev, I., Rau, V., Eger, E. I., Harris, R. A., and Fanselow, M. S. (2010). Amygdala transcriptome and cellular mechanisms underlying stress-enhanced fear learning in a rat model of posttraumatic stress disorder. *Neuropsychopharmacology* 35, 1402–1411. doi: 10.1038/npp.2010.10

Prut, L., and Belzung, C. (2003). The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *Eur. J. Pharmacol.* 463, 3–33. doi: 10.1016/s0014-2999(03)01272-x

Rabat, A., Bouyer, J. J., George, O., Le Moal, M., and Mayo, W. (2006). Chronic exposure of rats to noise: relationship between long-term memory deficits and slow wave sleep disturbances. *Behav. Brain Res.* 171, 303–312. doi: 10.1016/j.bbr.2006.04.007

Rodgers, R. J., and Cole, J. C. (1993). Influence of social isolation, gender, strain, and prior novelty on plus-maze behaviour in mice. *Physiol. Behav.* 54, 729–736. doi: 10.1016/0031-9384(93)90084-s

Romero, J. I., Hanschmann, E. M., Gellert, M., Eitner, S., Holubiec, M. I., Blanco-Calvo, E., et al. (2015). Thioredoxin 1 and glutaredoxin 2 contribute to maintain the phenotype and integrity of neurons following perinatal asphyxia. *Biochim. Biophys. Acta* 1850, 1274–1285. doi: 10.1016/j.bbagen.2015.02.015

Roozendaal, B. (2002). Stress and memory: opposing effects of glucocorticoids on memory consolidation and memory retrieval glucocorticoids and memory function. *Neurobiol. Learn. Mem.* 78, 578–595. doi: 10.1006/nlme.2002.4080

Säljö, A., Mayorga, M., Bolouri, H., Svensson, B., and Hamberger, A. (2011). Mechanisms and pathophysiology of the low-level blast brain injury in animal models. *Neuroimage* 54, S83–S88. doi: 10.1016/j.neuroimage.2010.05.050

Salomons, A. R., Arndt, S. S., and Ohl, F. (2012). Impact of anxiety profiles on cognitive performance in BALB/c and 129P2 mice. *Cogn. Affect. Behav. Neurosci.* 12, 794–803. doi: 10.3758/s13415-012-0109-7

Sampedro-Piquero, P., and Begega, A. (2017). Environmental enrichment as a positive behavioral intervention across the lifespan. *Curr. Neuropharmacol.* 15, 459–470. doi: 10.2174/1570159X14666160325115909

Samson, J., Wiktorek-Smagur, A., Politanski, P., Rajkowska, E., Pawlaczyk-Luszczynska, M., Dudarewicz, A., et al. (2008). Noise-induced time-dependent changes in oxidative stress in the mouse cochlea and attenuation by d-methionine. *Neuroscience* 152, 146–150. doi: 10.1016/j.neuroscience.2007.11.015

Sasse, S. K., Greenwood, B. N., Masini, C. V., Nyhuis, T. J., Fleshner, M., Day, H. E. W., et al. (2008). Chronic voluntary wheel running facilitates corticosterone response habituation to repeated audiogenic stress exposure in male rats. *Stress* 11, 425–437. doi: 10.1080/10253890801887453

Sathyasaikumar, K. V., Swapna, I., Reddy, P. V. B., Murthy, C. R. K., Dutta Gupta, A., Senthilkumaran, B., et al. (2007). Fulminant hepatic failure in rats induces oxidative stress differentially in cerebral cortex, cerebellum and pons medulla. *Neurochem. Res.* 32, 517–524. doi: 10.1007/s11064-006-9265-x

Schrijver, N. C. A., Bahr, N. I., Weiss, I. C., and Würbel, H. (2002). Dissociable effects of isolation rearing and environmental enrichment on exploration, spatial learning and HPA activity in adult rats. *Pharmacol. Biochem. Behav.* 73, 209–224. doi: 10.1016/s0091-3057(02)00790-6

Scott, K. A., de Kloet, A. D., Smeltzer, M. D., Krause, E. G., Flak, J. N., Melhorn, S. J., et al. (2017). Susceptibility or resilience? Prenatal stress predisposes male rats to social subordination, but facilitates adaptation to subordinate status. *Physiol. Behav.* 178, 117–125. doi: 10.1016/j.physbeh.2017.03.006

Sies, H. (2015). Oxidative stress: a concept in redox biology and medicine. *Redox Biol.* 4, 180–183. doi: 10.1016/j.redox.2015.01.002

Silva, R. C. B., and Brandão, M. L. (2000). Acute and chronic effects of gepirone and fluoxetine in rats tested in the elevated plus-maze: an ethological analysis. *Pharmacol. Biochem. Behav.* 65, 209–216. doi: 10.1016/s0091-3057(99)00193-8

Silva-Adaya, D., Gonsebatt, M. E., and Guevara, J. (2014). Thioredoxin system regulation in the central nervous system: experimental models and clinical evidence. *Oxid. Med. Cell. Longev.* 2014:590808. doi: 10.1155/2014/590808

Smith, B. L., Morano, R. L., Ulrich-Lai, Y. M., Myers, B., Solomon, M. B., and Herman, J. P. (2018). Adolescent environmental enrichment prevents behavioral and physiological sequelae of adolescent chronic stress in female (but not male) rats. *Stress* 21, 464–473. doi: 10.1080/10253890.2017.1402883

Spear, L. (2000). The adolescent brain and age-related behavioral manifestations. *Neurosci. Behav. Rev.* 24, 417–463. doi: 10.1016/S0149-7634(00)00014-2

Tamura, H., Ohgami, N., Yajima, I., Iida, M., Ohgami, K., Fujii, N., et al. (2012). Chronic exposure to low frequency noise at moderate levels causes impaired balance in mice. *PLoS One* 7:e39807. doi: 10.1371/journal.pone.0039807

Ten, V. S., and Starkov, A. (2012). Hypoxic-ischemic injury in the developing brain: the role of reactive oxygen species originating in mitochondria. *Neurol. Res. Int.* 2012:542976. doi: 10.1155/2012/542976

Trapanotto, M., Benini, F., Farina, M., Gobber, D., Magnavita, V., and Zacchello, F. (2004). Behavioural and physiological reactivity to noise in the newborn. *J. Paediatr. Child Health* 40, 275–281. doi: 10.1111/j.1440-1754.2004.00363.x

Uran, S. L., Aon-Bertolino, M. L., Caceres, L. G., Capani, F., and Guelman, L. R. (2012). Rat hippocampal alterations could underlie behavioral abnormalities induced by exposure to moderate noise levels. *Brain Res.* 1471, 1–12. doi: 10.1016/j.brainres.2012.06.022

Uran, S. L., Caceres, L. G., and Guelman, L. R. (2010). Effects of loud noise on hippocampal and cerebellar-related behaviors. Role of oxidative state. *Brain Res.* 1361, 102–114. doi: 10.1016/j.brainres.2010.09.022

Uran, S. L., Gómez-Casati, M. E., and Guelman, L. R. (2014). Long-term recovery from hippocampal-related behavioral and biochemical abnormalities induced by noise exposure during brain development. Evaluation of auditory pathway integrity. *Int. J. Dev. Neurosci.* 37, 41–51. doi: 10.1016/j.ijdevneu.2014.06.002

Uttara, B., Singh, A. V., Zamboni, P., and Mahajan, R. T. (2009). Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Curr. Neuropharmacol.* 7, 65–74. doi: 10.2174/157015909787602823

van Praag, H., Kempermann, G., and Gage, F. H. (1999). Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat. Neurosci.* 2, 266–270. doi: 10.1038/6368

Venero, C., Guadaño-Ferraz, A., Herrero, A. I., Nordström, K., Manzano, J., De Escobar, G. M., et al. (2005). Anxiety, memory impairment, and locomotor dysfunction caused by a mutant thyroid hormone receptor α1 can be ameliorated by T3 treatment. *Genes Dev.* 19, 2152–2163. doi: 10.1101/gad.346105

Vianna, M. R. M., Alonso, M., Viola, H., Quevedo, J., de Paris, F., Furman, M., et al. (2000). Role of hippocampal signaling pathways in long-term memory formation of a nonassociative learning task in the rat. *Learn. Mem.* 7, 333–340. doi: 10.1101/lm.34600

Violle, N., Balandras, F., Le Roux, Y., Desor, D., and Schroeder, H. (2009). Variations in illumination, closed wall transparency and/or extramaze space influence both baseline anxiety and response to diazepam in the rat elevated plus-maze. *Behav. Brain Res.* 203, 35–42. doi: 10.1016/j.bbr.2009.04.015

Walf, A. A., and Frye, C. A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat. Protoc.* 2, 322–328. doi: 10.1038/nprot.2007.44

WHO (1999). WHO guidelines for community noise. *Noise Vib. Worldw.* 31: 161. doi: 10.1260/0957456001497535

Wright, T. M., Fone, K. C. F., Langley-Evans, S. C., and Voigt, J. P. W. (2011). Exposure to maternal consumption of cafeteria diet during the lactation period programmes feeding behaviour in the rat. *Int. J. Dev. Neurosci.* 29, 785–793. doi: 10.1016/j.ijdevneu.2011.09.007

Wu, X., Li, L., Zhang, L., Wu, J., Zhou, Y., Zhou, Y., et al. (2015). Inhibition of thioredoxin-1 with siRNA exacerbates apoptosis by activating the ASK1-JNK/p38 pathway in brain of a stroke model rats. *Brain Res.* 1599, 20–31. doi: 10.1016/j.brainres.2014.12.033

Yamane, H., Nakai, Y., Takayama, M., Iguchi, H., Nakagawa, T., and Kojima, A. (1995). Appearance of free radicals in the guinea pig inner ear after noise-induced acoustic trauma. *Eur. Arch. Otorhinolaryngol.* 252, 504–508. doi: 10.1007/bf02114761

Yamashita, D., Jiang, H. Y., Schacht, J., and Miller, J. M. (2004). Delayed production of free radicals following noise exposure. *Brain Res.* 1019, 201–209. doi: 10.1016/j.brainres.2004.05.104

Yamasoba, T., Harris, C., Shoji, F., Lee, R. J., Nuttall, A. L., and Miller, J. M. (1998). Influence of intense sound exposure on glutathione synthesis in the cochlea. *Brain Res.* 804, 72–78. doi: 10.1016/s0006-8993(98)00660-x

Zeeni, N., Bassil, M., Fromentin, G., Chaumontet, C., Darcel, N., Tome, D., et al. (2015). Environmental enrichment and cafeteria diet attenuate the response to chronic variable stress in rats. *Physiol. Behav.* 139, 41–49. doi: 10.1016/j.physbeh.2014.11.003

Zheng, K. C., and Ariizumi, M. (2007). Modulations of immune functions and oxidative status induced by noise stress. *J. Occup. Health* 49, 32–38. doi: 10.1539/joh.49.32

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Δ^9-Tetrahydrocannabinol During Adolescence Attenuates Disruption of Dopamine Function Induced in Rats by Maternal Immune Activation

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The combination of prenatal, such as maternal infections, and postnatal environmental insults (e.g., adolescent drug abuse) increases risks for psychosis, as predicted by the two-hit hypothesis of schizophrenia. Cannabis abuse during adolescence is widespread and is associated with increased risk of psychoses later in life. Here, we hypothesized that adolescent Δ^9-tetrahydrocannabinol (THC) worsens the impact of prenatal maternal immune activation (MIA) on ventral tegmental area (VTA) dopamine cells in rat offspring. Additionally, since substance abuse disorder is particularly prevalent among schizophrenia patients, we also tested how VTA dopamine neurons in MIA offspring respond to acute nicotine and cocaine administration. We used a model of neurodevelopmental disruption based on prenatal administration of the polyriboinosinic-polyribocytidilic acid [poly (I:C)] in rats, which activates the maternal immune system by mimicking a viral infection and induces behavioral abnormalities and disruption of dopamine transmission relevant to psychiatric disorders in the offspring. Male offspring were administered THC (or vehicle) during adolescence (PND 45–55). Once adult (PND 70–90), we recorded the spontaneous activity of dopamine neurons in the VTA and their responses to nicotine and cocaine. MIA male offspring displayed reduced number, firing rate and altered activity pattern of VTA dopamine cells. Adolescent THC attenuated several MIA-induced effects. Both prenatal [poly (I:C)] and postnatal (THC) treatments affected the response to nicotine but not to cocaine. Contrary to our expectations, adolescent THC did not worsen MIA-induced deficits. Results indicate that the impact of cannabinoids in psychosis models is complex.

Keywords: dopamine neurons, maternal immune activation, cannabinoids, adolescence, electrophysiology, schizophrenia

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INTRODUCTION

Environmental factors, such as prenatal exposure to a variety of infectious agents and consequent maternal immune activation (MIA), can lead to aberrant brain development, emerging in pathological phenotypes, such as autism and schizophrenia (Hornig et al., 2018). An association between MIA and increased risks of developing psychiatric disorders in offspring later in life has been reported by preclinical investigations and epidemiological studies in humans (Meyer et al., 2011).

In utero exposure to polyriboinosinic-polyribocytidylic acid (Poly I:C), a double-stranded synthetic RNA that activates an innate immune response, induces MIA in rodents by mimicking a viral infection and has been shown to induce schizophrenia- or autism-like phenotypes in rodents (Zuckerman et al., 2003). Hence, offspring display behavioral abnormalities, e.g., impairment in recognition memory, in social interactions and in sensorimotor gating as well as alterations in brain regions key in the neuropathology of psychoses, such as the dopaminergic ventral tegmental area (VTA; Patterson, 2002, 2009; Meyer et al., 2005; Boksa, 2010). Indeed, previous studies reported an increase in the number of TH-immunoreactive neurons in the VTA, TH-positive terminals in the striatum (Meyer et al., 2008; Winter et al., 2009; Vuillermot et al., 2010), increases in evoked striatal dopamine release *ex vivo* (Zuckerman et al., 2003) and enhanced dopamine levels in the prefrontal cortex and lateral globus pallidus (Winter et al., 2009). In our previous studies we observed a marked alteration of VTA dopamine neuron activity (reduced firing rate, reduced number of spontaneously active cells and altered firing pattern) in male but not female offspring coupled with disruption of sensorimotor gating and of cognitive and social behavior, and increase in dopamine levels in the nucleus accumbens (Luchicchi et al., 2016; De Felice et al., 2019).

Besides the prenatal period, adolescence is also a critical window of enhanced vulnerability. During adolescence the brain is particularly susceptible to perturbations, such as exposure to drugs of abuse, which can disrupt cognitive, emotional, and social maturation (Crews et al., 2007). Cannabis is the most widely used illegal drug during adolescence and its consumption might induce neurobiological changes that affect adult brain function (Rubino and Parolaro, 2016).

The dopamine system is particularly sensitive to cannabinoids. Both Δ^9-tetrahydrocannabinol (THC) and synthetic cannabinoids induce increases in firing rate of mesolimbic and mesocortical VTA dopamine cells (Diana et al., 1998; Gessa et al., 1998) and in extracellular dopamine levels in terminal regions (Tanda et al., 1997). Accordingly, in humans, THC reduces [^{11}C]raclopride binding in the ventral striatum, consistent with a modest increase in dopamine release (Bossong et al., 2009, 2015) and exacerbates psychotic symptoms (Mason et al., 2009). We and others reported that adolescent THC administration induced long-lasting changes in the response to dopamine cells to drugs of abuse and enhanced behavioral responses and self-administration (Pistis et al., 2004; Scherma et al., 2016) which might extend across generations (Vassoler et al., 2013). Moreover, adolescent administration of cannabinoids is associated with schizophrenia-like deficits in adult rodents (Rubino et al., 2009; Leweke and Schneider, 2011).

Considering that in humans early marijuana intake is associated with increased risk of psychoses later in life (Arseneault et al., 2004; Fergusson, 2004; Degenhardt and Hall, 2006), our hypothesis is that cannabinoid administration during adolescence in male rats exposed to MIA would worsen the outcome, as the two-hits hypothesis of schizophrenia (genetic/prenatal plus postnatal environment factors) predicts. Additionally, since substance abuse disorder, specifically heavy tobacco smoking (Winterer, 2010) and stimulant use disorder (Hunt et al., 2018), is particularly prevalent among schizophrenia patients we also tested how VTA dopamine neurons in MIA offspring treated with THC and their controls respond to acute nicotine and cocaine administration.

MATERIALS AND METHODS

All procedures were performed in accordance with the European legislation EU Directive 2010/63 and were approved by the Animal Ethics Committee of the University of Cagliari and by Italian Ministry of Health (auth. n. 658/2015-PR). Animals were housed in groups of three to six in standard conditions of temperature (21 ± 1°C) and humidity (60%) under a 12 h light/dark cycle (lights on at 7:00 A.M.) with food and water available *ad libitum*. We made all efforts to minimize animal discomfort and to reduce the number of animals used.

Prenatal Treatment

Female Sprague–Dawley rats (Envigo, Italy) were mated at the age of 3 months. The first day after the copulation was defined as gestational day 1 (GD 1). MIA was induced at GD 15, following the procedure described by Zuckerman et al. (2003). Dams were anesthetized with isoflurane 2% and a single dose of Poly I:C (4.0 mg/kg, i.v.; InvivoGen, San Diego, CA, USA) or an equivalent volume of endotoxin-free saline solution was administered in the lateral vein of the tail. To assess the efficacy of Poly I:C injection, all pregnant rats were weighed for the first 3 days after the administration of either Poly I:C or saline to evaluate weight loss as underlined by previous investigations (Zuckerman et al., 2003; Wolff and Bilkey, 2010). After weaning, male offspring were housed with littermates and maintained undisturbed until adolescent treatment (PND 45–55) and experiments in adulthood (PND 70–90). Male rats were randomly assigned to the experimental procedures and care was taken to avoid assigning more than three animals from the same litter to the same experimental group (Kentner et al., 2019).

Adolescent Treatment

Male rats were intraperitoneally injected with THC (THC-Pharm GmbH) or vehicle (1% ethanol, 2% Tween 80 and saline) at PND 45, in the mid-adolescence period. Increasing doses of THC (2.5 mg/kg, PND 45–47; 5 mg/kg, PND 48–51; 10 mg/kg, PND 52–55) or vehicle were given twice/day for 11 consecutive days. Theses doses of THC were chosen according to the literature (Scherma et al., 2016). Body weight and food intake were monitored for the entire period of treatment.

In vivo Electrophysiological Experiments

In vivo electrophysiology experiments were carried out at PND 70–90. This age window, which corresponds to the young adulthood in humans, was selected as it is the most vulnerable age for the onset of schizophrenia (Häfner, 2003). Moreover, studies on the ontogeny of MIA-induced deficits showed that these are evident at PND 70 (Romero et al., 2010; Vuillermot et al., 2010).

In vivo electrophysiological recordings were performed as described previously (Melis et al., 2008, 2009; Luchicchi et al., 2016). At PND 70–90, male rats were anesthetized with urethane (1.3 g/kg, i.p.) and placed in the stereotaxic apparatus (Kopf, Tujunga, CA, USA) with their body temperature maintained at 37 ± 1°C by a heating pad.

For the placement of a recording electrode, the scalp was retracted, and one burr hole was drilled above the parabrachial pigmented nucleus (PBP) of the posterior VTA (AP, 5.8–6.2 mm posterior from Bregma, L, 0.4–0.6 mm lateral from midline) according to the Atlas of Rat Brain (Paxinos and Watson, 2007). We selected this subregion as it contains the largest density of dopamine cells as compared to the more medial portions of the posterior VTA.

Extracellular single-unit activity of dopamine neurons located in the VTA (V, 7.0–8.0 mm from the cortical surface) was recorded with glass micropipettes filled with 2% Pontamine sky blue (PSB) dissolved in 0.5 M sodium acetate (impedance 2.5–5 MΩ). The population spontaneous activity of VTA dopamine cells was determined in 6–9 predetermined tracks separated by 200 μm each other. Putative VTA dopamine neurons were selected when all criteria for identification were fulfilled: firing rate <10 Hz and duration of action potential >2.5 ms as measured from start to end (Grace and Bunney, 1983). At the end of the experimental session, inhibition of spontaneous activity by dopamine receptor agonists and subsequent reversal by dopamine receptor antagonists was tested. Bursts were defined as the occurrence of two spikes at interspike interval <80 ms, and terminated when the interspike interval exceeded 160 ms (Grace and Bunney, 1984). The electrical activity of each neuron was recorded for 2–3 min. Single-unit activity was filtered (bandpass 0.1–10,000 Hz) and individual action potentials were isolated and amplified (Neurolog System, Digitimer, Hertfordshire, UK), displayed on a digital storage oscilloscope (TDS 3012, Tektronics, Marlow, UK) and digitally recorded. Experiments were sampled on-line and off-line with Spike2 software (Cambridge Electronic Design, Cambridge, UK) by a computer connected to CED 1401 interface (Cambridge Electronic Design, Cambridge, UK). At the end of recording sessions, DC current (15 mA for 5 min) was passed through the recording micropipette in order to eject PSB for marking the recording site. Brains were then rapidly removed and frozen in isopentane cooled to −40°C. The position of the electrodes was microscopically identified on serial 60 μm sections stained with Neutral Red.

In separate experiments where the effects of nicotine and cocaine were assessed, after 5 min of stable baseline activity, cocaine (Akzo Pharma Division Diosynth, Oss, Netherlands) was administered i.v. at exponentially increasing cumulative doses (0.25–2 mg/kg) every 2 min or nicotine [(-)-nicotine hydrogen tartrate), Sigma-Aldrich, Italy] at a bolus dose of 0.2 mg/kg.

Statistical Analysis

Averaged data from different experiments are given as mean ± SEM. Data were checked for outliers (ROUT test) and statistical significance was assessed using Student's *t*-test, one-way ANOVA, two-way ANOVA and two-way ANCOVA, where appropriate. *Post hoc* multiple comparisons were made using the Sidak's test. Data were analyzed using GraphPad Prism (San Diego, CA, USA). The significance level was established at $P < 0.05$.

RESULTS

In agreement with previous studies (Zuckerman et al., 2003), rat dams underwent a significant weight loss in the 24 h following Poly I:C systemic administration (−4.9 ± 2.8 g $n = 8$; vs. controls +7.5 ± 2.4 g $n = 7$; $P < 0.01$, Student's *t*-test; data not shown). This weight loss indicates that Poly I:C treatment induced a flu-like syndrome in treated rats (Kentner et al., 2019). However, Poly I:C treatment did not affect litter size (controls: 11.6 ± 1.8 pups, $n = 8$; Poly I:C: 12.4 ± 1.2 pups, $n = 7$, $P = 0.72$, Student's *t*-test). As our previous studies determined that detrimental effects induced by MIA were only evident in males (De Felice et al., 2019), we evaluated the effect of pubertal THC solely in male rats. Hence, prenatal Poly I:C or vehicle male offspring were randomly assigned to the adolescent THC or vehicle groups, taking care that no more than three animals from the same litter were assigned to the same experimental group or procedure (Kentner et al., 2019). Therefore, electrophysiological experiments were carried out in four experimental groups: vehicle-vehicle, vehicle-THC, Poly I:C-vehicle and Poly I:C-THC.

We next determined if Poly I:C prenatal and THC postnatal treatments affect spontaneous activity of dopamine cells, by carrying out a population sample in the VTA. For these experiments we utilized $n = 14$ vehicle-vehicle (from 6 litters), $n = 19$ Poly I:C-vehicle (from 8 litters), $n = 8$ vehicle-THC (from 4 litters) and $n = 10$ Poly I:C-THC (from 5 litters) male offspring.

The number of cells/track (**Figure 1A**), which is an index of population activity of dopamine neurons in the VTA, was significantly reduced by Poly I:C treatment in vehicle-treated but not in THC-treated male offspring [two-way ANOVA: effect of Poly I:C, $F_{(1,45)} = 9.49$, $P < 0.01$; effect of THC, $F_{(1,45)} = 14.78$, $P < 0.001$; interaction between treatments, $F_{(1,45)} = 0.66$, $P > 0.05$; *post hoc* Sidak's test: significant effect only between vehicle-vehicle and Poly I:C-vehicle rats ($t_{(45)} = 3.2$, $P < 0.05$, **Figure 1A**)]. The firing rate was reduced by Poly I:C treatment in both vehicle- and THC-treated rats (**Figure 1B**; two-way ANOVA: effect of Poly I:C, $F_{(1,454)} = 13.53$, $P < 0.01$; effect of THC, $F_{(1,454)} = 1.98$, $P > 0.05$; interaction between treatments, $F_{(1,454)} = 0.10$, $P > 0.05$). The percentage of spikes per burst was reduced by both Poly I:C and THC treatments (**Figure 1C**) [two-way ANOVA: effect of Poly I:C, $F_{(1,386)} = 26.28$, $P < 0.001$; effect of THC, $F_{(1,386)} = 9.92$, $P < 0.01$; interaction between treatments, $F_{(1,386)} = 4.67$, $P < 0.05$; *post hoc* Sidak's test: significant effect between vehicle-vehicle and all other groups: ($t_{(386)} = 5.5$, $t_{(386)} = 3.9$, $t_{(386)} = 6.1$ for vehicle-vehicle vs. Poly I:C-vehicle, vehicle-THC, Poly I:C-THC, respectively, $P < 0.001$ for all comparisons, **Figure 1C**)]. The number of spikes per burst (**Figure 1D**) was significantly reduced by Poly I:C treatment only in vehicle-treated rats [two-way ANOVA: effect of Poly I:C, $F_{(1,334)} = 20.85$, $P < 0.0001$; effect of THC, $F_{(1,334)} = 6.32$, $P < 0.05$; interaction between

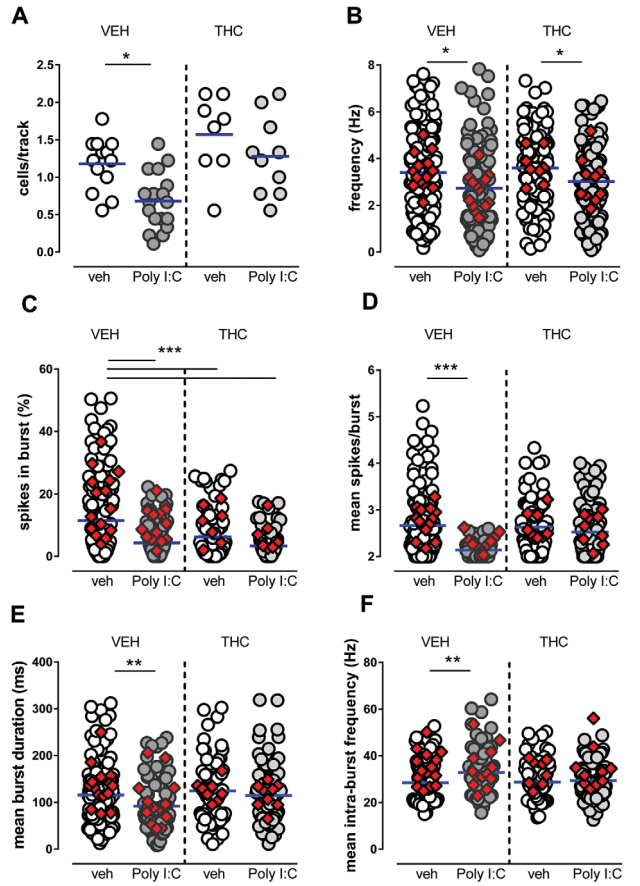

FIGURE 1 | Effects of maternal immune activation (MIA) and adolescent Δ^9-tetrahydrocannabinol (THC) administration on ventral tegmental area (VTA) dopamine neuron activity *in vivo*. Adolescent THC administration prevented the Poly I:C-induced decrease in the number of spontaneously active VTA dopamine neurons **(A)** but not the decrease in firing rate **(B)**. Graphs show the effect of poly IC and THC (or vehicles) in the percentage of spikes in burst **(C)**, mean burst duration **(D)**, mean number of spikes in bursts **(E)** and intra-burst frequency **(F)**. Superimposed colored diamonds show the averages for each individual rat. Both Poly I:C and THC, or their combination, induced a reduction in the percentage of spikes in bursts **(C)**, whereas THC prevented alterations induced by Poly I:C in the other electrophysiological parameters **(D,E)**. The number of cells for each group is: veh-veh, $n = 156$; Poly I:C-veh, $n = 121$; veh-THC, $n = 101$; Poly I:C-THC, $n = 117$. The horizontal blue line represents the mean. Statistical analysis was conducted with two-way ANOVA (Poly I:C and THC treatments as factors) and Sidak's multiple comparison test. Asterisks on graphs represent the result of the Sidak's multiple comparison test: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

treatments, $F_{(1,334)} = 9.30$, $P < 0.01$; *post hoc* Sidak's test: significant effect only between vehicle-vehicle and Poly I:C-vehicle rats ($t_{(334)} = 5.4$, $P < 0.0001$, **Figure 1D**)]. The mean burst duration (**Figure 1E**) was also significantly reduced by Poly I:C treatment in vehicle-treated but not in THC-treated rats [two-way ANOVA: effect of Poly I:C, $F_{(1,367)} = 3.58$, $P > 0.05$; effect of THC, $F_{(1,367)} = 3.42$, $P > 0.05$; interaction between treatments, $F_{(1,367)} = 5.62$, $P < 0.05$; *post hoc* Sidak's test: significant effect only between vehicle-vehicle and Poly I:C-vehicle rats ($t_{(367)} = 3.2$, $P < 0.01$, **Figure 1E**)]. Similarly, the mean intraburst frequency (**Figure 1F**) was significantly reduced by Poly I:C treatment in vehicle-treated but not in THC-treated offspring [two-way ANOVA: effect of Poly I:C, $F_{(1,338)} = 7.87$, $P < 0.01$; effect of THC, $F_{(1,338)} = 3.26$, $P > 0.05$; interaction between treatments, $F_{(1,338)} = 4.25$, $P < 0.05$; *post hoc* Sidak's test: significant effect only between vehicle-vehicle and Poly I:C-vehicle rats ($t_{(338)} = 3.6$, $P < 0.01$, **Figure 1F**)].

In summary, in Poly I:C-vehicle male offspring we detected a reduced number of spontaneously active cells, lower frequency, shorter bursts, a lower number of action potentials per burst, when compared with vehicle-vehicle offspring. Adolescent THC treatment in vehicle-THC rats did not exert significant effects, except for the percentage of spikes in burst, which was reduced when compared to vehicle-vehicle offspring, whereas in Poly I:C-THC offspring, THC reversed the effects of MIA on cells/track index, mean spikes/burst, mean burst duration and mean intra-burst frequency. Our data indicate that dopamine cells in prepubertal THC-treated offspring are less affected by MIA when compared with Poly I:C-vehicle rats.

Considering that we recorded several neurons from each individual rat and that each cell was considered as an independent replicate, a two-way ANCOVA was carried out with treatments as factors and individual subjects as covariate, to exclude that differences among individual rats had significant effects. The results indicated that individual subjects had no significant effect overall (two-way ANCOVA $P > 0.05$ for all parameters).

We next examined the response of VTA dopamine cells to a nicotine challenge and to cumulative doses of cocaine.

Figure 2A shows that pre- and postnatal treatments affect the response of VTA dopamine cells to nicotine (0.2 mg/kg, i.v.). The dose of nicotine was selected as it approximately corresponds to the i.p. dose of nicotine (0.4 mg/kg) that induces a robust conditioned place preference and, consistently, induces also a strong increase in firing rate of VTA dopamine cells in control animals (Melis et al., 2008; Mascia et al., 2011; Sagheddu et al., 2019). Spontaneous activity of VTA neurons was recorded for 5 min then a bolus dose of nicotine was injected intravenously. In vehicle-vehicle rats nicotine induced a robust increase in firing rate, amounting to ∼165% of baseline ($F_{(4,7)} = 6.7$, $P < 0.05$, one-way ANOVA), which remained stable across the recording time. On the other hand, nicotine did not significantly affect firing rate of VTA cells either in Poly I:C-vehicle, vehicle-THC nor in Poly I:C-THC rats ($F_{(4,5)} = 3.6$, $F_{(4,5)} = 2.8$, $F_{(4,5)} = 0.4$, respectively, $P > 0.05$, one-way ANOVA for all comparisons). When curves were compared across groups, two-way ANOVA revealed a significant interaction between factors (time and treatments; $F_{(12,88)} = 2.10$, $P < 0.05$) and *post hoc* analysis indicates that nicotine-induced effects were significantly different in Poly I:C-THC rats when compared to the vehicle-vehicle group ($t_{(110)} = 2.5$, $P < 0.05$, Sidak's multiple comparison test).

It is well established that cocaine inhibits dopamine neurons *via* increased somatodendritic dopamine release acting on D2 autoreceptors (Einhorn et al., 1988). As illustrated in **Figure 2B**, we confirmed that cocaine (0.25, 0.5, 1.0 and 2.0 mg/kg, i.v., expressed as the final cumulative doses at

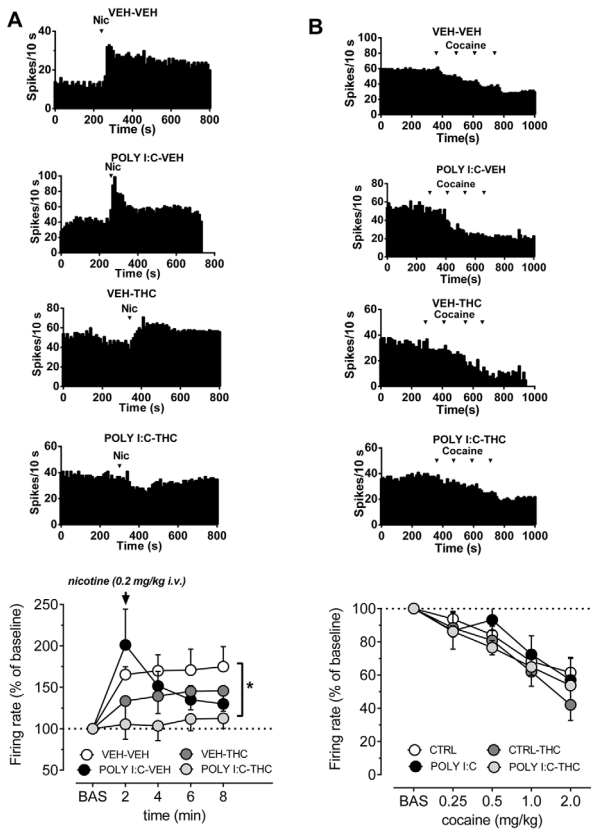

FIGURE 2 | Effects of nicotine and cocaine on firing rate of VTA dopamine neurons in prenatal Poly I:C and adolescent THC treated offspring and their controls. **(A)** Representative firing rate histograms of VTA dopamine neurons recorded from vehicle-vehicle, Poly I:C-vehicle, vehicle-THC and Poly I:C-THC rats showing the effects of a bolus dose of nicotine (0.2 mg/kg, i.v.). Arrows indicate the times of nicotine injection. The graph shows that the combination of prenatal Poly I:C and adolescent THC prevented nicotine-induced increase of firing rate (vehicle-vehicle, $n = 8$; Poly I:C-vehicle, $n = 6$; vehicle-THC, $n = 6$ and Poly I:C-THC, $n = 6$; two-way ANOVA and Sidak's test, $^*P < 0.05$). **(B)** Representative firing rate histograms of VTA dopamine neurons recorded from vehicle-vehicle, Poly I:C-vehicle, vehicle-THC and Poly I:C-THC rats showing the effects of cumulative doses of cocaine (0.25–2.0 mg/kg, i.v.). Arrows indicate the times of cocaine injections (0.25, 0.25, 0.5, 1.0 mg/kg). The bottom graph displays the dose–response curves of the effect of cumulative doses of cocaine on the firing rate of VTA DA neurons recorded from vehicle-vehicle ($n = 5$), Poly I:C-vehicle ($n = 6$), vehicle THC ($n = 4$) and Poly I:C-THC ($n = 4$). Results are presented as mean ± SEM of firing rate expressed as a percentage of baseline levels.

each point, as we injected 0.25, 0.25, 0.5 and 1 mg/kg, i.v.), dose-dependently reduced firing rate of dopamine cell to approximately 50% in vehicle-vehicle rats ($F_{(4,5)} = 17.29$, $P < 0.001$, one-way ANOVA). This inhibitory effect was similar to the control group and statistically significant also in Poly I:C-vehicle ($F_{(4,5)} = 4.5$, $P < 0.05$, one-way ANOVA), vehicle-THC ($F_{(4,3)} = 29.9$, $P < 0.01$, one-way ANOVA) and Poly I:C-THC rats ($F_{(4,3)} = 81.6$, $P < 0.01$, one-way ANOVA). The comparison across groups revealed that neither Poly I:C nor THC treatments, or their interaction with the doses of cocaine, changed the inhibitory effect of cumulative doses of cocaine onto VTA dopamine cells ($F_{(12,64)} = 0.63$, $P = 0.8$, two-way ANOVA).

DISCUSSION

The present findings confirm our previous studies that MIA, evoked by maternal exposure to Poly I:C, induces harmful effects in offspring, namely disruption of dopamine cell electrophysiological activity: (i) reduced number of spontaneously active cells; (ii) decrease in their firing rate; and (iii) profound alterations in their firing pattern (Luchicchi et al., 2016; De Felice et al., 2019). We and other groups showed that changes in dopamine transmission translate into abnormal behavior such as disrupted sensorimotor gating, deficits in cognition and social interactions (Zuckerman et al., 2003; Meyer et al., 2011; Luchicchi et al., 2016). The risk to develop schizophrenia has often been hypothesized with models requiring two hits in order to induce the clinical phenotype: an early priming in a genetically/prenatally predisposed individual and a second, likely environmental, insult (Davis et al., 2016). Consistent with this scenario, combining exposure to prenatal immune challenge and peripubertal stress in mice was shown to induce synergistic pathological effects on adult behavior and neurochemistry (Giovanoli et al., 2013, 2016).

Cannabis exposure during adolescence is consistently associated with an increased risk to develop schizophrenia later in life and with an earlier onset of the disease (Arseneault et al., 2004; Fergusson, 2004; Degenhardt and Hall, 2006). Preclinical findings consistently indicate that adolescent cannabinoid agonist intake induces long-term behavioral impairment and depressive-like signs (Rubino et al., 2009; Rubino and Parolaro, 2016). Therefore, it may represent a risk factor for developing psychotic-like symptoms in adulthood (Rubino et al., 2008).

Thus, our hypothesis was that exposure to THC during adolescence might exacerbate the disruption in VTA dopamine cell activity observed in offspring following MIA. Contrary to our expectations, adolescent THC did not induce effects in prenatal vehicle-treated animals, apart from a decrease in the bursting activity of dopamine cells, whereas in Poly I:C-treated offspring it attenuated several alterations induced by MIA. Notably, MIA with Poly I:C was shown to induce in rats persistent increases in cannabinoid CB1 receptor expression in adulthood in sensory cortex and hypothalamus assessed by PET (Verdurand et al., 2014). These findings indicate that prenatal Poly I:C leads to region-specific long-term alterations in the integrity of the endocannabinoid system that mirror those observed in patients with schizophrenia in post-mortem and *in vivo* PET studies (Köfalvi and Fritzsche, 2008). It is tempting to speculate that THC in adolescence might induce changes in CB1 receptor expression that, in our model, counteract those induced by MIA. As an example, in MIA-exposed male offspring we observed a decrease in the probability of glutamate and GABA release onto dopamine cells, indexed by an increase in the paired-pulse ratio of excitatory and inhibitory currents coupled with a reduced frequency of miniature inhibitory and excitatory postsynaptic currents (De Felice et al., 2019). As the release of GABA and

glutamate is tightly regulated by 2-arachidonoylglicerol (2-AG) acting on presynaptic CB1 receptors (Melis et al., 2004, 2014), we can speculate that this reduced neurotransmitter release might be caused by an increased expression or activity of CB1 receptors on GABA or glutamate terminals, consistent with the study by Verdurand et al. (2014). Alternatively, one possibility is that of an enhanced biosynthesis of 2-AG by DAG-lipase in dopamine cells or reduced degradation by MAG-lipase. How adolescent THC might reverse these changes is not known. One intriguing possibility is that adolescent subchronic THC might induce a long-lasting tolerance by reducing expression or activity of CB1 receptors, as shown in our previous studies (Pistis et al., 2004; Dudok et al., 2015).

To the best of our knowledge, this is the very first study carried out in a neurodevelopmental schizophrenia model with the phytocannabinoid THC and not with synthetic cannabinoids (Gomes et al., 2014; Aguilar et al., 2018). Interestingly, in line with our findings, the study by Gomes et al. (2014) reported that administration of the synthetic cannabinoid WIN55212 during adolescence did not exacerbate the behavioral and electrophysiological changes in methylazoxymethanol acetate (MAM)-treated rats but attenuated the enhanced locomotor response to amphetamine. On the other hand, in the study by Aguilar et al. (2018), pubertal exposure to WIN55212 or to the fatty acid amide hydrolase (FAAH) inhibitor URB597, which increases endogenous anandamide levels, augmented the proportion of second-generation MAM rats that develop schizophrenia-like deficits. In both studies, the synthetic cannabinoid treatment was able to increase the number of spontaneously active dopamine cells in vehicle-treated animals. Although we observed a trend toward an increase in the cell/track index in vehicle-THC offspring (**Figure 1A**), this effect did not reach statistical significance. These divergent results with our study might be due to different pharmacology of the cannabinoid agonists used (full vs. partial agonist), to the different length and protocol of adolescent cannabinoid treatment (11 vs. 25 days, continuous vs. intermittent), or to different neurodevelopmental models (MAM vs. MIA).

Epidemiological studies confirm that schizophrenia patients show enhanced prevalence of substance use disorders, particularly concerning nicotine dependence, psychostimulant and cannabis abuse (Kalman et al., 2005; Swendsen et al., 2010). In animal models of psychiatric disorders, responses to psychostimulant or nicotine is altered: locomotor response to psychostimulants is enhanced in neurodevelopmental models of schizophrenia (Gomes et al., 2014; Aguilar et al., 2018), whereas nicotine is more self-administered and ameliorated cognitive deficits in a lipopolysaccharide MIA model of schizophrenia (Waterhouse et al., 2018). Here we tested if prenatal and/or postnatal treatments affected responses of VTA dopamine neurons to nicotine and cocaine. We found a blunted effect of nicotine on VTA dopamine cells in all groups when compared to vehicle-vehicle animals, although this difference reached a statistical significance only in Poly I:C-THC offspring. These results suggest that adolescent THC and MIA, or the combination of both factors, induce persistent changes in neuronal response to nicotine. The reason for this effect requires further investigation. It can be speculated that a reduced response to nicotine in both THC- or MIA-exposed rats might be relevant for the high prevalence of heavy tobacco smoking reported in both cannabis abusers or schizophrenia patients (Kalman et al., 2005; Swendsen et al., 2010), as higher nicotine doses might be required to attain positive subjective effects. On the other hand, the inhibitory effect of cocaine did not change among the four experimental groups.

Our results, together with other previous studies, confirm that the effects of adolescent cannabinoid exposure in MIA-exposed individuals are more complex than expected and that the combination of prenatal and postnatal insults (the double hit hypothesis of schizophrenia) in neurodevelopmental models of schizophrenia needs to be further explored.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee of the University of Cagliari and by Italian Ministry of Health (auth. n. 658/2015-PR).

AUTHOR CONTRIBUTIONS

AL and SL contributed to the acquisition of animal data, performed data analysis, contributed to interpretation of results and provided critical revision of the manuscript. MS and PF assisted with acquisition of animal data, analysis and interpretation of findings. PF and AM provided critical revision of the manuscript for important intellectual content. MP was responsible for the study concept and design and drafted the manuscript. All authors critically reviewed the content and approved the final version for publication.

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REFERENCES

Aguilar, D. D., Giuffrida, A., and Lodge, D. J. (2018). Adolescent synthetic cannabinoid exposure produces enduring changes in dopamine neuron activity in a rodent model of schizophrenia susceptibility. *Int. J. Neuropsychopharmacol.* 21, 393–403. doi: 10.1093/ijnp/pyy003

Arseneault, L., Cannon, M., Witton, J., and Murray, R. M. (2004). Causal association between cannabis and psychosis: examination of the evidence. *Br. J. Psychiatry* 184, 110–117. doi: 10.1192/bjp.184.2.110

Boksa, P. (2010). Effects of prenatal infection on brain development and behavior: a review of findings from animal models. *Brain Behav. Immun.* 24, 881–897. doi: 10.1016/j.bbi.2010.03.005

Bossong, M. G., Mehta, M. A., van Berckel, B. N., Howes, O. D., Kahn, R. S., and Stokes, P. R. (2015). Further human evidence for striatal dopamine release induced by administration of Δ^9-tetrahydrocannabinol (THC): selectivity to limbic striatum. *Psychopharmacology* 232, 2723–2729. doi: 10.1007/s00213-015-3915-0

Bossong, M. G., van Berckel, B. N., Boellaard, R., Zuurman, L., Schuit, R. C., Windhorst, A. D., et al. (2009). Δ^9-tetrahydrocannabinol induces dopamine release in the human striatum. *Neuropsychopharmacology* 34, 759–766. doi: 10.1038/npp.2008.138

Crews, F., He, J., and Hodge, C. (2007). Adolescent cortical development: a critical period of vulnerability for addiction. *Pharmacol. Biochem. Behav.* 86, 189–199. doi: 10.1016/j.pbb.2006.12.001

Davis, J., Eyre, H., Jacka, F. N., Dodd, S., Dean, O., McEwen, S., et al. (2016). A review of vulnerability and risks for schizophrenia: beyond the two hit hypothesis. *Neurosci. Biobehav. Rev.* 65, 185–194. doi: 10.1016/j.neubiorev.2016.03.017

De Felice, M., Melis, M., Aroni, S., Muntoni, A. L., Fanni, S., Frau, R., et al. (2019). The PPARα agonist fenofibrate attenuates disruption of dopamine function in a maternal immune activation rat model of schizophrenia. *CNS Neurosci. Ther.* 25, 549–561. doi: 10.1111/cns.13087

Degenhardt, L., and Hall, W. (2006). Is cannabis use a contributory cause of psychosis? *Can. J. Psychiatry* 51, 556–565. doi: 10.1177/070674370605100903

Diana, M., Melis, M., and Gessa, G. L. (1998). Increase in meso-prefrontal dopaminergic activity after stimulation of CB1 receptors by cannabinoids. *Eur. J. Neurosci.* 10, 2825–2830. doi: 10.1046/j.1460-9568.1998.00292.x

Dudok, B., Barna, L., Ledri, M., Szabo, S. I., Szabadits, E., Pinter, B., et al. (2015). Cell-specific STORM super-resolution imaging reveals nanoscale organization of cannabinoid signaling. *Nat. Neurosci.* 18, 75–86. doi: 10.1038/nn.3892

Einhorn, L. C., Johansen, P. A., and White, F. J. (1988). Electrophysiological effects of cocaine in the mesoaccumbens dopamine system: studies in the ventral tegmental area. *J. Neurosci.* 8, 100–112. doi: 10.1523/jneurosci.08-01-00100.1988

Fergusson, D. M. (2004). Cannabis and psychosis: two kinds of limitations which attach to epidemiological research. *Addiction* 99, 512–513; author reply 515. doi: 10.1111/j.1360-0443.2004.00699.x

Gessa, G. L., Melis, M., Muntoni, A. L., and Diana, M. (1998). Cannabinoids activate mesolimbic dopamine neurons by an action on cannabinoid CB1 receptors. *Eur. J. Pharmacol.* 341, 39–44. doi: 10.1016/s0014-2999(97)01442-8

Giovanoli, S., Engler, H., Engler, A., Richetto, J., Feldon, J., Riva, M. A., et al. (2016). Preventive effects of minocycline in a neurodevelopmental two-hit model with relevance to schizophrenia. *Transl. Psychiatry* 6:e772. doi: 10.1038/tp.2016.38

Giovanoli, S., Engler, H., Engler, A., Richetto, J., Voget, M., Willi, R., et al. (2013). Stress in puberty unmasks latent neuropathological consequences of prenatal immune activation in mice. *Science* 339, 1095–1099. doi: 10.1126/science.1228261

Gomes, F. V., Guimaraes, F. S., and Grace, A. A. (2014). Effects of pubertal cannabinoid administration on attentional set-shifting and dopaminergic hyper-responsivity in a developmental disruption model of schizophrenia. *Int. J. Neuropsychopharmacol.* 18:pyu018. doi: 10.1093/ijnp/pyu018

Grace, A. A., and Bunney, B. S. (1983). Intracellular and extracellular electrophysiology of nigral dopaminergic neurons—1. Identification and characterization. *Neuroscience* 10, 301–315. doi: 10.1016/0306-4522(83)90135-5

Grace, A. A., and Bunney, B. S. (1984). The control of firing pattern in nigral dopamine neurons: burst firing. *J. Neurosci.* 4, 2877–2890. doi: 10.1523/jneurosci.04-11-02877.1984

Häfner, H. (2003). Gender differences in schizophrenia. *Psychoneuroendocrinology* 28, 17–54. doi: 10.1016/S0306-4530(02)00125-7

Hornig, M., Bresnahan, M. A., Che, X., Schultz, A. F., Ukaigwe, J. E., Eddy, M. L., et al. (2018). Prenatal fever and autism risk. *Mol. Psychiatry* 23, 759–766. doi: 10.1038/mp.2017.119

Hunt, G. E., Large, M. M., Cleary, M., Lai, H. M. X., and Saunders, J. B. (2018). Prevalence of comorbid substance use in schizophrenia spectrum disorders in community and clinical settings, 1990–2017: systematic review and meta-analysis. *Drug Alcohol Depend.* 191, 234–258. doi: 10.1016/j.drugalcdep.2018.07.011

Kalman, D., Morissette, S. B., and George, T. P. (2005). Co-morbidity of smoking in patients with psychiatric and substance use disorders. *Am. J. Addict.* 14, 106–123. doi: 10.1080/10550490590924728

Kentner, A. C., Bilbo, S. D., Brown, A. S., Hsiao, E. Y., McAllister, A. K., Meyer, U., et al. (2019). Maternal immune activation: reporting guidelines to improve the rigor, reproducibility and transparency of the model. *Neuropsychopharmacology* 44, 245–258. doi: 10.1038/s41386-018-0185-7

Köfalvi, A., and Fritzsche, M. (2008). "The endocannabinoid system is a major player in schizophrenia," in *Cannabinoids and the Brain*, ed. A. Köfalvi (Boston, MA: Springer US), 485–528.

Leweke, F. M., and Schneider, M. (2011). Chronic pubertal cannabinoid treatment as a behavioural model for aspects of schizophrenia: effects of the atypical antipsychotic quetiapine. *Int. J. Neuropsychopharmacol.* 14, 43–51. doi: 10.1017/s1461145710000842

Luchicchi, A., Lecca, S., Melis, M., De Felice, M., Cadeddu, F., Frau, R., et al. (2016). Maternal immune activation disrupts dopamine system in the offspring. *Int. J. Neuropsychopharmacol.* 19:pyw007. doi: 10.1093/ijnp/pyw007

Mascia, P., Pistis, M., Justinova, Z., Panlilio, L. V., Luchicchi, A., Lecca, S., et al. (2011). Blockade of nicotine reward and reinstatement by activation of alpha-type peroxisome proliferator-activated receptors. *Biol. Psychiatry* 69, 633–641. doi: 10.1016/j.biopsych.2010.07.009

Mason, O., Morgan, C. J., Dhiman, S. K., Patel, A., Parti, N., Patel, A., et al. (2009). Acute cannabis use causes increased psychotomimetic experiences in individuals prone to psychosis. *Psychol. Med.* 39, 951–956. doi: 10.1017/S0033291708004741

Melis, M., Pillolla, G., Luchicchi, A., Muntoni, A. L., Yasar, S., Goldberg, S. R., et al. (2008). Endogenous fatty acid ethanolamides suppress nicotine-induced activation of mesolimbic dopamine neurons through nuclear receptors. *J. Neurosci.* 28, 13985–13994. doi: 10.1523/jneurosci.3221-08.2008

Melis, M., Pillolla, G., Perra, S., Colombo, G., Muntoni, A. L., and Pistis, M. (2009). Electrophysiological properties of dopamine neurons in the ventral tegmental area of Sardinian alcohol-preferring rats. *Psychopharmacology* 201, 471–481. doi: 10.1007/s00213-008-1309-2

Melis, M., Pistis, M., Perra, S., Muntoni, A. L., Pillolla, G., and Gessa, G. L. (2004). Endocannabinoids mediate presynaptic inhibition of glutamatergic transmission in rat ventral tegmental area dopamine neurons through activation of CB1 receptors. *J. Neurosci.* 24, 53–62. doi: 10.1523/jneurosci.4503-03.2004

Melis, M., Sagheddu, C., De Felice, M., Casti, A., Madeddu, C., Spiga, S., et al. (2014). Enhanced endocannabinoid-mediated modulation of rostromedial tegmental nucleus drive onto dopamine neurons in Sardinian alcohol-preferring rats. *J. Neurosci.* 34, 12716–12724. doi: 10.1523/jneurosci.1844-14.2014

Meyer, U., Engler, A., Weber, L., Schedlowski, M., and Feldon, J. (2008). Preliminary evidence for a modulation of fetal dopaminergic development by maternal immune activation during pregnancy. *Neuroscience* 154, 701–709. doi: 10.1016/j.neuroscience.2008.04.031

Meyer, U., Feldon, J., and Dammann, O. (2011). Schizophrenia and autism: both shared and disorder-specific pathogenesis *via* perinatal inflammation? *Pediatr. Res.* 69, 26R–33R. doi: 10.1203/pdr.0b013e318212c196

Meyer, U., Feldon, J., Schedlowski, M., and Yee, B. K. (2005). Towards an immuno-precipitated neurodevelopmental animal model of schizophrenia. *Neurosci. Biobehav. Rev.* 29, 913–947. doi: 10.1016/j.neubiorev.2004.10.012

Patterson, P. H. (2002). Maternal infection: window on neuroimmune interactions in fetal brain development and mental illness. *Curr. Opin. Neurobiol.* 12, 115–118. doi: 10.1016/s0959-4388(02)00299-4

Patterson, P. H. (2009). Immune involvement in schizophrenia and autism: etiology, pathology and animal models. *Behav. Brain Res.* 204, 313–321. doi: 10.1016/j.bbr.2008.12.016

Paxinos, G., and Watson, C. (2007). *The Rat Brain in Stereotaxic Coordinates.* 7th Edn. London: Elsevier Academic Press.

Pistis, M., Perra, S., Pillolla, G., Melis, M., Muntoni, A. L., and Gessa, G. L. (2004). Adolescent exposure to cannabinoids induces long-lasting changes in the response to drugs of abuse of rat midbrain dopamine neurons. *Biol. Psychiatry* 56, 86–94. doi: 10.1016/j.biopsych.2004.05.006

Romero, E., Guaza, C., Castellano, B., and Borrell, J. (2010). Ontogeny of sensorimotor gating and immune impairment induced by prenatal immune challenge in rats: implications for the etiopathology of schizophrenia. *Mol. Psychiatry* 15, 372–383. doi: 10.1038/mp.2008.44

Rubino, T., and Parolaro, D. (2016). The impact of exposure to cannabinoids in adolescence: insights from animal models. *Biol. Psychiatry* 79, 578–585. doi: 10.1016/j.biopsych.2015.07.024

Rubino, T., Realini, N., Braida, D., Guidi, S., Capurro, V., Vigano, D., et al. (2009). Changes in hippocampal morphology and neuroplasticity induced by adolescent THC treatment are associated with cognitive impairment in adulthood. *Hippocampus* 19, 763–772. doi: 10.1002/hipo.20554

Rubino, T., Vigano, D., Realini, N., Guidali, C., Braida, D., Capurro, V., et al. (2008). Chronic Δ^9-tetrahydrocannabinol during adolescence provokes sex-dependent changes in the emotional profile in adult rats: behavioral and biochemical correlates. *Neuropsychopharmacology* 33, 2760–2771. doi: 10.1038/sj.npp.1301664

Sagheddu, C., Scherma, M., Congiu, M., Fadda, P., Carta, G., Banni, S., et al. (2019). Inhibition of N-acylethanolamine acid amidase reduces nicotine-induced dopamine activation and reward. *Neuropharmacology* 144, 327–336. doi: 10.1016/j.neuropharm.2018.11.013

Scherma, M., Dessi, C., Muntoni, A. L., Lecca, S., Satta, V., Luchicchi, A., et al. (2016). Adolescent Δ^9-tetrahydrocannabinol exposure alters WIN55,212–2 self-administration in adult rats. *Neuropsychopharmacology* 41, 1416–1426. doi: 10.1038/npp.2015.295

Swendsen, J., Conway, K. P., Degenhardt, L., Glantz, M., Jin, R., Merikangas, K. R., et al. (2010). Mental disorders as risk factors for substance use, abuse and dependence: results from the 10-year follow-up of the National Comorbidity Survey. *Addiction* 105, 1117–1128. doi: 10.1111/j.1360-0443.2010.02902.x

Tanda, G., Pontieri, F. E., and Di Chiara, G. (1997). Cannabinoid and heroin activation of mesolimbic dopamine transmission by a common mu1 opioid receptor mechanism. *Science* 276, 2048–2050. doi: 10.1126/science.276.5321.2048

Vassoler, F. M., Johnson, N. L., and Byrnes, E. M. (2013). Female adolescent exposure to cannabinoids causes transgenerational effects on morphine sensitization in female offspring in the absence of *in utero* exposure. *J. Psychopharmacol.* 27, 1015–1022. doi: 10.1177/0269881113503504

Verdurand, M., Dalton, V. S., Nguyen, V., Grégoire, M.-C., Zahra, D., Wyatt, N., et al. (2014). Prenatal poly I:C age-dependently alters cannabinoid type 1 receptors in offspring: a longitudinal small animal PET study using [18F]MK-9470. *Exp. Neurol.* 257, 162–169. doi: 10.1016/j.expneurol.2014.05.004

Vuillermot, S., Weber, L., Feldon, J., and Meyer, U. (2010). A longitudinal examination of the neurodevelopmental impact of prenatal immune activation in mice reveals primary defects in dopaminergic development relevant to schizophrenia. *J. Neurosci.* 30, 1270–1287. doi: 10.1523/JNEUROSCI.5408-09.2010

Waterhouse, U., Brennan, K. A., and Ellenbroek, B. A. (2018). Nicotine self-administration reverses cognitive deficits in a rat model for schizophrenia. *Addict. Biol.* 23, 620–630. doi: 10.1111/adb.12517

Winter, C., Djodari-Irani, A., Sohr, R., Morgenstern, R., Feldon, J., Juckel, G., et al. (2009). Prenatal immune activation leads to multiple changes in basal neurotransmitter levels in the adult brain: implications for brain disorders of neurodevelopmental origin such as schizophrenia. *Int. J. Neuropsychopharmacol.* 12, 513–524. doi: 10.1017/s1461145708009206

Winterer, G. (2010). Why do patients with schizophrenia smoke? *Curr. Opin. Psychiatry* 23, 112–119. doi: 10.1097/yco.0b013e3283366643

Wolff, A. R., and Bilkey, D. K. (2010). The maternal immune activation (MIA) model of schizophrenia produces pre-pulse inhibition (PPI) deficits in both juvenile and adult rats but these effects are not associated with maternal weight loss. *Behav. Brain Res.* 213, 323–327. doi: 10.1016/j.bbr.2010.05.008

Zuckerman, L., Rehavi, M., Nachman, R., and Weiner, I. (2003). Immune activation during pregnancy in rats leads to a postpubertal emergence of disrupted latent inhibition, dopaminergic hyperfunction and altered limbic morphology in the offspring: a novel neurodevelopmental model of schizophrenia. *Neuropsychopharmacology* 28, 1778–1789. doi: 10.1038/sj.npp.1300248

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Folic Acid and Risk of Preterm Birth: A Meta-Analysis

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The results from epidemiologic studies linking blood folate concentrations, folic acid supplementation, or dietary folate to the risk of preterm birth are inconsistent. In this study, we aimed to summarize the available evidence on these associations. A systematic search of the PubMed/MEDLINE, Google Scholar, Web of Science, and Cochrane Library databases up to October 20, 2018 was performed and reference lists of retrieved articles were screened. Pooled odds ratios (ORs) and 95% confidence intervals (CIs) for the highest vs. the lowest levels of folate concentrations, folic acid supplementation, and dietary folate were calculated using random-effects models. Subgroup analyses and univariate meta-regression were performed to explore the sources of heterogeneity. Ten studies (six prospective cohort studies and four case-control studies) were included on folate concentrations, 13 cohort studies were included about folic acid supplementation, and 4 cohort studies were included regarding dietary folate intake. Higher maternal folate levels were associated with a 28% reduction in the risk of preterm birth (OR 0.72, 95% CI 0.56–0.93). Higher folic acid supplementation was associated with 10% lower risk of preterm birth (OR 0.90, 95% CI 0.85–0.95). In addition, a significant negative association was observed between dietary folate intake and the risk of preterm birth (OR 0.68, 95% CI 0.55–0.84), but no significant relation was seen between dietary folate and the risk of spontaneous preterm birth (OR 0.89, 95% CI 0.57–1.41). In the subgroup analysis, higher maternal folate levels in the third trimester were associated with a lower risk of preterm birth (OR 0.58, 95% CI 0.36–0.94). To initiate taking folic acid supplementation early before conception was adversely associated with preterm birth risk (OR 0.89, 95% CI 0.83–0.95). In conclusion, higher maternal folate levels and folic acid supplementation were significantly associated with a lower risk of preterm birth. The limited data currently available suggest that dietary folate is associated with a significantly decreased risk of preterm birth.

Keywords: folate levels, folic acid supplementation, dietary folate intake, meta-analysis, preterm birth, preterm brain injury, sequelae of preterm birth

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INTRODUCTION

Preterm birth (PTB) and its associated complications, which include brain injury, retinopathy of prematurity, cerebral palsy, and developmental disabilities, are among the most serious global health issues. These complications directly affect the child's quality of life and are a huge burden both socially and economically (Goldenberg et al., 2008; Song et al., 2016; WHO, 2018). Thus, prevention of PTB is a global priority (Blencowe et al., 2013). Recent estimates of the incidence of PTB in most Europe countries range from 3.7 to 7.5% of live births (Poulsen et al., 2015). In the United States, the incidence is higher at about 9.62% (The Lancet, 2016). In Australia, the incidence was at 8.7% according to the latest annual report in 2015 (Hoh et al., 2019). In China, the incidence was at ∼7% in 2016 (Chen et al., 2018). However, despite ongoing research, there has been no significant reduction in PTB rates. This might be a result of an inadequate understanding of the pathological processes contributing to PTB. PTB is considered a multifactorial syndrome, with almost 70% of PTBs resulting from spontaneous labor and/or rupture of membranes and the remainder from iatrogenic causes. Hence, it can be broadly categorized into spontaneous PTB (sPTB) and indicated PTB (Goldenberg et al., 2008). It has been recognized that there are numerous biological mechanisms that vary between individuals and that might lead to PTB (Frey and Klebanoff, 2016). Therefore, the identification of modifiable risk factors is of great importance for PTB management and prevention.

Maternal nutrition is an important determinant of the duration of pregnancy and fetal growth, and thereby influences pregnancy outcomes. Experimental data from animal studies suggest that maternal nutritional status such as folate status might play a role in PTB (Zhao et al., 2013; Scholl and Chen, 2015). Folate is an essential B vitamin that plays a role in DNA synthesis and cell division to support growth and fetal development (Lucock, 2000). During pregnancy, there is an increased demand for folate due to the rapid fetal growth. A previous study reported that pregnant women had a 5- to 10-fold higher folate requirement than non-pregnant women (Antony, 2007). Blood folate levels, including serum/plasma or red blood cell (RBC) folate, are considered reliable indicators of folate status (World Health Organization, 2015). Some recent epidemiological studies have indicated that low blood folate levels during pregnancy are associated with an increased risk of PTB (Bergen et al., 2012; Chen et al., 2014), while some other studies have shown no association between blood folate levels and PTB (Dunlop et al., 2012; Heeraman, 2016). In addition, a meta-analysis has not yet been conducted to summarize the epidemiological evidence on this association.

Folate cannot be synthesized by the body, and humans are entirely dependent on dietary sources or dietary supplements for their folate supply. There have been a large number of studies describing the association between folate intake and preterm birth (Vahratian et al., 2004; Lassi et al., 2013; Li et al., 2014; Mantovani et al., 2014; Martinussen et al., 2015; Zheng et al., 2016); however, their results are conflicting. Two meta-analyses from 2015 assessed the possible association between folic acid supplementation and the risk of PTB. One incorporated data from five randomized trials and reported no statistically significant effects (Saccone and Berghella, 2016), while the other identified nine observational studies and showed a decreased risk of PTB when initiating folic acid supplementation after conception (Zhang et al., 2017). Additional larger cohort studies have been published since then that might enhance the statistical power (Liu et al., 2016; Zheng et al., 2016), and thus an updated meta-analysis is needed.

In this study, we aimed to evaluate the available evidence on the associations between blood folate levels, dietary folate intake, and folic acid supplementation and the risk of PTB.

MATERIALS AND METHODS

Literature Search

We conducted a literature search of PubMed (Medline), Google Scholar, Web of Science, and the Cochrane Library from their inception through October 2018. The search terms included "folic acid," "vitamin B9," "folate," "folate status," "folate levels," "folate concentrations," "serum folate," "red blood cell folate," "folic acid consumption," "folic acid supplementation," "folic acid intake," "food folate," or "dietary folate" combined with "preterm delivery," "premature birth," or "preterm birth." We adhered to the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) guidelines when undertaking this study (Stroup et al., 2000).

Inclusion Criteria

Articles were included if (1) the study design was observational, (2) the population was healthy women who had the intention to become pregnant or who were pregnant, (3) the exposure of interest was folate levels or dietary folate intake or folic acid supplementation, (4) the outcome of interest was preterm birth, which was defined as delivery at <37 weeks gestation, (5) the association between folate levels or dietary folate intake or folic acid supplementation and risk of PTB was evaluated, and (6) adjusted risk estimates [relative risks (RRs), hazard risks (HRs), or odds ratios (ORs)] with their corresponding 95% confidence intervals (CIs) or standard errors were reported. Additionally, we excluded reviews, editorials, non-human studies, randomized clinical trials, and letters without sufficient data. We excluded randomized clinical trials without folic acid in a control group because of limited publications and ethical issues. When multiple reports based on the same study were published, only the most recent or complete report was used.

Data Extraction

We extracted the following data from the included articles: the name of the first author, year of publication, country, study design, sample size, follow-up period, study period, assessment methods of folate levels, or dietary folate intake or folic acid supplementation, ascertainment of PTB, ORs and corresponding 95% CIs, and the confounding factors used for adjustment of the ORs. The ORs with more adjusted confounders were chosen when studies had different models for the calculation of estimated risks.

Quality Assessment

Two reviewers independently performed the quality assessment using the Newcastle-Ottawa Scale (for cohort and case-control studies; Stang, 2010), which is a nine-point system including the selection process of studies (0–4 points), the comparability of studies (0–2 points), and the identification of the exposure and the outcomes of the study participants (0–3 points). The quality of articles was first evaluated according to the established questions, which were scored as 1 if the item was considered in the study or 0 if the item was not considered or if it was impossible to determine whether it was considered or not. We assigned scores of 0–3, 4–6, and 7–9 points for low, moderate, and high-quality studies, respectively (**Supplementary Table 1**).

Statistical Analysis

Because most of included studies reported risk estimates as ORs, and because it was previously reported that ORs, HRs, and RRs provide similar estimates of risk when the incidence of outcome is very low (<10%) (Greenland, 1987), we chose ORs as the common effect size and combined HRs and RRs with ORs in the meta-analysis. The statistical analyses for the overall association between folate levels, folic acid supplementation/dietary folate intake, and PTB risk were based on comparisons of the highest category with the lowest. If the original studies did not provide corresponding data, the OR and its 95% CI were recalculated.

The ORs and corresponding 95% CIs were pooled using the DerSimonian and Laird random-effects model (DerSimonian and Laird, 1986), which considers both within-study and between-study variations. The summary measures were presented as forest plots where the sizes of the data markers (squares) correspond to the inverse of the variance of the natural logarithm of the OR from each study and the diamond indicates the pooled OR. Statistical heterogeneity among studies was quantified using the I^2 statistic (Higgins et al., 2003).

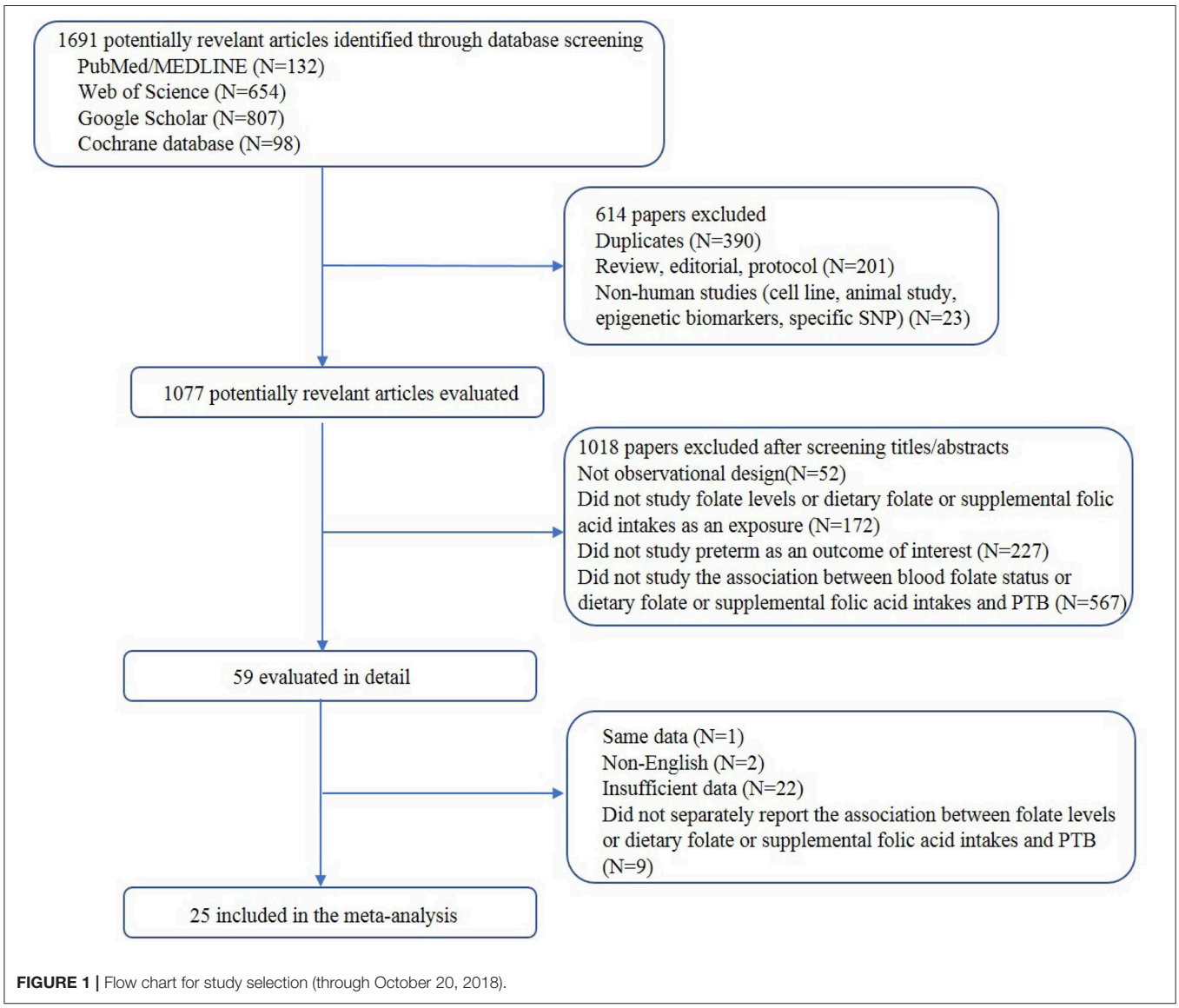


FIGURE 1 | Flow chart for study selection (through October 20, 2018).

To further evaluate the effects of heterogeneity, univariate meta-regression analyses were performed examining the effects of several key study characteristics. Stratified analyses by specimen gestational age, sample type, initiation time of folic acid supplementation, and dosage of folic acid were conducted to assess their impact on our estimates. Sensitivity analyses were employed to find potential origins of heterogeneity and to examine the influence of various exclusions on the combined OR. Funnel plots were used to assess small-study effects. Publication bias was assessed through the visual inspection of funnel plots and with tests of Begg rank correlation (Begg and Mazumdar, 1994). $P < 0.05$ was considered to be representative of a statistically significant publication bias. Forest plots were created to assess the overall association between folate levels, dietary folate, folic acid supplementation, and PTB.

All statistical analyses were performed with STATA version 12.0 software (Stata Corporation, College Station, TX, US). All reported probabilities (P-values) were two-sided, with $P < 0.05$ considered statistically significant, except for the Cochran's Q statistic in the heterogeneity test, in which the significance level was 0.10.

RESULTS

Characteristics of the Studies

A total of 25 articles were included. The process of study selection is depicted in **Figure 1**. A total of 9 studies assessed the association between blood folate levels and the risk of PTB, 12 examined folic acid supplementation and the risk of PTB, and 2 studies assessed the association between dietary folate intake and the risk of PTB. Moreover, 1 study examined the association of folic acid supplementation and dietary folate and the risk of PTB, and 1 study assessed the association of blood and dietary folate with the risk of PTB.

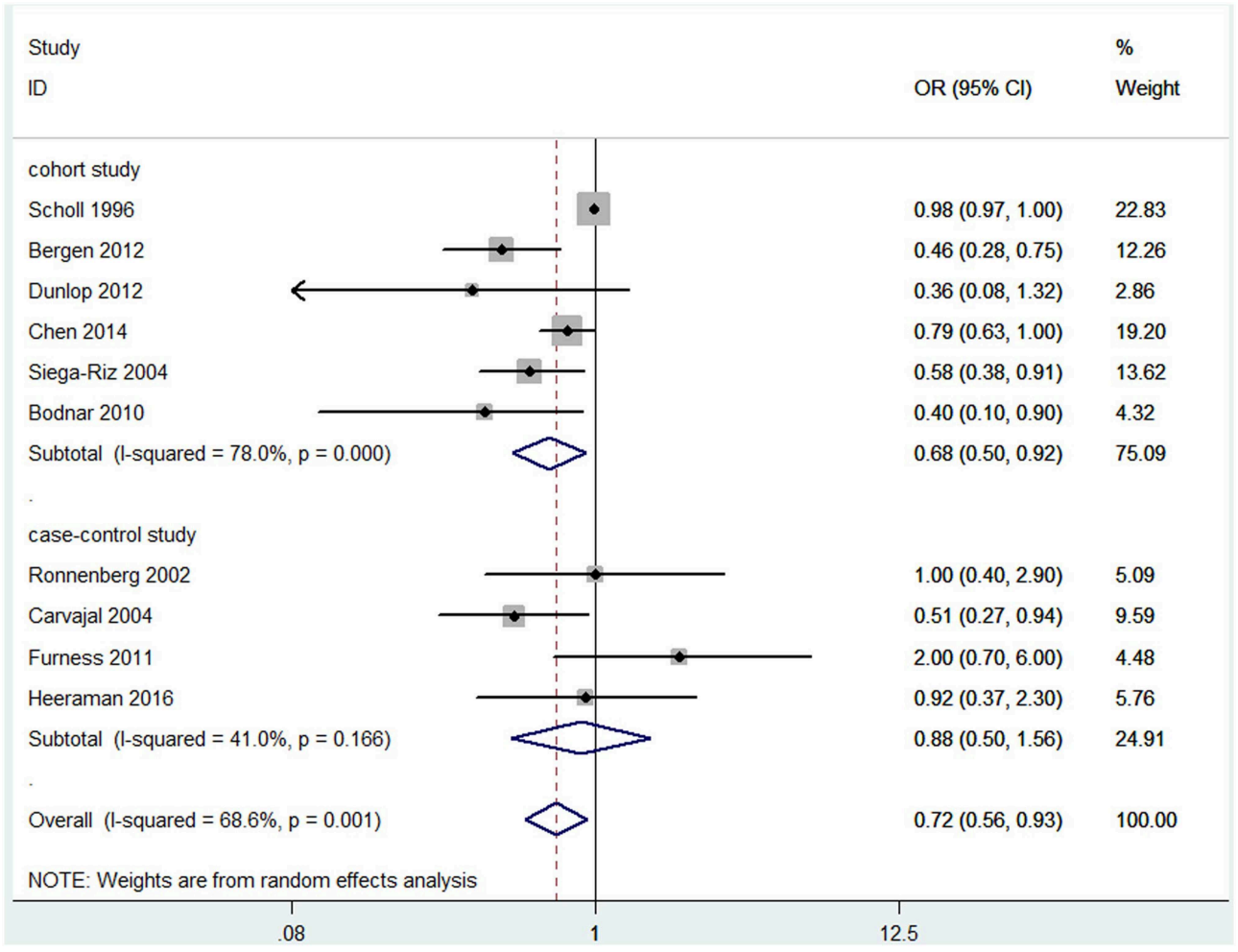


FIGURE 2 | Forest plot of the meta-analysis of PTB risk in relation to blood folate levels, comparing the highest category with the lowest. The solid diamonds and horizontal lines indicate the study-specific ORs and 95% CIs. The size of the gray area reflects the study-specific statistical weight. The hollow diamonds represent the pooled ORs and 95% CIs of each subgroup and the overall population. The vertical solid line shows the OR of 1, and the vertical red dashed line represents the combined effect estimate.

Blood Folate Levels and Risk of PTB

For the overall risk of PTB in relation to blood folate levels, we included 6 prospective cohort studies (Scholl et al., 1996; Siega-Riz et al., 2004; Bodnar et al., 2010; Bergen et al., 2012; Dunlop et al., 2012; Chen et al., 2014) and 4 case-control studies (Ronnenberg et al., 2002; Martí-Carvajal et al., 2004; Furness et al., 2012; Heeraman, 2016). A total of 7 studies measured folate levels in plasma/serum, and 3 studies measured folate levels in RBCs. Among these studies, 3 were sampled in the first trimester, 3 in the second trimester, and 3 in the third trimester. The main characteristics of these studies are summarized in **Supplementary Tables 2, 3**.

Overall, a significant negative association between blood folate levels and PTB risk was observed. The pooled OR (95% CI) for PTB risk in individuals with the highest level of blood folate compared with the lowest level was 0.72 (95% CI, 0.56–0.93) with a moderate to high degree of statistical heterogeneity (I^2 = 68.6%; **Figure 2**). For the 6 cohort studies, the negative association was consistent and the combined OR was 0.68 (95% CI, 0.50–0.92) with a high heterogeneity (I^2 = 78%). For the 4 case-control studies, no significant association was found between blood folate levels and the risk of PTB (OR 0.88, 95% CI 0.50–1.56). Heterogeneity was observed between studies (I^2 = 41%). To examine this heterogeneity, we conducted meta-regression analyses with type of design, gestational age of the specimen, geographical region, year of publication, and sample type as the independent variables. As shown in **Table 1**, no significant differences were found among these groups. No evidence of publication bias was observed when assessing the association between maternal folate levels and PTB (Begg's test, P = 0.592). A sensitivity analysis by omitting one study at a time did not dramatically influence the pooled ORs, suggesting that the combined OR was valid and credible.

TABLE 1 | Blood folate levels and the risk of preterm birth analyzed by univariate meta-regression model.

Covariate	Number of studies	β-coefficient	*P*-value
Type of design			
Cohort study, Case-control study	10	0.266	0.429
Specimen gestational age			
First trimester vs. Second trimester vs. Third trimester vs. preconception	10	−0.021	0.927
Geographical region			
Asia vs. US vs. Europe vs. Australia	10	0.084	0.597
Sample size			
≥1,000 vs. <1,000	10	0.444	0.083
Year of publication			
≥2010 vs. <2010	10	0.089	0.756
Type of control			
Hospital vs. Population	10	0.418	0.077
Sample type			
Serum/Plasma vs. Red blood cells	10	0.216	0.534

The β-coefficient represents the change in log OR per unit increase in the relevant variable.

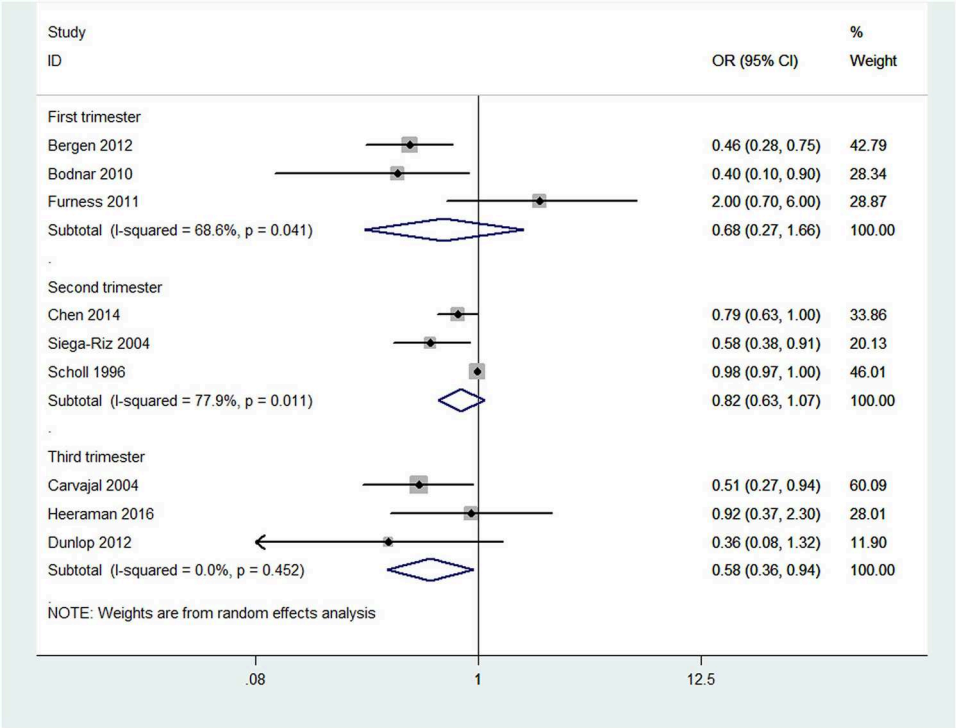
Subgroup analysis of specimen gestational age suggested no association between blood folate levels in the first (OR = 0.68, 95% CI 0.27–1.66, P = 0.393) and second trimester (OR = 0.82, 95% CI 0.63–1.07, P = 0.139) and the risk of PTB. However, the blood folate level in the third trimester was significantly inversely associated with the risk of PTB (OR = 0.58, 95% CI 0.36–0.94, P = 0.026; **Figure 3A**).

Subgroup analysis showed that higher plasma/serum folate was associated with 30% lower risk of PTB (OR = 0.70, 95% CI 0.52–0.93, P = 0.014), while RBC folate was not associated with the risk of PTB (OR = 0.89, 95% CI 0.45–1.79, P = 0.75; **Figure 3B**).

Folic Acid Supplementation and Risk of PTB

Of the 13 cohort studies that assessed folic acid supplementation and the overall risk of PTB (Scholl et al., 1997; Vahratian et al., 2004; Catov et al., 2007, 2011; Timmermans et al., 2009; Alwan et al., 2010; Czeizel et al., 2010; Papadopoulou et al., 2013; Li et al., 2014; Martinussen et al., 2015; Liu et al., 2016; Zheng et al., 2016; Baron et al., 2017), 6 studies reported two separate outcomes stratified by initiation time of supplementation (preconception and postconception). In this case, each of studies could be considered as two independent reports. Thus, there were 19 independent reports included in this meta-analysis. Among these studies, 4 used folic acid alone and the rest used folic acid containing multivitamins. A total of 9 stated the exact content of folic acid supplements, and 7 out of the 9 studies used folic acid at 400 μg daily (as suggested by WHO), and 2 used high doses of folic acid of more than 1,000 μg daily. Overall, the lowest category (reference category) observed in the included studies ranged from 0 to 200 μg daily, and the highest category ranged from any folic acid/folic acid-containing supplements consumption to ≥1,000 μg daily. Because we used the categories reported by the studies, these categories were not mutually exclusive. None of the studies described the mutual effects associated with multivitamins. The main characteristics of these studies are summarized in **Supplementary Table 4**.

The results combining the ORs comparing the highest and lowest category of folic acid supplementation for the risk of PTB are shown in **Figure 4**. An inverse association was found (OR = 0.90, 95% CI 0.86–0.95), and low to moderate heterogeneity across the studies was found (I^2 = 30.1%). To examine this heterogeneity, we conducted meta-regression analyses with initiation time of supplementation, geographical region, source of cohorts, and ascertainment of PTB, sample size, year of publication and dosage of folic acid intake. As shown in **Table 2**, no significant differences were observed among these subgroups. Visual inspection of the funnel plot showed little asymmetry for studies on folic acid supplementation and PTB risk (**Figure 5**). No evidence of publication bias was found across the included studies (Begg's test, P = 0.284), and sensitivity analyses using



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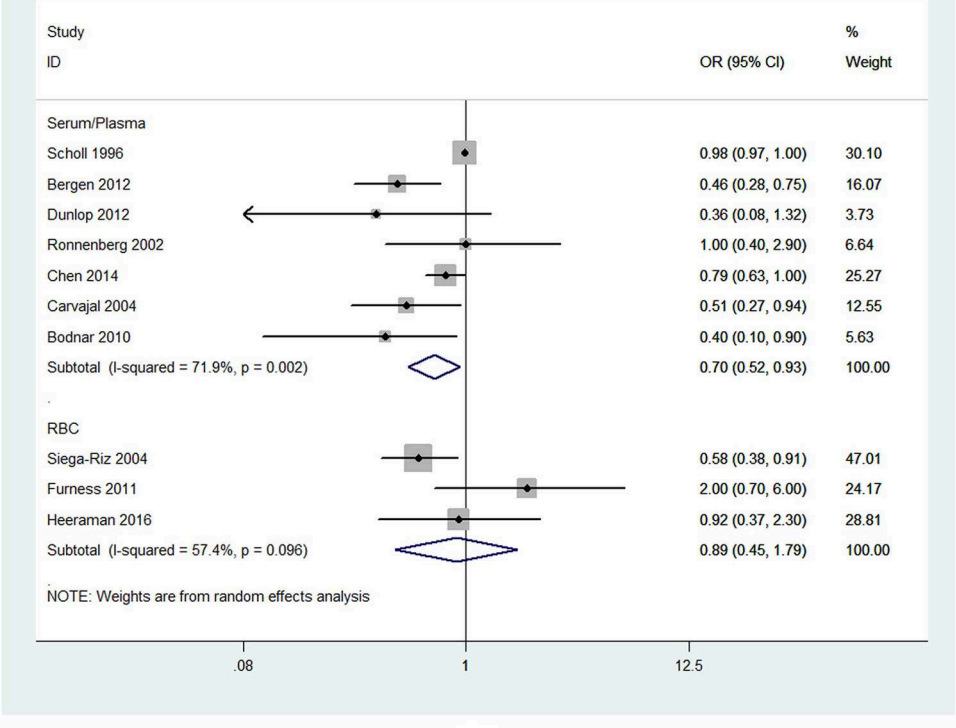

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FIGURE 3 | Forest plot of the meta-analysis of PTB risk in relation to blood folate stratified by gestational age of the specimen **(A)** and sample type **(B)**, comparing the highest category with the lowest. The diamonds and horizontal lines indicate the subgroup-specific ORs and 95% CIs. The size of the gray area reflects the study-specific statistical weight. The vertical solid line shows the OR of 1.

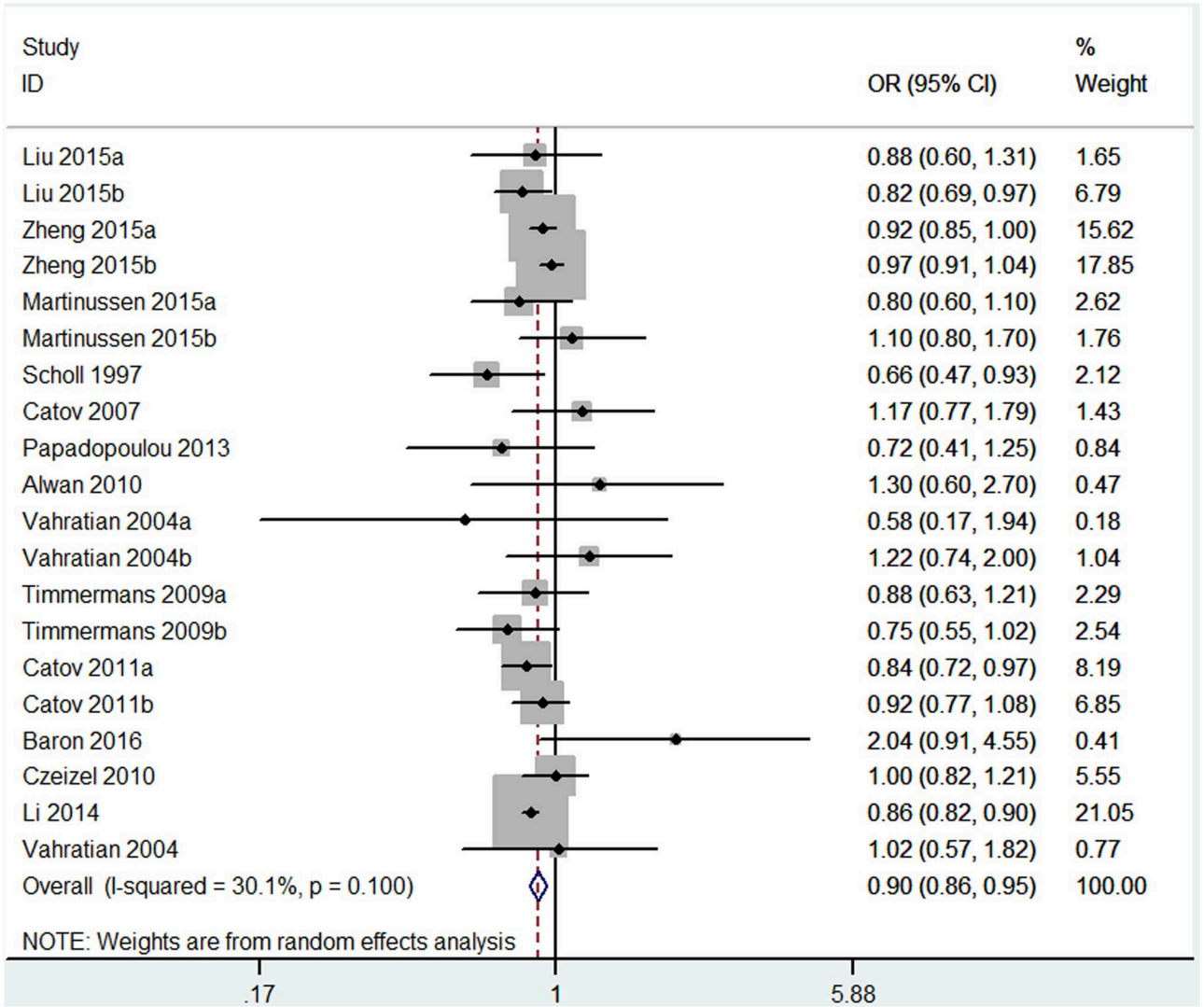


FIGURE 4 | Forest plot of the meta-analysis of PTB risk in relation to folic acid supplementation, comparing the highest category with the lowest. The diamonds and horizontal lines indicate the corresponding ORs and 95% CIs. The size of the gray area reflects the study-specific statistical weight. The vertical solid line shows the OR of 1, and the vertical red dashed line represents the combined effect estimate. The suffix "a" or "b" after the studies indicates two separate outcomes stratified by the initiation time of supplementation (preconception and post-conception) in the same study.

a fixed-effect model or omitting one study at a time did not substantially alter the pooled results.

Subgroup analysis of initiation time of folic acid supplementation showed that initiating folic acid supplements before conception was associated with a significant decreased risk of PTB (OR = 0.87, 95% CI: 0.84–0.91, $P < 0.001$), while starting folic acid supplementation at post-conception was associated with a marginal decreased risk of PTB (OR = 0.90, 95% CI: 0.80–1.00, $P = 0.049$; **Figure 6A**).

In the analysis stratified by dose of folic acid intake, a statistically significant protective effect was noted between folic acid supplementation at a daily dosage of <1,000 μg and PTB risk (OR = 0.90, 95% CI: 0.85–0.95, $P < 0.001$). However, taking folic acid supplementation at a daily dosage of more than 1,000 μg was not significantly associated with the risk of PTB (OR = 0.95, 95% CI: 0.74–1.20, $P = 0.65$; **Figure 6B**).

Dietary Folate Intake and Risk of PTB

Of the 4 cohort studies that assessed dietary folate intake and risk of PTB (Siega-Riz et al., 2004; Shaw et al., 2011; Sengpiel et al., 2014; Liu et al., 2016), 1 study reported two separate outcomes stratified by the initiation time of supplementation (preconception and post-conception). Thus, there were 5 independent reports, including 95,448 participants, in our meta-analysis. Out of these 5 studies, 2 studies reported the outcome of sPTB in addition to overall PTB, 2 studies just reported sPTB, and 1 study just reported overall PTB. In total, 3 studies reported the overall risk of PTB in relation to dietary folate intake, and 4

TABLE 2 | Folic acid supplementation and the risk of preterm birth analyzed by univariate meta-regression model.

Covariate	Number of studies	β-coefficient	*P*-value
Time of FA intake			
Preconception vs. Postconception vs. Periconception	19	0.014	0.829
Geographical region			
Asia vs. US vs. Europe	18*	−0.0008	0.98
Sample size			
≥1,000 vs. <1,000	19	−0.012	0.877
Year of publication			
≥2010 vs. <2010	19	−0.054	0.565
Source of Cohort			
Hospital vs. Population	19	0.012	0.875
Definition of GA			
LMP vs. Ultrasound vs. Both	19	−0.009	0.977
Dose of intake			
Moderate vs. High	19	0.067	0.571

**One study did not provide data for geographical region. The β-coefficient represents the change in log OR per unit increase in the relevant variable. FA, folic acid; LMP, the last normal menstrual period; GA, gestational age.*

studies reported sPTB. The main characteristics of these studies are summarized in **Supplementary Tables 5, 6**.

For the 3 studies about overall PTB, there was a significant inverse association observed between dietary folate intake and the overall risk of PTB, and the pooled OR and 95% CI for PTB when comparing the highest with the lowest levels of dietary folate intake was 0.68 (95% CI 0.55–0.84) with a moderate heterogeneity ($I^2 = 67.8\%$; **Figure 7A**). For the 4 studies about sPTB, a non-significant association was observed. The summary OR and 95% CI was 0.89 (95% CI 0.57–1.41) with a high heterogeneity ($I^2 = 88.1\%$; **Figure 7B**). We did not perform subgroup analysis or sensitivity analysis due to the small number of studies.

DISCUSSION

Principal Findings of This Study

The current meta-analysis is the first time to assess the association of PTB risk with blood folate levels and dietary folate intake. We found that blood folate levels, folic acid supplementation, and dietary folate intake were negatively associated with the overall risk of PTB. Furthermore, we found that dietary folate intake was not significantly associated with the risk of sPTB.

Compared to the previous meta-analysis on folic acid supplementation and risk of PTB, which included 9 studies, this updated meta-analysis included 13 studies and increased the sample size from 306,695 to 562,068 participants, which increased the statistical power of our analysis. Moreover, the included studies used modern methods of multivariate adjustment rather than raw data.

Evidence from biological studies supports a role of folate in PTB. First, folate contributes to oocyte maturation and early placentation (Jongbloet et al., 2008; Koukoura et al., 2012). Folate deficiency may lead to poor placentation and influence the development of and maintenance of uteroplacental circulation (Baker et al., 2017), which subsequently triggers poor pregnancy outcomes including PTB (Engel et al., 2006; Bailey, 2009). It has been observed that folate transporters, which transfer folate from maternal circulation to the fetus, are present at lower concentrations in preterm placentas compared to term placentas (Castaño et al., 2017). Second, folate is also a cofactor in the metabolism of homocysteine, which might be a contributing factor for placental vascular disease (Van der Molen et al., 2000), and epidemiological studies have shown that elevated homocysteine concentrations are associated with PTB (Bergen et al., 2012; Chen et al., 2014). Third, folate status during pregnancy might play an anti-inflammatory role. Many cases of PTB are associated with an abnormal inflammatory response, which is often caused by intrauterine infection and inflammation (Goldenberg et al., 2000). In a mouse model of lipopolysaccharide-induced PTB, folate reduced the levels of circulating biomarkers of inflammation, including interleukin (IL)-6 and keratinocyte-derived cytokine in the amniotic fluid of mice (Zhao et al., 2013).

Blood Folate Levels and Risk of PTB

We found an inverse association between blood folate levels in the third trimester and the risk of PTB, while no significant association was observed in the first and second trimester. This might be explained by the following mechanisms. Placental dysfunction is one of the risk factors for PTB (Romero et al., 2014), and it was observed that maternal blood folate levels decreased from the fifth month of pregnancy and plasma homocysteine concentrations increased in later pregnancy (Wang et al., 2016). Thus, we hypothesized that inadequate third trimester maternal folate levels impact fetal development by adversely affecting placental function during the period of maximal fetal development. Previous studies have reported that the persistence of placental dysfunction from the 24th week of pregnancy is associated with increased risks of adverse pregnancy outcomes (López-Quesada et al., 2004; Gaillard et al., 2013). On the other hand, folate might also be indirectly involved in placental development through its role in the homocysteine cycle. Folate deficiency may disrupt the function of the enzymes in homocysteine metabolism and lead to an increase in homocysteine levels. It was reported that elevated homocysteine concentration is also associated with oxidative stress, arteriolar constriction, endothelial damage, and placental thrombosis, all of which increase the risk of pregnancy complications (Maged et al., 2017). Moreover, folate is an important methyl-group vitamin, and maternal plasma folate levels are associated with offspring DNA methylation, which is in turn related to fetal development. It was reported that maternal folate levels in late pregnancy are more important than folate levels in early pregnancy for overall fetal growth (Sulaiman et al., 2017). Thus, an inverse association was evident between maternal folate levels in the third trimester and the risk of PTB, and maternal folate levels in the third trimester might be an indirect predictor of PTB.

In general, the RBC folate concentration is generally considered to reflect folate status during the preceding 3–4 months, and plasma or serum folate is a short-term measure

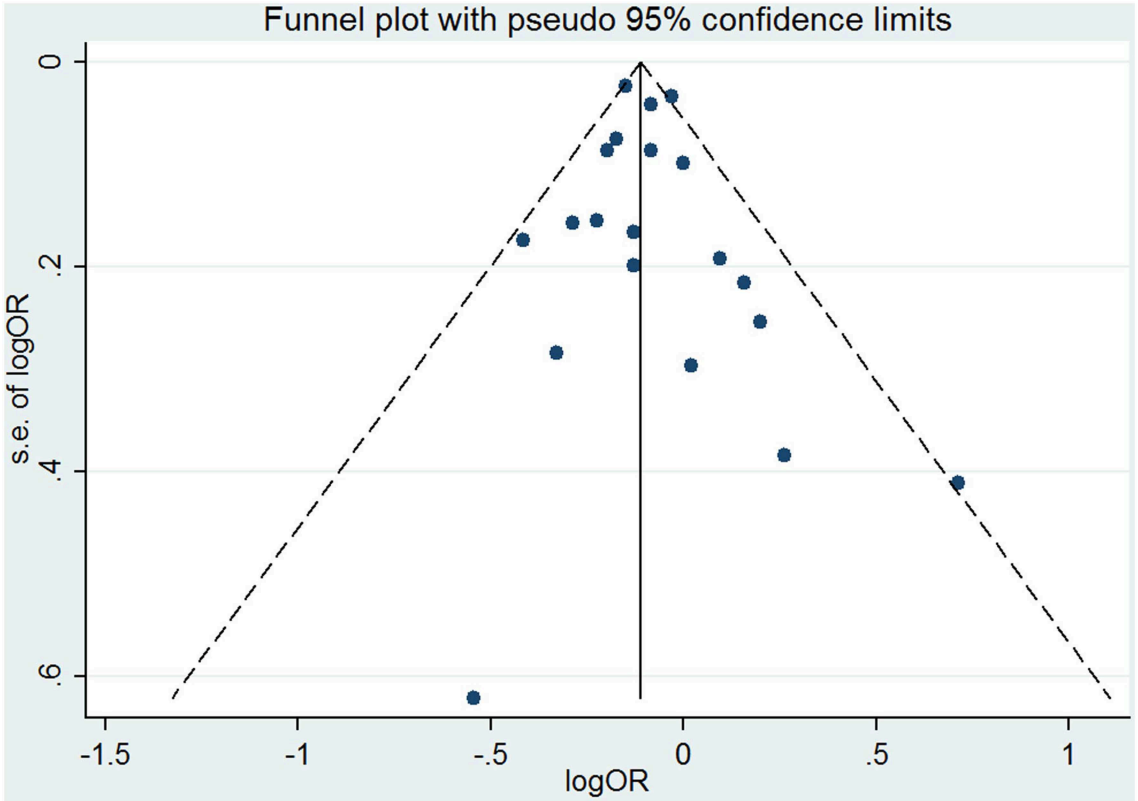


FIGURE 5 | Funnel plot for studies of folic acid supplementation in relation to PTB risk. The vertical solid line represents the summary effect estimates, and the dotted lines are pseudo 95% CIs.

reflecting fluctuation of dietary/supplement intakes over the past month (He et al., 2016). Theoretically, the two different folate indicators are likely to be one source of heterogeneity. Although no evidence was found that the sample type might have affected results, our meta-analysis suggested that there was no significant association between RBC folate and PTB risk, but a negative association was identified between plasma/serum folate and PTB risk. Additionally, the higher I^2 for the plasma/serum subgroup compared to RBCs suggests that different methods for measuring blood folate might be potential reasons for the heterogeneity, and the difference between serum/plasma and RBCs might had affected the I^2 results to some degree.

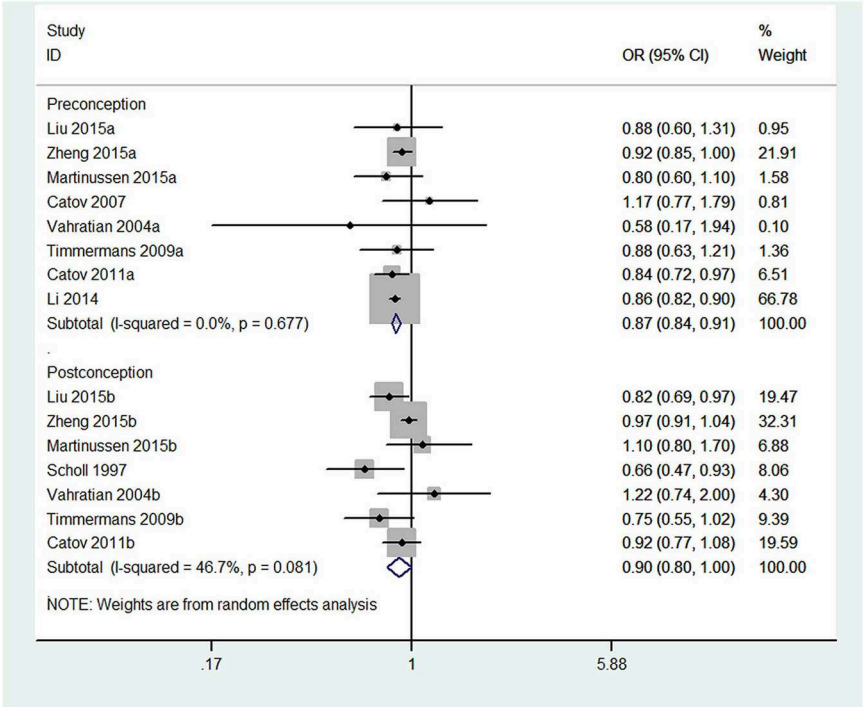
Folic Acid Supplementation and Risk of PTB

In accordance with a previous meta-analysis of observational studies, we found that folic acid supplementation reduced the risk for PTB. Moreover, we found that starting folic acid supplementation before conception was more effective in reducing the risk of PTB compared with post-conception. A population-based mega-cohort study came to the same conclusion as us (Nijhout et al., 2004). This seems biologically plausible given that folate has a half-life of 100 days (Tamura and Picciano, 2006). It has been well-established that folate concentrations in the circulation decline as pregnancy advances (Pickell et al., 2011), and it has been shown that starting folic acid supplementation before conception significantly increases maternal RBC folate concentrations and prevents the decline in serum folate concentration after pregnancy, and this might be beneficial to fetal growth (Bailey, 2009).

There have been concerns that high folic acid intake might be linked to abnormal embryonic development and long-term negative health outcomes in the offspring of mice (Dwarkanath et al., 2013) and humans (Shaw et al., 2004), therefore, it is necessary to evaluate the association between high folic acid intake and the risk of PTB. However, a meta-analysis of randomized controlled trials found that higher folic acid supplementation had no significant reduction of PTB risk (Saccone and Berghella, 2016). Another meta-analysis found that high folic acid reduced the risk of PTB (Zhang et al., 2017). In this meta-analysis, we found that folic acid supplementation was effective in reducing the risk of PTB only if the daily dose was <1,000 μg (moderate dose group), and folic acid supplementation ≥1,000 μg per day (high dose group) did not influence the risk of PTB. The cutoff of 1,000 μg of folic acid supplementation was chosen because most prenatal vitamins contain <1,000 μg folic acid (Bailey, 2009).

Dietary Folate Intake and Risk of PTB

In this meta-analysis, the included 3 studies were unable to differentiate between spontaneous preterm birth and iatrogenic



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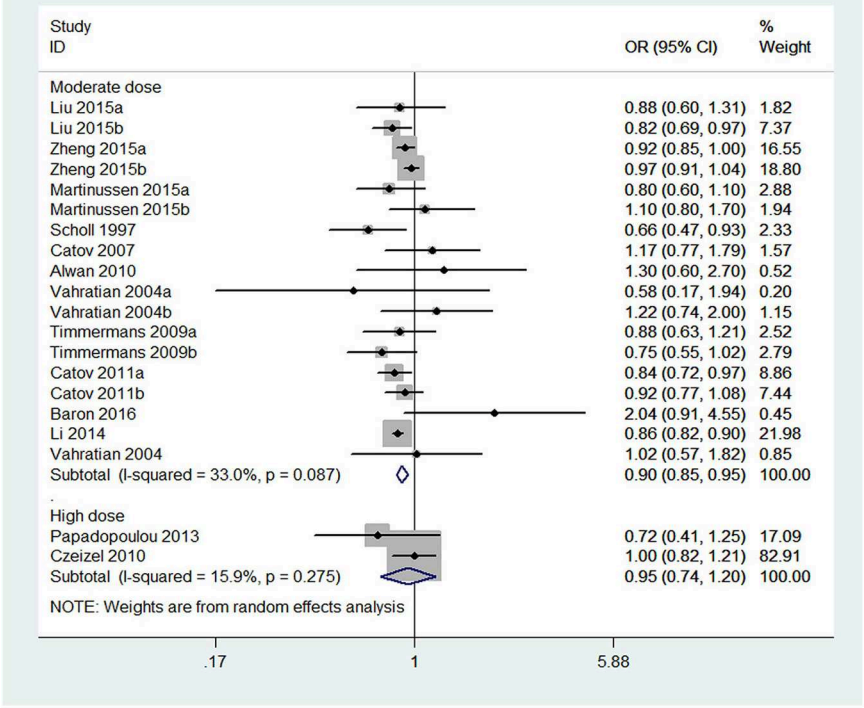

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FIGURE 6 | Forest plot of the meta-analysis of PTB risk in relation to folic acid supplementation stratified by initiation time **(A)** and dose of folic acid intake **(B)**, comparing the highest category with the lowest. The diamonds and horizontal lines indicate the subgroup-specific ORs and 95%CIs. The size of the gray area reflects the study-specific statistical weight. The vertical solid line shows the OR of 1. The suffix "a" or "b" after the studies indicates two separate outcomes stratified by initiation time of supplementation (preconception and post-conception) in the same study.

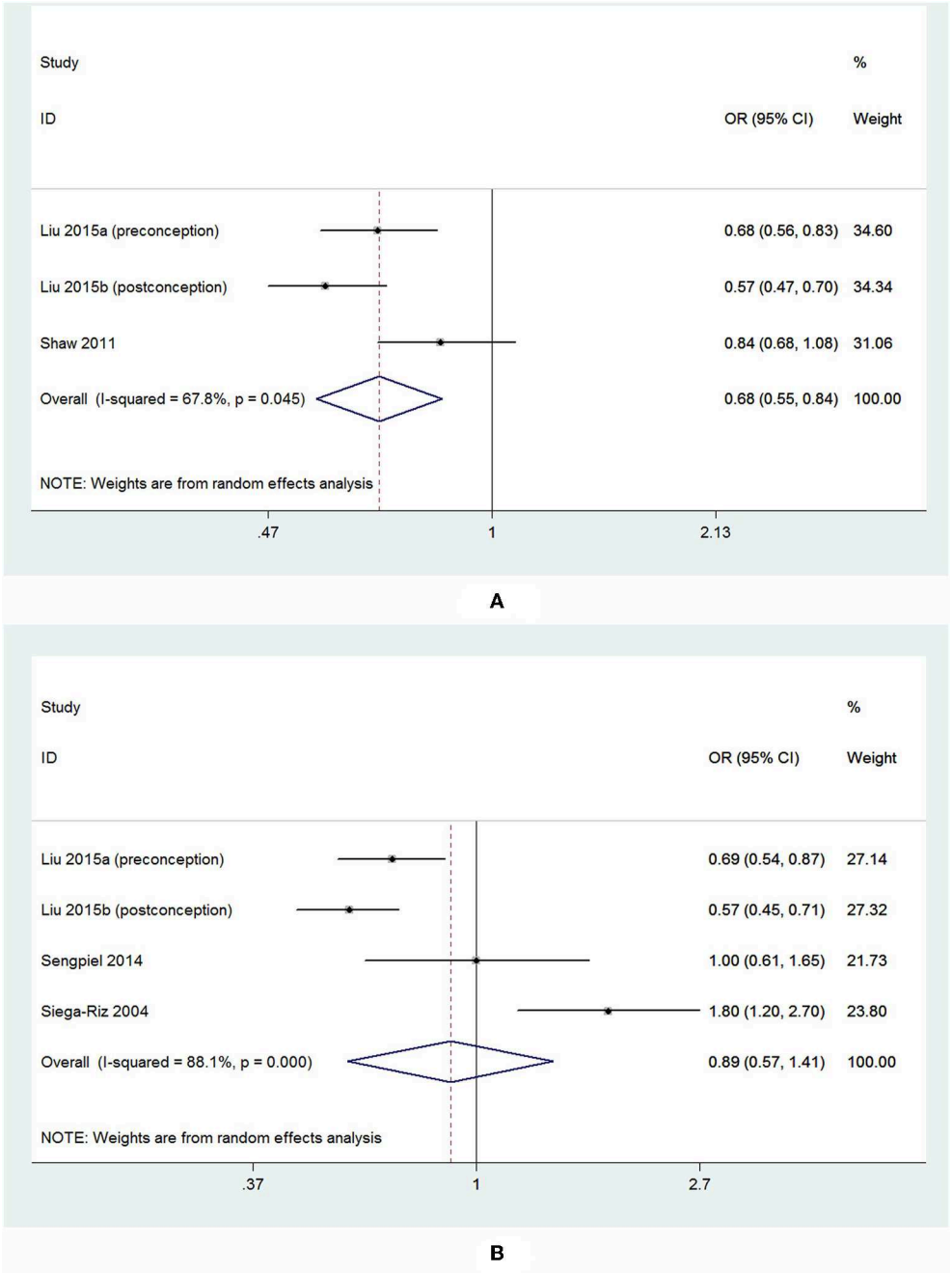

FIGURE 7 | Forest plot of the meta-analysis of PTB risk **(A)** and sPTB risk **(B)** in relation to dietary folate, comparing the highest category with the lowest. The diamonds and horizontal lines indicate the corresponding ORs and 95% CIs. The size of the gray area reflects the study-specific statistical weight. The vertical solid line shows the OR of 1, and the vertical red dashed line represents the combined effect estimate.

preterm birth and thus the two were equated. We found that dietary folate intake showed a strong inverse association with the overall risk of PTB, which was consistent with a previous a cross-sectional study (Deniz et al., 2018). As for the 4 studies specifically examining sPTB, dietary folate intake showed no significant reduction in the risk of sPTB. These results should be interpreted with caution due to the limited data, however, and future studies are required to address these issues.

Limitations of the Study

There were several limitations in the present meta-analysis. First, we did not include RCTs in the current meta-analysis. Because of ethical issues, few RCTs have been conducted that have studied the association between folic acid supplementation and PTB compared to placebo or to no supplementation. Second, there were not enough studies to explore the dose-response trend of blood folate levels and

folic acid supplementation in relation to PTB risk. Third, only a relatively small number of studies on the association between dietary folate intake and PTB risk have been published, so conclusions should be drawn with caution. Moreover, we defined folic acid supplementation as folic acid alone or folic acid-containing vitamins, and this might have led to the introduction of clinical heterogeneity. We were also unable to assess the mutual effect of multivitamins because of insufficient information. In addition, major risk factors for PTB, including socioeconomic status, lifestyle factors, and adverse health behaviors were difficult to control for in the included studies.

CONCLUSIONS

In summary, the results of the present meta-analysis suggest that higher folate levels and folic acid supplementation are significantly associated with a lower overall risk of overall PTB. Dietary folate intake seemed to be significantly associated with a decreased risk of overall PTB and was not associated with risk of sPTB. However, this should be interpreted with caution because of the small number of studies. Subgroup analyses indicated that higher maternal folate levels in late pregnancy are associated with lower PTB risk and that initiating folic acid supplementation early before conception has a significant protective effect against PTB.

Therefore, considering the increasing numbers of preterm infants and recent reports on the neuroprotective effect of folate intake during pregnancy (Julvez et al., 2009), pregnant women should reinforce and start folate intake early before conception in order to reduce the risk of PTB and subsequent risks for long-lasting neurodevelopmental impairments. Additionally, moderately decreased folate levels in late pregnancy might increase the risk of PTB, and this will help with clinical risk stratification and patient counseling.

AUTHOR CONTRIBUTIONS

BL conceptualized and designed the study, collected and organized the data, and drafted the initial manuscript. XZ collected and organized the data, reviewed the included articles, and conducted the analyses. XP and SZ collected and organized the data and reviewed the included articles. XW conceptualized and designed the study and critically reviewed and revised the manuscript. CZ conceptualized and designed the study, coordinated and supervised data collection, and critically reviewed and revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2019.01284/full#supplementary-material

REFERENCES

Alwan, N., Greenwood, D., Simpson, N., McArdle, H., and Cade, J. (2010). The relationship between dietary supplement use in late pregnancy and birth outcomes: a cohort study in British women. *BJOG* 117, 821–829. doi: 10.1111/j.1471-0528.2010.02549.x

Antony, A. C. (2007). In utero physiology: role of folic acid in nutrient delivery and fetal development. *Am. J. Clin. Nutr.* 85, 598S—603S. doi: 10.1093/ajcn/85.2.598S

Bailey, L. B. (2009). *Folate in Health and Disease*. Boca Raton, FL: CRC Press. doi: 10.1201/9781420071252

Baker, B. C., Mackie, F. L., Lean, S. C., Greenwood, S. L., Heazell, A. E., Forbes, K., et al. (2017). Placental dysfunction is associated with altered microRNA expression in pregnant women with low folate status. *Mol. Nutr. Food Res.* 61:1600646. doi: 10.1002/mnfr.201600646

Baron, R., Te Velde, S. J., Heymans, M. W., Klomp, T., Hutton, E. K., and Brug, J. (2017). The relationships of health behaviour and psychological characteristics with spontaneous preterm birth in nulliparous women. *Matern. Child Health J.* 21, 873–882. doi: 10.1007/s10995-016-2160-4

Begg, C. B., and Mazumdar, M. (1994). Operating characteristics of a rank correlation test for publication bias. *Biometrics* 50, 1088–1101. doi: 10.2307/2533446

Bergen, N., Jaddoe, V., Timmermans, S., Hofman, A., Lindemans, J., Russcher, H., et al. (2012). Homocysteine and folate concentrations in early pregnancy and the risk of adverse pregnancy outcomes: the Generation R Study. *BJOG* 119, 739–751. doi: 10.1111/j.1471-0528.2012.03321.x

Blencowe, H., Cousens, S., Chou, D., Oestergaard, M., Say, L., Moller, A. B., et al. (2013). Born too soon: the global epidemiology of 15 million preterm births. *Reprod. Health* 10:S2. doi: 10.1186/1742-4755-10-S1-S2

Bodnar, L. M., Himes, K. P., Venkataramanan, R., Chen, J. Y., Evans, R. W., Meyer, J. L., et al. (2010). Maternal serum folate species in early pregnancy and risk of preterm birth. *Am. J. Clin. Nutr.* 92, 864–871. doi: 10.3945/ajcn.2010.29675

Castaño, E., Caviedes, L., Hirsch, S., Llanos, M., Iñiguez, G., and Ronco, A. M. (2017). Folate transporters in placentas from preterm newborns and their relation to cord blood folate and vitamin B12 levels. *PLoS ONE* 12:e0170389. doi: 10.1371/journal.pone.0170389

Catov, J. M., Bodnar, L. M., Ness, R. B., Markovic, N., and Roberts, J. M. (2007). Association of periconceptional multivitamin use and risk of preterm or small-for-gestational-age births. *Am. J. Epidemiol.* 166, 296–303. doi: 10.1093/aje/kwm071

Catov, J. M., Bodnar, L. M., Olsen, J., Olsen, S., and Nohr, E. A. (2011). Periconceptional multivitamin use and risk of preterm or small-for-gestational-age births in the Danish National Birth Cohort. *Am. J. Clin. Nutr.* 94, 906–912. doi: 10.3945/ajcn.111.012393

Chen, C., Zhang, J., Xia, H., Zhang, H., Betran, A. P., Zhang, L., et al. (2018). Epidemiology of preterm birth in China in 2015 and 2016: a nationwide survey. *Lancet* 392:S73. doi: 10.1016/S0140-6736(18)32702-8

Chen, L. W., Lim, A. L., Colega, M., Tint, M.-T., Aris, I. M., Tan, C. S., et al. (2014). Maternal folate status, but not that of vitamins B-12 or B-6, is sssociated with gestational age and preterm birth risk in a multiethnic Asian population. *J. Nutr.* 145, 113–120. doi: 10.3945/jn.114.196352

Czeizel, A., Puho, E., Langmar, Z., Acs, N., and Banhidy, F. (2010). Possible association of folic acid supplementation during pregnancy with reduction of preterm birth: a population-based study. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 148, 135–140. doi: 10.1016/j.ejogrb.2009.10.016

Deniz, B. F., Confortim, H. D., Deckmann, I., Miguel, P. M., Bronauth, L., de Oliveira, B. C., et al. (2018). Folic acid supplementation during pregnancy prevents cognitive impairments and BDNF imbalance in the hippocampus of the offspring after neonatal hypoxia-ischemia. *J. Nutr. Biochem.* 60, 35–46. doi: 10.1016/j.jnutbio.2018.06.008

DerSimonian, R., and Laird, N. (1986). Meta-analysis in clinical trials. *Control Clin. Trials* 7, 177–188. doi: 10.1016/0197-2456(86)90046-2

Dunlop, A. L., Taylor, R. N., Tangpricha, V., Fortunato, S., and Menon, R. (2012). Maternal micronutrient status and preterm versus term birth for black and white US women. *Reprod. Sci.* 19, 939–948. doi: 10.1177/1933719112438442

Dwarkanath, P., Barzilay, J. R., Thomas, T., Thomas, A., Bhat, S., and Kurpad, A. V. (2013). High folate and low vitamin B-12 intakes during pregnancy are associated with small-for-gestational age infants in South Indian women: a prospective observational cohort study. *Am. J. Clin. Nutr.* 98, 1450–1458. doi: 10.3945/ajcn.112.056382

Engel, S. M., Olshan, A. F., Siega-Riz, A. M., Savitz, D. A., and Chanock, S. J. (2006). Polymorphisms in folate metabolizing genes and risk for spontaneous preterm and small-for-gestational age birth. *Am. J. Obstet. Gynecol.* 195, 1231.e1-11. doi: 10.1016/j.ajog.2006.07.024

Frey, H. A., and Klebanoff, M. A. (2016). The epidemiology, etiology, and costs of preterm birth. *Semin. Fetal Neonatal Med.* 21, 68–73. doi: 10.1016/j.siny.2015.12.011

Furness, D. L., Yasin, N., Dekker, G. A., Thompson, S. D., and Roberts, C. T. (2012). Maternal red blood cell folate concentration at 10–12 weeks gestation and pregnancy outcome. *J. Matern. Fetal Neonatal Med.* 25, 1423–1427. doi: 10.3109/14767058.2011.636463

Gaillard, R., Arends, L. R., Steegers, E. A., Hofman, A., and Jaddoe, V. W. (2013). Second-and third-trimester placental hemodynamics and the risks of pregnancy complications: the Generation R Study. *Am. J. Epidemiol.* 177, 743–754. doi: 10.1093/aje/kws296

Goldenberg, R. L., Culhane, J. F., Iams, J. D., and Romero, R. (2008). Epidemiology and causes of preterm birth. *Lancet* 371, 75–84. doi: 10.1016/S0140-6736(08)60074-4

Goldenberg, R. L., Hauth, J. C., and Andrews, W. W. (2000). Intrauterine infection and preterm delivery. *New Engl. J. Med.* 342, 1500–1507. doi: 10.1056/NEJM200005183422007

Greenland, S. (1987). Quantitative methods in the review of epidemiologic literature. *Epidemiol. Rev.* 9, 1–30. doi: 10.1093/oxfordjournals.epirev.a036298

He, Y., Pan, A., Hu, F. B., and Ma, X. (2016). Folic acid supplementation, birth defects, and adverse pregnancy outcomes in Chinese women: a population-based mega-cohort study. *Lancet* 388:S91. doi: 10.1016/S0140-6736(16)32018-9

Heeraman, C. (2016). *Association between folate levels and preterm birth in Tampa, Florida* (Thesis). University of South Florida, Tampa, FL, United States.

Higgins, J. P., Thompson, S. G., Deeks, J. J., and Altman, D. G. (2003). Measuring inconsistency in meta-analyses. *BMJ* 327:557. doi: 10.1136/bmj.327.7414.557

Hoh, J. K., Lappas, M., Liu, C., Qiao, C., Pallavi, K., Takeda, J., et al. (2019). Preterm birth rate and dilemma of preterm labor treatment in Asia. *Placenta* 79, 68–71. doi: 10.1016/j.placenta.2019.01.005

Jongbloet, P. H., Verbeek, A. L., den Heijer, M., and Roeleveld, N. (2008). Methylenetetrahydrofolate reductase (MTHFR) gene polymorphisms resulting in suboptimal oocyte maturation: a discussion of folate status, neural tube defects, schizophrenia, and vasculopathy. *J. Exp. Clin. Assist. Reprod.* 5:5. doi: 10.1186/1743-1050-5-5

Julvez, J., Fortuny, J., Mendez, M., Torrent, M., Ribas-Fitó, N., and Sunyer, J. (2009). Maternal use of folic acid supplements during pregnancy and four-year-old neurodevelopment in a population-based birth cohort. *Paediatr. Perinat. Epidemiol.* 23, 199–206. doi: 10.1111/j.1365-3016.2009.01032.x

Koukoura, O., Sifakis, S., and Spandidos, D. A. (2012). DNA methylation in the human placenta and fetal growth. *Mol. Med. Rep.* 5, 883–889. doi: 10.3892/mmr.2012.763

Lassi, Z. S., Salam, R. A., Haider, B. A., and Bhutta, Z. A. (2013). Folic acid supplementation during pregnancy for maternal health and pregnancy outcomes. *Cochrane Database Syst. Rev.* CD006896. doi: 10.1002/14651858.CD006896.pub2

Li, Z., Ye, R., Zhang, L., Li, H., Liu, J., and Ren, A. (2014). Periconceptional folic acid supplementation and the risk of preterm births in China: a large prospective cohort study. *Int. J. Epidemiol.* 43, 1132–1139. doi: 10.1093/ije/dyu020

Liu, X., Lv, L., Zhang, H., Zhao, N., Qiu, J., He, X., et al. (2016). Folic acid supplementation, dietary folate intake and risk of preterm birth in China. *Eur. J. Nutr.* 55, 1411–1422. doi: 10.1007/s00394-015-0959-1

López-Quesada, E., Vilaseca, M. A., Vela, A., and Lailla, J. M. (2004). Perinatal outcome prediction by maternal homocysteine and uterine artery Doppler velocimetry. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 113, 61–66. doi: 10.1016/j.ejogrb.2003.05.003

Lucock, M. (2000). Folic acid: nutritional biochemistry, molecular biology, and role in disease processes. *Mol. Genet. Metab.* 71, 121–138. doi: 10.1006/mgme.2000.3027

Maged, A. M., Saad, H., Meshaal, H., Salah, E., Abdelaziz, S., Omran, E., et al. (2017). Maternal serum homocysteine and uterine artery Doppler as predictors of preeclampsia and poor placentation. *Arch. Gynecol. Obstet.* 296, 475–482. doi: 10.1007/s00404-017-4457-y

Mantovani, E., Filippini, F., Bortolus, R., and Franchi, M. (2014). Folic acid supplementation and preterm birth: results from observational studies. *BioMed Res. Int.* 2014:481914. doi: 10.1155/2014/481914

Martí-Carvajal, A., Peña-Martí, G., Comunián-Carrasco, G., Muñoz-Navarro, S., Luco, M., Martí-Peña, A., et al. (2004). Prematurity and maternal folate deficiency: anemia during pregnancy study group results in Valencia, Venezuela. *Arch. Latinoam. Nutr.* 54, 45–49.

Martinussen, M. P., Bracken, M. B., Triche, E. W., Jacobsen, G. W., and Risnes, K. R. (2015). Folic acid supplementation in early pregnancy and the risk of preeclampsia, small for gestational age offspring and preterm delivery. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 195, 94–99. doi: 10.1016/j.ejogrb.2015.09.022

Nijhout, H. F., Reed, M. C., Budu, P., and Ulrich, C. M. (2004). A mathematical model of the folate cycle new insights into folate homeostasis. *J. Biol. Chem.* 279, 55008–55016. doi: 10.1074/jbc.M410818200

Papadopoulou, E., Stratakis, N., Roumeliotaki, T., Sarri, K., Merlo, D. F., Kogevinas, M., et al. (2013). The effect of high doses of folic acid and iron supplementation in early-to-mid pregnancy on prematurity and fetal growth retardation: the mother–child cohort study in Crete, Greece (Rhea study). *Eur. J. Nutr.* 52, 327–336. doi: 10.1007/s00394-012-0339-z

Pickell, L., Brown, K., Li, D., Wang, X. L., Deng, L., Wu, Q., et al. (2011). High intake of folic acid disrupts embryonic development in mice. *Birth Defects Res. A Clin. Mol. Teratol.* 91, 8–19. doi: 10.1002/bdra.20754

Poulsen, G., Strandberg-Larsen, K., Mortensen, L., Barros, H., Cordier, S., Correia, S., et al. (2015). Exploring educational disparities in risk of preterm delivery: a comparative study of 12 European birth cohorts. *Paediatr. Perinat. Epidemol.* 29, 172–183. doi: 10.1111/ppe.12185

Romero, R., Dey, S. K., and Fisher, S. J. (2014). Preterm labor: one syndrome, many causes. *Science* 345, 760–765. doi: 10.1126/science.1251816

Ronnenberg, A. G., Goldman, M. B., Chen, D., Aitken, I. W., Willett, W. C., Selhub, J., et al. (2002). Preconception homocysteine and B vitamin status and birth outcomes in Chinese women. *Am. J. Clin. Nutr.* 76, 1385–1391. doi: 10.1093/ajcn/76.6.1385

Saccone, G., and Berghella, V. (2016). Folic acid supplementation in pregnancy to prevent preterm birth: a systematic review and meta-analysis of randomized controlled trials. *Eur. J. Obstet. Gynecol. Repprod. Biol.* 199, 76–81. doi: 10.1016/j.ejogrb.2016.01.042

Scholl, T. O., and Chen, X. (2015). "Maternal nutrition and preterm delivery," in *Preventive Nutrition. Nutrition and Health,* eds A. Bendich and R. Deckelbaum (Cham: Springer), 705–731. doi: 10.1007/978-3-319-22431-2_33

Scholl, T. O., Hediger, M. L., Bendich, A., Schall, J. I., Smith, W. K., and Krueger, P. M. (1997). Use of multivitamin/mineral prenatal supplements: influence on the outcome of pregnancy. *Am. J. Epidemiol.* 146, 134–141. doi: 10.1093/oxfordjournals.aje.a009244

Scholl, T. O., Hediger, M. L., Schall, J. I., Khoo, C. S., and Fischer, R. L. (1996). Dietary and serum folate: their influence on the outcome of pregnancy. *Am. J. Clin. Nutr.* 63, 520–525. doi: 10.1093/ajcn/63.4.520

Sengpiel, V., Bacelis, J., Myhre, R., Myking, S., Pay, A. S. D., Haugen, M., et al. (2014). Folic acid supplementation, dietary folate intake during pregnancy and risk for spontaneous preterm delivery: a prospective observational cohort study. *BMC Pregnancy Childbirth* 14:375. doi: 10.1186/s12884-014-0375-1

Shaw, G. M., Carmichael, S. L., Nelson, V., Selvin, S., and Schaffer, D. M. (2004). Occurrence of low birthweight and preterm delivery among California infants before and after compulsory food fortification with folic acid. *Public Health Rep.* 119, 170–173. doi: 10.1177/003335490411900210

Shaw, G. M., Carmichael, S. L., Yang, W., and Siega-Riz, A. M. (2011). Periconceptional intake of folic acid and food folate and risks of preterm delivery. *Am. J. Perinatol.* 28, 747–752. doi: 10.1055/s-0031-1280855

Siega-Riz, A. M., Savitz, D. A., Zeisel, S. H., Thorp, J. M., and Herring, A. (2004). Second trimester folate status and preterm birth. *Am. J. Obstet. Gynecol.* 191, 1851–1857. doi: 10.1016/j.ajog.2004.07.076

Song, J., Sun, H., Xu, F., Kang, W., Gao, L., Guo, J., et al. (2016). Recombinant human erythropoietin improves neurological outcomes in very preterm infants. *Ann. Neurol.* 80, 24–34. doi: 10.1002/ana.24677

Stang, A. (2010). Critical evaluation of the Newcastle-Ottawa scale for the assessment of the quality of nonrandomized studies in meta-analyses. *Eur. J. Epidemiol.* 25, 603–605. doi: 10.1007/s10654-010-9491-z

Stroup, D. F., Berlin, J. A., Morton, S. C., Olkin, I., Williamson, G. D., Rennie, D., et al. (2000). Meta-analysis of observational studies in epidemiology: a proposal for reporting. *JAMA* 283, 2008–2012. doi: 10.1001/jama.283.15.2008

Sulaiman, S. A., De Blasio, M. J., Harland, M. L., Gatford, K. L., and Owens, J. A. (2017). Maternal methyl donor and cofactor supplementation in late pregnancy increases β-cell numbers at 16 days of life in growth-restricted twin lambs. *Am. J. Physiol. Endocrinol. Metab.* 313, E381–E90. doi: 10.1152/ajpendo.00033.2017

Tamura, T., and Picciano, M. F. (2006). Folate and human reproduction. *Am. J. Clin. Nutr.* 83, 993–1016. doi: 10.1093/ajcn/83.5.993

The Lancet (2016). The unfinished agenda of preterm births. *Lancet* 388:2323. doi: 10.1016/S0140-6736(16)32170-5

Timmermans, S., Jaddoe, V. W., Hofman, A., Steegers-Theunissen, R. P., and Steegers, E. A. (2009). Periconception folic acid supplementation, fetal growth and the risks of low birth weight and preterm birth: the Generation R Study. *Br. J. Nutr.* 102, 777–785. doi: 10.1017/S0007114509288994

Vahratian, A., Siega-Riz, A. M., Savitz, D. A., and Thorp, J. M. (2004). Multivitamin use and the risk of preterm birth. *Am. J. Epidemiol.* 160, 886–892. doi: 10.1093/aje/kwh305

Van der Molen, E., Verbruggen, B., Novakova, I., Eskes, T., Monnens, L., and Blom, H. (2000). Hyperhomocysteinemia and other thrombotic risk factors in women with placental vasculopathy. *BJOG* 107, 785–791. doi: 10.1111/j.1471-0528.2000.tb13341.x

Wang, S., Ge, X., Zhu, B., Xuan, Y., Huang, K., Rutayisire, E., et al. (2016). Maternal continuing folic acid supplementation after the first trimester of pregnancy increased the risk of large-for-gestational-age birth: a population-based birth cohort study. *Nutrients* 8:493. doi: 10.3390/nu8080493

WHO (2018). *Preterm Birth. Fact Sheet.* 363.

World Health Organization (2015). *Serum and Red Blood Cell Folate Concentrations for Assessing Folate Status in Populations. Vitamin and Mineral Nutrition Information System*. Geneva: WHO.

Zhang, Q., Wang, Y., Xin, X., Zhang, Y., Liu, D., Peng, Z., et al. (2017). Effect of folic acid supplementation on preterm delivery and small for gestational age births: a systematic review and meta-analysis. *Reprod. Toxicol.* 67, 35–41. doi: 10.1016/j.reprotox.2016.11.012

Zhao, M., Chen, Y. H., Dong, X. T., Zhou, J., Chen, X., Wang, H., et al. (2013). Folic acid protects against lipopolysaccharide-induced preterm delivery and intrauterine growth restriction through its anti-inflammatory effect in mice. *PLoS ONE* 8:e82713. doi: 10.1371/journal.pone.0082713

Zheng, J. S., Guan, Y., Zhao, Y., Zhao, W., Tang, X., Chen, H., et al. (2016). Pre-conceptional intake of folic acid supplements is inversely associated with risk of preterm birth and small-for-gestational-age birth: a prospective cohort study. *Br. J. Nutr.* 115, 509–516. doi: 10.1017/S0007114515004663

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Sex-Dimorphic Interactions of *MAOA* Genotype and Child Maltreatment Predispose College Students to Polysubstance Use

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Polysubstance use (PSU) is highly prevalent among college students. Recent evidence indicates that PSU is based on gene x environment (G×E) interactions, yet the specific biosocial factors underlying this problem remain elusive. We recently reported that lifetime use of tobacco and cannabis in college students is influenced by the interaction of the X-linked *MAOA* (monoamine oxidase A) gene and child maltreatment. Building on these premises, here we evaluated whether the same G×E interaction may also predict PSU in this population. Students of a large Midwestern university (n = 470; 50.9% females) took part in a computer survey for substance use, as well as childhood trauma exposure, using the Child Trauma Questionnaire (CTQ). DNA was extracted from their saliva samples and genotyped for *MAOA* variable-number of tandem repeat (VNTR) variants. Findings indicated that the highest number of substances were used by male students harboring low-activity *MAOA* alleles with a history of childhood emotional abuse. In contrast, female homozygous high-activity *MAOA* carriers with a history of emotional and physical abuse reported consumption of the greatest number of substances. Our results indicate that PSU among college students is influenced by the interaction of *MAOA* and child maltreatment in a sex-specific fashion. Further studies are warranted to understand the mechanisms of sex differences in the biosocial interplays underlying PSU in this at-risk group.

Keywords: polysubstance use, MAOA, child maltreatment, sex differences, gene × environment interactions

INTRODUCTION

Polysubstance use (PSU) is a major health concern that has garnered much attention from clinicians and researchers, due to its robust association with substance use disorders and other negative outcomes throughout the lifespan (McCabe et al., 2006; Trenz et al., 2012; Moss et al., 2014). Recent surveys have ascertained that PSU risk is particularly high among college students (Gledhill-Hoyt et al., 2000; Johnston et al., 2004; Mohler-Kuo et al., 2003; Barrett et al., 2006; National Center on

Addiction and Substance Abuse at Columbia University, 2007; O'Grady et al., 2008) with alcohol, tobacco, and cannabis being the three most widely used substances in this population (Lipari and Jean-Francois, 2016). Indeed, these drugs share similar trajectories of use among emerging adults, with high rates of comorbidity (Jackson et al., 2008) and simultaneous consumption (Martin et al., 1992; Baggio et al., 2014).

Vulnerability to PSU, and more generally to substance use disorders and related behavioral phenotypes (including externalizing psychopathology), is strongly influenced by both genetic (Uhl et al., 2001; Dick et al., 2009) and environmental factors. Several genes implicated in the predisposition to substance use disorders have been shown to be related to monoamine neurotransmitters, such as serotonin, dopamine, and norepinephrine (Guo et al., 2007; Ducci and Goldman, 2012); these molecules are known to serve a pivotal role in the pathophysiology of drug abuse (Volkow et al., 2007; Fitzgerald, 2013; Müller and Homberg, 2015). Early-life adversity, and particularly child maltreatment is another well-known variable associated with high risk of PSU (Galaif et al., 2001; Leeb et al., 2008; Goldstein et al., 2013; Cohen et al., 2017). It has been estimated that ~70% of adolescents receiving substance abuse treatment have a history of trauma (Funk et al., 2003), and that maltreated children are 300% more likely to develop substance abuse (Kilpatrick et al., 2003). According to recent conceptual frameworks, the pathogenic influence of child maltreatment and other forms of early stress on PSU is moderated by genetic factors (Vink, 2016). However, only limited data are available on the specific interactions of heritable factors and child maltreatment with respect to PSU predisposition.

We recently showed that, among college students, tobacco and cannabis consumption is influenced by the interaction of child maltreatment and the gene *MAOA*, the X-linked gene encoding for monoamine oxidase A (Fite et al., 2018). In line with our report, Stogner and Gibson (2013) also documented that the interplay of this gene with lifetime stress increases the risk for initiation to alcohol and cannabis use in male adolescents. Monoamine oxidase A catalyzes the degradation of serotonin, norepinephrine and dopamine (Bortolato et al., 2008). The best-characterized *MAOA* functional polymorphism is a 30-bp variable number tandem repeat located in its promoter region (*uVNTR*) (Sabol et al., 1998). The six alleles of this genotype feature different numbers of repeats (2, 3, 3.5, 4, 5, and 6) (Huang et al., 2004), in association with different transcriptional efficiency and enzyme activity. The two- and three-repeat variants, which are associated with low activity (Sabol et al., 1998; Deckert et al., 1998; Denney et al., 1999), confer a greater risk for externalizing psychopathology in male carriers with a history of maltreatment (Caspi et al., 2002; Kim-Cohen et al., 2006; Williams et al., 2009; Fergusson et al., 2011).

A large body of evidence has documented that *MAOA uVNTR* variants exert a sex-dimorphic influence on the overall risk and specific clinical manifestations of alcohol use disorders, both *per se* and in interaction with early-life adversity (Samochowiec et al., 1999; Schmidt et al., 2000; Vanyukov et al., 2004; Guindalini et al., 2005; Herman et al., 2005; Ducci et al., 2008; Nilsson et al., 2011). Low-activity *uVNTR* alleles (hereafter designated as *MAOA-L*), for example, are associated with a younger age of onset of alcohol dependence (Vanyukov et al., 1995; Vanyukov et al., 2004) and antisocial alcoholism (Samochowiec et al., 1999) in males. A history of maltreatment predisposes female carriers of high-activity alleles (*MAOA-H*) or male MAOA-L carriers to a greater risk of alcohol use (Nilsson et al., 2011). In alignment with these findings, we found that greater lifetime tobacco use was predicted by the interaction of childhood maltreatment and *MAOA-L* variants in males and *MAOA-H* alleles in females (Fite et al., 2018).

Given these premises, the present study tested the hypothesis that the same gene x environment (G×E) interactions may predispose to PSU in college students and analyzed whether the influence of these biosocial interplays may follow a sex-dimorphic pattern.

METHODS

Participants

Participants were 470 students (239 females and 231 males; see **Table 1**) enrolled in undergraduate psychology courses at a large Midwestern university. Recruitment was based on SONA, an online system that allows students to electronically sign up to participate in active studies at the university. Most students (71.1%) identified as Caucasians, attended the first year of college (61.1%) and reported that their parents had a higher educational level than high school (80.9% of fathers and 79.7% of mothers).

Procedures

All study procedures were approved by the researchers' Institutional Review Board. All participants were instructed to abstain from eating for 1 h before the study, and refrain from the use of any drug (including prescription medicines and caffeinated beverages) for at least 3 h before the study. Upon arrival, they were given a complete summary of the study and provided informed consent. Subsequently, participants rinsed their mouth with water and, ten minutes later, were instructed to give 2 ml of saliva in a tube for genetic analyses. Then, they provided demographic information, including their age and race/ethnicity, and completed a Qualtrics online survey in about 1 h. At the end of the study, participants were compensated with a $5 debit card for the saliva sample and 3 SONA credits for the survey. To keep the identity of participants anonymous, survey responses and saliva samples were assigned a unique ID without any identifying information.

Questionnaires

The survey included the following questionnaires:

1. the *Child Trauma Questionnaire* (CTQ), a standardized self-report instrument for the retrospective assessment of trauma exposure during childhood (Bernstein and Fink, 1998). The CTQ consists of 5 subscales of trauma (physical abuse,

TABLE 1 | Participant demographics and descriptive statistics.

	Overall Sample (n = 470)	Males (n = 231)	Females (n = 239)
M (*SD*) Age	18.95 (1.19)	19.14 (1.25)	18.76 (1.10)
Year in school			
% 1st year student	61.1	55.8	66.1
% 2nd year student	27.4	29.4	25.5
% 3rd year student	8.9	11.7	6.3
% 4th year student	1.9	2.6	1.3
% 5th year or more student	0.7	0.5	0.8
Race/Ethnicity			
% Caucasian	71.1	72.7	69.5
% African American	3.6	3.0	4.2
% Hispanic/Latino	6.2	4.8	7.5
% Native American	1.3	.9	1.7
% Asian	10.6	10.4	10.9
% Mixed or other	7.2	8.2	6.2
Medical History			
% Psychological Disorder	13.2	10.4	15.9
% Current Illness/Injury	3.4	3.5	3.3
% Currently Medications	43.4	25.1	61.1
Parental Education at birth			
% Fathers greater than high school	80.9	81.0	78.4
% Mothers greater than high school	79.7	83.8	78.2

emotional abuse, sexual abuse, physical neglect, and emotional neglect) with multiple items based on a 5-point Likert scale format. Mean scores for each subscale, as well as an overall child maltreatment score, were calculated. The physical neglect subscale yielded the lowest reliability coefficient (α = 0.56) in the current sample; internal consistencies for the remaining four subscales were good (with all α's > 0.81);

2. A *substance use questionnaire*. based on three items from the Center for Substance Abuse Prevention (CSAP) Student Survey (Pentz et al., 1989), a self-report instrument assessing lifetime tobacco (i.e., "Have you ever smoked a cigarette, even just a few puffs, or used chewing tobacco, snuff, or dip), alcohol (i.e., "Have you ever had a drink of alcohol?"), and cannabis use (i.e., "Have you ever tried marijuana?"). The number of substances used by each participant was calculated (ranging from 0 to 3).

MAOA uVNTR Variants Genotyping

DNA extracted and *MAOA-uVNTR* genotyping were performed as previously described (Fite et al., 2018). All laboratory procedures were carried out by personnel blind to the demographic and psychological characteristics of the subject (other than gender). All genotype data of participants are shown in **Table 2**. Given that the *MAOA* gene is located on the X chromosome, males were designated as either low-activity (*MAOA-L*) or high-activity (*MAOA-H*) hemizygous, depending on the number of repeats of their allelic variant (2 and 3 vs 3.5 and 4, respectively). Conversely, females were either homozygous for either allele (*MAOA-LL* or *MAOA-HH*) or heterozygous carriers (*MAOA-LH*). In line with previous studies on *MAOA* (Byrd and Manuck, 2014), carriers of 5-repeat *uVNTR* alleles were excluded from the analyses, as the actual functional significance of this variant remains controversial (Sabol et al., 1998; Deckert et al., 1998). To allow for comparability between males and females, *MAOA-LL* and *MAOA-LH* female participants were combined (n = 165), in agreement with previous functional studies on sex-dimorphic effects of *MAOA uVNTR* variants (Fan et al., 2003; Meyer-Lindenberg et al., 2006; Frazzetto et al., 2007; Buckholtz et al., 2008; Dannlowski et al., 2009) The validity of this approach was confirmed by analyzing the interactions of *MAOA* genotype variants (MAOA-LL, MAOA-HH, and MAOA-LH) and maltreatment types in female participants. The results of these analyses indicated that *MAOA-LH* genotype operated consistent with the *MAOA-LL* genotype in its interaction with maltreatment types to predict PSU. All genotypic and phenotypic data are presented as **Supplementary Materials**.

Data Analysis

Of the original 500 students recruited for the study, M*AOA* genotyping could not be performed for 11 participants, while 11 participants were missing CTQ and/or substance use data. We further excluded 8 participants (4 males and 4 females) carrying 5-repeat *uVNTR* alleles. Based on power tables (Aiken and West, 1991), it was determined that the current sample had adequate power (α = 0.80) to detect moderate to large, but not small, *MAOA* × maltreatment interaction effects for males and females. No differences in sex or age (*ps* > 0.48) or in child maltreatment scores (*ps* > 0.16) were found in the comparison between the participants included in and excluded from the analyses. Multiple regression models were used to evaluate proposed associations. Substance use count was the dependent variable in each model, with sex, *MAOA* variant, and maltreatment types included as independent variables. All five maltreatment types were included in each model to evaluate unique associations. Three-way interactions (e.g., sex × *MAOA* variant × maltreatment type) were then evaluated one at a time to determine if child maltreatment-MAOA interactive effects

TABLE 2 | Genotypic data of all participants. Genotypes containing 5-repeat variants were not included in either MAOA low-activity (MAOA-L) or high-activity (MAOA-H) allele groups. For more details, see text.

	MALES		
	Number of repeats	**Number**	**Percentage**
MAOA-L	2	1	0.43%
	3	93	39.57%
MAOA-H	3.5	10	4.26%
	4	127	54.04%
Excluded genotypes	5	4	1.70%
	FEMALES		
	Number of repeats	**Number**	**Percentage**
MAOA-LL	2-2	1	0.41%
	2-3	1	0.41%
	3-3	42	17.28%
MAOA-LH	2-3.5	0	0%
	2-4	1	0.41%
	3-3.5	2	0.82%
	3-4	118	48.56%
MAOA-HH	3.5-3.5	0	0%
	3.5-4	4	1.65%
	4-4	70	28.81%
Excluded genotypes	2-5	0	0%
	3-5	0	0%
	3.5-5	0	0%
	4-5	4	1.65%

depended on sex. All independent variables were mean centered prior to analyses to aid in the interpretation of interaction effects. Statistically significant interactions were probed based on sex (male vs. female) and for *MAOA* variants to determine the nature of the interactions, consistent with standard procedures (Aiken and West, 1991).

RESULTS

Approximately 11.5% of the sample had not used any substance, 28.9% of the sample had used one substance, 23.2% had used two substances, and 36.4% of the sample had used three substances. Based on the clinical cutoff scores recommended by Bernstein and Fink (1998), ~46.5% of the sample reported at least low levels of one or more maltreatment types. This percentage is consistent with previous data on undergraduate, emerging adult samples (Reichert and Flannery-Schroeder, 2014).

Regression analyses indicated a significant three-way interaction when examining any experience of maltreatment (B = 1.36, $p = 0.00$; see **Table 3**). Additionally, a significant three -way interaction was found for physical abuse (B = 1.37, $p = 0.00$) as well as emotional abuse (B = 0.58, $p = 0.04$). However, no significant three -way interactions were found for any other child maltreatment type: physical neglect (B = 0.54, $p = 0.26$), emotional neglect (B = 0.40, $p = 0.15$), or sexual abuse (B = 0.43, $p = 0.39$). Additionally, no significant two-way interactions between maltreatment variables and *MAOA* alleles were evident (ps > 0.12).

TABLE 3 | Three-way interaction regression analyses. Significant results are indicated in bold.

	SU Count	
	B	***p***
Sexual Abuse	0.43	0.39
Emotional Neglect	0.40	0.15
Physical Abuse	1.37	**0.00**
Emotional Abuse	0.58	**0.04**
Physical Neglect	0.54	0.26
Any Maltreatment	1.36	**0.00**

The statistically significant three -way interactions with any maltreatment type, physical abuse, and emotional abuse were further evaluated by conducting tests of the simple slopes (**Table 4**). Specifically, the models were conditioned at *MAOA-H* and *MAOA-L* for both males and females to determine the patterns of associations. For *MAOA-L* males, there was a marginally statistically trend for any maltreatment type (B = 0.42, $p = 0.08$) (**Figure 1A**) and statistically significant effect for and emotional abuse (B = 0.38, $p = 0.03$) (**Figure 1C**) to be positively associated with the number of substances used. However, an association between physical abuse and number of substances used was not found (B = 0.26, $p = 0.17$) (**Figure 1B**). For *MAOA-H* males, any maltreatment type was marginally statistically negatively associated (B = -0.42, $p = 0.07$) (**Figure 1A**) and physical abuse was statistically negatively associated (B = -0.33, $p = 0.03$) with the number of substances used (**Figure 1B**). Emotional abuse (B = -0.05, $p = 0.77$)

TABLE 4 | Simple-slope analyses of three-way interactions. SE, standard error. *p < 0.05; +p < 0.09.

	Males				Females			
	MAOA – L		*MAOA – H*		*MAOA – LL+ MAOA-LH*		*MAOA – HH*	
	B	SE	B	SE	B	SE	B	SE
Any Maltreatment	0.42+	0.24	–0.42+	0.23	0.00	0.16	0.52*	0.26
Physical Abuse	0.26	0.19	–0.33*	0.15	–0.25	0.19	0.54*	0.24
Emotional Abuse	0.38*	0.17	–0.05	0.17	0.19	0.14	0.34*	0.17

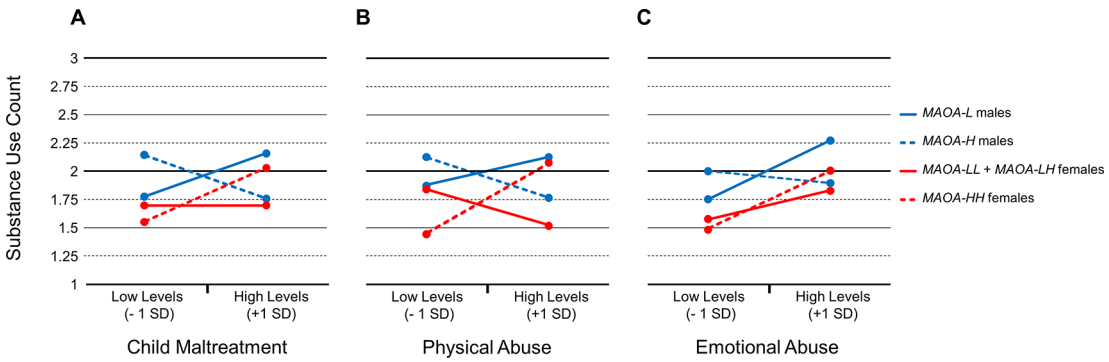

FIGURE 1 | Associations between child maltreatment types and substance use count for male and female carriers of *MAOA uVNTR* variants. **(A)** Overall associations with child maltreatment scores. **(B)** Associations with physical abuse scores. **(C)** Associations with emotional abuse scores.

was statistically unrelated to number of substances used for *MAOA-H* males (**Figure 1C**).

In contrast, for female carriers of low-activity MAOA variants (*MAOA-LL* and *MAOA-LH),* there was no association evident between any maltreatment type (B = 0.00, $p = 0.99$) (**Figure 1A**), physical abuse (B = -0.25, $p = 0.18$) (**Figure 1B**), or emotional abuse (B = 0.19, $p = 0.17$) (**Figure 1C**) and number of substances used. For homozygous *MAOA-H* females, there was a statistically significant positive association between any maltreatment type (B = 0.52, $p = 0.04$) (**Figure 1A**), and physical abuse (B = 0.54, $p = 0.03$) (**Figure 1B**), and emotional abuse (B = 0.34, $p = 0.04$) (**Figure 1C**) and number of substances used.

DISCUSSION

The results of the current study showed that, in a sample of students enrolled in a large Midwestern university, PSU was predicted by the interaction of *MAOA uVNTR* allelic variants, sex, and specific child maltreatment types. The highest number of substances used was found in *MAOA-L* male and *MAOA-HH* female carriers with a history of emotional abuse (as well as physical abuse in women). To our knowledge, this is the first report documenting a key role of *MAOA* as a mediator of child maltreatment with respect to PSU. While previous studies have shown the importance of G×E interactions in PSU (Vaughn et al., 2009; Rende, 2011), the specific genetic factors implicated in such biosocial interplays remain mostly elusive; if confirmed by future studies, our results may point to *MAOA* as a key molecular basis for PSU.

The present findings extend our previous report of sex-dimorphic influences of G×E interactions in the lifetime use of tobacco (Fite et al., 2018) among college students. Furthermore, these results are consistent with previous evidence indicating sex differences in the interactive influence of these G×E interactions with respect to antisocial conduct (Nikulina et al., 2012; Stogner and Gibson, 2013; Byrd and Manuck, 2014; Harro and Oreland, 2016) and alcohol use (Nilsson et al., 2011). The interaction of *MAOA* alleles and child maltreatment can be interpreted from the perspective of the diathesis-stress model, which postulates that the predisposition to specific neurobehavioral deficits is the result of a synergistic combination of genetic and environmental untoward factors (Zuckerman, 1999). Another alternative interpretation follows the differential susceptibility hypothesis, which posits that specific genetic variables may sensitize to both the positive and the negative influence of early experiences (Ellis et al., 2011). This possibility is partially supported by Belsky and colleagues (Belsky et al., 2009; Belsky and Beaver, 2011), who have conceptualized that *MAOA* variants may act as plasticity factors in the predisposition to substance use and other psychopathological conditions.

In line with previous data (Armour et al., 2014), the current results highlight the importance of examining specific maltreatment types in relation to PSU. Our findings suggest that, although physical abuse and emotional abuse interact with *MAOA* variants to predict PSU even when statistically controlling

for the other maltreatment types, no interaction effects were found for sexual abuse, physical neglect, or emotional neglect. This evidence is partially consistent with a previous study by Nikulina and colleagues (2012) suggesting that *MAOA* does not serve as a protective or risk factor for substance use outcomes among individuals who have experienced childhood sexual abuse. However, in contrast with our results, the results of that investigation showed that alcohol use was not predicted by the interaction of *MAOA* with either physical abuse or neglect. Given that the participants of that study ranged between 31 and 51 years of age, it is possible that the discrepancy with those results may reflect age differences; accordingly, the moderating effect of *MAOA* on child maltreatment and negative outcomes has been hypothesized to be age-dependent (Huizinga et al., 2006). Alternatively, these divergent findings may result from other differences between our studies, including the substance use outcomes (i.e., PSU vs alcohol abuse) and measurement of child maltreatment (i.e., self-report vs official records). Nevertheless, research shows that experiences of child maltreatment are associated with decreased propensity for reward selection, which could be due to lower reward sensitivity (Guyer et al., 2006). In turn, this dual risk might increase the risk of PSU. Thus, child maltreatment types, physical abuse and emotional abuse may be more saliently associated with blunted reward sensitivity.

The existence of sex-dimorphic G×E interactions involving *MAOA uVNTR* alleles has been attested in other psychopathological states. For example, male carriers of *MAOA-L* alleles with a history of child maltreatment have a significantly higher risk of antisocial, aggressive, and violent behavior (Caspi et al., 2002; Kim-Cohen et al., 2006; Beaver et al., 2010; Aslund et al., 2011; Fergusson et al., 2011; Fergusson et al., 2012; Byrd and Manuck, 2014; Godar et al., 2016). Notably, the same G×E interaction has been reproduced in mouse models, further supporting the biological nature of this biosocial interplay (Godar et al., 2019). Conversely, female carriers of *MAOA-H* alleles with a positive history for early-life adversity display a higher proclivity for antisocial and violent responses (Sjöberg et al., 2007; McGrath et al., 2012; Verhoeven et al., 2012). It has been hypothesized that this effect may reflect the enhancement of emotional reactivity during adolescence (Byrd et al., 2018). Furthermore, these effects may reflect sex- and genotype-specific differences in the effects of MAOA on monoamine metabolism (Jönsson et al., 2000; Aklillu et al., 2009). Notably, aggression and delinquency have been extensively linked to PSU, particularly in boys (McCormick and Smith, 1995; Mason and Windle, 2002; Martinotti et al., 2009). This concurrence strongly suggests that the G×E interaction of *MAOA* genotype and child maltreatment may predispose to a broad set of externalizing responses, ranging from antisocial personality to PSU propensity. In line with this interpretation, neuroimaging studies have pointed to *MAOA* as a key molecule to influence the function of the anterior cingulate cortex (ACC) (Passamonti et al., 2008). This region plays a major role in the regulation of self-regulation (Posner et al., 2007), the key domain implicated in the ontogeny of antisocial behavior (Gardner et al., 2008; Trentacosta and Shaw, 2009; Gillespie et al., 2018), as well as in the role of G×E interactions in PSU (Vaughn et al., 2009). The effects of *MAOA* on ACC activation patterns are sex-dimorphic; specifically, *MAOA-L* male and *MAOA-H* female carriers with a history of early stress display impairments in the activation of the ACC in response inhibition (Holz et al., 2016), a process directly related to self-regulation (Posner and Rothbart, 1998; Blair and Ursache, 2011; Hofmann et al., 2012). It should be noted that functional deficits of the ACC are associated with a reduction in inhibitory control (Bush et al., 2000; Chan et al., 2011), as well as a facilitation of ventral striatal responses to incentive stimuli, which in turn increases drug use propensity (Holmes et al., 2016; Koyama et al., 2017). Notably, these deficits may be particularly overt in young individuals (and therefore highly relevant in the age range of college students), due to their incomplete myelination of the ACC as well as the development of the dopaminergic system, which further exacerbates their proclivity to engage in impulsive and risky actions and heightens their reward sensitivity (Casey et al., 2008; Steinberg, 2008). At least in females, the presence of *MAOA-H* alleles may further reduce dopamine levels, ultimately promoting the ontogeny of reward deficiency syndrome (Blum, 2017; Blum et al., 2018). From this perspective, these results suggest that the interaction of *MAOA-L* alleles in males and *MAOA-H* in females and early-life maltreatment may interfere with the development of inhibitory control in emerging adulthood, ultimately increasing PSU risk.

Several limitations of this study should be acknowledged. First, our analyses focused exclusively on *MAOA* polymorphisms, yet several studies point to the importance of many other genes in the vulnerability to PSU, such as those encoding for dopamine receptor 2 and 4 as well as dopamine and serotonin transporters (Blum et al., 2010); further studies are needed to evaluate the potential interaction of child maltreatment with these vulnerability factors. Second, although rich literature has documented that *MAOA* variants interact with childhood maltreatment to increase the propensity for externalizing behaviors, our findings need to be replicated in larger samples from multiple colleges and with less skewed ethnic distribution. Indeed, our sample comprised of predominantly Caucasian youth, which may limit the generalizability of current results. Second, this study relied solely on self-reports of constructs, with a low internal consistency associated with our measure of physical neglect. Future research examining associations in other samples (e.g., clinical and criminal) using multiple, psychometrically sound assessments of constructs would be useful for establishing generalizability of findings. Finally, our research combined two- and three-repeat variant carriers in the *MAOA-L* group; however, previous studies, however, have shown that, in males, two-repeat alleles resulted in much lower levels of promoter activity as well as stronger phenotypic effects than the three-repeat genotype (Sabol et al., 1998; Guo et al., 2008). Notably, two-repeat variants have shown to increase antisocial phenotypes, including the propensity to engage in particularly violent conduct (such as shooting and stabbing), in African-American males (Beaver et al., 2013; Beaver

et al., 2014). Unfortunately, given that only one male participant was found to carry the two-repeat alleles, our analyses were not sufficiently powered to differentiate across specific genotypes; however, future studies will be needed to verify whether specific differences may be identified with respect to the interaction of specific variants with early maltreatment.

Despite these limitations, the current study contributes to the growing literature indicating sex differences in genetic risk of MAOA in addition to the importance of the interactive influences of genetic and environmental risk for PSU. Further, findings indicate the importance of evaluating specific maltreatment types to better understand *MAOA* and maltreatment interactive risks for substance use.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB University of Kansas. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PF conceptualized the project, supervised data collection and analysis, and drafted the first draft of the manuscript. SB helped with data collection and analysis and helped draft the first version of the manuscript. WH performed genotyping analyses and drafted part of the method sections. AM and MGB contributed to the conceptualization of the study and reviewed genotyping data. MB conceptualized the project, reviewed data analysis, and wrote the final version of the manuscript. All authors have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.01314/full#supplementary-material

REFERENCES

Aiken, L. S., and West, S. G. (1991). *Multiple regression: Testing and interpreting interactions* (Newbury Park, CA: Sage Publication).

Aklillu, E., Karlsson, S., Zachrisson, O. O., Ozdemir, V., and Agren, H. (2009). Association of MAOA gene functional promoter polymorphism with CSF dopamine turnover and atypical depression. *Pharmacogenet. Genomics* 19, 267–275. doi: 10.1097/FPC.0b013e328328d4d3

Armour, C., Shorter, G. W., Elhai, J. D., Elklit, A., and Christoffersen, M. N. (2014). Polydrug use typologies and childhood maltreatment in a nationally representative survey of Danish young adults. *J. Stud. Alcohol Drugs* 75 (1), 170–178. doi: 10.15288/jsad.2014.75.170

Aslund, C., Nordquist, N., Comasco, E., Leppert, J., Oreland, L., and Nilsson, K. W. (2011). Maltreatment, MAOA, and delinquency: sex differences in gene-environment interaction in a large population-based cohort of adolescents. *Behav. Genet.* 41, 262–272. doi: 10.1007/s10519-010-9356-y

Baggio, S., Deline, S., Studer, J., N'Goran, A., Mohler-Kuo, M., Daeppen, J. B., et al. (2014). Concurrent versus simultaneous use of alcohol and non-medical use of prescription drugs: is simultaneous use worse for mental, social, and health issues? *J. Psychoactive Drugs* 46, 334–339. doi: 10.1080/02791072.2014.921747

Barrett, S. P., Darredeau, C., and Pihl, R. O. (2006). Patterns of simultaneous polysubstance use in drug using university students. *Hum. Psychopharmacol.* 21 (4), 255– 263. doi: 10.1002/hup.766

Beaver, K. M., DeLisi, M., Vaughn, M. G., and Barnes, J. C. (2010). Monoamine oxidase a genotype is associated with gang membership and weapon use. *Compr. Psychiatry* 51, 130–134. doi: 10.1016/j.comppsych.2009.03.010

Beaver, K. M., Wright, J. P., Boutwell, B. B., Barnes, J. C., DeLisi, M., and Vaughn, M. G. (2013). Exploring the association between the 2-repeat allele of the MAOA gene promoter polymorphism and psychopathic personality traits, arrests, incarceration, and lifetime antisocial behavior. *Pers. Individ Dif.* 54, 164–168. doi: 10.1016/j.paid.2012.08.014

Beaver, K. M., Barnes, J. C., and Boutwell, B. B. (2014). The 2-repeat allele of the MAOA gene confers an increased risk for shooting and stabbing behaviors. *Psychiatr. Q.* 85, 257–265. doi: 10.1007/s11126-013-9287-x

Belsky, J., and Beaver, K. M. (2011). Cumulative-genetic plasticity, parenting and adolescent self-regulation. *J. Child Psychol. Psychiatry* 52, 619–626. doi: 10.1111/j.1469-7610.2010.02327.x

Belsky, J., Jonassaint, C., Pluess, M., Stanton, M., Brummett, B., and Williams, R. (2009). Vulnerability genes or plasticity genes? *Mol. Psychiatry* 14, 746–754. doi: 10.1038/mp.2009.44

Bernstein, D. P., and Fink, L. (1998). Childhood trauma questionnaire: a retrospective self-report: manual. (San Antonio, TX: The Psychological Corporation).

Blair, C., and Ursache, A. (2011). "A bidirectional model of executive functions and self-regulation," in *Handbook of self-regulation: Research, theory, and applications*. Eds. K. D. Vohs and R. F. Baumeister (New York, NY, US: Guilford Press), 300–320.

Blum, K., Giordano, J., Morse, S., Liu, Y., Tian, J., Bowirrat, A., et al. (2010). Genetic Addiction Risk Score (GARS) analysis: exploratory development of polymorphic risk alleles in poly-drug addicted males. *Integr. Omics Appl. Biotechnol.* 1, 1–14. doi: 10.5772/20067

Blum, K., Gondré-Lewis, M. C., Baron, D., Thanos, P. K., Braverman, E. R., Neary, J., et al. (2018). Introducing Precision Addiction Management of Reward Deficiency Syndrome, the Construct That Underpins All Addictive Behaviors. *Front. Psychiatry* 9, 548. doi: 10.3389/fpsyt.2018.00548

Blum, K. (2017). "Reward deficiency syndrome," in *The Sage Encyclopedia of Abnormal Clinical Psychology*. Ed. A. Wenzel (Newbury Park, CA: Sage Publication).

Bortolato, M., Chen, K., and Shih, J. C. (2008). Monoamine oxidase inactivation: from pathophysiology to therapeutics. *Adv. Drug Deliv. Rev.* 60, 1527–1533. doi: 10.1016/j.addr.2008.06.002

Buckholtz, J. W., Callicott, J. H., Kolachana, B., Hariri, A. R., Goldberg, T. E., Genderson, M., et al. (2008). Genetic variation in MAOA modulates ventromedial prefrontal circuitry mediating individual differences in human personality. *Mol. Psychiatry* 13, 313–324. doi: 10.1038/sj.mp.4002020

Bush, G., Luu, P., and Posner, M. I. (2000). Cognitive and emotional influences in anterior cingulate cortex. *Trends Cogn. Sci.* 4, 215–222. doi: 10.1016/s1364-6613(00)01483-2

Byrd, A. L., and Manuck, S. B. (2014). MAOA, childhood maltreatment, and antisocial behavior: meta-analysis of a gene-environment interaction. *Biol. Psychiatry* 75, 9–17. doi: 10.1016/j.biopsych.2013.05.004

Byrd, A. L., Manuck, S. B., Hawes, S. W., Vebares, T. J., Nimgaonkar, V., Chowdari, K. V., et al. (2018). The interaction between monoamine oxidase A (MAOA) and childhood maltreatment as a predictor of personality pathology in females: Emotional reactivity as a potential mediating mechanism. *Dev. Psychopathol.* 22, 1–17. doi: 10.1017/S0954579417001900

Casey, B. J., Jones, R. M., and Hare, T. A. (2008). The adolescent brain. *Ann. N Y Acad. Sci.* 1124, 111–126. doi: 10.1196/annals.1440.010

Caspi, A., McClay, J., Moffitt, T. E., Mill, J., Martin, J., Craig, I. W., et al. (2002). Role of genotype in the cycle of violence in maltreated children. *Science* 297, 851–854. doi: 10.1126/science.1072290

Chan, A. S., Han, Y. M., Leung, W. W., Leung, C., Wong, V. C., and Cheung, M. (2011). Abnormalities in the anterior cingulate cortex associated with attentional and inhibitory control deficits: a neurophysiological study on children with autism spectrum disorders. *Res. Autism Spectr. Disord.* 5, 254–266. doi: 10.1016/j.rasd.2010.04.007

Cohen, J. R., Menon, S. V., Shorey, R. C., Le, V. D., and Temple, J. R. (2017). The distal consequences of physical and emotional neglect in emerging adults: a person-centered, multi-wave, longitudinal study. *Child Abuse Negl.* 63, 151–161. doi: 10.1016/j.chiabu.2016.11.030

Dannlowski, U., Ohrmann, P., Konrad, C., Domschke, K., Bauer, J., Kugel, H., et al. (2009). Reduced amygdala-prefrontal coupling in major depression: association with MAOA genotype and illness severity. *Int. J. Neuropsychopharmacol.* 12, 11–22. doi: 10.1017/S1461145708008973

Deckert, J., Catalano, M., Syagailo, Y. V., Bosi, M., Okladnova, O., Di Bella, D., et al. (1998). Excess of high activity monoamine oxidase a gene promoter alleles in female patients with panic disorder. *Hum. Mol. Genet.* 8, 621–624. doi: 10.1093/hmg/8.4.621

Denney, R. M., Koch, H., and Craig, I. W. (1999). Association between monoamine oxidase A activity in human male skin fibroblasts and genotype of the MAOA promoter-associated variable number tandem repeat. *Hum. Genet.* 105, 542–551. doi: 10.1007/s004399900183

Dick, D. M., Prescott, C. A., and McGue, M. (2009). "The genetics of substance use and substance use disorders," in *Handbook of Behavior Genetics*, Ed. Y. K. Kim (New York: Springer), 433–453.

Ducci, F., and Goldman, D. (2012). The genetic basis of addictive disorders. *Psychiatr. Clin. North Am.* 35, 495–519.

Ducci, F., Enoch, M. A., Hodgkinson, C., Xu, K., Catena, M., Robin, R. W., et al. (2008). Interaction between a functional MAOA locus and childhood sexual abuse predicts alcoholism and antisocial personality disorder in adult women. *Mol. Psychiatry* 13, 334–347.

Ellis, B. J., Boyce, W. T., Belsky, J., Bakermans-Kranenburg, M. J., and van Ijzendoorn, M. H. (2011). Differential susceptibility to the environment: an evolutionary–neurodevelopmental theory. *Dev. Psychopathol.* 23, 7–28. doi: 10.1017/S0954579410000611

Fan, J., Fossella, J., Sommer, T., Wu, Y., and Posner, M. I. (2003). Mapping the genetic variation of executive attention onto brain activity. *Proc. Natl. Acad. Sci. U.S.A.* 100, 7406–7411. doi: 10.1073/pnas.0732088100

Fergusson, D. M., Boden, J. M., Horwood, L. J., Miller, A. L., and Kennedy, M. A. (2011). MAOA, abuse exposure and antisocial behaviour: 30-year longitudinal study. *Br. J. Psychiatry* 198, 457–463.

Fergusson, D. M., Boden, J. M., Horwood, L. J., Miller, A., and Kennedy, M. A. (2012). Moderating role of the MAOA genotype in antisocial behaviour. *Br. J. Psychiatry* 200, 116–123. doi: 10.1192/bjp.bp.111.093328

Fite, P. J., Brown, S., Hossain, W., Manzardo, A., Butler, M. G., and Bortolato, M. (2018). Tobacco and cannabis use in college students are predicted by sex-dimorphic interactions between MAOA genotype and child abuse. *CNS Neurosci. Ther.* 25 (1), 101–11. doi: 10.1111/cns.13002

Fitzgerald, P. J. (2013). Elevated norepinephrine may be a unifying etiological factor in the abuse of a broad range of substances: alcohol, nicotine, marijuana, heroin, cocaine, and caffeine. *Subst. Abuse.* 7, 171–183. doi: 10.4137/SART.S13019

Frazzetto, G., Di Lorenzo, G., Carola, V., Proietti, L., Sokolowska, E., Siracusano, A., et al. (2007). Early trauma and increased risk for physical aggression during adulthood: The moderating role of MAOA genotype. *PloS One* 2, e486. doi: 10.1371/journal.pone.0000486

Funk, R. R., McDermeit, M., Godley, S. H., and Adams, L. (2003). Maltreatment issues by level of adolescent substance abuse treatment: the extent of the problem at intake and relationship to early outcomes. *Child Maltreat* 8, 36–45. doi: 10.1177/1077559502239607

Galaif, E. R., Stein, J. A., Newcomb, M. D., and Bernstein, D. P. (2001). Gender differences in the prediction of problem alcohol use in adulthood: Exploring the influence of family factors and childhood maltreatment. *J. @ Stud. Alcohol.* 62 (4), 486–493. doi: 10.15288/jsa.2001.62.486

Gardner, T. W., Dishion, T. J., and Connell, A. M. (2008). Adolescent self-regulation as resilience: resistance to antisocial behavior within the deviant peer context. *J. Abnorm. Child Psychol.* 36, 273–284. doi: 10.1007/s10802-007-9176-6

Gillespie, S. M., Brzozowski, A., and Mitchell, I. J. (2018). Self-regulation and aggressive antisocial behaviour: insights from amygdala-prefrontal and heart-brain interactions. *Psychol. Crime Law* 24, 243–257.). doi: 10.1080/1068316X.2017.1414816

Gledhill-Hoyt, J., Lee, H., Strote, J., and Wechsler, H. (2000). Increased use of marijuana and other illicit drugs at US colleges in the 1990s: results of three national surveys. *Addiction* 95, 1655–1667. 10.1046/j.1360-0443.2000.951116556.x

Godar, S. C., Fite, P. J., McFarlin, K. M., and Bortolato, M. (2016). The role of monoamine oxidase a in aggression: current translational developments and future challenges. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 69, 90–100. doi: 10.1016/j.pnpbp.2016.01.001

Godar, S. C., Mosher, L. J., Scheggi, S., Devoto, P., Moench, K. M., Strathman, H. J., et al. (2019). Gene-environment interactions in antisocial behavior are mediated by early-life 5-HT(2A) receptor activation. *Neuropharmacol.* 159, 107513. doi: 10.1016/j.neuropharm.2019.01.028

Goldstein, A. L., Faulkner, B., and Wekerle, C. (2013). The relationship among internal resilience, smoking, alcohol use, and depression symptoms in emerging adults transitioning out of child welfare. *Child Abuse Negl.* 37 (1), 22–32. doi: 10.1016/j.chiabu.2012.08.007

Guindalini, C., Scivoletto, S., Ferreira, R. G., Nishimura, A., Zilberman, M. L., Peluso, M. M., et al. (2005). Association of MAO A polymorphism and alcoholism in Brazilian females. *Psychiatr. Genet.* 15, 141–144. doi: 10.1097/00041444-200506000-00011

Guo, G., Wilhelmsen, K., and Hamilton, N. (2007). Gene-lifecourse interaction for alcohol consumption in adolescence and young adulthood: five monoamine genes. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 144B (4), 417–423. doi: 10.1002/ajmg.b.30340

Guo, G., Ou, X. M., Roettger, M., and Shih, J. C. (2008). The VNTR 2 repeat in MAOA and delinquent behavior in adolescence and young adulthood: associations and MAOA promoter activity. *Eur. J. Hum. Genet.* 16, 626–634. doi: 10.1038/sj.ejhg.5201999

Guyer, A. E., Nelson, E. E., Perez-Edgar, K., Hardin, M. G., Roberson-Nay, R., Monk, C. S., et al. (2006). Striatal functional alteration in adolescents characterized by early childhood behavioral inhibition. *J. Neurosci.* 26, 6399–6405. doi: 10.1523/JNEUROSCI.0666-06.2006

Harro, J., and Oreland, L. (2016). The role of MAO in personality and drug use. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 69, 101–111. doi: 10.1016/j.pnpbp.2016.02.013

Herman., A. I., Kaiss, K. M., Ma, R., Philbeck, J. W., Hasan, A., Dasti, H., et al. (2005). Serotonin transporter promoter polymorphism and monoamine oxidase type A VNTR allelic variants together influence alcohol binge drinking risk in young women. *Am. J. Med. Genet. B. Neuropsychiatr. Genet.* 133B, 74–78. doi: 10.1002/ajmg.b.30135

Hofmann, W., Schmeichel, B. J., and Baddeley, A. D. (2012). Executive functions and self-regulation. *Trends Cogn. Sci.* 16, 174–180. doi: 10.1016/j.tics.2012.01.006

Holmes, A. J., Hollinshead, M. O., Roffman, J. L., Smoller, J. W., and Buckner, R. L. (2016). Individual differences in cognitive control circuit anatomy link sensation seeking, impulsivity, and substance use. *J. Neurosci.* 36, 4038–4049. doi: 10.1523/JNEUROSCI.3206-15.2016

Holz, N., Boecker, R., Buchmann, A. F., Blomeyer, D., Baumeister, S., Hohmann, S., et al. (2016). Evidence for a sex-dependent maoa× childhood stress interaction in the neural circuitry of aggression. *Cereb. Cortex* 26, 904–914. doi: 10.1093/cercor/bhu249

Huang, Y. Y., Cate, S. P., Battistuzzi, C., Oquendo, M. A., Brent, D., and Mann, J. J. (2004). An association between a functional polymorphism in the monoamine

oxidase a gene promoter, impulsive traits and early abuse experiences. *Neuropsychopharmacology* 29, 1498–1505. doi: 10.1038/sj.npp.1300455

Huizinga, D., Haberstick, B. C., Smolen, A., Menard, S., Young, S. E., Corley, R. P., et al. (2006). Childhood maltreatment, subsequent antisocial behavior, and the role of monoamine oxidase a genotype. *Biol. Psychiatry* 60, 677–683. doi: 10.1016/j.biopsych.2005.12.022

Jönsson, E. G., Norton, N., Gustavsson, J. P., Oreland, L., Owen, M. J., and Sedvall, G. C. (2000). A promoter polymorphism in the monoamine oxidase a gene and its relationships to monoamine metabolite concentrations in CSF of healthy volunteers. *J. Psychiatr. Res.* 34, 239–244. doi: 10.1016/s0022-3956(00)00013-3

Jackson, K. M., Sher, K. J., and Schulenberg, J. E. (2008). Conjoint developmental trajectories of young adult substance use. *Alcohol Clin. Exp. Res.* 32, 723–737. doi: 10.1111/j.1530-0277.2008.00643.x

Johnston, L. D., O'Malley, P. M., and Bachman, J. G. (2004). *Monitoring the future: National survey results on drug use, 1975-2002. Volume II, college students and adults*. Ages 19–45. (Bethesda, MD: National Institute on Drug Abuse), 19–40.

Kilpatrick, D. G., Saunders, B. E., and Smith, D. W. (2003). *Youth Victimization: Prevalence and Implications [Electronic]* (Office of Justice Program, National Institute of Justice: U.S. Department of Justice).

Kim-Cohen, J., Caspi, A., Taylor, A., Williams, B., Newcombe, R., Craig, I. W., et al. (2006). MAOA, maltreatment, and gene-environment interaction predicting children's mental health: new evidence and a meta-analysis. *Mol. Psychiatry* 11, 903–913. doi: 10.1038/sj.mp.4001851

Koyama, M. S., Parvaz, M. A., and Goldstein, R. Z. (2017). The adolescent brain at risk for substance use disorders: a review of functional MRI research on motor response inhibition. *Curr. Opin. Behav. Sci.* 13, 186–195. doi: 10.1016/j.cobeha.2016.12.006

Leeb, R. T., Paulozzi, L., Melanson, C., Simon, T., and Arias, I. (2008). *Child maltreatment surveillance: Uniform definitions for public health and recommended data elements, Version 1.0* (Atlanta, GA): Centers for Disease Control and Prevention, National Center for Injury Prevention and Control).

Lipari, R. N., and Jean-Francois, B. A. (2016). *Day in the Life of College Students Aged 18 to 22*, Substance use facts. The CBHSQ Report: May 26 (2016). Rockville, MD Center for Behavioral Health Statistics and Quality, Substance Abuse and Mental Health Services Administration.

Müller, C. P., and Homberg, J. R. (2015). The role of serotonin in drug use and addiction. *Behav. Brain Res.* 277, 146–192. doi: 10.1016/j.bbr.2014.04.007

Martin, C. S., Clifford, P. R., and Clapper, R. L. (1992). Patterns and predictors of simultaneous and concurrent use of alcohol, tobacco, marijuana, and hallucinogens in first-year college students. *J. Subst. Abuse.* 4 (3), 319–326. doi: 10.1016/0899-3289(92)90039-z

Martinotti, G., Carli, V., Tedeschi, D., Di Giannantonio, M., Roy, A., Janiri, L., et al. (2009). Mono- and polysubstance dependent subjects differ on social factors, childhood trauma, personality, suicidal behaviour, and comorbid Axis I diagnoses. *Addict. Behav.* 34, 790–793. doi: 10.1016/j.addbeh.2009.04.012

Mason, W. A., and Windle, M. (2002). Reciprocal relations between adolescent substance use and delinquency: a longitudinal latent variable analysis. *J. Abnorm. Psychol.* 111, 63–76.

McCabe, S. E., Cranford, J. A., Morales, M., and Young, A. (2006). Simultaneous and concurrent polydrug use of alcohol and prescription drugs: prevalence, correlates, and consequences. *J. Stud. Alcohol.* 67, 529–537. doi: 10.15288/jsa.2006.67.529

McCormick, R. A., and Smith, M. (1995). Aggression and hostility in substance abusers: the relationship to abuse patterns, coping style, and relapse triggers. *Addict. Behav.* 20, 555–562. doi: 10.1016/0306-4603(95)00015-5

McGrath, L. M., Mustanski, B., Metzger, A., Pine, D. S., Kistner-Griffin, E., Cook, E., et al. (2012). A latent modeling approach to genotype-phenotype relationships: maternal problem behavior clusters, prenatal smoking, and MAOA genotype. *Arch. Womens Ment. Health* 15, 269–282. doi: 10.1007/s00737-012-0286-y

Meyer-Lindenberg, A., Buckholtz, J. W., Kolachana, B. R., Hariri, A., Pezawas, L., Blasi, G., et al. (2006). Neural mechanisms of genetic risk for impulsivity and violence in humans. *Proc. Natl. Acad. Sci. U.S.A.* 103, 6269–6274. doi: 10.1073/pnas.0511311103

Mohler-Kuo, M., Lee, J. E., and Wechsler, H. (2003). Trends in marijuana and other illicit drug use among college students: results from 4 Harvard School of Public Health College Alcohol Study surveys: 1993-2001. *J. Am. Coll. Health* 52, 17–24. doi: 10.1080/07448480309595719

Moss, H. B., Chen, C. M., and Yi, H. (2014). Early adolescent patterns of alcohol, cigarette, and marijuana polysubstance use and young adulth substance use outcomes in a nationally representative sample. *Drug Alcohol Dep.* 136, 51–62. doi: 10.1016/j.drugalcdep.2013.12.011

National Center on Addiction and Substance Abuse at Columbia University (2007). *Wasting the Best and the Brightest: Substance Abuse at America"s Colleges and Universities* (New York: CASA).

Nikulina, V., Widom, C. S., and Brzustowicz, L. M. (2012). Child abuse and neglect, MAOA, and mental health outcomes: a prospective examination. *Biol. Psychiatry* 71, 350–357. doi: 10.1016/j.biopsych.2011.09.008

Nilsson, K. W., Comasco, E., Åslund, C., Nordquist, N., Leppert, J., and Oreland, L. (2011). MAOA genotype, family relations and sexual abuse in relation to adolescent alcohol consumption. *Addict. Biol.* 16, 347–355. doi: 10.1111/j.1369-1600.2010.00238.xf

O'Grady, K. E., Arria, A. M., Fitzelle, D. M., and Wish, E. D. (2008). Heavy drinking and polydrug use among college students. *J. Drug Issues* 38, 445–466. doi: 10.1177/002204260803800204

Passamonti, L., Cerasa, A., Gioia, M. C., Magariello, A., Muglia, M., Quattrone, A., et al. (2008). Genetically dependent modulation of serotonergic inactivation in the human prefrontal cortex. *Neuroimage* 40, 1264–1273. doi: 10.1016/j.neuroimage.2007.12.028.

Pentz, M. A., Dwyer, J. H., MacKinnon, D. P., Flay, B. R., Hansen, W. B., Wang, E. Y., et al. (1989). A multicommunity trial for primary prevention of adolescent drug abuse: effects on drug use prevalence. *J. Am. Med. Assoc.* 262 (22), 3259–3266.

Posner, M. I., and Rothbart, M. K. (1998). Attention, self-regulation and consciousness. *Philos. Trans. R. Soc Lond. B. Biol. Sci.* 353, 1915–1927. doi: 10.1098/rstb.1998.0344

Posner, M. I., Rothbart, M. K., Sheese, B. E., and Tang, Y. (2007). The anterior cingulate gyrus and the mechanism of self-regulation. *Cogn. Affect. Behav. Neurosci.* 7, 391–395. doi: 10.3758/cabn.7.4.391

Reichert, E. L., and Flannery-Schroeder, E. (2014). Posttraumatic cognitions as mediators between childhood maltreatment and poorer mental health among young adults. *J. Child Adol. Trauma.* 7, 153–162. doi: 10.1007/s40653-014-0021-0

Rende, R. (2011). Interaction of genes and environment in adolescent substance use: opportunities and implications for intervention. *Mind Brain* 2 (1), 50–55.

Sabol, S. Z., Hu, S., and Hamer, D. (1998). A functional polymorphism in the monoamine oxidase a gene promoter. *Hum. Genet.* 103, 273–279. doi: 10.1007/s004390050816

Samochowiec, J., Lesch, K. P., Rottmann, M., Smolka, M., Syagailo, Y. V., Okladnova, O., et al. (1999). Association of a regulatory polymorphism in the promoter region of the monoamine oxidase a gene with antisocial alcoholism. *Psychiatry Res.* 86, 67–72. doi: 10.1016/s0165-1781(99)00020-7

Schmidt, L. G., Sander, T., Kuhn, S., Smolka, M., Rommelspacher, H., Samochowiec, J., et al. (2000). Different allele distribution of a regulatory MAOA gene promoter polymorphism in antisocial and anxious-depressive alcoholics. *J. Neural Transm.* 107, 681–689. doi: 10.1007/s007020070069

Sjöberg, R. L., Nilsson, K. W., Wargelius, H. L., Leppert, J., Lindström, L., and Oreland, L. (2007). Adolescent girls and criminal activity: Role of MAOA-LPR genotype and psychosocial factors. *Am. J. Med. Genet. B. Neuropsychiatr. Genet.* 144B, 159–164. doi: 10.1002/ajmg.b.30360

Steinberg, L. (2008). A social neuroscience perspective on adolescent risk-taking. *Dev. Rev.* 28, 78–106. doi: 10.1016/j.dr.2007.08.002

Stogner, J. M., and Gibson, C. L. (2013). Stressful life events and adolescent drug use: moderating influences of the MAOA gene. *J. Crim. Just.* 41, 357–363. doi: 10.1016/j.jcrimjus.2013.06.003

Trentacosta, C. J., and Shaw, D. S. (2009). Emotional self-regulation, peer rejection, and antisocial behavior: developmental associations from early childhood to early adolescence. *J. Appl. Dev. Psychol.* 30, 356–365. doi: 10.1016/j.appdev.2008.12.016

Trenz, R. C., Scherer, M., Harrell, P., Zur, J., Sinha, A., and Latimer, W. (2012). Early onset of drug and polysubstance use as predictors of injection drug use among adult drug users. *Addictive Beh.* 37, 367–372. doi: 10.1016/j.addbeh.2011.11.011

Uhl, G. R., Liu, Q. R., Walther, D., Hess, J., and Naiman, D. (2001). Polysubstance abuse-vulnerability genes: genome scans for association, using 1,004 subjects and 1,494 single-nucleotide polymorphisms. *Am. J. Hum. Genet.* 69, 1290–1300. doi: 10.1086/324467

Vanyukov, M. M., Moss, H. B., Yu, L. M., Tarter, R. E., and Deka, R. (1995). Preliminary evidence for an association of a dinucleotide repeat polymorphism at the MAOA gene with early onset al.coholism/substance abuse. *Am. J. Med. Genet. Neuropsychiat Genet.* 60, 122–126. doi: 10.1002/ajmg.1320600207

Vanyukov, M. M., Maher, B. S., Devlin, B., Tarter, R. E., Kirillova, G. P., Yu, L. M., et al. (2004). Haplotypes of the monoamine oxidase genes and the risk for substance use disorders. *Am. J. Med. Genet. B. Neuropsychiatr. Genet.* 125B, 120–125. doi: 10.1002/ajmg.b.20105

Vaughn, M. G., Beaver, K. M., DeLisi, M., Perron, B. E., and Schelbe, L. (2009). Gene-environment interplay and the importance of self-control in predicting polydrug use and substance-related problems. *Addict. Behav.* 34, 112–116. doi: 10.1016/j.addbeh.2008.08.011

Verhoeven, F. E., Booij, L., Kruijt, A. W., Cerit, H., Antypa, N., and Does, W. (2012). The effects of MAOA genotype, childhood trauma, and sex on trait and state-dependent aggression. *Brain Behav.* 2, 806–813. doi: 10.1002/brb3.96

Vink, J. M. (2016). Genetics of addiction: future focus on gene × environment interaction? *J. Stud. Alcohol Drugs* 77, 684–687. doi: 10.15288/jsad.2016.77.684

Volkow, N. D., Fowler, J. S., Wang, G. J., Swanson, J. M., and Telang, F. (2007). Dopamine in drug abuse and addiction: results of imaging studies and treatment implications. *Arch. Neurol.* 64, 1575–1579. doi: 10.1001/archneur.64.11.1575

Williams, L. M., Gatt, J. M., Kuan, S. A., Dobson-Stone, C., Palmer, D. M., Paul, R. H., et al. (2009). A polymorphism of the MAOA gene is associated with emotional brain markers and personality traits on an antisocial index. *Neuropsychopharmacology* 34, 1797–1809. doi: 10.1038/npp.2009.1

Zuckerman, M. (1999). "Diathesis-stress models," in *Vulnerability to psychopathology: A biosocial model.* Ed. M. Zuckerman (Washington, DC, US: American Psychological Association), 3–23.

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Environmental Tobacco Smoke During the Early Postnatal Period of Mice Interferes With Brain ^{18}F-FDG Uptake From Infancy to Early Adulthood – A Longitudinal Study

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Exposure to environmental tobacco smoke (ETS) is associated with high morbidity and mortality, mainly in childhood. Our aim was to evaluate the effects of postnatal ETS exposure in the brain 2-deoxy-2-[^{18}F]-fluoro-D-glucose (^{18}F-FDG) uptake of mice by positron emission tomography (PET) neuroimaging in a longitudinal study. C57BL/6J mice were exposed to ETS that was generated from 3R4F cigarettes from postnatal day 3 (P3) to P14. PET analyses were performed in male and female mice during infancy (P15), adolescence (P35), and adulthood (P65). We observed that ETS exposure decreased ^{18}F-FDG uptake in the whole brain, both left and right hemispheres, and frontal cortex in both male and female infant mice, while female infant mice exposed to ETS showed decreased ^{18}F-FDG uptake in the cerebellum. In addition, all mice showed reduced ^{18}F-FDG uptake in infancy, compared to adulthood in all analyzed VOIs. In adulthood, ETS exposure during the early postnatal period decreased brain ^{18}F-FDG uptake in adult male mice in the cortex, striatum, hippocampus, cingulate cortex, and thalamus when compared to control group. ETS induced an increase in ^{18}F-FDG uptake in adult female mice when compared to control group in the brainstem and cingulate cortex. Moreover, male ETS-exposed animals showed decreased ^{18}F-FDG uptake when compared to female ETS-exposed in the whole brain, brainstem, cortex, left amygdala, striatum, hippocampus, cingulate cortex, basal forebrain and septum, thalamus, hypothalamus, and midbrain. The present study shows that several brain regions are vulnerable to ETS exposure during the early postnatal period and these

effects on ^{18}F-FDG uptake are observed even a long time after the last exposure. This study corroborates our previous findings, strengthening the idea that exposure to tobacco smoke in a critical period interferes with brain development of mice from late infancy to early adulthood.

Keywords: environmental tobacco smoke, passive smoke, neuroimaging, positron emission tomography, ^{18}F-FDG uptake, glucose metabolism, longitudinal study, brain

INTRODUCTION

Exposure to environmental tobacco smoke (ETS), one of the most common indoor pollutants, is composed of both mainstream and sidestream smoke. Approximately 40% of children in the world are exposed to ETS, which is related to allergic reactions in the short-term, while it is associated to acute myocardial infarction, lung cancer, and chronic obstructive pulmonary disease in the long term (Oberg et al., 2011).

Clinical studies show that ETS leads to behavioral disorders and deleterious effects on the brain. The exposure to ETS is related to attention deficits and hyperactive behavior during childhood (Pagani, 2014), while maternal smoke during lactation causes sleep and wake disruption (Banderali et al., 2015). Also, paternal smoke in the early postnatal period of childhood has been linked with perinatal mortality, respiratory disease, neurobehavioral problems, decreased academic performance, and brain tumors (Plichart et al., 2008; Hwang et al., 2012). Adolescents exposed to tobacco smoke during prenatal period show distinct brain function in the working memory and alterations in the brain volume, especially in the cerebellum (de Zeeuw et al., 2012; Bennett et al., 2013). In rodents, exposure to mainstream smoke during a critical period of brain development leads to hyperactivity and aggressive behavior (Yochum et al., 2014), while exposure to ETS disturbs cognitive functions, synaptic proteins, and myelination process from late infancy to early adulthood (Torres et al., 2015a,b).

Positron emission tomography (PET) is a molecular imaging technique that enables studying brain function *in vivo*. The 2-deoxy-2-[^{18}F]-fluoro-D-glucose (^{18}F-FDG) has been widely used to evaluate changes in cerebral glucose metabolism. ^{18}F-FDG is required in metabolically active tissues, and the metabolic activity of a brain region is directly proportional to the amount of ^{18}F-FDG that accumulates in this region (Sokoloff et al., 1977; Welch et al., 2013).

Relatively few studies evaluated the effects of tobacco smoke on brain glucose metabolism by PET imaging and focuses on dependence by nicotine in humans. In a context of tobacco craving and exposure to cues that are related to tobacco, heavy smokers showed rise in glucose metabolism in the anterior cingulate gyrus, orbitofrontal cortex, dorsolateral prefrontal cortex, anterior insula, and sensorimotor cortex (Brody et al., 2002). In addition, smokers treated with bupropion, a norepinephrine and dopamine reuptake inhibitor, showed decrease in glucose metabolism in the anterior cingulate cortex (Brody et al., 2004). Costello et al. (2010) reported that bupropion and practical group counseling reduce glucose metabolism in the posterior cingulate gyrus, with association between cigarette use and ^{18}F-FDG uptake in the occipital gyrus and parietal–temporal junction (Costello et al., 2010). However, there is still a lack of studies evaluating the effects of tobacco smoke on glucose metabolism during the brain development period. Thus, our aim was to investigate the effects of ETS during the early postnatal period on glucose metabolism in a longitudinal preclinical study, by ^{18}F-FDG PET imaging during mice infancy, adolescence, and adulthood.

MATERIALS AND METHODS

Animals

C57BL/6 mice were obtained from the animal facility of the School of Medicine of University of São Paulo and were housed at 20–22°C with a 12 h/12 h light/dark cycle with water and commercial pellet food for small rodents from Nuvital (Nuvilab CR-1; Colombo, Brazil) *ad libitum*. All of the procedures were approved by the Ethics Committee of the School of Medicine (027/14) and the School of Pharmaceutical Sciences (P446/14), University of São Paulo.

Experimental Design

The size of each litter was randomly adjusted to six to seven pups within the first day after delivery, as previously described by Torres et al. (2015b). The C57BL/6 pups were exposed to ETS as described by Lobo-Torres et al. (2012). Briefly, the pups, together with their mothers, were subjected to two exposure sessions per day of 1-h each (1 h at 8 a.m. and 1 h at 5 p.m.) to a mixture of mainstream and sidestream tobacco smoke from reference cigarettes 3R4F (College of Agriculture, University of Kentucky). The exposure was performed from the 3rd (P3) to the 14th (P14) days of life, within a chamber measuring 564 × 385 × 371 mm. The levels of CO in the chamber during the exposure (470.2 ± 90.93 ppm) and measurements of the exposure biomarkers (COHb: 21.62 ± 1.80%; plasma nicotine: 139.94 ± 13.02 ng/mL; plasma cotinine: 113.65 ± 16.78 ng/mL) were similar to previous studies from our group (Torres et al., 2015a,b). Control subjects were exposed to the same experimental conditions but inhaled compressed air only.

Based on Vanhove et al. (2015), the number of animals required for imaging studies for changes about 20–25% is four to six animals. Thus, in the present study, we opted to use five animals of each sex in each group. Nonetheless, we used the isogenic C57Bl/6 mice in order to reduce intra-animal variability.

After the exposure period, 19 animals were used from P15 to P65 to evaluate the regional brain metabolism of the animals with ^{18}F-FDG on PET/CT during infancy (P15; n = 5 females

ETS-exposed and $n = 4$ females control; $n = 5$ males ETS-exposed and $n = 5$ males control), adolescence (P35; $n = 4$ females ETS-exposed and $n = 4$ females control; $n = 5$ males ETS-exposed and $n = 5$ males control), and adulthood (P65; $n = 5$ females ETS-exposed and $n = 4$ females control; $n = 5$ males ETS-exposed and $n = 5$ males control) in a longitudinal study.

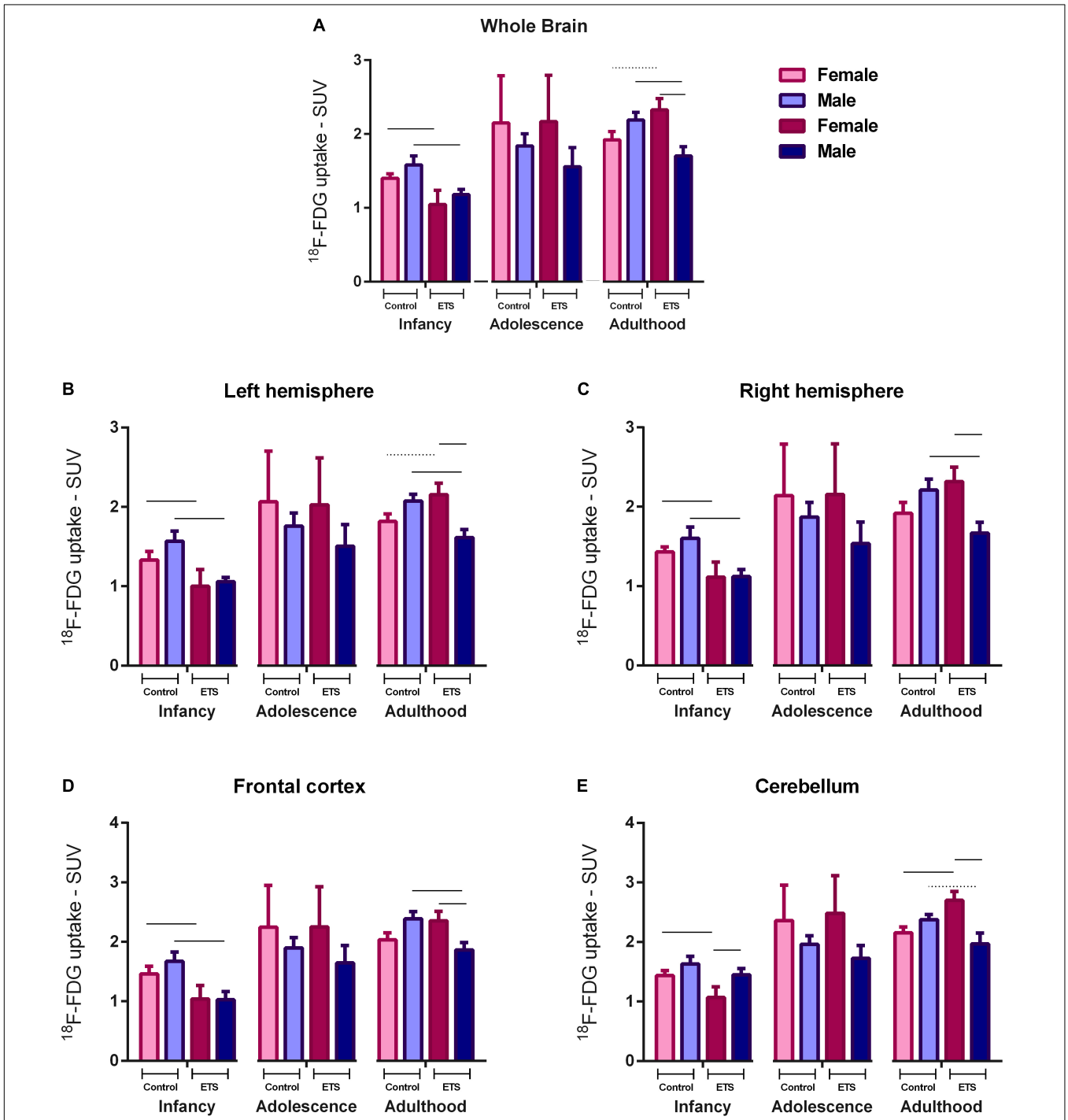

FIGURE 1 | ^{18}F-FDG uptake in the whole brain **(A)**, left hemisphere **(B)**, right hemisphere **(C)**, frontal cortex **(D)**, and cerebellum **(E)** for female and male infant ($n = 5$ females ETS-exposed and $n = 4$ females control; $n = 5$ males ETS-exposed and $n = 5$ males control), adolescent ($n = 4$ females ETS-exposed and $n = 4$ females control; $n = 5$ males ETS-exposed and $n = 5$ males control), and adult ($n = 5$ females ETS-exposed and $n = 4$ females control; $n = 5$ males ETS-exposed and $n = 5$ males control) mice exposed to ETS during the early postnatal period. Three-way mixed ANOVA with repeated measures (groups × VOIs) and *post hoc* paired *t*-test (Bonferroni) with multiple comparison correction. *Continuous bar:* $p < 0.05$. *Dashed bar:* trend toward statistical significance.

^{18}F-FDG-PET/CT Imaging

Positron emission tomography/CT images were acquired using a protocol modified from Welch et al. (2013). Briefly, animals received about 19 MBq (18.86 ± 2.70) of ^{18}F-FDG intraperitoneally (i.p.). After 65 (67 ± 5) min of injection (biodistribution period of the radiotracer), animals were anesthetized with isoflurane (2% in O_2) and positioned in an equipment bed with the brain in the center of the field of view (FOV). The scanner used was an Albira PET-SPECT-CT (Bruker Biospin, Valencia, Spain), for small animals. The static PET image was acquired for 50 min with 94.4 mm of trans-axial FOV. A CT scan was acquired immediately after, with 400 projections, 45 kVp, and 400 μA and magnification factor of 1.46. After acquisitions, PET images were reconstructed using maximum-likelihood expectation–maximization (MLEM), with 12 iterations, and corrected for radioactive decay, scatter, and random, but not for attenuation. CT was reconstructed using filtered back projection (FBP) algorithm.

PET Image Analysis

Positron emission tomography image analysis was performed with PMOD 3.4 software (PMOD^{TM} Technologies Ltd., Switzerland). The scans were manually co-registered to: (1) own animal CT for analysis in the different animals' age and (2) to a T2 weighted MRI template (available in the PMOD software) in the adult animals' analysis to facilitate the identification of different brain regions.

In the analysis of the brain in the different ages, manual volumes of interest (VOIs) were drawn in the PET images fused to the CT (Zovein et al., 2004). Due to the small size of the infant animals' brain, to allow comparison with adolescent and adult mice, the VOIs for all ages were defined as whole brain, left and right brain hemispheres, frontal cortex, and cerebellum, always using the skull defined by the CT as a border line. When analysis was restricted to adult animals, PET image was co-registered to the MRI template and different brain regions considered in the analysis (whole brain, brainstem, cortex, cerebellum, left and right amygdala left and right striatum, left and right hippocampus, cingulate cortex, basal forebrain and septum, thalamus, hypothalamus, and left and right midbrain).

The ^{18}F-FDG uptake is presented as a standardized uptake value (SUV) which is calculated as radioactivity concentration (kBq/cc) divided by the ratio between injected dose (kBq) and animal body weight (g).

Statistical Analysis

As in young animals it is difficult to analyze small brain areas, due to the limited PET imaging spatial resolution, and in order to compare infancy, adolescence, and adulthood, we analyzed brain glucose metabolism in the following brain areas: whole brain, left and right brain hemispheres, frontal cortex, and cerebellum. Thus, we performed a three-way mixed ANOVA with repeated measures, considering groups as between and time and VOIs (whole brain, frontal cortex, cerebellum, right hemisphere, and left hemisphere) in infancy, adolescence, and adulthood as within-subject factors. Bonferroni *post hoc* test with multiple comparison correction was performed to test ^{18}F-FDG uptake differences between the time points and groups for each VOI (**Figure 1**). PET imaging of adult animals was analyzed by a two-way ANOVA, considering groups as between and VOIs (whole brain, brainstem, cortex, cerebellum, amygdala, striatum, hippocampus, cingulate cortex, basal forebrain and septum, thalamus, hypothalamus, and midbrain) as within-subject factors. Bonferroni *post hoc* with multiple comparison correction was performed to test ^{18}F-FDG uptake differences between the groups for each VOI (**Figure 3**). The data were analyzed using SPSS Statistics 20 Software, Armonk, NY: IBM Corp., United States and data were plotted using GraphPad Prism 6 Software, La Jolla, CA, United States. Results are presented as mean ± standard error. Differences with a probability of 95% ($p < 0.05$) were considered statistically significant.

RESULTS

ETS During the Early Postnatal Period Decreased Brain ^{18}F-FDG Uptake in Infant Mice

Positron emission tomography scan data of glucose uptake for male and female infant, adolescent, and adult mice exposed to ETS during the early postnatal period were analyzed by a three-way mixed ANOVA with repeated measures (groups × VOIs)

TABLE 1 | Detailed description of the statistical analysis of ^{18}F-FDG uptake in infancy, adolescence, and adulthood mice in distinct brain regions.

VOI	^{18}F-FDG uptake		
	ETS-exposed	ETS-exposed vs. control	
	Male vs. female	Male	Female
Infancy			
Whole brain	ns	↓ $p = 0.020$	↓ $p = 0.013$
Left hemisphere	ns	↓ $p = 0.010$	↓ $p = 0.026$
Right hemisphere	ns	↓ $p = 0.013$	↓ $p = 0.036$
Frontal cortex	ns	↓ $p = 0.010$	↓ $p = 0.031$
Cerebellum	↓ $p = 0.014$	ns	↓ $p = 0.021$
Adolescence			
Whole brain	ns	ns	ns
Left hemisphere	ns	ns	ns
Right hemisphere	ns	ns	ns
Frontal cortex	ns	ns	ns
Cerebellum	ns	ns	ns
Adulthood			
Whole brain	↓ $p = 0.060$	↓ $p = 0.019$	↑ $p = 0.068$
Left hemisphere	↓ $p = 0.060$	↓ $p = 0.011$	↑ $p = 0.072$
Right hemisphere	↓ $p = 0.015$	↓ $p = 0.024$	ns
Frontal cortex	↓ $p = 0.026$	↓ $p = 0.015$	ns
Cerebellum	↓ $p = 0.040$	↓ $p = 0.062$	↑ $p = 0.029$

Each p-value is adjusted for multiple comparisons. ↓, decreased ^{18}F-FDG uptake; ↑, increased ^{18}F-FDG uptake.

and Bonferroni *post hoc* test with multiple comparison correction. We found a significant effect for the factors VOIs ($F_{4,56} = 33.7333$; $p < 0.00010$) and time ($F_{2,8} = 10.99$; $p < 0.0001$), and a significant VOIs × time interaction ($F_{8,112} = 4.592$, $p < 0.0001$). We also had a significant VOIs × time × group interaction ($F_{24,112} = 1.753$; $p < 0.05$).

The *post hoc* analysis revealed that all mice showed reduced ^{18}F-FDG uptake in infancy, compared to adulthood in all analyzed VOIs. Regarding the females of ETS group, the ^{18}F-FDG uptake was also lower during infancy when compared to adolescence. As detailed at **Table 1**, in infancy, both male and female mice ETS-exposed had lower ^{18}F-FDG uptake in the whole brain, left and right hemisphere and frontal cortex, compared to the control group. Females showed decreased ^{18}F-FDG uptake in the cerebellum (**Figure 1** and **Table 1**). When mice reached adulthood, males showed a reduction in ^{18}F-FDG uptake when compared to controls in all VOIs analyzed (**Figure 1** and **Table 1**). However, in the females a different pattern occurred, as they had higher ^{18}F-FDG uptake in the whole brain, left hemisphere, and cerebellum, when compared to the control group (**Figure 1** and **Table 1**). When both ETS-exposed groups were compared, the *post hoc* analysis showed a reduction in ^{18}F-FDG uptake in the males in all the VOIs evaluated when compared to

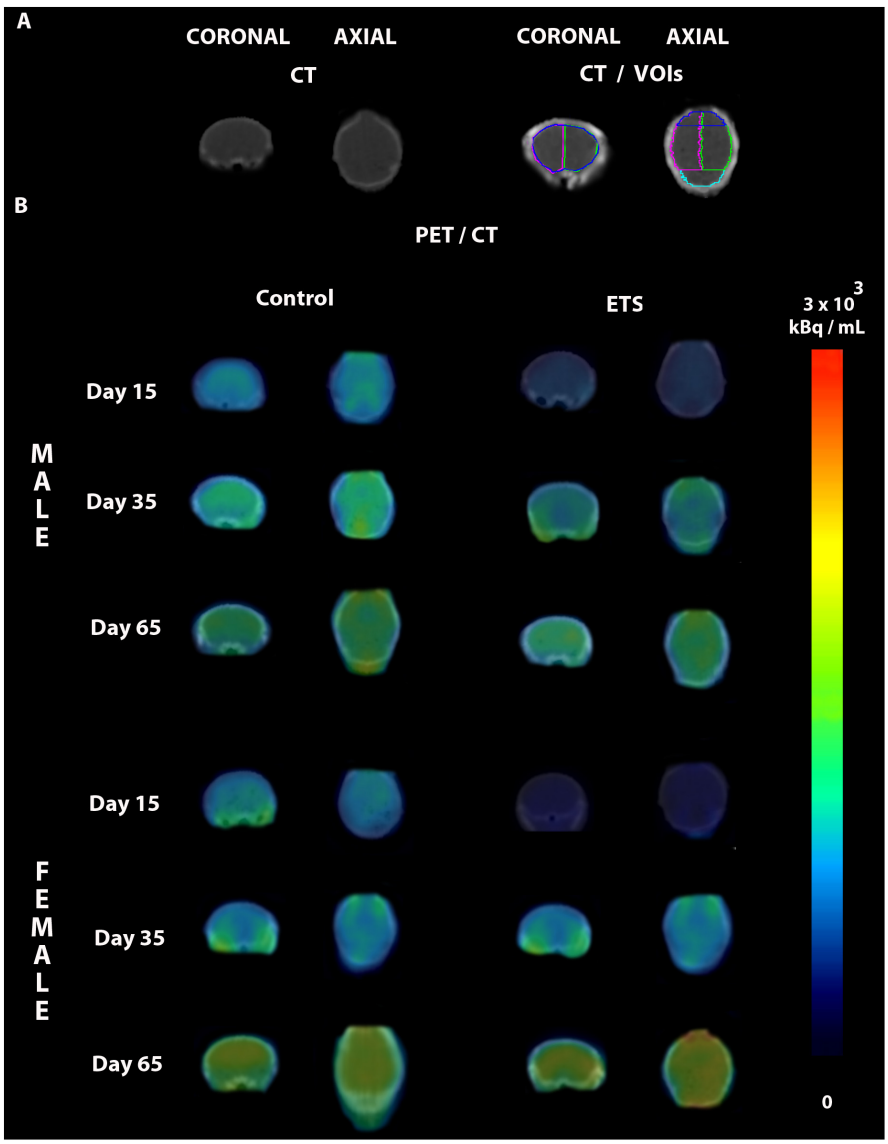

FIGURE 2 | Representative PET/CT brain images of male and female mice exposed to ETS during the early postnatal period and the control group. **(A)** CT images in different anatomical planes in the top left, and CT with the drawn volumes of interest (VOIs) used for quantification in the top right (whole brain, left hemisphere, right hemisphere, frontal cortex, and cerebellum). **(B)** PET images fused to the CT images of infant (Day 15), adolescent (Day 35), and adult (Day 65) male and female mice of the control and ETS groups.

females (**Figure 1** and **Table 1**). **Figure 2** shows a representative brain PET/CT scans of mice exposed to ETS during the early postnatal period and the control group during infancy, adolescence, and adulthood.

ETS During the Early Postnatal Period Decreased ^{18}F-FDG Uptake in Adult Male Mice in Distinct Brain Regions

Positron emission tomography scan data of glucose uptake for male and female adult mice exposed to ETS during the early postnatal period were analyzed by two-way ANOVA (VOIs × treatment) with Bonferroni *post hoc* test with *p*-values corrected for multiplicity. We found a significant effect for VOIs ($F_{15,240} = 7.101$, $p < 0.0001$) and treatment ($F_{3,240} = 59.5$, $p < 0.0001$) factors; however, the interaction between them was not significant ($F_{45,240} = 0.033$, $p > 0.999$; see **Table 2** for detailed description of the statistical analysis).

The *post hoc* analysis showed that exposure to ETS during the early postnatal period decreased ^{18}F-FDG uptake in adult male mice when compared with adult female mice in the whole brain, brainstem, left amygdala, left and right striatum, left and right hippocampus, cingulate cortex, basal forebrain and septum, thalamus, hypothalamus, and left and right midbrain. There was a trend of statistical significance in the cortex (**Figure 3** and **Table 2**). We also observed that adult male mice exposed to ETS showed a decrease in glucose metabolism when compared with male mice from the control group in the left and right striatum, left hippocampus, cingulate cortex, and thalamus (**Figure 3** and **Table 2**). It was also detected a trend toward statistical significance in the cortex, and right hippocampus (**Figure 3** and **Table 2**). Regarding adult female mice exposed to ETS, we observed an increase in glucose uptake when compared with female mice from the control group in the brainstem, with trend toward statistical significance in the cingulate cortex. **Figure 4** shows brain PET/CT average scans of female and male adult mice exposed to ETS during the early postnatal period and the control group.

TABLE 2 | Detailed description of the statistical analysis of ^{18}F-FDG uptake in adult mice in distinct brain regions.

VOI	^{18}F-FDG uptake		
	ETS-exposed	ETS-exposed vs. control	
	Male vs. female	Male	Female
Whole brain	↓ $p = 0.033$	ns	ns
Brainstem	↓ $p = 0.020$	ns	↑ $p = 0.021$
Cortex	↓ $p = 0.058$	↓ $p = 0.058$	ns
Cerebellum	ns	ns	ns
Left amygdala	↓ $p = 0.012$	ns	ns
Right amygdala	ns	ns	ns
Left striatum	↓ $p = 0.003$	↓ $p = 0.038$	ns
Right striatum	↓ $p = 0.019$	↓ $p = 0.033$	ns
Left hippocampus	↓ $p = 0.001$	↓ $p = 0.024$	ns
Right hippocampus	↓ $p = 0.008$	↓ $p = 0.053$	ns
Cingulate cortex	↓ $p = 0.0003$	↓ $p = 0.028$	↑ $p = 0.069$
Basal forebrain and spetum	↓ $p = 0.049$	ns	ns
Thalamus	↓ $p = 0.0007$	↓ $p = 0.022$	ns
Hypothalamus	↓ $p = 0.033$	ns	ns
Left midbrain	↓ $p = 0.0008$	ns	ns
Right midbrain	↓ $p = 0.0007$	ns	ns

Each p-value is adjusted for multiple comparisons. ↓, decreased ^{18}F-FDG uptake; ↑, increased ^{18}F-FDG uptake.

DISCUSSION

To the best of our knowledge, this is the first study that investigated the effects of ETS exposure during brain development on ^{18}F-FDG uptake in the brain of mice. By PET imaging, we observed that ETS exposure during the early postnatal period decreased brain ^{18}F-FDG uptake in both male and female infant mice and in adult male mice in distinct brain regions and increased ^{18}F-FDG uptake in adult female mice in the brainstem and cingulate cortex. In addition, male ETS-exposed mice showed decreased ^{18}F-FDG uptake when compared to female ETS-exposed. These results are in accordance with previous studies of our group and with studies that show that exposure to tobacco smoke during brain development can affect the central nervous system. Exposure to tobacco smoke extract during gestational period of Sprague–Dawley rats decreased nicotinic and serotonin receptors in different brain regions (Slotkin et al., 2017). In addition, postnatal exposure to tobacco smoke leads to impairment in the myelination, learning, and memory, and induces oxidative stress and lower brain-derived neurotrophic factor (BDNF) and synaptic proteins levels (Stangherlin et al., 2009; Lobo-Torres et al., 2012; Torres et al., 2015a,b).

The exposure biomarkers of the present study were similar to Obot et al. (2004), Amos-Kroohs et al. (2013), and Torres et al. (2015a,b, 2019a,b). Nwosu and Kum-Nji (2018) suggested that the classification as passive or active smoking could be done according to serum cotinine levels. Thus, serum cotinine levels between 0.05 and 10 ng/mL can be considered as passive smokers and >10 ng/mL as active smoking. Although cotinine concentration of the present study could be considered as active smoker by Nwosu and Kum-Nji (2018), the classification was based in children and adolescent under 17 years old and not in rodents. Moreover, the authors did not mention how long after tobacco smoke exposure the blood was collected. In the present study, due to the weaker binding affinity of CO for mouse hemoglobin when compared to that of human hemoglobin (Watson et al., 1987), blood collection for the quantification of the biological markers was performed immediately after the ETS exposure. Thus, the cotinine levels data reflect the peak of cotinine, as the half-life of plasma nicotine in rodents is 0.9–1.1 h.

Neuronal activity requires high energy, mainly in the level of synaptic connections and signaling transduction pathways (Sokoloff, 1999). In this scenario, ^{18}F-FDG brain uptake correlates with brain metabolic activity, since we can predict brain function through the relationship between energy

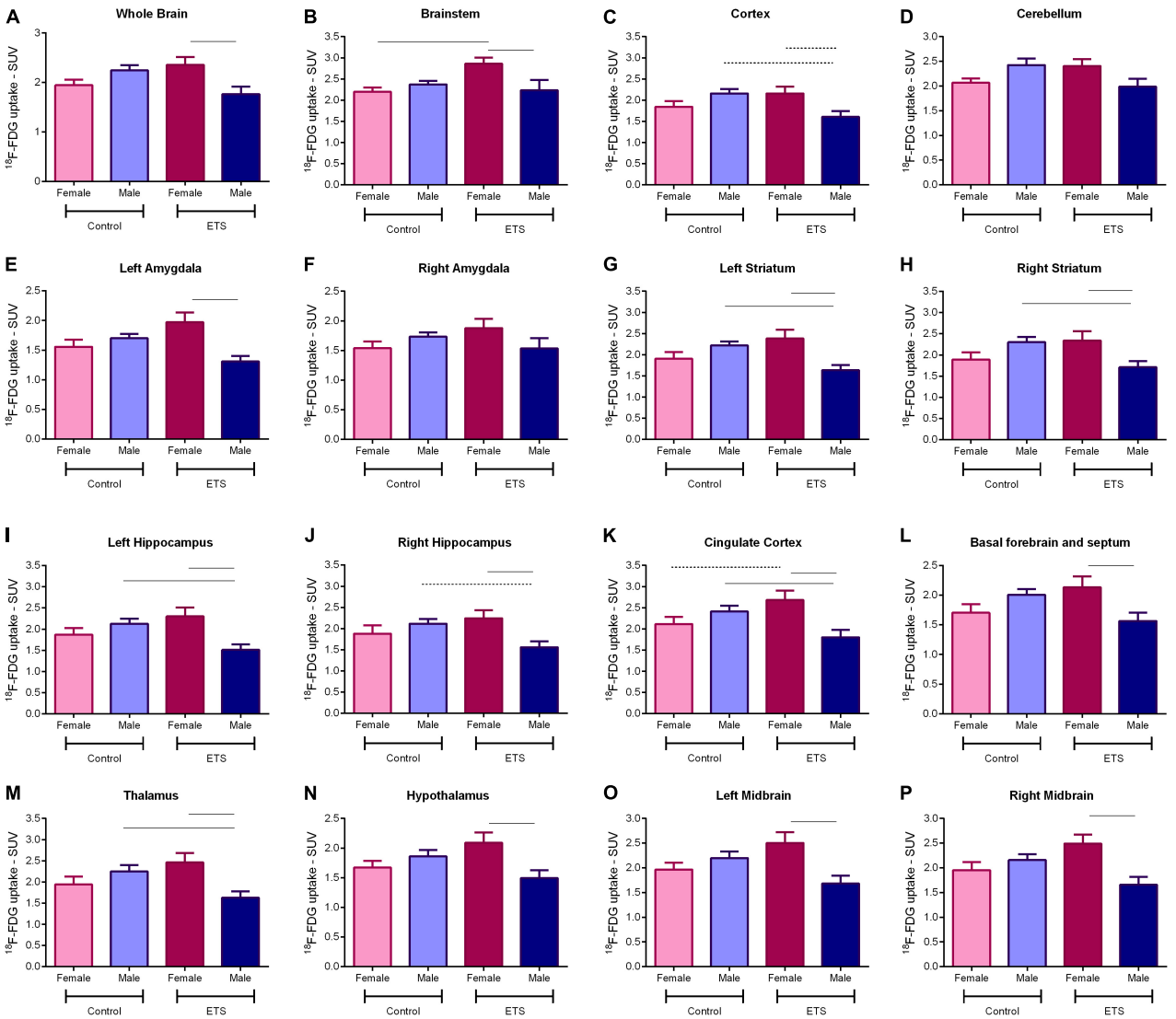

FIGURE 3 | ^{18}F-FDG uptake for adult mice (n = 5 females ETS-exposed and n = 4 females control; n = 5 males ETS-exposed and n = 5 males control) in the whole brain **(A)**, brainstem **(B)**, cortex **(C)**, cerebellum **(D)**, left amygdala **(E)**, right amygdala **(F)**, left striatum **(G)**, right striatum **(H)**, left hippocampus **(I)**, right hippocampus **(J)**, cingulate cortex **(K)**, basal forebrain and septum **(L)**, thalamus **(M)**, hypothalamus **(N)**, left midbrain **(O)**, and right midbrain **(P)**. Two-way ANOVA with Bonferroni *post hoc* test and adjusted p-values for multiple comparisons. *Continuous bar:* $p < 0.05$. *Dashed bar:* trend toward statistical significance.

consumption and neuronal activity (Shulman et al., 2004). Small animal PET imaging in longitudinal studies allows *in vivo* quantification of brain metabolic activity in the same animal during different periods of life making possible to analyze how brain behave in different stages and how xenobiotics might be able to interfere in the homeostatic state. Our data showed decreased ^{18}F-FDG uptake in infancy, suggesting that ETS exposure is affecting brain neuronal activity during this important period of brain development. In fact, it is known that in humans, childhood is a period in which the central nervous system is under active development, maturation, and with open critical periods of synaptic plasticity, with the higher levels of metabolism, reaching a peak on the fourth year of life (Chugani and Phelps, 1991). Until the ninth year of life there is a plateau, followed by a steady decline until adulthood, in the second decade of life, when the prefrontal cortex has completed its maturation (Kennedy and Sokoloff, 1957). During normal aging, brain passes through structural and function changes in white and gray matters, reflecting in a declined metabolic activity (Moeller et al., 1996).

It is interesting to note that even a long time after the last exposure, adult mice showed changes in ^{18}F-FDG uptake in distinct brain regions, which were sex dependent. Adult male mice exposed to ETS during brain development showed decreased ^{18}F-FDG uptake compared with adult female mice or with male controls in different brain regions. In fact, sex seems to be an important factor in brain response to ETS. A clinical study

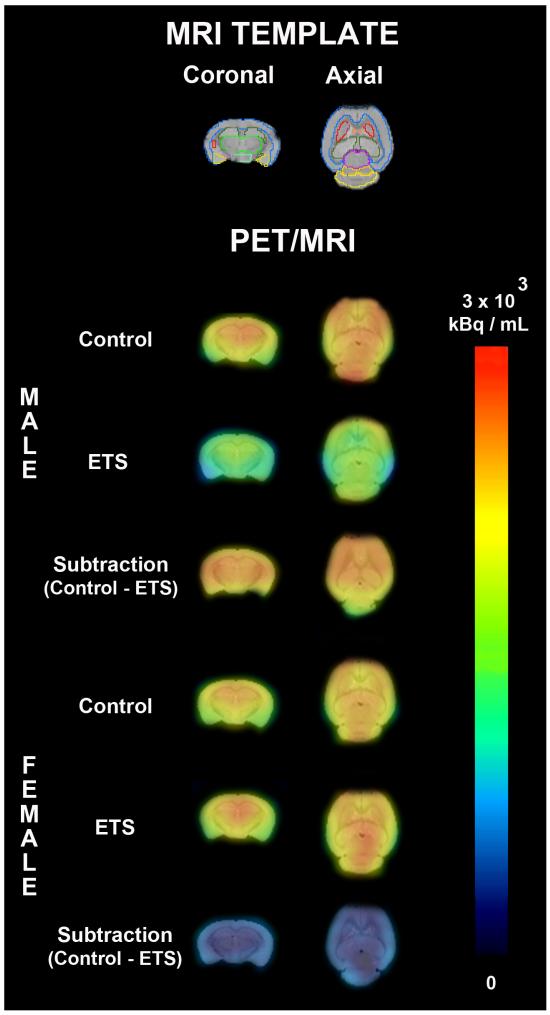

FIGURE 4 | PET average images of female and male adult mice exposed to ETS during the early postnatal period and the control group. On the top, a MRI template used for drawing volumes of interest (whole brain, brainstem, cortex, cerebellum, left amygdala, right amygdala, left striatum, right striatum, left hippocampus, right hippocampus, cingulate cortex, basal forebrain and septum, thalamus, hypothalamus, left midbrain, and right midbrain). The first two rows represent PET average images fused to MRI template and the third row represent the difference of PET average image fused to MRI template between control group and ETS exposed mice.

showed that nicotine administered by patch induced increased brain ^{18}F-FDG uptake in females than males during a Continuous Performance Task or the Bushman Competition and Retaliation Task tests (Fallon et al., 2005). A previous study of our group also showed that the effects of ETS exposure during the early postnatal period are sex-dependent, as infant female mice showed poorer performance in learning and memory tests than males (Torres et al., 2015b).

Garcia et al. (2013) revealed that sex is determinant for post-hypoxic depression and recovery. Persistent post-hypoxic depression is more recurrent in male mice than female and glucose supplementation improves post-hypoxic recovery rhythmogenesis only in female mice (Garcia et al., 2013). These data are relevant, since post-hypoxic depression is involved in the pathogenesis of sudden infant death syndrome (SIDS). About 60% of the children affected by SIDS are male (Richardson et al., 2010). The exposure to ETS is related to higher risk of SIDS, a syndrome that has no known mechanism, but it requires immature cardiorespiratory control and impairments in sleep arousal (Mitchell and Milerad, 2006; Moon et al., 2016). The brainstem is associated with respiratory and cardiovascular responses, therefore is related to pathogenesis of SIDS and it is susceptible to ETS exposure. Previous studies showed that exposure to ETS in the early postnatal period decreased myelin-specific proteins and alter receptors and enzymes of the endocannabinoid system in the brainstem (Torres et al., 2015a, 2019a). The present study corroborates these findings since we observed that ETS exposure decreased ^{18}F-FDG uptake in ETS-exposed male mice compared with female mice and increased ^{18}F-FDG uptake in ETS-exposed female mice compared with control group.

Environmental tobacco smoke exposure during the early postnatal period induced a similar result in the left amygdala, which have a key role in the acquisition of memory related to fear conditioning (Fujisaki et al., 2004). Amygdala is associated to emotional experience, including fear and anxiety (De Bellis et al., 2000; de Oliveira et al., 2013). In fact, pediatric generalized anxiety disorder was associated to higher amygdala volumes (De Bellis et al., 2000). Active smokers have significantly reduced amygdala volumes compared with non-smokers (Luhar et al., 2013). In line with these results, preclinical studies have shown that the exposure to ETS during postnatal periods leads to anxiety-like behavior in a short- and long-term withdrawal (Abreu-Villaça et al., 2015; de la Peña et al., 2016; Torres et al., 2019b). Taken together, these data suggest that the anxiety behavioral disorders related to tobacco smoke may be associated with amygdala alterations.

In the present study, we observed that exposure to ETS decreased ^{18}F-FDG uptake in striatum of adult male mice. This data are consistent with studies that revealed that ETS exposure during a critical period induced oxidative stress, decreased synaptic proteins levels and BDNF, and modified elements of the endocannabinoid system in the striatum (Lobo-Torres et al., 2012; Torres et al., 2019a,b). The striatum, constituted by the caudate nucleus and putamen, is involved in motor, cognitive, and limbic functions, and is involved in the neurobiology of addiction (Burton et al., 2015). Addictive drugs increase dopamine levels in mesolimbic system, especially in the dorsal and ventral striatum/nucleus accumbens (Hyman et al., 2006). Romoli et al. (2019) reported that exposure to nicotine during the early postnatal period increased nicotine consumption during adulthood, effect mediated by dopaminergic neurons in the midbrain, that contains dopaminergic neurons that are located in the ventral tegmental area and in the substantia nigra, regions that are important for the development of drug addiction (Björklund and Dunnett, 2007; Romoli et al., 2019).

Weinstein et al. (2010) observed that smokers showed increased craving scores after watching a videotape with smoking

scenes, which were associated with brain ^{18}F-FDG uptake in the ventral striatum, anterior cingulate, orbitofrontal cortex, middle temporal lobe, hippocampus, insula, midbrain, and thalamus (Weinstein et al., 2010). In addition, Domino et al. (2000) showed that nicotine increases regional cerebral blood flow, evaluated by PET, in the thalamus, pons, primary visual cortex, and cerebellum of tobacco smokers. These individuals also showed reduction in the hippocampal area (Domino et al., 2000; Hanlon et al., 2014). We observed that ETS decreased glucose metabolism in ETS-exposed male mice compared with female mice and with control group in the hippocampus, one of the main areas that is involved in learning and memory. Indeed, ETS exposure during the early postnatal period decreased synaptic proteins and BDNF in hippocampus and induced impairment in the learning and memory from late infancy to early adulthood (Torres et al., 2015b).

It is important to point out the limitations of the present protocol. In order to evaluate the long-lasting effect of tobacco smoke exposure during the early postnatal period, we used a longitudinal study to measure ^{18}F-FDG uptake from infancy to adulthood. Although longitudinal studies have the advantage of evaluating the same animal throughout life, this type of protocol does not allow other measures to be performed, since euthanasia is only done when the animal reaches adulthood.

In summary, we showed that several brain regions are vulnerable to ETS exposure during the early postnatal period and these effects on ^{18}F-FDG uptake were observed even a long time after the last exposure. This study corroborates our previous studies, supporting the hypothesis that exposure to tobacco smoke in a critical period interferes with brain development of mice from late infancy to early adulthood.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of the School of Medicine (027/14) and the School of Pharmaceutical Sciences (P446/14), University of São Paulo.

AUTHOR CONTRIBUTIONS

LT and TM conceived and designed the project. LT, LS, AS, and TA performed all the experiments related to exposure to tobacco smoke and biomarkers quantification under TM supervision. LT, WT, LP, and PS performed the experiments related to PET/CT image acquisition. WT, CR, and DP performed the image processing and analysis. LT, WT, MP, CR, DP, and TM wrote and edited the manuscript. All authors contributed to the manuscript revision, and read and approved the submitted version of the manuscript.

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REFERENCES

Abreu-Villaça, Y., Filgueiras, C. C., Correa-Santos, M., Cavina, C. C., Naiff, V. F., Krahe, T. E., et al. (2015). Tobacco smoke containing high or low levels of nicotine during adolescence: effects on novelty-seeking and anxiety-like behaviors in mice. *Psychopharmacology* 232, 1693–1703. doi: 10.1007/s00213-014-3801-1

Amos-Kroohs, R. M., Williams, M. T., Braun, A. A., Graham, D. L., Webb, C. L., Birtle, T. S., et al. (2013). Neurobehavioral phenotype of C57BL/6J mice prenatally and neonatally exposed to cigarette smoke. *Neurotoxicol. Teratol.* 35, 34–45. doi: 10.1016/j.ntt.2013.01.001

Banderali, G., Martelli, A., Landi, M., Moretti, F., Radaelli, G., Lassandro, C., et al. (2015). Short and long term health effects of parental tobacco smoking during pregnancy and lactation: a descriptive review. *J. Transl. Med.* 13:327. doi: 10.1186/s12967-015-0690-y

Bennett, D. S., Mohamed, F. B., Carmody, D. P., Malik, M., Faro, S. H., and Lewis, M. (2013). Prenatal tobacco exposure predicts differential brain function during working memory in early adolescence: a preliminary investigation. *Brain Imaging Behav.* 7, 49–59. doi: 10.1007/s11682-012-9192-1

Björklund, A., and Dunnett, S. B. (2007). Dopamine neuron systems in the brain: an update. *Trends Neurosci.* doi: 10.1016/j.tins.2007.03.006

Brody, A. L., Mandelkern, M. A., Lee, G., Smith, E., Sadeghi, M., Saxena, S., et al. (2004). Attenuation of cue-induced cigarette craving and anterior cingulate cortex activation in bupropion-treated smokers: a preliminary study. *Psychiatry Res. Neuroimaging* 130, 269–281. doi: 10.1016/j.pscychresns.2003.12.006

Brody, A. L., Mandelkern, M. A., London, E. D., Childress, A. R., Lee, G. S., Bota, R. G., et al. (2002). Brain Metabolic changes during cigarette craving. *Arch. Gen. Psychiatry* 59, 1162–1172. doi: 10.1001/archpsyc.59.12.1162

Burton, A. C., Nakamura, K., and Roesch, M. R. (2015). From ventral-medial to dorsal-lateral striatum: neural correlates of reward-guided decision-making. *Neurobiol. Learn. Mem.* 117, 51–59. doi: 10.1111/j.1743-6109.2008.01122.x. Endothelial

Chugani, H. T., and Phelps, M. E. (1991). Imaging human brain development with positron emission tomography. *J. Nucl. Med.* 32, 23–26.

Costello, M. R., Mandelkern, M. A., Shoptaw, S., Shulenberger, S., Baker, S. K., Abrams, A. L., et al. (2010). Effects of treatment for tobacco dependence on resting cerebral glucose metabolism. *Neuropsychopharmacology* 35, 605–612. doi: 10.1038/npp.2009.165

De Bellis, M. D., Casey, B. J., Dahl, R. E., Birmaher, B., Williamson, D. E., Thomas, K. M., et al. (2000). A pilot study of amygdala volumes in pediatric generalized anxiety disorder. *Biol. Psychiatry* 48, 51–57. doi: 10.1016/S0006-3223(00)00835-0

de la Peña, J. B., Ahsan, A. H., Botanas, C. J., dela Peña, I. J., Woo, T., and Kim, H. J. (2016). Cigarette smoke exposure during adolescence but not adulthood induces anxiety-like behavior and locomotor stimulation in rats during withdrawal. *Int. J. Dev. Neurosci.* 55, 49–55. doi: 10.1016/j.ijdevneu.2016.09.007

de Oliveira, A. R., Reimer, A. E., Reis, F. M., and Brandão, M. L. (2013). Conditioned fear response is modulated by a combined action of the hypothalamic–pituitary–adrenal axis and dopamine activity in the basolateral amygdala. *Eur. Neuropsychopharmacol.* 23, 379–389. doi: 10.1016/J.EURONEURO.2012.05.007

de Zeeuw, P. D., Zwart, F., Schrama, R., Engeland, H. V., and Durston, S. (2012). Prenatal exposure to cigarette smoke or alcohol and cerebellum volume in attention-deficit/hyperactivity disorder and typical development. *Transl. Psychiatry* 2, e84–e89. doi: 10.1038/tp.2012.12

Domino, E. F., Minoshima, S., Guthrie, S., Ohl, L., Ni, L., Koeppe, R. A. et al. (2000). Nicotine effects on regional cerebral, blood flow in awake, resting tobacco smokers. *Synapse* 38, 313–321. doi: 10.1002/1098-2396(20001201)38:3<313::AID-SYN10>3.0.CO;2-6

Fallon, J. H., Keator, D. B., Mbogori, J., Taylor, D., and Potkin, S. G. (2005). Gender: a major determinant of brain response to nicotine. *Int. J. Neuropsychopharmacol.* 8, 17–26. doi: 10.1017/S1461145704004730

Fujisaki, M., Hashimoto, K., Iyo, M., and Chiba, T. (2004). Role of the amygdalo-hippocampal transition area in the fear expression: evaluation by behavior and immediate early gene expression. *Neuroscience* 124, 247–260. doi: 10.1016/j.neuroscience.2003.11.022

Garcia, A. J., Rotem-Kohavi, N., Doi, A., and Ramirez, J. M. (2013). Post-hypoxic recovery of respiratory rhythm generation is gender dependent. *PLoS One* 8:e60695. doi: 10.1371/journal.pone.0060695

Hanlon, C. A., Dowdle, L. T., Naselaris, T., Canterberry, M., and Cortese, B. M. (2014). Visual cortex activation to drug cues: a meta-analysis of functional neuroimaging papers in addiction and substance abuse literature. *Drug Alcohol Depend.* 143, 206–212. doi: 10.1016/J.DRUGALCDEP.2014.07.028

Hwang, S., Hwang, J. H., Moon, J. S., and Lee, D. (2012). Environmental tobacco smoke and children's health. *Korean J. Pediatr.* 55, 35–41. doi: 10.3345/kjp.2012.55.2.35

Hyman, S. E., Malenka, R. C., and Nestler, E. J. (2006). Neural mechanisms of addiction: the role of reward-related learning and memory. *Ann. Rev. Neurosci.* 29, 565–98. doi: 10.1146/annurev.neuro.29.051605.113009

Kennedy, C., and Sokoloff, L. (1957). An adaptation of the nitrous oxide method to the study of the cerebral circulation in children: normal values for cerebral blood flow and cerebral metabolic rate in childhood. *J. Clin. Invest.* 36, 1130–1137. doi: 10.1172/JCI103509

Lobo-Torres, L. H., Moreira, W. L., Garcia, R. C. T., Annoni, R., Carvalho, A. L. N., Teixeira, S. A., et al. (2012). Environmental tobacco smoke induces oxidative stress in distinct brain regions of infant mice. *J. Toxicol. Environ. Health Part A* 75, 971–980. doi: 10.1080/15287394.2012.695985

Luhar, R. B., Sawyer, K. S., Gravitz, Z., Ruiz, S. M., and Oscar-Berman, M. (2013). Brain volumes and neuropsychological performance are related to current smoking and alcoholism history. *Neuropsychiatr. Dis. Treat.* 9, 1767–1784. doi: 10.2147/NDT.S52298

Mitchell, E. A., and Milerad, J. (2006). Smoking and the sudden infant death syndrome. *Rev. Environ. Health* 21, 81–103. doi: 10.1515/REVEH.2006.21.2.81

Moeller, J. R., Ishikawa, T., Dhawan, V., Spetsieris, P. G., Mandel, F., Alexander, G. E., et al. (1996). The metabolic topography of normal aging. *J. Cereb. Blood Flow Metab.* 16, 385–398. doi: 10.1097/00004647-199605000-00005

Moon, R. Y., Darnall, R. A., Feldman-Winter, L., Goodstein, M. H., and Hauck, F. R. (2016). SIDS and other sleep-related infant deaths: evidence base for 2016 updated recommendations for a safe infant sleeping environment. *Pediatrics* 138:e20162940. doi: 10.1542/peds.2016-2940

Nwosu, B. U., and Kum-Nji, P. (2018). Tobacco smoke exposure is an independent predictor of vitamin D deficiency in US children. *PLoS One* 13:e0205342. doi: 10.1371/journal.pone.0205342

Oberg, M., Jaakkola, M. S., Woodward, A., Peruga, A., and Prüss-Ustün, A. (2011). Worldwide burden of disease from exposure to second-hand smoke: a retrospective analysis of data from 192 countries. *Lancet* 377, 139–146. doi: 10.1016/S0140-6736(10)61388-8

Obot, C. J., Lee, K. M., Fuciarelli, A. F., Renne, R. A., and McKinney, W. J. (2004). Characterization of mainstream cigarette smoke-induced biomarker responses in ICR and C57BI/6 mice. *Inhal. Toxicol.* 16, 701–719. doi: 10.1080/08958370490476604

Pagani, L. S. (2014). Environmental tobacco smoke exposure and brain development: the case of attention deficit/hyperactivity disorder. *Neurosci. Biobehav. Rev.* 44, 195–205. doi: 10.1016/j.neubiorev.2013.03.008

Plichart, M., Menegaux, F., Lacour, B., Hartmann, O., Frappaz, D., Doz, F., et al. (2008). Parental smoking, maternal alcohol, coffee and tea consumption during pregnancy and childhood malignant central nervous system tumours: the ESCALE study (SFCE). *Eur. J. Cancer Prev.* 17, 376–383. doi: 10.1097/CEJ.0b013e3282f75e6f

Richardson, H. L., Walker, A. M., and Horne, R. S. C. (2010). Sleeping like a baby – does gender influence infant arousability? *Sleep* 33, 1055–1060. doi: 10.1093/sleep/33.8.1055

Romoli, B., Lozada, A. F., Sandoval, I. M., Manfredsson, F. P., Hnasko, T. S., and Berg, D. K. (2019). Neonatal nicotine exposure primes midbrain neurons to a dopaminergic phenotype and increases adult drug consumption. *Biol. Psychiatry* 86, 344–355. doi: 10.1016/j.biopsych.2019.04.019

Shulman, R. G., Rothman, D. L., Behar, K. L., and Hyder, F. (2004). Energetic basis of brain activity: implications for neuroimaging. *Trends Neurosci.* 27, 489–495. doi: 10.1016/j.tins.2004.06.005

Slotkin, T. A., Stadler, A., Skavicus, S., Card, J., Ruff, J., and Levin, E. D. (2017). Is there a critical period for the developmental neurotoxicity of low-level tobacco smoke exposure? *Toxicol. Sci.* 155, 75–84. doi: 10.1093/toxsci/kfw180

Sokoloff, L. (1999). Energetics of functional activiation in neural tissues. *Neurochem. Res.* 24, 321–329. doi: 10.1023/a:1022534709672

Sokoloff, L., Reivich, M., Kennedy, C., Rosiers, M. H. D., Patlak, C. S., Pettigrew, K. D., et al. (1977). The [14 C]deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anesthetized albino rat. *J. Neurochem.* 28, 897–916. doi: 10.1111/j.1471-4159.1977.tb10649.x

Stangherlin, E. C., Luchese, C., Ardais, A. P., and Nogueira, C. W. (2009). Passive smoke exposure induces oxidative damage in brains of rat pups: protective role of diphenyl diselenide. *Inhal. Toxicol.* 21, 868–874. doi: 10.1080/08958370802526881

Torres, L. H., Annoni, R., Balestrin, N. T., Coleto, P. L., Duro, S. O., Garcia, R. C. T., et al. (2015a). Environmental tobacco smoke in the early postnatal period induces impairment in brain myelination. *Arch. Toxicol.* 89, 2051–2058. doi: 10.1007/s00204-014-1343-2

Torres, L. H., Balestrin, N. T., Spelta, L. E. W., Duro, S. O., Pistis, M., and Marcourakis, T. (2019a). Exposure to tobacco smoke during the early postnatal period modifies receptors and enzymes of the endocannabinoid system in the brainstem and striatum in mice. *Toxicol. Lett.* 302, 35–41. doi: 10.1016/j.toxlet.2018.12.002 .

Torres, L. H., Garcia, R. C. T., Blois, A. M. M., Dati, L. M. M., Durão, A. C., Alves, A. S., et al. (2015b). Exposure of neonatal mice to tobacco smoke disturbs synaptic proteins and spatial learning and memory from late Infancy to early adulthood. *PLoS One* 10:e0136399. doi: 10.1371/journal.pone.0136399

Torres, L. H., Garcia, R. C. T., Blois, A. M. M., Pacheco-Neto, M., Camarini, R., and Britto, L. R. (2019b). Early postnatal tobacco smoke exposure triggers anxiety-like behavior and decreases synaptic proteins even after a long exposure-free period in mice. *Brain Res.* 1707, 99–106. doi: 10.1016/j.brainres.2018.11.022

Vanhove, C., Bankstahl, J. P., Kramer, S. D., Visser, E., Belcari, N., and Vandenberghe, S. (2015). Accurate molecular imaging of small animals taking into account animal models, handling, anaesthesia, quality control and imaging system. *EJNMMI Phys.* 2:31. doi: 10.1186/s40658-015-0135-y

Watson, E. S., Jones, A. B., Ashfaq, M. K., and Barrett, J. T. (1987). Spectrophotometric evaluation of carboxyhemoglobin in blood of mice after exposure to marijuana or tobacco smoke in a modified walton horizontal smoke exposure machine. *J. Anal. Toxicol.* 11, 19–23. doi: 10.1093/jat/11.1.19

Weinstein, A., Greif, J., Yemini, Z., Lerman, H., Weizman, A., and ven-Sapir, E. E. (2010). Attenuation of cue-induced smoking urges and brain reward activity in smokers treated successfully with bupropion. *J. Psychopharmacol.* 24, 829–838. doi: 10.1177/0269881109105456

Welch, K. D., Pfister, J. A., Lima, F. G., Green, B. T., and Gardner, D. R. (2013). Effect of α7 nicotinic acetylcholine receptor agonists and antagonists on motor

function in mice. *Toxicol. Appl. Pharmacol.* 266, 366–374. doi: 10.1016/j.taap.2012.11.024

Yochum, C., Doherty-Lyon, S., Hoffman, C., Hossain, M. M., Zelikoff, J. T., and Richardson, J. R. (2014). Prenatal cigarette smoke exposure causes hyperactivity and aggressive behavior: role of altered catecholamines and BDNF. *Exp. Neurol.* 254, 145–152. doi: 10.1016/j.expneurol.2014.01.016

Zovein, A., Fowers-Ziegler, J., Thamotharan, S., Shin, D., Sankar, R., Nguyen, K., et al. (2004). Postnatal hypoxic-ischemic brain injury alters mechanisms mediating neuronal glucose transport. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 286, R273–R282.

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Targeting the Stress System During Gestation: Is Early Handling a Protective Strategy for the Offspring?

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The perinatal window is a critical developmental time when abnormal gestational stimuli may alter the development of the stress system that, in turn, influences behavioral and physiological responses in the newborns. Individual differences in stress reactivity are also determined by variations in maternal care, resulting from environmental manipulations. Despite glucocorticoids are the primary programming factor for the offspring's stress response, therapeutic corticosteroids are commonly used during late gestation to prevent preterm negative outcomes, exposing the offspring to potentially aberrant stress reactivity later in life. Thus, in this study, we investigated the consequences of one daily s.c. injection of corticosterone (25 mg/kg), from gestational day (GD) 14–16, and its interaction with offspring early handling, consisting in a brief 15-min maternal separation until weaning, on: (i) maternal behavior; and (ii) behavioral reactivity, emotional state and depressive-like behavior in the adolescent offspring. Corticosterone plasma levels, under non-shock- and shock-induced conditions, were also assessed. Our results show that gestational exposure to corticosterone was associated with diminished maternal care, impaired behavioral reactivity, increased emotional state and depressive-like behavior in the offspring, associated with an aberrant corticosterone response. The early handling procedure, which resulted in increased maternal care, was able to counteract the detrimental effects induced by gestational corticosterone exposure both in the behavioral- and neurochemical parameters examined. These findings highlight the potentially detrimental consequences of targeting the stress system during pregnancy as a vulnerability factor for the occurrence of emotional and affective distress in the adolescent offspring. Maternal extra-care proves to be a protective strategy that confers resiliency and restores homeostasis.

Keywords: prenatal exposure, glucocorticoid, early handling, stress reactivity, depressive-like behavior, emotionality

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INTRODUCTION

Numerous studies across a wide range of species have shown that prenatal exposure to different conditions such as infections, nutritional deficiencies, teratogenic substances, and emotional distress, predisposes the newborns to a spectrum of different disorders characterized by deficits in cognitive functioning, motor, and visuospatial abilities and to the genesis of chronic systemic diseases (Cannizzaro et al., 2002, 2005, 2006b, 2007, 2008; Hellemans et al., 2010; Leggio et al., 2014; Sarro et al., 2014; Martines et al., 2016; Moukarzel et al., 2018). Notably, maternal stress during pregnancy could dispose of the offspring toward vulnerability to neurobehavioral disorders. As mediators of the stress response, glucocorticoids are among the main primary programming factors conveying maternal stress to the fetus *via* the placenta (Zarrow et al., 1970; Schmidt et al., 2019), through the activation of the glucocorticoid receptors (GR), whose ontogenetic pattern has been detected in human from the early prenatal life stages (Kitraki et al., 1997; Diaz et al., 1998; Kemp et al., 2016). Thus, glucocorticoids, by a receptor-mediated regulatory role during ontogenic development, could affect normal brain neurogenesis (Cintra et al., 1993). In this regard, prospective animal- and retrospective human studies have revealed that antenatal glucocorticoid administration in late gestation can lead to lifelong alterations on brain structures and functionality and may produce long-lasting modifications in the maturation of the hypothalamic-pituitary-adrenal (HPA) axis (Heim et al., 1997; French et al., 1999, 2004; Sloboda et al., 2005; de Vries et al., 2007; Charil et al., 2010; Fowden and Forhead, 2015). Indeed, exposure to glucocorticoids during pregnancy, reducing negative-feedback on HPA axis, increases cortisol release in the progeny (Alexander et al., 2012): this leads to a slower recovery from stressors, reducing coping strategy in aversive situations (Welberg et al., 2001; Plescia et al., 2013). This evidence represents a key issue in the therapeutic administration of antenatal corticosteroids, which are commonly used when at risk of preterm delivery to ensure the survival of the preterm infant (Singh et al., 2012). Indeed, last-trimester administration of synthetic glucocorticoids also "programs" outcomes comparable to those elicited by prenatal stress in humans (Seckl et al., 2000). Accordingly, treating pregnant rodents with synthetic glucocorticoids leads to offspring with similar HPA axis- and behavioral changes as prenatally stressed offspring (Schmidt et al., 2019).

The gestational experiences may also affect the maternal-infant dyad (Tarullo et al., 2017; Reck et al., 2018). Indeed, pregnant women who experience social and emotional stress may divest themselves of maternal bonding (Baker et al., 2008; Azhari et al., 2019). Importantly, these conditions appear to have a major impact on child cognitive, emotional and physical development (Cogill et al., 1986; Bhagwanani et al., 1997; Smith et al., 2004). Alterations in maternal caregiving behavior after maternal stress, or exogenous administration of glucocorticoids, occur also in rodent models (Darnaudéry et al., 2004; Koehl et al., 2012; Jafari et al., 2017; Gemmel et al., 2018). For instance, acutely and repeatedly stressed dams spend less time in activities directed towards the pups rather than in self-oriented behaviors (Patin et al., 2002; Smith et al., 2004; Boero et al., 2018). After birth, the infant is dependent on the primary caregiver, not only for nursing and protection but also for the normal development of emotional behavior (Bella et al., 2018). Indeed, deficiency of motherly care during infancy affects the development of stress reactivity, contributing to the raising of the individual distinctness in emotional responses (Cannizzaro et al., 2005, 2006b). On the other hand, early handling procedures are able to significantly affect the development of the offspring's emotional behavior and HPA axis physiology. In particular, extensive research has shown that brief periods of maternal separation of the pups during the nursing stage result in offspring decreased adrenal reactivity in response to stressors (Liu et al., 1997; Cannizzaro et al., 2005, 2006b, 2007; Plescia et al., 2014b), as well as fear-oriented behavior and emotionality (Cannizzaro et al., 2005, 2006b). The majority of these behavioral and neuroendocrine studies have been carried out on the adult progeny exposed to repeated prenatal stress. However, none of them has yet investigated the influence of the gestational exposure to corticosterone on emotional behaviors in adolescence, which emerges as a "critical" phase in the development of stress responsiveness (Cannizzaro et al., 2006b).

Thus, given these premises, the aim of the current study was to investigate the consequences of prenatal corticosterone exposure on maternal and offspring outcomes, during a timeframe when a relatively high expression of GR in multiple brain areas of the pups occurs (Cintra et al., 1993). In particular, we assessed maternal behavior, behavioral reactivity, emotionality and depressive-like behavior in the adolescent male offspring employing, respectively, the open field test (OFT), the acoustic startle reflex (ASR) and the forced swim test (FST). Offspring corticosterone plasma levels, under non-shock- and shock-induced conditions were evaluated as a measure of HPA axis activity. Early handling procedure, as a brief maternal separation, was also carried out as a putative protective strategy able to restore homeostasis.

MATERIALS AND METHODS

Animals and Pharmacological Treatment

Wistar rats (Harlan, Udine, Italy) housed with free access to food and water were maintained on a 12 h on/off cycle (8:00–20:00 h) at a constant temperature (22 ± 2°C) and humidity (55 ± 10%). Pairs of primiparous females of 120 days of age were mated with one male of 150 days of age. The day on which sperm was detected in the vaginal smear was designed as gestational day (GD) 1. Pregnancy was determined by weighing and palpation. The pregnant dams' weight on GD 14 was approximately 300 g. From GD 14 through GD 16, a period of time during which corticosterone can interact with GR expressed in the last week of gestation, the dams received a single daily subcutaneous injection of corticosterone (Ct; Sigma–Aldrich, Italy; 25 mg/kg) or vehicle (Vh; 100 mM DMSO in 0.9% saline solution) in a volume of 1 ml/kg. The pregnant dams were individually housed in standard rat cages (40 cm × 60 cm, 20 cm in height) for

at least 7 days before delivery. All litters born within a 2-day period were reduced to ten pups (five males and five females) Forty male pups in total were used in our investigations; they were divided into the following experimental 10-rat (five rats per litter) groups: vehicle-non-handled (Vh); corticosterone-non-handled (Ct); vehicle-handled (Vh-H); corticosterone-handled (Ct-H). At weaning time, postnatal day (PND) 22, rats were randomly assigned two per cage accordingly to each experimental condition. The experiments were performed on adolescent rats—from PND 32 to 43. On the test day, each group of rats was brought into the laboratory and allowed to acclimate for at least 60 min prior to the experimental session. The experiments were performed in a sound isolated room between 9:00 and 14:00 and the animals were tested randomly, regardless of the group they belonged to. Animal performance during the different experimental sessions was recorded on the computer and then analyzed by an experimenter unaware of the different treatments. All the experiments were conducted in accordance with the regulations of the Committee for the Protection and Use of Animals of the University of Palermo, Italy, in accordance with current Italian legislation on animal experimentation (D.L. 26/2014) and the European Directive (2010/63/EU) on the care and use of laboratory animals. All efforts were made to minimize the number of animals used and possible distress.

Early Handling and Pups Body Weight

Half of Ct- and Vh-treated litters remained undisturbed during the post-weaning period (i.e., non-handled, Ct and Vh groups), and half of prenatally Ct- and Vh-treated litters underwent early handling procedure (Ct-H and Vh-H groups), from PND 2 until PND 21. Early handling procedure consisted of removing the dam from the nest for 15 min during which she was temporarily placed in a separate cage. Simultaneously, pups were moved into a different room and individually placed into sawdust-containing small plastic cups for 15 min. In the end, mothers and pups were brought together in their home cages. Early handling procedure was performed in the same room, at the same time (10:00 h) and by the same experimenter. From PND 2 to PND 21 pups' body weight was also evaluated.

Maternal Behavior Assessment

Dam's behavior in the presence of the offspring was assessed by direct periodic observations under undisturbed conditions in their home cages (Capone et al., 2005), from PND 2 to PND 21. Each animal was subjected to four assessments a day, during the diurnal time (9:00 am, 11:30 am, 01:30 pm, and 03:00 pm) when animals behave more maternally (Ader and Grota, 1970); instantaneous 20-s sampling was repeated three times at each time, for a total of 12 instantaneous observations per animal per day (3 observations × 4 times per day × 20 days = 240 observations per dam). The 20-s time of observation allows for an exact identification of the on-going behavioral patterns: retrieval, nursing (arched-back, blanket, passive), pup care (licking, anogenital licking), dam self-care (self-grooming, eating, drinking), and others (rearing, moving, resting, standing out of nest). Original data were recorded using dichotomous scores (0/1): score 0 was assigned when the behavior was not shown in the interval of observation; score 1 was assigned when the behavior was performed. Thus, a daily score ranged between 0 and 12. In order to gain a comprehensive framework of the behavioral measurements, a daily index of overall maternal behavior (MB-I) was calculated as follows: (maternal score) − (non-maternal score)/(maternal score) + (non-maternal score). The index ranges from −1 (totally non-maternal behaviors) to +1 (totally maternal behaviors; Brancato et al., 2016).

Open Field Test

Locomotor activity and explorative behavior were assessed in the open-field arena with a contrast-sensitive, video tracking system, ANY MAZE (Ugo Basile, Gemonio, Italy), in a mean light intensity (100 lx) illuminated room (Brancato et al., 2014). The apparatus consisted in a square box (44 × 44 × 20 cm) and produced a quality-quantitative mapping of the ambulatory patterns, measuring simultaneously: total distance traveled (TDT) in centimeters, number of transition from peripheral to central squares of the arena (NCT) and amount of time spent on the central areas (ATC) in second. The 5-min recording and measurement of each experimental session started after 1-min habituation in the arena, to allow the rats to acclimatize, and was displayed on a personal computer (Cacace et al., 2011). The test was performed at PND 32.

Acoustic Startle Reflex Test

The ASR provides a useful readout of the neural processing that might underpin an organism's response to an emotional context or stressor (Hoffman, 2016; Hantsoo et al., 2018). The ASR response was measured using a Responder-X apparatus (Columbus Instruments, USA) at PND 34. The peak amplitude of the responses was recorded and displayed on a personal computer. A 10-min test session started by placing the rat in a 28 cm long, 16 cm wide, 15 cm high device with a stainless-steel grid floor, into a ventilated, sound-attenuated and darkroom, in which the animal was left undisturbed for the first 5 min period and was subsequently subjected to the startle stimulus for 5 min. The startle stimulus consisted of a 110 dB, 8 kHz tone superimposed on a continuous 50 dB white noise background; the stimulus duration was 200 ms, with a fixed 10-s interval. Sound levels in the test room were measured with a Bruel and Kjaer 2209 sound level meter. The maximum force exerted by the rat on-grid floor during the 200 ms period was designated as peak amplitude. The amplitude of ASR was measured in units, over the range of 60–550 g (1 unit = 2.1 g of force); maximum output was 255 units. The experimental session consisted of 10 trials.

Forced Swim Test

We employed the FST as described by Porsolt et al. (1977) with some modifications, in order to test depressive-like behavior at PND 38. The test was composed of a pre-test stage (15 min) and, 24 h later, of a test stage (5 min), for both pre-test and test sessions, conducted under low illumination (12 lx), the animals were placed inside a transparent Plexiglas cylinder

(50 cm high, 20 cm inside diameter) filled with tap clean water at 23 ± 1°C, adjusting the water depth according to the rat's size, so that it cannot touch the bottom of the container with its hind legs (Yankelevitch-Yahav et al., 2015). A video camera was placed above the tank and connected to a video recorder to register each stage for subsequent scoring. An experimenter, unaware of the different treatments, scored the specific behavioral parameters from the videotape. Behavioral categories considered were as follows: immobility time, defined as floating in the water, making only the movement necessary to keep the head above water; swimming time, defined as making swimming motions and moving around the cylinder. Following either pre-test stage or test stage, the rats were dried with a towel and kept warm on a heating pad for 30 min in their home cages.

Stress Procedure

At PND 43, rats from each experimental group were individually placed in a cage with an electrified grid floor through which shock could be delivered. The session started immediately after placing the rat into the shock-delivering apparatus. Rats (five per group) received an inescapable mild footshock (0.6 mA for 3 s) every 20 s, along 1 min. Control animals (non-stressed, five per group) were placed into the apparatus for the same time but were not shocked (Cannizzaro et al., 2006b).

Plasma Corticosterone Assay

Rats were killed by decapitation 30 min after being placed into the shock-delivering apparatus. Trunk blood was collected into heparinized tubes. After centrifugation at 3,000 rpm at 4°C for 5 min, plasma samples were separated and stored at −80°C prior to assay. Plasma corticosterone concentration was assayed in duplicate using the RIA kit for rats (IDS Limited, Boldon, UK). The inter-and intra-assay coefficient of variation was 8% and 3% respectively, with a detection limit of 0.5 ng/mL. All measures were in the linear range of the standard curve (0.5–62.5 ng/mL).

Statistical Analysis

Statistical data from bodyweight were carried out by a three-way ANOVA followed by Tukey's test post-test ($\alpha = 0.05$).

Statistical analysis of the data from the OFT, ASR, FST, maternal behavior scores and from non-shock- and shock-induced corticosterone plasma levels were carried out using a two-way ANOVA for unpaired measures. When necessary, *post hoc* comparisons were calculated with Tukey's multiple comparison post-test ($\alpha = 0.05$). Data are reported as mean ± SD. Statistical significance was set at $p < 0.05$.

RESULTS

Pups Body Weight

Rats' body weight was recorded from PND 2 to PND 21 in order to obtain data related to the influence of a single daily corticosterone administration and early handling procedure on weight gain during the pre-weaning period. No significant differences in number, weight, morbidity or mortality were observed among the different experimental groups. The results of a three-way ANOVA performed on body weight as a dependent variable, and days, prenatal corticosterone exposure and early handling as independent variables are shown in **Table 1**. The table indicates that: the factors days, prenatal corticosterone treatment, and early handling were significant. Moreover, the interaction between days- and prenatal treatment-with early handling was significant. The results of Tukey's multiple comparisons test performed on each single day showed a reduction in body weight in Ct treated rats on days 10, 11 and 12 ($q = 6.29$, $p = 0.04340$; $q = 6.25$; $p = 0.0463$; $q = 6.219$, $p = 0.0495$), with respect to Vh groups; and a decrease in body weight on days 19 and 21 ($q = 5.825$, $p = 0.0270$; $q = 6.341$; $p = 0.0380$) in Ct with respect to Ct-H groups (**Figure 1**). No statistical difference was observed when Ct-H was compared to Vh and Vh-H groups.

Dams Spontaneous Maternal Behavior

In order to evaluate the impact of gestational manipulation by corticosterone, the influence of a daily 15-min early handling procedure on dams spontaneous behavior, retrieval, nursing (arched-back, blanket, passive), pup care (licking, anogenital licking, digging), dam self-care (self-grooming, eating, drinking), and other behaviors (rearing, moving, resting, standing out of nest) were scored. Results from a two-way ANOVA performed on MB-I as dependent variable and prenatal corticosterone and early handling as independent variables, showed that: the factor prenatal corticosterone ($F_{(1,76)} = 19.77$; $p < 0.0001$) early handling ($F_{(1,76)} = 64.58$; $p < 0.0001$) and their interaction ($F_{(1,76)} = 8.646$; $p = 0.0043$) were significant. In detail, *post hoc* analysis conducted by Tukey's multiple comparison post-test highlighted a significant lower maternal behavior in Ct treated

TABLE 1 | Pups body weight: results of three-way ANOVA performed on body weight as dependent variable and days (1), prenatal treatment with corticosterone (2), and early handling (3) as independent variables.

Source of Variation	DF	SS	MS	F	*P*-level
1-days	19	922,580	48,557	89.39	<0.001
2-prenatal treatment	1	54,686	54,686	100.7.83	<0.001
3-early handling	1	77,176	77,176	142.1.33	<0.001
1:2	19	9,384	492	0.9057	=0.5775
1:3	19	17,917	943	1.736.79.1	=0.0468
2:3	1	42,968	42,968	0.6584	<0.001
1:2:3	19	6,795	357.7		=0.8477
Residuals	80	43,457	543.2		

Pups' body weight (g) was expressed as the weight for the entire litter.

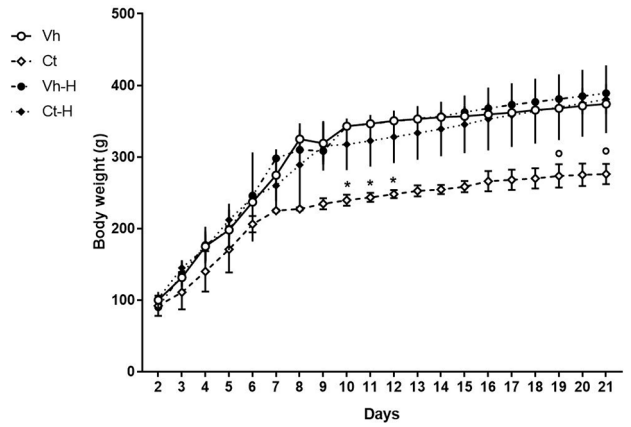

FIGURE 1 | Graph showing the effect of prenatal corticosterone on body weight from postnatal day 2 until 21. Each value represents the mean ± SD of 10 rats. $^{*}p < 0.05$ vs. Vh, $^{\circ}p < 0.05$ vs. Vh-H.

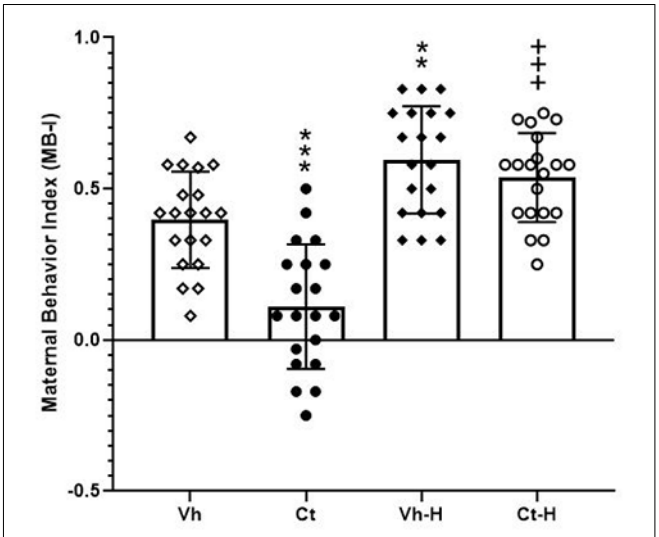

FIGURE 2 | Maternal Behavior Index (MB-I). Influence of a daily 15-min early handling procedure on dams spontaneous behavior (retrieval, nursing, pup care, dam self-care). Each value represents the mean of ± SD of 20 measures. $^{***}p < 0.001$, $^{**}p < 0.01$, vs. Vh, $^{+++}p < 0.001$ vs. Ct.

dams ($q = 7.386$, $p < 0.0001$) with respect to Vh group. Moreover, early handling was able to increase dams maternal behavior in both Vh-H ($q = 5.096$, $p < 0.0031$) and Ct-H ($q = 10.98$, $p < 0.0001$) groups, when compared to respective control groups (**Figure 2**).

Open Field Test

Rats were tested in the OFT in order to assess the influence of prenatal corticosterone exposure and early handling on behavioral reactivity. Results obtained by a two-way ANOVA performed on total distance travelled, number of transition from peripheral to central squares of the arena, and amount of time spent on the central areas as dependent variables, and prenatal corticosterone and early handling as independent variables, showed that prenatal corticosterone, early handling and their interaction were significant for TDT ($F_{(1,36)} = 91.79$, $p < 0.0001$; $F_{(1,36)} = 66.16$, $p < 0.0001$; $F_{(1,36)} = 8.866$, $p = 0.0052$), and ATC ($F_{(1,36)} = 6.388$; $p = 0.0160$; $F_{(1,36)} = 87.15$; $p < 0.0001$; $F_{(1,36)} = 4.494$; $p = 0.0410$). *Post hoc* analysis conducted by Tukey's multiple comparison post-test showed that prenatal Ct induced a decrease in both TDT and in ATC ($q = 12.56$; $p < 0.001$; $q = 4.647$; $p < 0.0116$) when compared to Vh groups. On the contrary, the early handling procedure induced an increase in TDT and in ATC in both Vh ($q = 5.156$, $p < 0.0044$; $q = 7.216$, $p < 0.001$) and Ct ($q = 17.71$, $p < 0.001$; = 11.46, $p < 0.001$) treated rats when compared to respective controls (**Figure 3**). No statistical difference was observed when Ct-H was compared to Vh-H group. No statistical differences were found on a number of transitions from peripheral to central squares of the arena.

Acoustic Startle Reflex Test

The effects of prenatal corticosterone exposure and the influence of early handling procedure on the response to an anxiety-inducing intense stimulus, were evaluated measuring startle amplitude in the ASR test. The results of a two-way ANOVA performed on the peak amplitude as dependent variable, and prenatal corticosterone and early handling as independent variables, indicated that: the factor prenatal corticosterone ($F_{(1,36)} = 29.48$; $p < 0.0001$) early handling ($F_{(1,36)} = 503.4$; $p < 0.0001$) and their interaction ($F_{(1,36)} = 78.57$; $p < 0.0001$) were significant. In detail, Tukey's multiple comparison post-test analysis showed that the prenatal treatment with Ct induced an increase in startle amplitude ($q = 14.29$; $p < 0.001$) when compared to Vh. Interestingly, early handling was able to reduce startle amplitude in both Vh ($q = 13.57$; $p < 0.001$) and in Ct ($q = 31.30$; $p < 0.001$) treated rats, when compared to respective controls. No statistical difference was observed when Ct-H was compared to Vh-H group ($q = 16.70$; $p > 0.05$; **Figure 4**).

Forced Swim Test

Rats were tested in the Porsolt test in order to evaluate the effects of prenatal exposure to corticosterone and the influence of early handling procedure on depressive-like behavior. Rats were first exposed to the pre-stage and, 24 h after, underwent the 5-min stage test, when immobility-, swimming-time were recorded. A two-way ANOVA performed on time spent in immobility, swimming, as a dependent variable, and prenatal corticosterone and early handling as independent variables. The results indicate that prenatal corticosterone, early handling and their interaction were significant for both immobility ($F_{(1,36)} = 5.327$; $p = 0.0269$); ($F_{(1,36)} = 299.9$; $p < 0.0001$); ($F_{(1,36)} = 10.81$; $p = 0.0023$) and swimming ($F_{(1,36)} = 7.119$; $p = 0.0114$); ($F_{(1,36)} = 242.9$; $p < 0.0001$); ($F_{(1,36)} = 11.32$; $p = 0.0018$). Tukey's multiple comparison post-test analysis showed that the prenatal treatment with corticosterone induced an increase in immobility time in Ct ($q = 5.596$; $p < 0.0019$) with respect to Vh, and a significant decrease on immobility time in both Vh-H ($q = 14.03$; $p < 0.001$) and in Ct-H ($q = 20.6$; $p < 0.001$) when compared respectively with Vh and Ct groups (**Figure 5A**). In agreement with these results, *post hoc* analysis showed a significant decrease in Ct ($q = 6.033$; $p = 0.0008$) compared to Vh-rats and an increase in

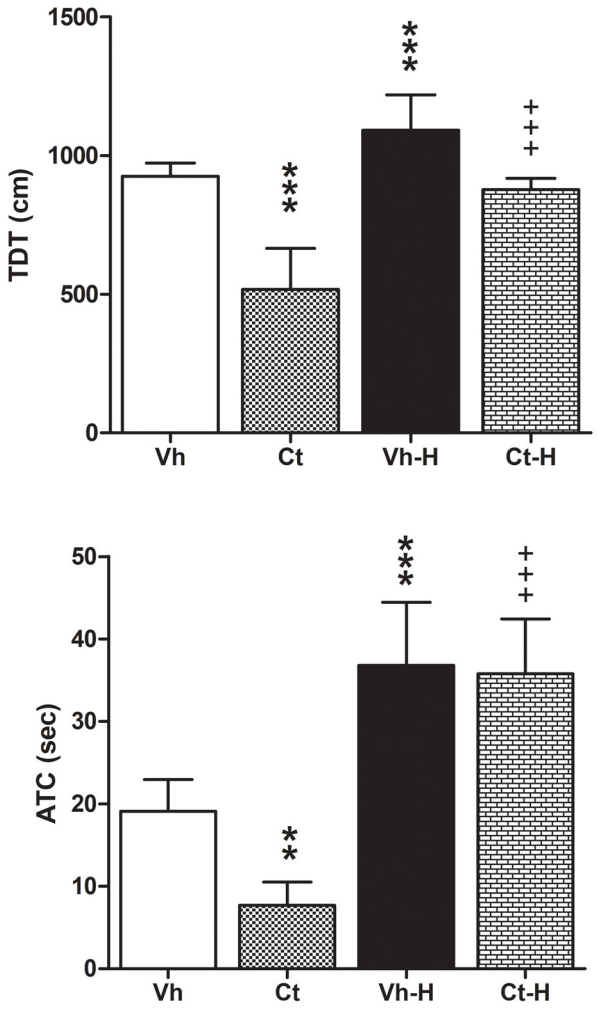

FIGURE 3 | Open field test (OFT). Effects of prenatal corticosterone exposure and early handling procedure on total distance traveled (TDT), and amount of time spent (ATC) in the central area of the arena. Each value represents the mean ± SD of 10 rats. $^{***}p < 0.001$, $^{**}p < 0.01$, vs. Vh, $^{+++}p < 0.001$ vs. Ct.

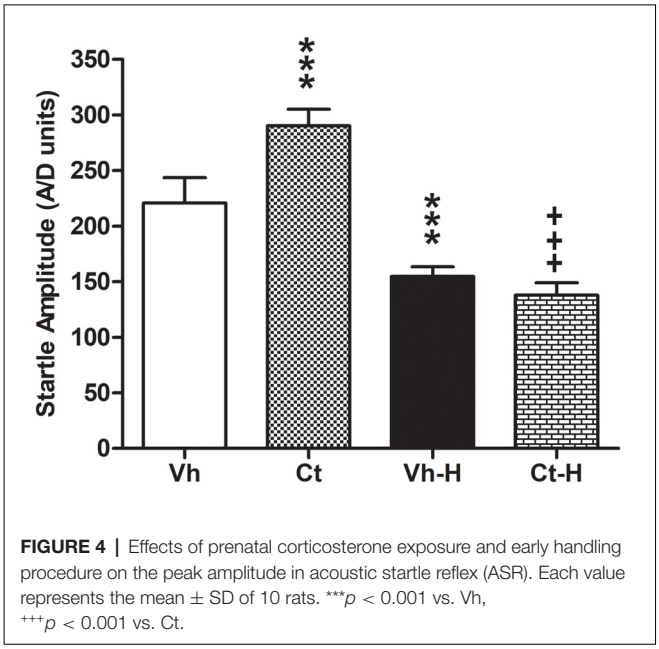

FIGURE 4 | Effects of prenatal corticosterone exposure and early handling procedure on the peak amplitude in acoustic startle reflex (ASR). Each value represents the mean ± SD of 10 rats. $^{***}p < 0.001$ vs. Vh, $^{+++}p < 0.001$ vs. Ct.

swimming time in both Vh-H ($q = 12.22$; $p < 0.001$) and in Ct-H ($q = 18.95$; $p < 0.001$) with respect to their non-handled controls, and (**Figure 5B**).

Corticosterone Plasma Levels

The effects of prenatal exposure to corticosterone, early handling and their mutual influence on corticosterone plasma levels in rats under non-shock- or shock-induced stress conditions were also investigated. A two-way ANOVA performed on the levels of corticosterone under non-shock-induced conditions as dependent variables, and prenatal corticosterone treatment, early handling as independent variables indicate that: prenatal corticosterone ($F_{(1,36)} = 5.311$; $p = 0.0271$) early handling ($F_{(1,36)} = 515.9$; $p < 0.0001$) and their interaction ($F_{(1,36)} = 4.502$; $p = 0.004$), were significant under non-shock-induced conditions. The results of Tukey's multiple comparisons test showed that non-shock-induced corticosterone plasma levels increased in Ct-exposed offspring compared to vehicle group ($q = 4.426$; $p < 0.0174$) and that early handling reduced corticosterone plasma levels in both Vh-H ($q = 20.59$, $p < 0.0001$; $q = 24.84$, $p < 0.0001$) and in Ct-H ($q = 26.23$, $p < 0.001$; $q = 28.67$, $p < 0.001$) when compared with respective control groups (**Figure 6A**).

When rats were exposed to shock-induced stress conditions in order to evaluate the corticosterone plasma levels under stressful conditions, the results of a two-way ANOVA performed respectively on the levels of corticosterone as dependent variables, and prenatal corticosterone treatment, early handling as independent variables showed a significant effect for early handling ($F_{(1,36)} = 913.3$; $p < 0.0001$) and interaction between corticosterone treatment and early handling ($F_{(1,36)} = 4.325$; $p = 0.0447$), but no for prenatal corticosterone treatment ($F_{(1,36)} = 2.584$; $p = 0.1167$). In detail, Tukey's multiple comparison post-test analysis showed that; shock exposure did not modify corticosterone levels in Ct-exposed offspring ($q = 0.472$, $p = 0.9870$), and that early handling reduced corticosterone plasma levels in both Vh-H ($q = 28.14$, $p < 0.0001$) and in Ct-H ($q = 32.30$, $p < 0.0001$) when compared with respective control groups (**Figure 6B**).

DISCUSSION

In agreement with previous animal studies, we here show that exposure to corticosterone, during the 3rd week of rat gestation, can affect maternal care and program an abnormal neuroendocrine and behavioral profile of the adolescent offspring that resembles a vulnerable phenotype for affective disorders (French et al., 1999; Shoener et al., 2006). Notably, early handling as a brief maternal separation during the

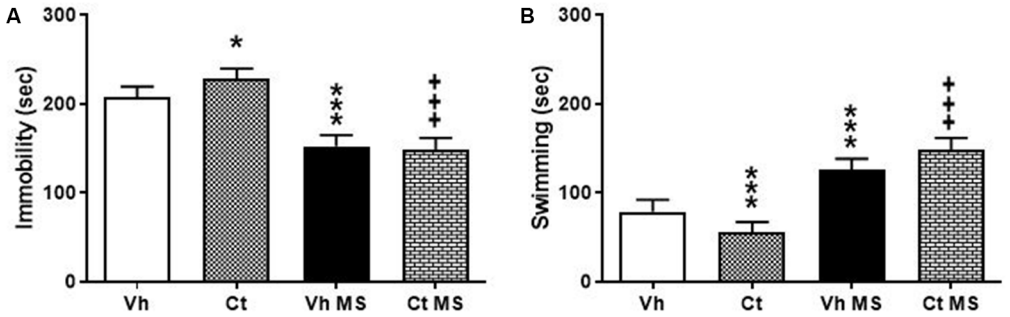

FIGURE 5 | Effects of prenatal corticosterone exposure and early handling procedure on immobility **(A)** and swimming **(B)** in the Forced swim test (FST). Each value represents the mean ± SD of 10 rats. $^{***}p < 0.001$, $^{*}p < 0.05$ vs. Vh, $^{+++}p < 0.001$ vs. Ct.

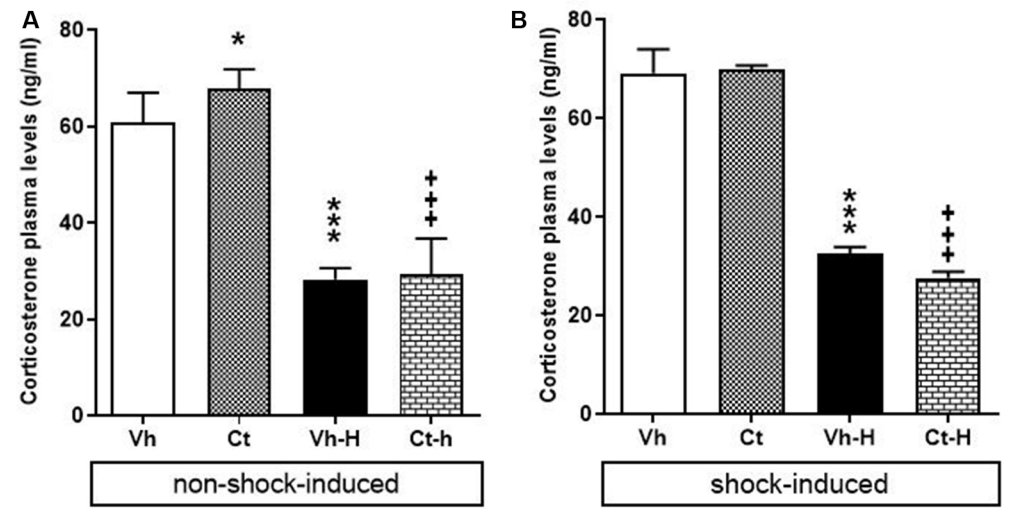

FIGURE 6 | Effects of prenatal corticosterone exposure and early handling procedure on: non-shock- **(A)** and shock-induced **(B)** corticosterone plasma levels. Each value represents the mean ± SD of five rats. $^{***}p < 0.001$, $^{*}p < 0.05$ vs. Vh, $^{+++}p < 0.001$ vs. Ct.

early stages of postnatal life, promoted an increase in maternal care and counterbalanced the detrimental effects induced by the prenatal glucocorticoid manipulation in all the investigated parameters.

Effects of Prenatal Exposure to Corticosterone

The first evidence following the manipulation of the intrauterine environment by corticosterone injection from GD 14 to 16 was a reduction in weight during the pre-weaning time. Our data are in accordance with studies showing that glucocorticoid treatment during pregnancy reduces offspring birth weight and body weight throughout adolescence (Smith and Waddell, 2000; Manojlović-Stojanoski et al., 2012) as well as the reduction on birth weight appears more evident when glucocorticoids are administered during the 3rd week of gestation and not earlier, indicating a late gestational window of sensitivity to glucocorticoids (Nyirenda et al., 1998; Seckl, 2004). Although the reduced body weight of the offspring as a consequence of gestational corticosterone exposure is still not fully clear, Iwasa et al. (2014) suggested a possible alteration of serum leptin and hypothalamic neuropeptide Y (NPY) mRNA levels, two peptides playing pivotal roles in the regulation of appetite and calories intake, as well as in the modulation of emotionality (Velísek, 2006; Iwasa et al., 2014; Plescia et al., 2014a).

A critical outcome of glucocorticoid exposure in early life is the programming of emotional and affective homeostasis. In the rat, *in utero* glucocorticoids, either from an exogenous source or *via* maternal extra-release, induce a decrease in behavioral reactivity in the open field and an increase in anxiety-like behavior in the elevated plus-maze in the offspring (Harris and Seckl, 2011). These alterations may be associated with an impairment in offspring's capacity to cope under a stressful situation in adolescence (Vallée et al., 1997; Dickerson et al., 2005; Harris and Seckl, 2011), enhancing the risk of emerging

psychological disorders (Casey et al., 2010). Accordingly, our data demonstrate that the prenatal Ct-treatment during a sensitive time window, was able to induce an overall impairment of locomotor activity in the adolescent offspring, as shown by a reduction in TDT and in the exploration of the central areas of the arena. The reduction in behavioral reactivity might reflect an increased emotional response to the novel environment. Consistently, adolescent rats exposed *in utero* to corticosterone exhibited an increase in the peak amplitude of the ASR, as a proof of their negative emotional state (Lang et al., 1990; Lang, 1995; Bradley and Sabatinelli, 2003; McMillan et al., 2012). Indeed, The ASR, a reflexive movement occurring after sudden exposure to loud noise, represents a valid behavioral model to study the emotional response of the animals. An increase in the amplitude of the ASR is ascribed to a rise in emotionality, which mirrors a higher sensitivity of the animals towards an anxiogenic environment (Hijzen et al., 1995; Cannizzaro et al., 2002). These results are in accordance with our data on the FST the most commonly used assay to test the efficacy of chronic antidepressant treatments (Detke et al., 1997). Our findings indicate that prenatal Ct treatment was able to increase immobility time and decrease swimming in the adolescent offspring, promoting the occurrence of a depressive-like phenotype (Yankelevitch-Yahav et al., 2015).

Indeed, over-exposure to glucocorticoids and impaired GR signaling can result in degeneration and functional impairment of brain regions critically involved in mood processing and contribute to the induction of depressive symptoms later in life (Anacker et al., 2011; Brancato et al., 2017; Di Liberto et al., 2017; Shishkina and Dygalo, 2017).

During development, there is a relatively high expression of GR from midgestation onwards (Diaz et al., 1998), which are essential for normal brain development and offspring survival (Kapoor et al., 2008). In the rat, antenatal stress or maternal administration of glucocorticoids during this time window results in offspring with decreased expression of GR mRNA in specific brain areas involved in glucocorticoid feedback such as the hippocampus, hypothalamus, and pituitary (Levitt et al., 1996; Liu et al., 2001). This reduction could promote pups grow up with altered negative feedback response, manifested as a chronic elevation of corticosterone (Maccari and Morley-Fletcher, 2007). Indeed, the behavioral outcomes here observed are supported by the results from plasma corticosterone level assessment in non-shock-induced conditions. Specifically, prenatally exposed adolescent offspring showed an increase in non-shock-induced plasma corticosterone levels, in line with findings in rodents and non-human primates (Welberg et al., 2001; de Vries et al., 2007; Rakers et al., 2017). It has been shown previously that differences in HPA axis activity are associated with differences in locomotor activity in response to novelty (Gancarz et al., 2012). Prenatal stress induces a prolonged corticosterone secretion, which is negatively correlated with lower levels of explorative behavior in the open field (Rosecrans, 1970; Iuvone and Van Hartesveldt, 1976; Vallée et al., 1997). Moreover, a significant correlation between plasma corticosterone levels and the behavioral scores in the FST was observed (Morley-Fletcher et al., 2003).

Plasma corticosterone levels in the non-shock-induced group do not differ from levels in the shock-induced group. This may be for that non-shocked group plasma corticosterone levels do not reflect baseline activity of the HPA axis, but rather HPA axis reactivity in response to novelty of the electrified grid floor cage (Friedman et al., 1967; Bassett et al., 1973). Furthermore, differently from plasma corticosterone levels in shock-induced condition, we found that corticosterone release after shock administration did not differ between prenatally exposed adolescent offspring and Vh group. This can be due to an altered drive of the HPA axis programming that may result from the combination of *in utero* Ct-treatment and stress exposure in adolescence. Indeed, we may speculate that prenatal corticosterone treatment was able to reduce the density of corticosteroid receptors that, through the attenuation of HPA axis feedback sensitivity, set the release of corticosterone to a ceiling set point already at basal conditions (Pornsawad, 2013). This might prevent the physiological rise in stress-related glucocorticoid release as we have observed in this study and might represent a vulnerable factor for the development of emotional and affective disorders (Harris and Seckl, 2011; Constantinof et al., 2016).

Effects of Early Brief Maternal Separation

In most mammalian species, the maternal environment represents the developmental context within which mothers shape socio-emotional maturation of the progeny, serving as essential external regulators of infant physiology, neurodevelopment, and behavioral responses. Thus, manipulating quality and consistency of maternal care during the early stages of life can influence and, also revert developmental processes that set emotional and physiological responses in adulthood (Drury et al., 2016).

Numerous studies have shown that at least some of the long-term effects of early-life exposure to an adverse environment are mediated by low levels of parent-child linking and decreased parental investment during early childhood. For instance, poor parental ties are usually associated with increased risk for several psychological vulnerabilities, whereas an increase in parental care improved behavioral outcomes, cognitive performance and also boost resiliency to stress (Canetti et al., 1997; Meaney, 2001; Kaffman and Meaney, 2007). Accordingly, early handling procedure, consisting in a short maternal separation of the mother from the pups, represents a particular event for the dam that is able to produce higher level of interest by the mother in the offspring and, in turn, elicits more maternal care upon reunion (Rees and Fleming, 2001; Kosten and Kehoe, 2010; Zimmerberg and Sageser, 2011; Own and Patel, 2013; Orso et al., 2018). These observations are consistent with those obtained in the present research where the effects on the maternal-infant dyad were investigated. Indeed, our results show that early handling procedure produced an increase in maternal care, as shown by a higher MB-I, that in turn, improved the response to stressful situations and reduced emotionality in the offspring. Specifically, when compared to non-handled counterparts, briefly maternal separated adolescent rats showed increased locomotor activity, reduced avoidance of the center of

the arena in the open field, and decreased peak amplitude in ASR. At the same time, early-handled offspring displayed a reduction in immobility time and an increase in swimming time in the FST, together with a reduction in corticosterone plasma levels, under non-shock- and shock-induced conditions.

The mitigated emotional profile observed in early handled rats in this study may be dependent upon modifications of the developing HPA axis (Kaffman and Meaney, 2007). In particular, the effect of early handling on behavioral reactivity and emotionality may be due to a dampening of HPA axis response in the progeny that better cope with the task administered (Cannizzaro et al., 2006b). Indeed, maternal behaviors, such as licking, grooming and arched-back, lead to increased GR mRNA expression in the brain, glucocorticoid negative feedback sensitivity, and decreased hypothalamic corticotropin-releasing factor mRNA levels (Meaney, 2001; Edelmann et al., 2016). Taken together these data suggest that postnatal maternal care is able to affect the magnitude of the HPA axis response to stress, "hardening" the pups which display a blunting in corticosterone release and in emotional profile (Meaney et al., 1985, 1988; Liu et al., 1997). On the other hand, the variations in the early postnatal environment can interact with the effects of prenatal exposure to stressors in a complex, mutually interacting process (Cannizzaro et al., 2006b). Indeed, whether early exposure to corticosterone is associated with elevation of non-shock-induced conditions corticosterone release and with a vulnerable phenotype for emotional and affective disturbances, early handling procedure induces opposite modifications in the stress-behavioral responses and corticosterone release that are associated to the occurrence of a "rescued" profile. Although we believe that the rodent model used in this study will be helpful to identify physiological mechanisms underlying the neuroendocrine functional response to stress induced by early handling in prenatal corticosterone condition, this issue deserves further insight in future researches on many distinct players which may take part to the interplay between maternal care and the regulation of the HPA axis, such as oxytocin (Cannizzaro et al., 2006a; Kojima et al., 2012; Cox et al., 2015; Zinni et al., 2018). However, it is evident that increasing the intensity of maternal care, could serve as a source for the enhancement of neuronal plasticity able to promote adaptive behavioral responses.

CONCLUSION

These findings highlight a brief prenatal exposure to glucocorticoids during the 3rd week of gestation as a signal able to produce behavioral and neuroendocrine abnormalities later in life, contributing to the programming of a vulnerable phenotype to emotional- and affective-like disorders. This issue is particularly relevant due to the common practice of multiple administrations of glucocorticoids to pregnant women during late gestation to ensure the survival of the preterm newborns. Even though synthetic glucocorticoids, such as dexamethasone (DEX) or betamethasone, have been extensively used rather than cortisol or hydrocortisone (Jobe, 2003; Oliveira et al., 2006; Singh et al., 2012), the natural glucocorticoid is increasingly considered as an alternative therapy during pregnancy (Crowther et al., 2019). Therefore, a long-term follow-up in children who were treated *in utero* with glucocorticoids is strongly recommended. As expected, we here show that enhanced maternal care plays a primary role in setting pro-adaptive behavioral and neuroendocrine responses and may re-route aberrant trajectories during neurodevelopment, emphasizing the role of an optimal mother-infant dyad as a protective factor for healthy development of the offspring.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee for the Protection and Use of Animals of the University of Palermo.

AUTHOR CONTRIBUTIONS

FP has formulated evolution of overarching research goals and aims and has coordinated the research activity planning and execution. He has provided statistical analyzses, has written the article and has acquired the financial support for the project leading to this publication. VC has carried out research and investigation activity, performed the experiments, and has collaborated on the writing of the manuscript. GL and AB has carried out research and investigation activity, performed the experiments.

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REFERENCES

Ader, R., and Grota, L. J. (1970). Rhythmicity in the maternal behaviour of Rattus norvegicus. *Anim. Behav.* 18, 144–150. doi: 10.1016/0003-3472(70)90083-7

Alexander, N., Rosenlöcher, F., Stalder, T., Linke, J., Distler, W., Morgneret, J., et al. (2012). Impact of antenatal synthetic glucocorticoid exposure on endocrine stress reactivity in term-born children. *J. Clin. Endocrinol. Metab.* 97, 3538–3544. doi: 10.1210/jc.2012-1970

Anacker, C., Zunszain, P. A., Carvalho, L. A., and Pariante, C. M. (2011). The glucocorticoid receptor: pivot of depression and of antidepressant treatment? *Psychoneuroendocrinology* 36, 415–425. doi: 10.1016/j.psyneuen.2010.03.007

Azhari, A., Leck, W. Q., Gabrieli, G., Bizzego, A., Rigo, P., Setoh, P., et al. (2019). Parenting stress undermines mother-child brain-to-brain synchrony: a hyperscanning study. *Sci. Rep.* 9:11407. doi: 10.1038/s41598-019-47810-4

Baker, S., Chebli, M., Rees, S., Lemarec, N., Godbout, R., and Bielajew, C. (2008). Effects of gestational stress: 1. Evaluation of maternal and juvenile offspring behavior. *Brain Res.* 1213, 98–110. doi: 10.1016/j.brainres.2008.03.035

Bassett, J. R., Cairncross, K. D., and King, M. G. (1973). Parameters of novelty, shock predictability and response contigency in corticosterone release in the rat. *Physiol. Behav.* 10, 901–907. doi: 10.1016/0031-9384(73)90060-7

Bella, A. F., Andersson, E., Goding, K., and Vonderheid, S. C. (2018). The birth experience and maternal caregiving attitudes and behavior: a systematic review. *Sex. Reprod. Healthc.* 16, 67–77. doi: 10.1016/j.srhc.2018.02.007

Bhagwanani, S. G., Seagraves, K., Dierker, L. J., and Lax, M. (1997). Relationship between prenatal anxiety and perinatal outcome in nulliparous women: a prospective study. *J. Natl. Med. Assoc.* 89, 93–98.

Boero, G., Biggio, F., Pisu, M. G., Locci, V., Porcu, P., and Serra, M. (2018). Combined effect of gestational stress and postpartum stress on maternal care in rats. *Physiol. Behav.* 184, 172–178. doi: 10.1016/j.physbeh.2017.11.027

Bradley, M. M., and Sabatinelli, D. (2003). ''Startle reflex modulation: perception, attention, and emotion,'' in *Experimental Methods in Neuropsychology. Neuropsychology and Cognition, vol 21*, ed. K. Hugdahl (Boston, MA: Springer), 65–87.

Brancato, A., Bregman, D., Ahn, H. F., Pfau, M. L., Menard, C., Cannizzaro, C., et al. (2017). Sub-chronic variable stress induces sex-specific effects on glutamatergic synapses in the nucleus accumbens. *Neuroscience* 350, 180–189. doi: 10.1016/j.neuroscience.2017.03.014

Brancato, A., Plescia, F., Lavanco, G., Cavallaro, A., and Cannizzaro, C. (2016). Continuous and intermittent alcohol free-choice from pre-gestational time to lactation: focus on drinking trajectories and maternal behavior. *Front. Behav. Neurosci.* 10:31. doi: 10.3389/fnbeh.2016.00031

Brancato, A., Plescia, F., Marino, R. A. M., Maniaci, G., Navarra, M., and Cannizzaro, C. (2014). Involvement of dopamine D2 receptors in addictive-like behaviour for acetaldehyde. *PLoS One* 9:e99454. doi: 10.1371/journal.pone.0099454

Cacace, S., Plescia, F., La Barbera, M., and Cannizzaro, C. (2011). Evaluation of chronic alcohol self-administration by a 3-bottle choice paradigm in adult male rats. *Behav. Brain Res.* 219, 213–220. doi: 10.1016/j.bbr.2011.01.004

Canetti, L., Bachar, E., Galili-Weisstub, E., De-Nour, A. K., and Shalev, A. Y. (1997). Parental bonding and mental health in adolescence. *Adolescence* 32, 381–394.

Cannizzaro, C., D'Amico, M., Preziosi, P., and Martire, M. (2006a). Presynaptic effects of anandamide and WIN55,212–2 on glutamatergic nerve endings isolated from rat hippocampus. *Neurochem. Int.* 48, 159–165. doi: 10.1016/j.neuint.2005.10.009

Cannizzaro, C., Martire, M., Steardo, L., Cannizzaro, E., Gagliano, M., Mineo, A., et al. (2002). Prenatal exposure to diazepam and alprazolam, but not to zolpidem, affects behavioural stress reactivity in handling-naïve and handling-habituated adult male rat progeny. *Brain Res.* 953, 170–180. doi: 10.1016/s0006-8993(02)03282-1

Cannizzaro, C., Plescia, F., Martire, M., Gagliano, M., Cannizzaro, G., Mantia, G., et al. (2006b). Single, intense prenatal stress decreases emotionality and enhances learning performance in the adolescent rat offspring: interaction with a brief, daily maternal separation. *Behav. Brain Res.* 169, 128–136. doi: 10.1016/j.bbr.2005.12.010

Cannizzaro, C., Plescia, F., Gagliano, M., Cannizzaro, G., Mantia, G., La Barbera, M., et al. (2008). Perinatal exposure to 5-metoxytryptamine, behavioural-stress reactivity and functional response of 5-HT1A receptors in the adolescent rat. *Behav. Brain Res.* 186, 98–106. doi: 10.1016/j.bbr.2007.07.036

Cannizzaro, C., Plescia, F., Gagliano, M., Cannizzaro, G., Provenzano, G., Mantia, G., et al. (2007). Effects of pre- and postnatal exposure to 5-methoxytryptamine and early handling on an object-place association learning task in adolescent rat offspring. *Neurosci. Res.* 59, 74–80. doi: 10.1016/j.neures.2007.05.012

Cannizzaro, E., Martire, M., Gagliano, M., Plescia, F., La Barbera, M., Mantia, G., et al. (2005). Reversal of prenatal diazepam-induced deficit in a spatial-object learning task by brief, periodic maternal separation in adult rats. *Behav. Brain Res.* 161, 320–330. doi: 10.1016/j.bbr.2005.02.022

Capone, F., Bonsignore, L. T., and Cirulli, F. (2005). Methods in the analysis of maternal behavior in the rodent. *Curr. Protoc. Toxicol.* Chapter 13:Unit 13.9. doi: 10.1002/0471140856.tx1309s26

Casey, B. J., Jones, R. M., Levita, L., Libby, V., Pattwell, S. S., Ruberry, E. J., et al. (2010). The storm and stress of adolescence: insights from human imaging and mouse genetics. *Dev. Psychobiol.* 52, 225–235. doi: 10.1002/dev.20447

Charil, A., Laplante, D. P., Vaillancourt, C., and King, S. (2010). Prenatal stress and brain development. *Brain Res. Rev.* 65, 56–79. doi: 10.1016/j.brainresrev.2010.06.002

Cintra, A., Solfrini, V., Bunnemann, B., Okret, S., Bortolotti, F., Gustafsson, J. A., et al. (1993). Prenatal development of glucocorticoid receptor gene expression and immunoreactivity in the rat brain and pituitary gland: a combined *in situ* hybridization and immunocytochemical analysis. *Neuroendocrinology* 57, 1133–1147. doi: 10.1159/000126480

Cogill, S. R., Caplan, H. L., Alexandra, H., Robson, K. M., and Kumar, R. (1986). Impact of maternal depression in cognitive development of young children. *Br. Med. J.* 292, 1165–1167. doi: 10.1136/bmj.292.6529.1165

Constantinof, A., Moisiadis, V. G., and Matthews, S. G. (2016). Programming of stress pathways: a transgenerational perspective. *J. Steroid. Biochem. Mol. Biol.* 160, 175–180. doi: 10.1016/j.jsbmb.2015.10.008

Cox, E. Q., Stuebe, A., Pearson, B., Grewen, K., Rubinow, D., and Meltzer-Brody, S. (2015). Oxytocin and HPA stress axis reactivity in postpartum women. *Psychoneuroendocrinology* 55, 164–172. doi: 10.1016/j.psyneuen.2015.02.009

Crowther, C. A., Middleton, P. F., Voysey, M., Askie, L., Zhang, S., Martlow, T. K., et al. (2019). Effects of repeat prenatal corticosteroids given to women at risk of preterm birth: an individual participant data meta-analysis. *PLoS Med.* 16:e1002771. doi: 10.1371/journal.pmed.1002771

Darnaudéry, M., Dutriez, I., Viltart, O., Morley-Fletcher, S., and Maccari, S. (2004). Stress during gestation induces lasting effects on emotional reactivity of the dam rat. *Behav. Brain Res.* 153, 211–216. doi: 10.1016/j.bbr.2003.12.001

de Vries, A., Holmes, M. C., Heijnis, A., Seier, J. V., Heerden, J., Louw, J., et al. (2007). Prenatal dexamethasone exposure induces changes in nonhuman primate offspring cardiometabolic and hypothalamic- pituitary-adrenal axis function. *J. Clin. Invest.* 117, 1058–1067. doi: 10.1172/JCI30982

Detke, M. J., Johnson, J., and Lucki, I. (1997). Acute and chronic antidepressant drug treatment in the rat forced swimming test model of depression. *Exp. Clin. Psychopharmacol.* 5, 107–112. doi: 10.1037/1064-1297.5.2.107

Di Liberto, V., Frinchi, M., Verdi, V., Vitale, A., Plescia, F., Cannizzaro, C., et al. (2017). Anxiolytic effects of muscarinic acetylcholine receptors agonist oxotremorine in chronically stressed rats and related changes in BDNF and FGF2 levels in the hippocampus and prefrontal cortex. *Psychopharmacology* 234, 559–573. doi: 10.1007/s00213-016-4498-0

Diaz, R., Brown, R. W., and Seckl, J. R. (1998). Distinct ontogeny of glucocorticoid and mineralocorticoid receptor and 11β-hydroxysteroid dehydrogenase types I and II mRNAs in the fetal rat brain suggest a complex control of glucocorticoid actions. *J. Neurosci.* 18, 2570–2580. doi: 10.1523/JNEUROSCI.18-07-02570.1998

Dickerson, P. A., Lally, B. E., Gunnel, E., Birkle, D. L., and Salm, A. K. (2005). Early emergence of increased fearful behavior in prenatally stressed rats. *Physiol. Behav.* 86, 586–593. doi: 10.1016/j.physbeh.2005.08.025

Drury, S. S., Sánchez, M. M., and Gonzalez, A. (2016). When mothering goes awry: challenges and opportunities for utilizing evidence across rodent, nonhuman primate and human studies to better define the biological consequences of negative early caregiving. *Horm. Behav.* 77, 182–192. doi: 10.1016/j.yhbeh.2015.10.007

Edelmann, M. N., Sandman, C. A., Glynn, L. M., Wing, D. A., and Davis, E. P. (2016). Antenatal glucocorticoid treatment is associated with diurnal cortisol regulation in term-born children. *Psychoneuroendocrinology* 72, 106–112. doi: 10.1016/j.psyneuen.2016.06.012

Fowden, A. L., and Forhead, A. J. (2015). Glucocorticoids as regulatory signals during intrauterine development. *Exp. Physiol.* 100, 1477–1487. doi: 10.1113/ep085212

French, N. P., Hagan, R., Evans, S. F., Godfrey, M., and Newnham, J. P. (1999). Repeated antenatal corticosteroids: size at birth and subsequent development. *Am. J. Obstet. Gynecol.* 180, 114–121. doi: 10.1016/s0002-9378(99)70160-2

French, N. P., Hagan, R., Evans, S. F., Mullan, A., and Newnham, J. P. (2004). Repeated antenatal corticosteroids: effects on cerebral palsy and childhood behavior. *Am. J. Obstet. Gynecol.* 190, 588–595. doi: 10.1016/j.ajog.2003.12.016

Friedman, S. B., Ader, R., Grota, L. J., and Larson, T. (1967). Plasma corticosterone response to parameters of electric shock stimulation in the rat. *Psychosom. Med.* 29, 323–328. doi: 10.1097/00006842-196707000-00003

Gancarz, A. M., Robble, M. A., Kausch, M. A., Lloyd, D. R., and Richards, J. B. (2012). Association between locomotor response to novelty and light reinforcement: sensory reinforcement as a rodent model of sensation seeking. *Behav. Brain Res.* 230, 380–388. doi: 10.1016/j.bbr.2012.02.028

Gemmel, M., Harmeyer, D., Bögi, E., Fillet, M., Hill, L. A., Hammond, G. L., et al. (2018). Perinatal fluoxetine increases hippocampal neurogenesis and reverses the lasting effects of pre-gestational stress on serum corticosterone, but not on maternal behavior, in the rat dam. *Behav. Brain Res.* 339, 222–231. doi: 10.1016/j.bbr.2017.11.038

Hantsoo, L., Golden, C. E. M., Kornfield, S., Grillon, C., and Epperson, C. N. (2018). Startling differences: using the acoustic startle response to study sex differences and neurosteroids in affective disorders. *Curr. Psychiatry Rep.* 20:40. doi: 10.1007/s11920-018-0906-y

Harris, A., and Seckl, J. (2011). Glucocorticoids, prenatal stress and the programming of disease. *Horm. Behav.* 59, 279–289. doi: 10.1016/j.yhbeh.2010.06.007

Heim, C., Owens, M. J., Plotsky, P. M., and Nemeroff, C. B. (1997). The role of early adverse life events in the etiology of depression and posttraumatic stress disorder. Focus on corticotropin-releasing factor. *Ann. N Y Acad. Sci.* 821, 194–207. doi: 10.1111/j.1749-6632.1997.tb48279.x

Hellemans, K. G. C., Verma, P., Yoon, E., Yu, W. K., Young, A. H., and Weinberg, J. (2010). Prenatal alcohol exposure and chronic mild stress differentially alter depressive- and anxiety-like behaviors in male and female offspring. *Alcohol. Clin. Exp. Res.* 34, 633–645. doi: 10.1111/j.1530-0277.2009.01132.x

Hijzen, T. H., Houtzager, S. W. I., Joordens, R. J., Olivier, B., and Slangen, J. L. (1995). Predictive validity of the potentiated startle response as a behavioral model for anxiolytic drugs. *Psychopharmacology* 118, 150–154. doi: 10.1007/bf02245833

Hoffman, K. L. (2016). New dimensions in the use of rodent behavioral tests for novel drug discovery and development. *Expert Opin. Drug Discov.* 11, 343–353. doi: 10.1517/17460441.2016.1153624

Iuvone, P. M., and Van Hartesveldt, C. (1976). Locomotor activity and plasma corticosterone in rats with hippocampal lesions. *Behav. Biol.* 16, 515–520. doi: 10.1016/s0091-6773(76)91699-0

Iwasa, T., Matsuzaki, T., Munkhzaya, M., Tungalagsuvd, A., Kawami, T., and Murakami, M. (2014). Prenatal exposure to glucocorticoids affects body weight, serum leptin levels, and hypothalamic neuropeptide-Y expression in pre-pubertal female rat offspring. *Int. J. Dev. Neurosci.* 36, 1–4. doi: 10.1016/j.ijdevneu.2014.03.011

Jafari, Z., Mehla, J., Afrashteh, N., Kolb, B. E., and Mohajerani, M. H. (2017). Corticosterone response to gestational stress and postpartum memory function in mice. *PLoS One* 12:e0180306. doi: 10.1371/journal.pone.0180306

Jobe, A. H. (2003). Animal models of antenatal corticosteroids: clinical implications. *Clin. Obstet. Gynecol.* 46, 174–189. doi: 10.1097/00003081-200303000-00021

Kaffman, A., and Meaney, M. J. (2007). Neurodevelopmental sequelae of postnatal maternal care in rodents: clinical and research implications of molecular insights. *J. Child Psychol. Psychiatry* 48, 224–244. doi: 10.1111/j.1469-7610.2007.01730.x

Kapoor, A., Petropoulos, S., and Matthews, S. G. (2008). Fetal programming of hypothalamic-pituitary-adrenal (HPA) axis function and behavior by synthetic glucocorticoids. *Brain Res. Rev.* 57, 586–595. doi: 10.1016/j.brainresrev.2007.06.013

Kemp, M. W., Newnham, J. P., Challis, J. G., Jobe, A. H., and Stock, S. J. (2016). The clinical use of corticosteroids in pregnancy. *Hum. Reprod. Update* 22, 240–259. doi: 10.1093/humupd/dmv047

Kitraki, E., Kittas, C., and Stylianopoulou, F. (1997). Glucocorticoid receptor gene expression during rat embryogenesis. *Differentiation* 62, 21–31. doi: 10.1046/j.1432-0436.1997.6210021.x

Koehl, M., van der Veen, R., Gonzales, D., Piazza, P. V., and Abrous, D. N. (2012). Interplay of maternal care and genetic influences in programming adult hippocampal neurogenesis. *Biol. Psychiatry* 72, 282–289. doi: 10.1016/j.biopsych.2012.03.001

Kojima, S., Stewart, R. A., Demas, G. E., and Alberts, J. R. (2012). Maternal contact differentially modulates central and peripheral oxytocin in rat pups during a brief regime of mother-pup interaction that induces a filial huddling preference. *J. Neuroendocrinol.* 24, 831–840. doi: 10.1111/j.1365-2826.2012.02280.x

Kosten, T. A., and Kehoe, P. (2010). Immediate and enduring effects of neonatal isolation on maternal behavior in rats. *Int. J. Dev. Neurosci.* 28, 53–61. doi: 10.1016/j.ijdevneu.2009.09.005

Lang, P. J. (1995). The emotion probe: studies of motivation and attention. *Am. Psychol.* 50, 372–385. doi: 10.1037/0003-066x.50.5.372

Lang, P. J., Bradley, M. M., and Cuthbert, B. N. (1990). Emotion, attention, and the startle reflex. *Psychol. Rev.* 97, 377–395. doi: 10.1111/j.1469-8986.1990.tb01966.x

Leggio, G. M., Camillieri, G., Platania, C. B., Castorina, A., Marrazzo, G., Torrisi, S. A., et al. (2014). Dopamine D3 receptor is necessary for ethanol consumption: an approach with buspirone. *Neuropsychopharmacology* 39, 2017–2028. doi: 10.1038/npp.2014.51

Levitt, N. S., Lindsay, R. S., Holmes, M. C., and Seckl, J. R. (1996). Dexamethasone in the last week of pregnancy attenuates hippocampal glucocorticoid receptor gene expression and elevates blood pressure in the adult offspring in the rat. *Neuroendocrinology* 64, 412–418. doi: 10.1159/000127146

Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., et al. (1997). Maternal care, hippocampal glucocorticoid receptors and hypothalamic- pituitary-adrenal responses to stress. *Science* 277, 1659–1662. doi: 10.1126/science.277.5332.1659

Liu, L., Li, A., and Matthews, S. G. (2001). Maternal glucocorticoid treatment programs HPA regulation in adult offspring: sex-specific effects. *Am. J. Physiol. Endocrinol. Metab.* 280, E729–E739. doi: 10.1152/ajpendo.2001.280.5.E729

Maccari, S., and Morley-Fletcher, S. (2007). Effects of prenatal restraint stress on the hypothalamus-pituitary-adrenal axis and related behavioural and neurobiological alterations. *Psychoneuroendocrinology* 32, S10–S15. doi: 10.1016/j.psyneuen.2007.06.005

Manojlović-Stojanoski, M., Nestorović, N., and Milošević, V. (2012). Prenatal glucocorticoids: short-term benefits and long-term risks. Glucocorticoids – New Recognition of Our Familiar Friend, Xiaoxiao Qian, IntechOpen. doi: 10.5772/51106

Martines, F., Salvago, P., Ferrara, S., Messina, G., Mucia, M., Plescia, F., et al. (2016). Factors influencing the development of otitis media among Sicilian children affected by upper respiratory tract infections. *Braz. J. Otorhinolaryngol.* 82, 215–222. doi: 10.1016/j.bjorl.2015.04.002

McMillan, K. A., Asmundson, G. J., Zvolensky, M. J., and Carleton, R. N. (2012). Startle response and anxiety sensitivity: subcortical indices of physiologic arousal and fear responding. *Emotion* 12, 1264–1272. doi: 10.1037/a0029108

Meaney, M. J. (2001). Maternal care, gene expression and the transmission of individual differences in stress reactivity across generations. *Annu. Rev. Neurosci.* 24, 1161–1192. doi: 10.1146/annurev.neuro.24.1.1161

Meaney, M. J., Aitken, D. H., Bodnoff, S. R., Iny, L. J., Tatarewicz, J. E., and Sapolsky, R. M. (1985). Early postnatal handling alters glucocorticoid receptor concentrations in selected brain regions. *Behav. Neurosci.* 99, 765–770. doi: 10.1037/0735-7044.99.4.765

Meaney, M. J., Aitken, D. H., van Berkel, C., Bhatngar, S., and Sapolsky, R. M. (1988). Effect of neonatal handling on age-related impairments associated with the hippocampus. *Science* 239, 766–768. doi: 10.1126/science.3340858

Morley-Fletcher, S., Darnaudery, M., Koehl, M., Casolini, P., Van Reeth, O., and Maccari, S. (2003). Prenatal stress in rats predicts immobility behavior in the forced swim test. Effects of a chronic treatment with tianeptine. *Brain Res.* 989, 246–251. doi: 10.1016/s0006-8993(03)03293-1

Moukarzel, S., Ozias, M., Kerling, E., Christifano, D., Wick, J., Colombo, J., et al. (2018). Maternal Vitamin D Status and infant infection. *Nutrients* 10:E111. doi: 10.3390/nu10020111

Nyirenda, M. J., Lindsay, R. S., Kenyon, C. J., Burchell, A., and Seckl, J. R. (1998). Glucocorticoid exposure in late gestation permanently programs

rat hepatic phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes glucose intolerance in adult offspring. *J. Clin. Invest.* 101, 2174–2181. doi: 10.1172/jci1567

Oliveira, M., Bessa, J. M., Mesquita, A., Tavares, H., Carvalho, A., Silva, R., et al. (2006). Induction of a hyperanxious state by antenatal dexamethasone: a case for less detrimental natural corticosteroids. *Biol. Psychiatry* 59, 844–852. doi: 10.1016/j.biopsych.2005.08.020

Orso, R., Wearick-Silva, L. E., Creutzberg, K. C., Centeno-Silva, A., Roithmann, L. G., Pazzin, R., et al. (2018). Maternal behavior of the mouse dam toward pups: implications for maternal separation model of early life stress. *Stress* 21, 19–27. doi: 10.1080/10253890.2017.1389883

Own, L. S., and Patel, P. D. (2013). Maternal behavior and offspring resiliency to maternal separation in C57Bl/6 mice. *Horm. Behav.* 63, 411–417. doi: 10.1016/j.yhbeh.2012.11.010

Patin, V., Lordi, B., Vincent, A., Thoumas, J. L., Vaudry, H., and Caston, J. (2002). Effects of prenatal stress on maternal behavior in the rat. *Dev. Brain Res.* 139, 1–8. doi: 10.1016/s0165-3806(02)00491-1

Plescia, F., Brancato, A., Marino, R. A., and Cannizzaro, C. (2013). Acetaldehyde as a drug of abuse: insight into AM281 administration on operant-conflict paradigm in rats. *Front. Behav. Neurosci.* 7:64. doi: 10.3389/fnbeh.2013.00064

Plescia, F., Brancato, A., Marino, R. A., Vita, C., Navarra, M., and Cannizzaro, C. (2014a). Effect of acetaldehyde intoxication and withdrawal on NPY expression: focus on endocannabinoidergic system involvement. *Front. Psychiatry* 5:138. doi: 10.3389/fpsyt.2014.00138

Plescia, F., Marino, R. A., Navarra, M., Gambino, G., Brancato, A., Sardo, P., et al. (2014b). Early handling effect on female rat spatial and non-spatial learning and memory. *Behav. Processes* 103, 9–16. doi: 10.1016/j.beproc.2013.10.011

Pornsawad, P. (2013). "The feedforward-feedback system of the hypothalamus-pituitary-adrenal axis," in *International Conference on Advances in Computing, Communications and Informatics (ICACCI)*, (Mysore, India: IEEE), 1374–1379.

Porsolt, R. D., Le Pichon, M., and Jalfre, M. (1977). Depression: a new animal model sensitive to antidepressant treatments. *Nature* 266, 730–732. doi: 10.1038/266730a0

Rakers, F., Rupprecht, S., Dreiling, M., Bergmeier, C., Witte, O. W., and Schwab, M. (2017). Transfer of 691 maternal psychosocial stress to the fetus. *Neurosci. Biobehav. Rev.* doi: 10.1016/j.neubiorev.2017.02.019 [Epub ahead of print].

Reck, C., Tietz, A., Muller, M., Seibold, K., and Tronick, E. (2018). The impact of maternal anxiety disorder on mother-infant interaction in the postpartum period. *PLoS One* 13:e0194763. doi: 10.1371/journal.pone.0194763

Rees, S. L., and Fleming, A. S. (2001). How early maternal separation and juvenile experience with pups affect maternal behavior and emotionality in adult postpartum rats. *Anim. Learn. Behav.* 29, 221–233. doi: 10.3758/bf03192889

Rosecrans, J. A. (1970). Brain serotonin and pituitary-adrenal function in rats of different emotionalities. *Arch. Int. Pharmacodyn. Ther.* 187, 349–366.

Sarro, E. C., Sullivan, R. M., and Barr, G. (2014). Unpredictable neonatal stress enhances adult anxiety and alters amygdala gene expression related to serotonin and GABA. *Neuroscience* 258, 147–161. doi: 10.1016/j.neuroscience.2013.10.064

Schmidt, M., Lax, E., Zhou, R., Cheishvili, D., Ruder, A. M., Ludiroet, A., et al. (2019). Fetal glucocorticoid receptor (Nr3c1) deficiency alters the landscape of DNA methylation of murine placenta in a sex-dependent manner and is associated to anxiety-like behavior in adulthood. *Transl. Psychiatry* 9:23. doi: 10.1038/s41398-018-0348-7

Seckl, J. R. (2004). Prenatal glucocorticoids and long-term programming. *Eur. J. Endocrinol.* 151, U49–U62. doi: 10.1530/eje.0.151u049

Seckl, J. R., Cleasby, M., and Nyirenda, M. J. (2000). Glucocorticoids, 11β-hydroxysteroid dehydrogenase, and fetal programming. *Kidney Int.* 57, 1412–1417. doi: 10.1046/j.1523-1755.2000.00984.x

Shishkina, G. T., and Dygalo, N. N. (2017). The glucocorticoid hypothesis of depression: history and prospects. *Russ. J. Genet. Appl. Res.* 7, 128–133. doi: 10.1134/s2079059717010142

Shoener, J. A., Baig, R., and Page, K. C. (2006). Prenatal exposure to dexamethasone alters hippocampal drive on hypothalamic-pituitary-adrenal axis activity in adult male rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 290, R1366–R1373. doi: 10.1152/ajpregu.00757.2004

Singh, R. R., Cuffe, J. S. M., and Moritz, K. M. (2012). Short and long term effects of exposure to natural and synthetic glucocorticoids during development. *Clin. Exp. Pharmacol. Physiol.* 39, 979–989. doi: 10.1111/1440-1681.12009

Sloboda, D. M., Challis, J. R., Moss, T. J., and Newnham, J. P. (2005). Synthetic glucocorticoids: antenatal administration and long-term implications. *Curr. Pharm. Des.* 11, 1459–1472. doi: 10.2174/1381612053507873

Smith, J. W., Seckl, J. R., Evans, A. T., Costall, B., and Smythe, J. W. (2004). Gestational stress induces post-partum depression-like behaviour and alters maternal care in rats. *Psychoneuroendocrinology* 29, 227–244. doi: 10.1016/s0306-4530(03)00025-8

Smith, J. T., and Waddell, B. J. (2000). Increased fetal glucocorticoid exposure delays puberty onset in postnatal life. *Endocrinology* 141, 2422–2428. doi: 10.1210/endo.141.7.7541

Tarullo, A. R., St John, A. M., and Meyer, J. S. (2017). Chronic stress in the mother-infant dyad: maternal hair cortisol, infant salivary cortisol and interactional synchrony. *Infant. Behav. Dev.* 47, 92–102. doi: 10.1016/j.infbeh.2017.03.007

Vallée, M., Mayo, W., Dellu, F., Le Moal, M., Simon, H., and Maccari, S. (1997). Prenatal stress induces high anxiety and postnatal handling induces low anxiety in adult offspring: correlation with stress-induced corticosterone secretion. *J. Neurosci.* 17, 2626–2636. doi: 10.1523/jneurosci.17-07-02626.1997

Velísek, L. (2006). Prenatal exposure to betamethasone decreases anxiety in developing rats: hippocampal neuropeptide y as a target molecule. *Neuropsychopharmacology* 31, 2140–2149. doi: 10.1038/sj.npp.1301016

Welberg, L. A. M., Seckl, J. R., and Holmes, M. C. (2001). Prenatal glucocorticoid programming of brain corticosteroid receptors and corticotrophin-releasing hormone: possible implications for behavior. *Neuroscience* 104, 71–79. doi: 10.1016/s0306-4522(01)00065-3

Yankelevitch-Yahav, R., Franko, M., Huly, A., and Doron, R. (2015). The forced swim test as a model of depressive-like behavior. *J. Vis. Exp.* 97:e52587. doi: 10.3791/52587

Zarrow, M., Philpott, J., and Denenberg, V. (1970). Passage of 14C-corticosterone from the rat mother to the foetus and neonate. *Nature* 226, 1058–1059. doi: 10.1038/2261058a0

Zimmerberg, B., and Sageser, K. A. (2011). Comparison of two rodent models of maternal separation on juvenile social behavior. *Front. Psychiatry* 2:39. doi: 10.3389/fpsyt.2011.00039

Zinni, M., Colella, M., Batista Novais, A. R., Baud, O., and Mairesse, J. (2018). Modulating the oxytocin system during the perinatal period: a new strategy for neuroprotection of the immature brain? *Front. Neurol.* 9:229. doi: 10.3389/fneur.2018.00229

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Expression of Behavioral Phenotypes in Genetic and Environmental Mouse Models of Schizophrenia

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Schizophrenia is a neuropsychiatric disorder characterized by multifactorial etiology involving complex interactions among genetic and environmental factors. "Multiple-hit" models of the disorder can explain its variable incidence and prevalence in related individuals. Hence, there is a dire need to understand these interactions in the emergence of schizophrenia. To test these factors in the emergence of schizophrenia-like behaviors, we employed a genetic mouse model of the disorder (harboring the DISC1 mutation) along with various environmental insults, such as early life stress (maternal separation of pups) and/or pharmacological interventions (ketamine injections). When assessed on a battery of behavioral tests, we found that environmental interventions affect the severity of behavioral phenotypes in terms of increased negative behavior, as shown by reduced mobility in the forced swim and tail suspension tests, and changes to positive and cognitive symptoms, such as increased locomotion and disrupted PPI along with reduced working memory, respectively. Among the various interventions, the genetic mutation had the most profound effect on behavioral aberrations, followed by an environmental intervention by ketamine injections and ketamine-injected animals that were maternally separated during early postnatal days. We conclude that although environmental factors increased the prevalence of aberrant behavioral phenotypes, genetic background is still the predominant influence on phenotypic alterations in these mouse models of schizophrenia.

Keywords: DISC1 (disrupted-in-schizophrenia 1), maternal separation (MS), NMDAR hypofunction, ketamine injections, schizophrenia-like psychoses, gene-environment (G-E) interaction

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INTRODUCTION

Schizophrenia is a neuropsychiatric disorder whose etiology encompasses the interaction of several genetic and environmental factors. Heritability of the disorder is as high as 80% (Sullivan et al., 2003), with considerable ecogenetic variation in the prevalence of the disease among related individuals (Ettinger et al., 2004). Such variation correlates with the degree of genetic relatedness of affected individuals; prevalence in first degree relatives (4%–8%), second-degree relatives (2%–3.5%), and children of affected individuals (one parent affected, 13.6%; and both the parents affected, 37%) is indicative of the genetic heritability of the disorder (Salleh, 2004). The most pronounced variations exist in twin studies with a concordance of 50% (Cardno and Gottesman, 2000), suggesting a multifactorial etiology for schizophrenia and related disorders beyond genetic predisposition.

Epidemiological studies of the disease show pronounced interactions between genetic and environmental factors, which can explain variable degrees of onset, prevalence, and severity of disorders in different individuals with a genetic predisposition for the disorder (Karl and Arnold, 2014). Among the environmental factors, maternal separation, early life stress, drug abuse, and season and place of birth are related to the clinical presentation of schizophrenia (Tsuang, 2000; Tsuang et al., 2001; Morgan and Fisher, 2006). Such an interplay of genetics and environment has given rise to "multiple-hit" models of schizophrenia and associated psychotic disorders, where both genes and environment are important factors for disease expression in humans, as well as in animal models of psychotic disorders (Bayer et al., 1999; Maynard et al., 2001; McGrath et al., 2003; Feigenson et al., 2014).

Several candidate genes have been associated with schizophrenia through genome-wide association (GWA) and single nucleotide polymorphisms (SNPs) studies (McClellan et al., 2007; Gejman et al., 2010). Among these, the *DISC1* (Disrupted in Schizophrenia 1) gene confers a 2% risk of schizophrenia in carriers (Callicott et al., 2005; Song et al., 2008; Williams et al., 2009). DISC1 is a scaffolding protein that interacts with several genes, such as NDE, NUDEL, PDE4, ATF4 and PCM etc. (Blackwood et al., 2001; Brandon et al., 2009; Porteous and Millar, 2009; Bradshaw and Porteous, 2012; Teng et al., 2018). In particular, its interaction with PDE4 in dendritic spines serves as a molecular brake to maintain levels of cAMP to restore synaptic connectivity in the PFC (Soares et al., 2011). Due to its synaptic localization with PDE4 and HCN channels it plays a vital role in maintaining working memory and other related behavioral phenotypes (Niwa et al., 2010; Gamo et al., 2013; Paspalas et al., 2013).

Several human and animal studies have further demonstrated the role of mutations in the *DISC1* gene that lead to differential disease phenotypes with variable prevalence (Gottesman and Shields, 1976; Munafò et al., 2005; Van Winkel et al., 2010; Uher, 2014). In humans, a focused study of a Scottish family with this mutation, 33.3% of individuals exhibited symptoms of schizophrenia, major depression (47%), adolescent misconduct (9.5%), bipolar and minor depression, respectively (4.7%; Hennah et al., 2006). Furthermore, a frameshift mutation of the *DISC1* gene in an American family was associated with schizophrenic and schizotypic affective disorders (Sachs et al., 2005; Zhang et al., 2006). In animal studies, such as in the 129S inbred strain of mouse (with a spontaneous, native truncation in the C-terminal of DISC1), L100 and Q31l mutations of the gene result in schizophrenia-like phenotypes (Clapcote and Roder, 2006; Clapcote et al., 2007; Niwa et al., 2010; Sultana et al., 2019). With its relative prevalence and concordant disease expression, DISC1 mutations are an important genetic factor in the etiology of schizophrenia pathogenesis.

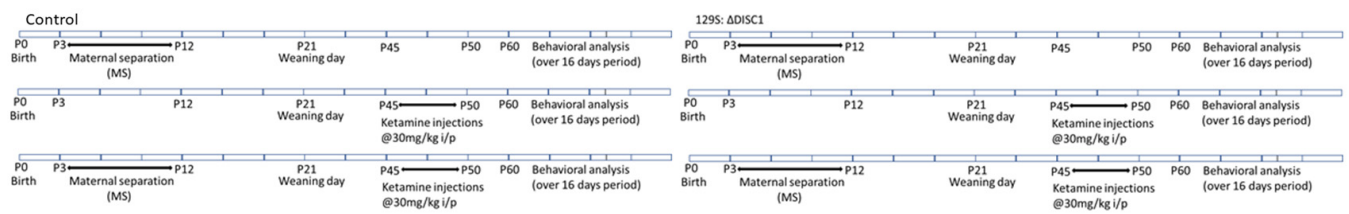
Despite the advances in understanding the genetic and environmental factors involved in the etiology of schizophrenia and a plethora of molecular interactions of DISC1 protein at presynaptic, synaptic and/or postsynaptic sites (Hikida et al., 2012; Weng et al., 2018; Barnett et al., 2019) it remains unclear the degree to which the *DISC1* gene interacts with environmental stressors and how such interactions impact the disease presentation in affected individuals. Therefore to understand the behavioral impact of DISC1 interactions with environmental insults, in the present study, we utilized a mouse model of schizophrenia (Jones et al., 2011) to test the effects of an environmental stressor (maternal separation) and/or pharmacological intervention (ketamine; **Table 1**) on the severity of behavioral phenotypes in genetically predisposed animals (with *DISC1* mutation) compared with controls. We found that although environmental variables increased the number of animals exhibiting aberrant behaviors, the genetic composition of the animals was still the major driver in the expression of schizophrenia-related phenotypes.

MATERIALS AND METHODS

Animal Care and Housing

A total of 12 animals were used in each group: 129SvEv (129S:ΔDISC1) a DISC1 mutation model and C57BL/6J

TABLE 1 | Description of all the intervention groups used.



Interventions	No treatment	Maternal separation (MS)	Ketamine injections	Maternal Separation (MS)+ Ketamine injection
Groups	Control	Control+MS	Control+Ketamine injection	Control+MS+Ketamine injection
	129S:ΔDISC1	DISC1+MS	DISC1+Ketamine injection	DISC1+MS+Ketamine injection

(control), and the intervention groups described below. Mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). These animals were assessed on a battery of behavioral tests when 8 weeks old, with the least stressful tests performed first (Sultana et al., 2019). Initially, control and 129S:ΔDISC1 animals were characterized by behavior without any interventions. Animals were housed in a temperature and humidity-controlled room with a 12 h light/dark cycle with lights on at 7:00 am and food and water provided *ad libitum*. All the experiments were conducted according to NIH guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of the Louisiana State University School of Veterinary Medicine.

Animal Models and Interventions

Maternal Separation (MS; Early Life Environmental Stress)

Maternal separation of newborns, young children have shown a strong correlation with psychotic disorder precipitation in human subjects (Mäki et al., 2003; Paksarian et al., 2015). Maternal separation in mouse and rat pups, in particular, has been used to model and study schizophrenia (Lehmann et al., 2000; Fabricius et al., 2008). In this study, maternal separation of pups was performed with slight modifications to previously described procedures (Roceri et al., 2002; Ellenbroek and Riva, 2003). The pups were separated from dams for 4 h a day from postnatal day (PND) 3 to PND12 (critical period of brain development at these stages; Rice and Barone, 2000) daily from 10:00 a.m. to 2:00 p.m. and weaned at PND21. The animals were tested after PND60.

NMDAR Hypofunction (i.p. Ketamine Injection)

NMDAR hypofunction is a convergent molecular deficit found in instances of schizophrenia. To induce a pharmacologically targeted behavioral deficit, we used a previously established model of schizophrenia (Ogundele and Lee, 2018) To induce NMDAR hypofunction similar to molecular findings in schizophrenia, both 129S:ΔDISC1 and control animals were injected with a subanesthetic dose of ketamine (30 mg/kg) for 5 days from day 45–50, as described previously (Becker et al., 2003; Frohlich and Van Horn, 2014; Ogundele and Lee, 2018) and were tested behaviorally starting at 5–7 days following the last ketamine injection.

Maternal Separation (PND3–PND12) With i.p. Ketamine Injection During Adulthood

Both 129S:ΔDISC1 and control animals were separated maternally (from PND3-PND12; 4 h a day). In adulthood, they were injected with ketamine (i.p. 30 mg/kg), as described above. These animals were then tested under the behavioral test battery as follows.

Behavioral Test Battery

All behavioral experiments were performed by the same investigator during the late morning. The behavioral procedures have been described in detail in our previous work (Sultana et al., 2019). One set of experiments was performed per day over a 16 days period with resting days in between. Experiments were performed in the order as we have described in our prior study (Sultana et al., 2019). The following tests were included.

Open Field Test for Thigmotaxis and Overall Activity

The total distance traveled in the apparatus was calculated and used as a measure of overall activity (Foshee et al., 1965). In addition, this test was also used as a measure of anxiety-like behavior in terms of thigmotaxis, i.e., time spent near the periphery of the chamber (Simon et al., 1994; Seibenhener and Wooten, 2015; Walz et al., 2016). Thus, we also determined the percent time the test animal spent at the periphery vs. center (Sultana et al., 2019) during the total 5 min test duration.

Sociability and Novelty

As a measure of social interaction, sociability and social novelty were tested as previously described (Kaidanovich-Beilin et al., 2011). On the test day, animals were assessed for sociability, as defined by the percent time that the test animal spent socializing with stranger 1 (S1) i.e., (S1/S1+E) $*$ 100. Social novelty was assessed as the percent time spent with stranger 2 (S2) as (S1/S1+S2) $*$ 100.

Modified Porsolt Forced Swimming Test

As a metric of despair, we utilized the modified Porsolt forced swimming test, derived from the procedure of Can et al. (2012a,b). The camera (1080 HD, Logitech, Newark, CA, USA) was positioned with a side view of the beaker to record the leg movements of the animal. Scoring of the movements was done as previously described by Can et al. (2012a,b). Percent mobility time was calculated from a total 4 min testing period, following an initial 2 min acclimation period which was later excluded from calculations. Measurements included when the animal was actively struggling to escape from the water container, whereas the propelling movement was not considered in the mobility calculations.

Tail Suspension Test

This test was used as another metric of negative behavior, animals were suspended by a custom holder and percent mobility during suspension was assessed (Can et al., 2012b). The total test duration was 6 min, but the latter 4 min were analyzed to remove any bias involving acclimation.

Stress Calls

When interpreted contextually, in certain psychotic disorders like schizophrenia and schizotypic affective disorders, ultrasonic vocalization (USV) patterns can provide an indicator of the affective state of the animal (Knutson et al., 2002; Schwarting and Wöhr, 2012; Mun et al., 2015). Stress calls were recorded simultaneously to the tail-suspension test, as described previously. An AT125 bat call recorder (Binary Acoustics, Carlisle, PA, USA) and digital recording software SPECT'R (Binary Acoustics, Carlisle, PA, USA) was used. Calls were analyzed offline using SCAN'R software (Binary Acoustics, Carlisle, PA, USA). Calls above 30 kHz (typical of adult mice) with a minimum duration of 5 ms were considered and analyzed. The number of calls per 6 min period was calculated along with

the mean and maximum frequency and the duration of each call. The entire 6 min period was analyzed since no difference was found when examining these data compared to the 4 min period post-acclimation.

Y-Maze Test

Working memory includes the ability to rapidly form a memory trace and the exclusion of old information from that which is currently valid. This task was used as a tool to assess schizophrenia-related cognitive impairment. Videos were recorded using a Logitech HD 1180 camera and later analyzed with ANY-maze (ANY-maze, Wood Dale, IL, USA). Percent entries into the correct arm were calculated using the formula described previously (Sultana et al., 2019).

Habituation to Acoustic Startle and Pre-pulse Inhibition (PPI)

As a measure of pre-attentive deficits, this test is also used to assess sensorimotor gating in human subjects with schizophrenia (Swerdlow et al., 2006). Following the protocol described by Valsamis and Schmid (2011), responses to acoustic startle stimuli were used to measure habituation and pre-pulse inhibition (PPI). The apparatus and protocols were followed as described in our prior study (Sultana et al., 2019). For stimulus delivery and recording of the startle signal, Audacity software 2.2.2 (Carnegie Mellon University, Pittsburgh, PA, USA) was used. The startle data were exported into Excel (Microsoft, Redmond, WA, USA) using Python. Further analysis was done using Excel followed by statistical analysis with GraphPad Prism 5 (LaJolla, CA, USA). The data were expressed as mean ± SEM.

Statistical Analysis

ANOVA followed by Tukey's *post hoc* test for multiple comparisons was used to determine significant differences among groups. All the data were expressed as mean ± SEM. A p-value < 0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Thigmotaxis, Anxiety-Related Behavior

As noted previously, time spent by animals along the walls of the open field provides an index of anxiety (Sultana et al., 2019). Here, we calculated the percent time that animal groups spent at the periphery. We found that control animals spent a significantly higher time in the center vs. control+MS+ketamine ($p \leq 0.05$; see **Figure 1A**), while animals with the *DISC1* mutation showed a variable degree of time at the periphery differing from control animals (DISC1 alone at $p \leq 0.001$ and DISC1+ketamine at $p \leq 0.01$). Different interventions on the control background did not affect this behavior, except in the control+MS+ketamine group (at $p \leq 0.05$; **Figure 1A**). The variable degree of anxiety-related behavior was intriguing, since not all the interventions on the DISC1 and/or control group background exhibited this behavior (37.5% of the population among all the groups tested here), compared to schizophrenia subjects, anxiety-related behavior is not the endophenotype, with a 38% prevalence of this behavior in human schizophrenia patients (Temmingh and Stein, 2015).

Exploratory Behavior and Activity

While in the open field, the total distance covered by all groups was assessed. Control animals traveled a significantly greater distance as compared to other groups, i.e., DISC1 animals alone and with interventions ($p \leq 0.001$; **Figure 1B**). Environmental interventions on the DISC1 background did not affect exploratory behavior when compared to the DISC1 without intervention animals. Moreover, control animals with maternal separation, ketamine injection showed significantly decreased exploration ($p \leq 0.01$ and $p \leq 0.001$, respectively). These results support the interpretation that all the environmental interventions affect animal behavior differently depending on the genetic background and their specific interactions.

Sociability and Novelty

Control animals exhibited significantly higher time socializing compared with other groups, with the exception of maternally separated (MS) controls (where different groups differed at p-values control+ketamine at $p \leq 0.001$, control+MS+ketamine at $p \leq 0.01$, DISC1 at $p \leq 0.01$, along with DISC1+MS, DISC1+ketamine, DISC1+MS+ketamine at $p \leq 0.001$, respectively; see **Figure 1C**). The intervention groups did not exhibit an intragroup difference in percent time with S1 (stranger 1) when compared amongst themselves, as shown in **Figure 1C**. These results indicate that environmental interventions equally affect social withdrawal in all the models, with a higher degree of social isolation in genetic mutation animals (significant at $p \leq 0.01$ in all *DISC1* background groups). However, environmental interventions on a DISC1 mutation background did not change the sociability behavior of these animals significantly.

Social novelty (i.e., percent time with S2) exhibited a different outcome (**Figure 1D**), with control animals differing from the DISC1 ($p \leq 0.05$), maternally separated DISC1 ($p \leq 0.01$), MS+DISC1 injected with ketamine at adulthood ($p \leq 0.01$), and the DISC1+ ketamine injection animals exhibited an increased social novelty vs. DISC1 animals alone ($p \leq 0.01$), suggesting a complex interplay in DISC1 animals with pharmacologically induced NMDAR hypofunction (using ketamine; **Figure 1D**). Additionally, other environmental interventions in DISC1 animals did not differ significantly from those with no interventions. Control animals did not show a significant difference when environmental interventions were imposed on this background, indicating that the genetic mutation in DISC1 animals influenced their behavior towards social novelty.

Y Maze: As an Index of Working Memory

In this behavioral task, control animals exhibited significantly higher percent time in the novel /correct arm (previously blocked arm) as compared to DISC1 (DISC1 alone and with interventions at $p \leq 0.001$) and other groups (control+MS and control+MS+ketamine at $p \leq 0.001$ and control+MS at

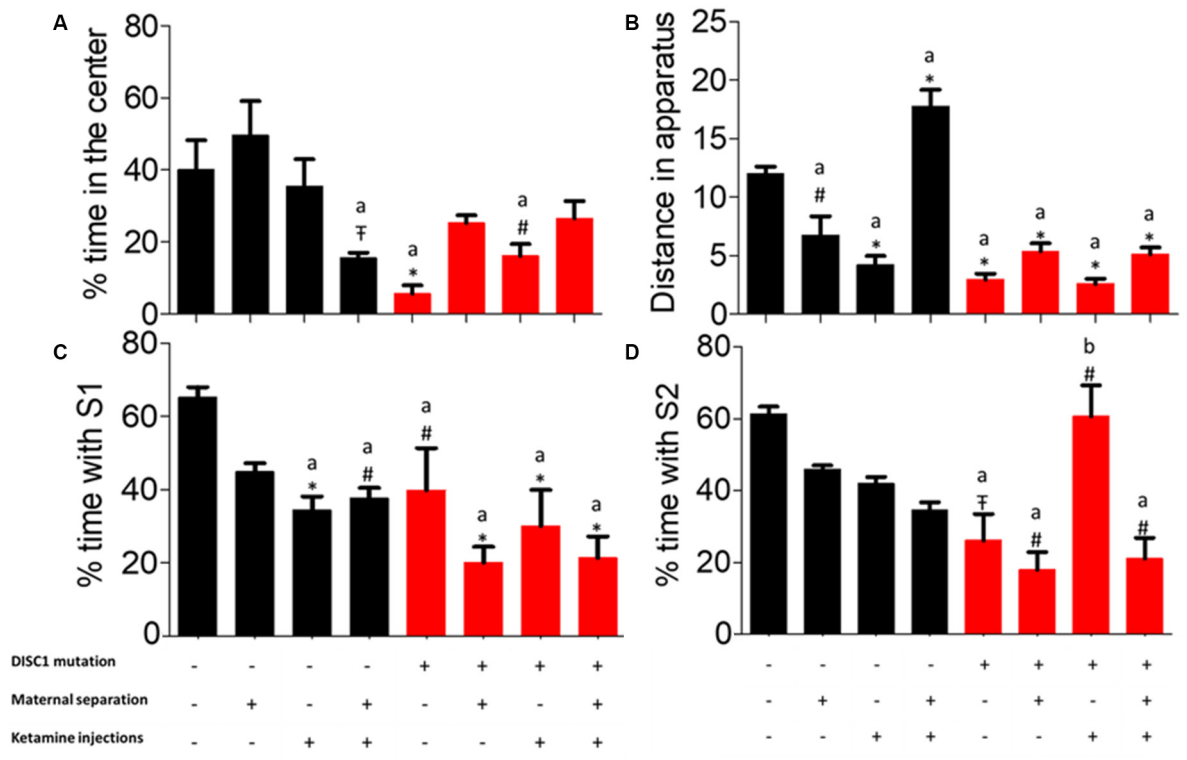


FIGURE 1 | Three-chambered test for **(A)** percent time spent at the center of open field. **(B)** Total distance traveled throughout the apparatus. **(C)** Sociability. **(D)** Novelty. All data presented here are presented as mean ± SEM, where a* compared to control at $p \leq 0.001$; a# as compared to control at $p \geq 0.05$; a∓ as compared to control at $p \leq 0.01$ and b with above symbols show comparison of groups with DISC1 mutation animals.

$p \leq 0.01$; **Figure 2**). It is important to note that environmental interventions on a DISC1 mutation background did not significantly disrupt the results of the working memory task when compared to DISC1 animals without interventions. These results demonstrate that working memory is affected in all the control animal groups with various environmental interventions, exhibiting the effect of these stressors on the hippocampus (Malhotra et al., 1997; de Azeredo et al., 2017). Percent novel arm entries did not show any significant difference among control vs. intervention groups (data not shown).

Porsolt Forced Swim Test (FST)

Mobility during forced swim test (FST) can be used as a measure of the degree of despair in animal models of behavioral disorders (Can et al., 2012a). When test groups were compared for mobility timing in FST, we found a variation in the number of animals exhibiting depressive behavior within each group, whereas when statistically compared, there was a significant reduction in mobility timing (DISC1, DISC1+MS, DISC1+MS+ketamine and control+ketamine, control+MS+ketamine at $p \leq 0.001$; and at $p \leq 0.01$ for control+MS; as shown in **Figure 3**). The environmental factors over a DISC1 mutation background did not show a significant reduction in mobility time when compared to DISC1 mutation animals without intervention. Although maternal separation (Millstein and Holmes, 2007), ketamine injections, and combinations of both interventions affect the animals of each group to a variable degree, these groups still exhibit an overall depressed phenotype (Elk et al., 1986).

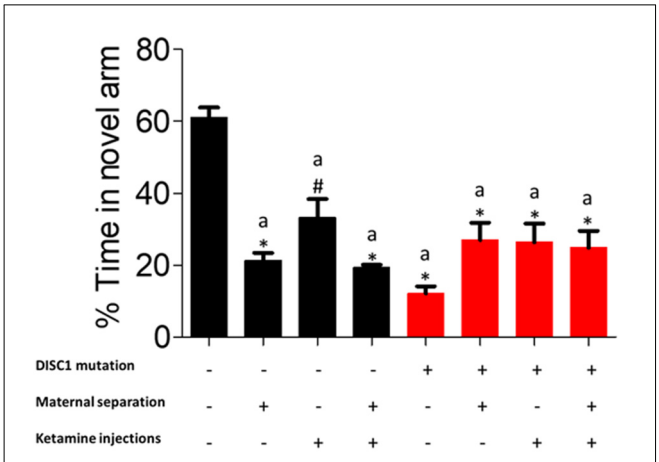


FIGURE 2 | Y-maze task for working memory: showing percent time each group spent in the novel arm. All data presented here are presented as mean ± SEM, where a* as compared to control at $p \leq 0.001$; a# as compared to control at $p \leq 0.05$.

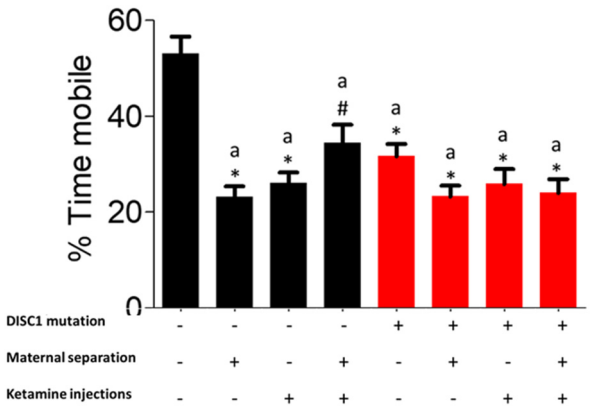


FIGURE 3 | Forced Swim Test (FST) for depressive behavior: exhibiting the percent mobility time by all the groups. All the presented here are presented as mean ± SEM, where a* as compared to controls at $p \leq 0.001$; a# as compared to controls at $p \leq 0.05$.

Tail Suspension Test and Stress Calls

The tail suspension test was used as another measure of despair and resulted in a similar outcome as the FST. Control animals showed the highest mobility, which was significantly different from other groups (at $p \leq 0.001$ for all the groups and $p \leq 0.01$ for DISC1+MS+Ketamine vs. control; see **Figure 4A**). DISC1 mutation animals vs. DISC1 with other interventions did not show significant differences amongst themselves. Thus, various environmental stressors led to decreased mobility in the groups which was not significantly different from each other (**Figure 4**).

While the animals were suspended, we also recorded the USV emitted by these animals, as a measure of calls produced under stress. We found that *DISC1* mutation animals produced fewer calls compared to control animals ($p \leq 0.01$; **Figure 4B**; Zimmerberg et al., 2003; Yin et al., 2016). Control animals also differed significantly from DISC1, with maternal separation and DISC1 maternally separated with ketamine injection ($p \leq 0.01$; **Figure 4B**). Affective vocalizations differed to varying degrees in control animals that were maternally separated and/or treated with ketamine (for traces of USVs see also **Figure 4C**). On the other hand, DISC1 animals with interventions did not exhibit a reduction in calls when compared to DISC1 animals without interventions.

Habituation to Acoustic Startle Response (ASR)

Habituation to acoustic startle response (ASR) was measured to determine sensorimotor gating and pre-attentive deficits. As previously described, the first test block measured habituation (Sultana et al., 2019). We found that maternal separation of the control pups and DISC1 pups did not affect habituation to ASR vs. control, exhibiting habituation of 65%, 53% and 42% (respectively for control, control+MS and DISC1+MS; **Figures 5A,D**). The magnitude of habituation was not significantly different in these animal groups. However, all other groups (see **Figure 5B**, control and DISC1 with ketamine, control and DISC1 with MS+ketamine, and DISC1 alone) did not show habituation but instead exhibited sensitization to the acoustic stimulus (**Figures 5B,D**). The animals exhibited increased startle, indicating that pharmacologically induced NMDAR hypofunction might be affecting the ability of the brain to habituate to the repeated acoustic stimulus.

Prepulse Inhibition (PPI)

Similar to the habituation test, the PPI responses exhibited a similar pattern of inhibition to different inter-stimulus interval (ISI) and pre-pulse intensity combinations. We found that control animals showed inhibition to all trial (ISI-PP intensity) combinations (see **Figures 6A–D**), significantly differing from control+MS+ketamine ($p \leq 0.001$), DISC1 ($p \leq 0.05$) and DISC1+ketamine ($p \leq 0.01$; at ISI of 30 ms with intensity of 75 dB depicted as 30_75 shown in **Figure 6A**), control+ketamine ($p \leq 0.05$) and control+MS+ketamine [$p \leq 0.01$; at 30 (ISI)_85 (Prepulse intensity); **Figure 6B**], and control vs. control+ketamine, control+MS+ketamine [$p \leq 0.05$ at 100 ms (ISI)_75 dB (Prepulse Intensity); **Figure 6C** and $p \leq 0.001$ at 100 ms (ISI)_85 dB (Prepulse Intensity) combinations; **Figure 6D**]. Control animals also differed from DISC1+MS [$p \leq 0.01$ at 100 ms (ISI)_85 dB (Prepulse Intensity; **Figure 6D**)]. Thus, unlike habituation to acoustic startle stimulus, maternal deprivation alone in these animals did not cause aberrations in PPI (Ellenbroek and Cools, 2002). Both control and DISC1 animals with ketamine injections exhibited aberrations in PPI behavior, showing an impact of NMDAR hypofunction on sensorimotor gating mechanism of brain circuitry (Cilia et al., 2007).

DISCUSSION

Schizophrenia is a neuropsychiatric disorder associated with multiple genetic and environmental etiologies (Tsuang et al., 2001). Although no single gene or environmental factor is known to be completely causal (Choi et al., 2009; van de Leemput et al., 2016; Howes et al., 2017), interactions among multiple factors increase the emergence of the disorder. We focused our studies on two different genetic backgrounds, control group (C57BL/6J) which does not have a genetic predisposition to schizophrenia or schizotypic disorders, and the test group that is genetically predisposed (129S strain with C-terminal truncation of *DISC1* gene) on a behavioral test battery (Brixey et al., 1993; Krystal et al., 1994; Ellenbroek and Riva, 2003; Koike et al., 2006).

We have previously observed that this 129S: ΔDISC1 strain differs behaviorally from several other common inbred and outbred mouse strains (Sultana et al., 2019). Our prior findings indicate that the inherent DISC1 mutation in the 129SvEv mice has a penetrant effect on behavioral phenotype above various genetic backgrounds. Other mouse strains (Balb/c, CBA/J, etc.) could potentially serve as an appropriate behavioral control strain here, since they are all similar behaviorally and distinct from the 129SvEv strain, putatively as a result of the DISC1 genetic mutation. This is supported by findings that DISC1 mutations on the same background strain do not differ

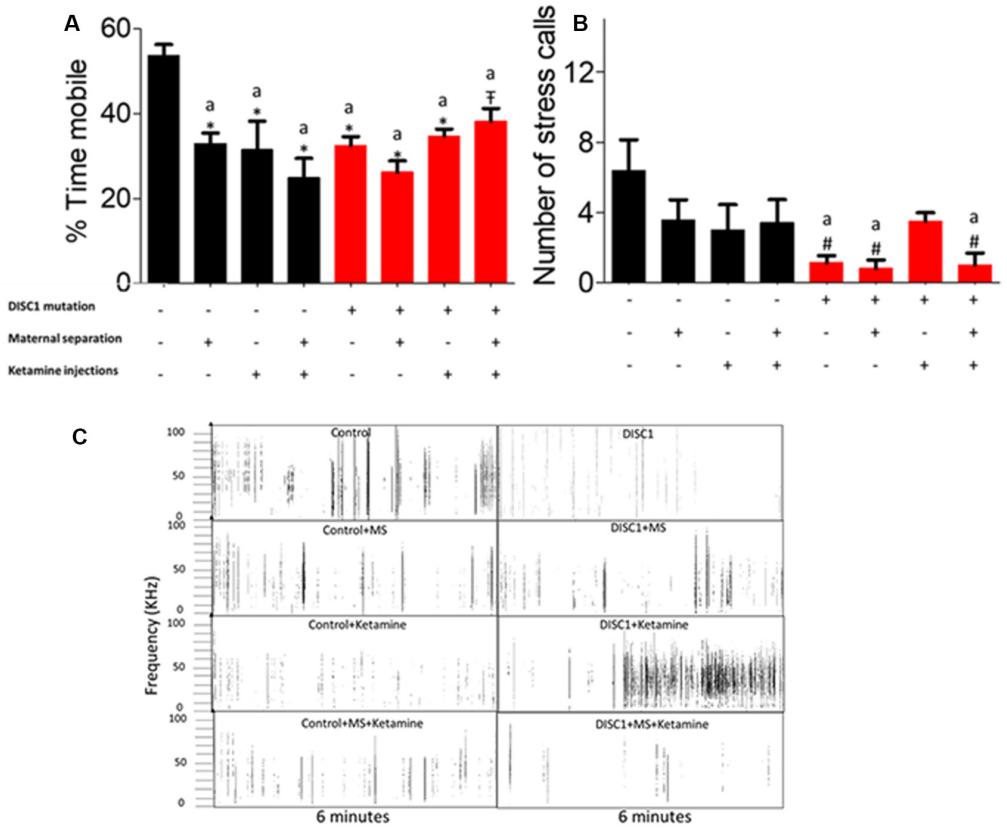


FIGURE 4 | Tail Suspension Test (TST) for depressive behavior: **(A)** exhibiting the percent mobility time by all the groups. **(B)** Stress calls recorded/6 min period while under tail suspension. **(C)** Representative stress calls in all the groups; with y-axis showing frequency/call and x-axis showing duration (6 min). All data presented here are presented as mean ± SEM, where a* as compared to control at $p \leq 0.001$; a# as compared to control at $p \leq 0.05$. a$\dagger$ as compared to control at $p \leq 0.01$ and *b* with above symbols shows comparison of groups with DISC1 mutation animals.

when compared across strains (Lee et al., 2011). Nevertheless, the comparisons employed here do add a potential caveat to our results, which must be considered in their interpretation.

DISC1 mutations affect behavior by its interactions with pathways such as PDE4, upregulation of SK2 (calcium-activated small potassium channels at the PSD; Sultana et al., 2018), and HCN (Paspalas et al., 2013). These interactions take place at the dendritic synaptic densities, where NMDARs acts as a convergence point for DISC1 and its interacting partners such as PDE4. NMDAR hypofunction alone or in combination with DISC1 mutations aggravate behavioral phenotypes, as discussed below (see also **Figures 1–3**).

We assessed the effects of genetic predisposition (DISC1 mutation), environmental factors (maternal separation and ketamine injections), and interactions of these factors (see **Table 1**). Among these factors, our results suggest that genetic factors play the predominant role in the presentation of behavioral phenotypes associated with the disease, while pharmacological intervention (ketamine injections) and maternal separation showed incremental effects, particularly on the genetically predisposed animals. Interestingly, maternal separation did not show a significant effect in terms of sociability novelty, overall activity (**Figure 1**), USV (**Figure 4B**), and habituation to acoustic startle (**Figure 5**) on control animals suggesting that genetic predisposition might be necessary for this stressor to contribute to disease pathology to exhibit above mentioned behavioral syndrome.

In many of the behavioral tasks, environmental interventions on the DISC1 background did not increase the severity of behavioral phenotypes. We suggest that the severity of symptoms in many of the behavioral task have a lower/upper limit. However, an interesting finding from our data is that the animals harboring the DISC1 mutation often exhibit a bimodal distribution in the expression of behavioral phenotypes, which is not found following environmental interventions. Thus, we propose that the effects of the environmental interventions may not necessarily be on the magnitude of the behavioral effects, but rather the probability that these animals may develop schizophrenia-related behavioral phenotypes.

As models to study schizophrenia, these interventions are argued to have faced, construct and/or predictive validity (Jones et al., 2011). The DISC1 mutation is known to associate with a neurological disorder in about 33.3% of the large Scottish population where the mutation is present, with members of the

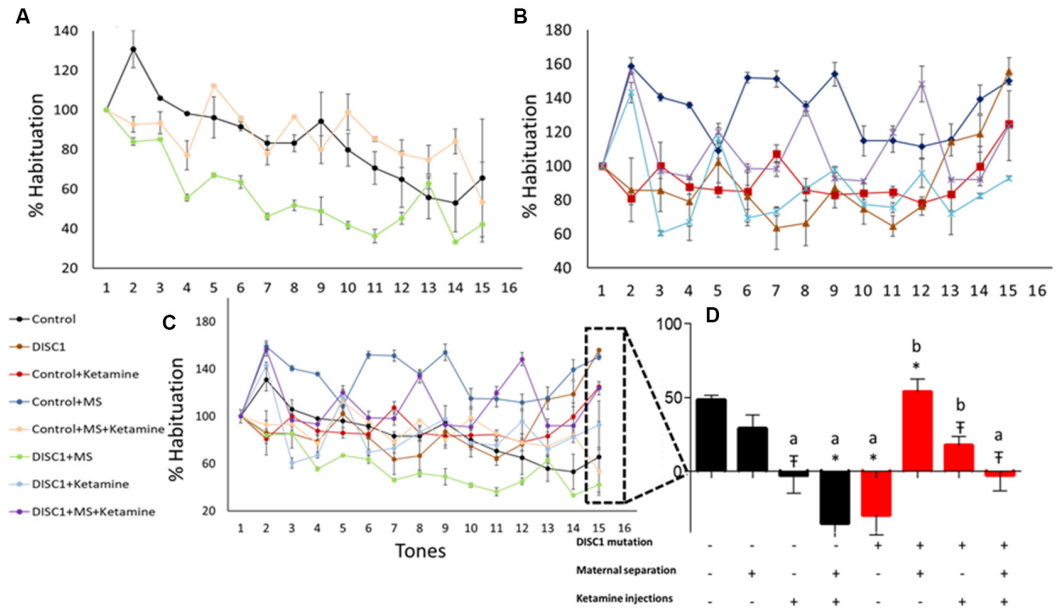


FIGURE 5 | Habituation to acoustic startle response (ASR). **(A)** Groups showing habituation; control (black), control+MS (green) and DISC1+MS (pink). **(B)** Groups that did not show habituation. **(C)** Merge showing all the groups with and without habituation. **(D)** Percent habituation in different groups towards the last tone. All data presented here are presented as mean ± SEM, where a* as compared to control at $p \leq 0.001$; a# as compared to control at $p \leq 0.05$; a∓ as compared to control at $p \leq 0.01$ and *b* with above symbols shows comparison of groups with DISC1 mutation animals.

family exhibiting schizophrenia, bipolar and major depression disorders etc. In the present study, DISC1 mutation animals exhibited decreased sociability, novelty (**Figure 1**), mobility time in forced swim and tail suspension test (**Figures 3**, **4A**). When compared with prior studies, animal models of DISC1 mutation produced using various methods, e.g., shRNA, use of chemicals, backcrossing 129S on C57BL6 background, report similar results to our findings (Jaaro-Peled, 2009; Johnstone et al., 2011; Tomoda et al., 2016). Moreover, disruption of sensorimotor gating has been observed in various models of schizophrenia including the DISC1 genetic mutation (Tomoda et al., 2017).

Additionally, NMDAR hypofunction is a key finding in human postmortem studies of schizophrenia and bolsters the glutamate dysfunction theory of schizophrenia pathogenesis (Snyder and Gao, 2013). Furthermore, it is known that DISC1 and NMDARs interact dynamically with each other (Wang and Zhu, 2014), such that DISC1 dependent changes in NMDAR synaptic responses are speculated to affect cognition in individuals with schizophrenia (Ramsey et al., 2011; Wei et al., 2014). Behaviorally, previous results from our lab also found that there is a reduced interaction of test mice in terms of sociability, social novelty, reduced spatial/working memory (Ogundele and Lee, 2018), similar to our results in ketamine-treated animals (control as well as DISC1 mutation background). We also found that ketamine injection in the DISC1 mutation animals resulted in increased hypo-frontality, leading to enhanced negative signs, such as more depressed behavior (**Figures 3**, **4**), as indicated by decreased mobility in FST and TST as well as the reduced number of stress calls in these animals.

Genetic mutation and environmental stress affect the behavioral emergence of schizophrenia. However, when there is a combination of factors, we found that genetic background has the biggest influence as shown by reduced sociability and novelty in the DISC1 mutation background animals (**Figures 1C,D**), when compared with the same insults on the control background highlighted in terms of anxiety-like behavior, stress calls and habituation to ASR as well as PPI (**Figures 1A**, **4B**, **5**, **6**). Environmental factors such as pharmacological interventions that cause direct NMDAR hypofunction (ketamine injections) results in similar behavioral outcomes (such as reduced sociability and mobility in FST and TST) for both control and DISC1 mutation animals, showing that NMDAR hypofunction is a convergence point for the molecular mechanism behind core symptoms of schizophrenia. On the other hand, maternal separation of pups leads to more negative symptoms, i.e., reduced mobility in FST and TST and spatial memory, but it does not affect the social recognition behavior of these animals (**Figures 1C,D**). All other combinations of interventions influenced the behavioral phenotype to a variable degree with DISC1+MS+ketamine animals showing more aberrant behaviors when compared with the control+MS+ketamine group, clearly indicating the effects of genetic predisposition.

Overall, our study supports the most recent theories of gene-environment interactions and their effects on the behavioral phenotype of nervous disorders, such as schizophrenia. Interactions between multiple components affect behaviors at various levels for positive (aberrant PPI, reduced habituation to acoustic startle) and negative symptoms (decreased mobility

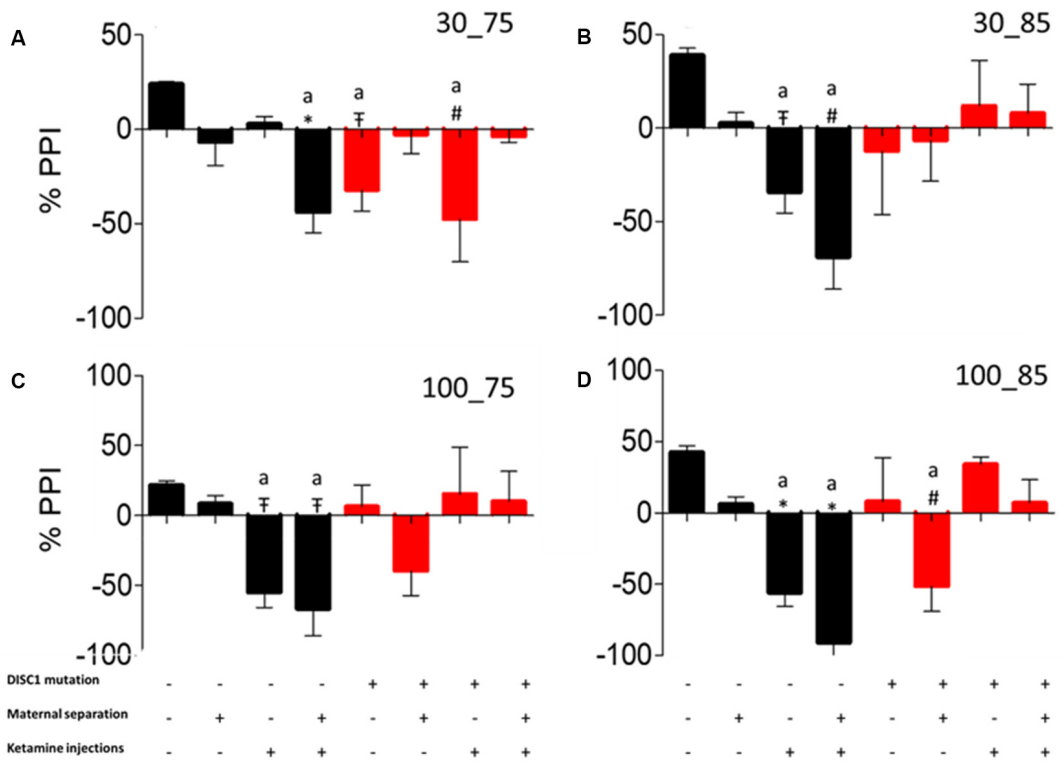


FIGURE 6 | Pre-pulse Inhibition (PPI). Prepulse inhibition at different inter-stimulus interval and pulse intensity. **(A)** 30_75. **(B)** 30_85. **(C)** 100_75. **(D)** 100_85, respectively. All data presented here are presented as mean ± SEM, where a* as compared to control at $p \leq 0.001$; a# as compared to control at $p \leq 0.05$; a∓ as compared to control at $p \leq 0.01$ and *b* with above symbols shows comparison of groups with DISC1 mutation animals.

timing on FST and TST tests; **Figures 3**, **4**), including cognitive tasks with learning and memory deficits (as shown in Y maze test; **Figure 2**; Ellenbroek and Cools, 2002; Powell and Miyakawa, 2006; Gómez-Sintes et al., 2014). We also emphasize the finding that, although DISC1 mutation animals with various environmental interventions did not change the severity of the behavioral profile of these animals when compared to DISC1 mutation alone, the prevalence of animals exhibiting aberrant behavioral phenotype increased due to gene-environmental interaction. We propose then that this effect could be due to environmental intervention acting as a second hit to increase the chances of disease development in genetically predisposed animals with the DISC1 mutation.

CONCLUSION

These behavioral changes suggest several aberrant molecular interactions must be occurring at the cellular, subcellular and/or extracellular levels. Here, we have first attempted to assess the combination of environment and genetics in the development of behavioral phenotype. The results of our present study suggest that the DISC1 genetic mutation predominates over the environmental factors used in our study in the presentation of schizophrenia-like behavioral phenotypes. The molecular and neural factors that lead to these behaviors remain to be examined, as are any potential epigenetic changes that these stressors may bring about in healthy individuals (Roth et al., 2009).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care & Use Committee (IACUC) of the Louisiana State University School of Veterinary Medicine.

AUTHOR CONTRIBUTIONS

CL designed the study, edited and finalized the manuscript. RS designed, executed the experiments, collected data, statistically analyzed and wrote the manuscript.

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REFERENCES

Barnett, B. R., Torres-Velázquez, M., Yi, S. Y., Rowley, P. A., Sawin, E. A., Rubinstein, C. D., et al. (2019). Sex-specific deficits in neurite density and white matter integrity are associated with targeted disruption of exon 2 of the Disc1 gene in the rat. *Transl. Psychiatry* 9:82. doi: 10.1038/s41398-019-0429-2

Bayer, T. A., Falkai, P., and Maier, W. (1999). Genetic and non-genetic vulnerability factors in schizophrenia: the basis of the "two hit hypothesis". *J. Psychiatr. Res.* 33, 543–548. doi: 10.1016/s0022-3956(99)00039-4

Becker, A., Peters, B., Schroeder, H., Mann, T., Huether, G., and Grecksch, G. (2003). Ketamine-induced changes in rat behaviour: a possible animal model of schizophrenia. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 27, 687–700. doi: 10.1016/S0278-5846(03)00080-0

Blackwood, D. H., Fordyce, A., Walker, M. T., St Clair, D. M., Porteous, D. J., and Muir, W. J. (2001). Schizophrenia and affective disorders—cosegregation with a translocation at chromosome 1q42 that directly disrupts brain-expressed genes: clinical and P300 findings in a family. *Am. J. Hum. Genet.* 69, 428–433. doi: 10.1086/321969

Bradshaw, N. J., and Porteous, D. J. (2012). DISC1-binding proteins in neural development, signalling and schizophrenia. *Neuropharmacology* 62, 1230–1241. doi: 10.1016/j.neuropharm.2010.12.027

Brandon, N. J., Millar, J. K., Korth, C., Sive, H., Singh, K. K., and Sawa, A. (2009). Understanding the role of DISC1 in psychiatric disease and during normal development. *J. Neurosci.* 29, 12768–12775. doi: 10.1523/JNEUROSCI.3355-09.2009

Brixey, S. N., Gallagher, B. J. III., McFalls, J. A. Jr., and Parmelee, L. F. (1993). Gestational and neonatal factors in the etiology of schizophrenia. *J. Clin. Psychol.* 49, 447–456. doi: 10.1002/1097-4679(199305)49:3<447::aid-jclp2270490321>3.0.co;2-4

Callicott, J. H., Straub, R. E., Pezawas, L., Egan, M. F., Mattay, V. S., Hariri, A. R., et al. (2005). Variation in DISC1 affects hippocampal structure and function and increases risk for schizophrenia. *Proc. Natl. Acad. Sci. U S A* 102, 8627–8632. doi: 10.1073/pnas.0500515102

Can, A., Dao, D., Arad, M., Terrillion, C. E., Piantadosi, S. C., and Gould, T. D. (2012a). The mouse forced swim test. *J. Vis. Exp.* 59:e3638. doi: 10.3791/3638

Can, A., David, T., Terrillion, C., Piantadosi, S., Bhat, S., and Gould, T. (2012b). The tail suspension test. *J. Vis. Exp.* 28:3769. doi: 10.3791/3769

Cardno, A. G., and Gottesman, I. I. (2000). Twin studies of schizophrenia: from bow-and-arrow concordances to Star Wars Mx and functional genomics. *Am. J. Med. Genet.* 97, 12–17. doi: 10.1002/(sici)1096-8628(200021)97:1<12::aid-ajmg3,,3.0.co;2-u

Choi, K. H., Zepp, M. E., Higgs, B. W., Weickert, C. S., and Webster, M. J. (2009). Expression profiles of schizophrenia susceptibility genes during human prefrontal cortical development. *J. Psychiatry Neurosci.* 34, 450–458.

Cilia, J., Hatcher, P., Reavill, C., and Jones, D. N. C. (2007). ± Ketamine-induced prepulse inhibition deficits of an acoustic startle response in rats are not reversed by antipsychotics. *J. Psychopharmacol.* 21, 302–311. doi: 10.1177/0269881107077718

Clapcote, S. J., Lipina, T. V., Millar, J. K., Mackie, S., Christie, S., Ogawa, F., et al. (2007). Behavioral phenotypes of disc1 missense mutations in mice. *Neuron* 54, 387–402. doi: 10.1016/j.neuron.2007.04.015

Clapcote, S., and Roder, J. (2006). Deletion polymorphism of disc1 is common to all 129 mouse substrains: implications for gene-targeting studies of brain function. *Genetics* 173, 2407–2410. doi: 10.1534/genetics.106.060749

de Azeredo, L. A., Wearick-Silva, L. E., Viola, T. W., Tractenberg, S. G., Centeno-Silva, A., Orso, R., et al. (2017). Maternal separation induces hippocampal changes in cadherin-1 (CDH-1) mRNA and recognition memory impairment in adolescent mice. *Neurobiol. Learn. Mem.* 141, 157–167. doi: 10.1016/j.nlm.2017.04.006

Elk, R., Dickman, B. J., and Teggin, A. F. (1986). Depression in schizophrenia: a study of prevalence and treatment. *Br. J. Psychiatry* 149, 228–229. doi: 10.1192/bjp.149.2.228

Ellenbroek, B. A., and Cools, A. R. (2002). Early maternal deprivation and prepulse inhibition: the role of the postdeprivation environment. *Pharmacol. Biochem. Behav.* 73, 177–184. doi: 10.1016/s0091-3057(02)00794-3

Ellenbroek, B. A., and Riva, M. A. (2003). Early maternal deprivation as an animal model for schizophrenia. *Clin. Neurosci. Res.* 3, 297–302. doi: 10.1016/s1566-2772(03)00090-2

Ettinger, U., Kumari, V., Crawford, T. J., Corr, P. J., Das, M., Zachariah, E., et al. (2004). Smooth pursuit and antisaccade eye movements in siblings discordant for schizophrenia. *J. Psychiatr. Res.* 38, 177–184. doi: 10.1016/s0022-3956(03)00105-5

Fabricius, K., Wörtwein, G., and Pakkenberg, B. (2008). The impact of maternal separation on adult mouse behaviour and on the total neuron number in the mouse hippocampus. *Brain Struct. Funct.* 212, 403–416. doi: 10.1007/s00429-007-0169-6

Feigenson, K. A., Kusnecov, A. W., and Silverstein, S. M. (2014). Inflammation and the two-hit hypothesis of schizophrenia. *Neurosci. Biobehav. Rev.* 38, 72–93. doi: 10.1016/j.neubiorev.2013.11.006

Foshee, D., Vierck, C. J., Meier, G. W., and Federspiel, C. (1965). Simultaneous measure of general activity and exploratory behavior. *Percept. Mot. Skills* 20, 445–451. doi: 10.2466/pms.1965.20.2.445

Frohlich, J., and Van Horn, J. D. (2014). Reviewing the ketamine model for schizophrenia. *J. Psychopharmacol.* 28, 287–302. doi: 10.1177/0269881113512909

Gamo, N. J., Duque, A., Paspalas, C. D., Kata, A., Fine, R., Boven, L., et al. (2013). Role of disrupted in schizophrenia 1 (DISC1) in stress-induced prefrontal cognitive dysfunction. *Transl. Psychiatry* 3:e328. doi: 10.1038/tp.2013.104

Gejman, P. V., Sanders, A. R., and Duan, J. (2010). The role of genetics in the etiology of schizophrenia. *Psychiatr. Clin. North Am.* 33, 35–66. doi: 10.1016/j.psc.2009.12.003

Gómez-Sintes, R., Kvajo, M., Gogos, J. A., and Lucas, J. J. (2014). Mice with a naturally occurring DISC1 mutation display a broad spectrum of behaviors associated to psychiatric disorders. *Front. Behav. Neurosci.* 8:253. doi: 10.3389/fnbeh.2014.00253

Gottesman, I. I., and Shields, J. (1976). A critical review of recent adoption, twin, and family studies of schizophrenia: behavioral genetics perspectives. *Schizophr. Bull.* 2, 360–401. doi: 10.1093/schbul/2.3.360

Hennah, W., Thomson, P., Peltonen, L., and Porteous, D. (2006). Genes and schizophrenia: beyond schizophrenia: the role of DISC1 in major mental illness. *Schizophr. Bull.* 32, 409–416. doi: 10.1093/schbul/sbj079

Hikida, T., Gamo, N. J., and Sawa, A. (2012). DISC1 as a therapeutic target for mental illnesses. *Expert Opin. Ther. Targets* 16, 1151–1160. doi: 10.1517/14728222.2012.719879

Howes, O. D., McCutcheon, R., Owen, M. J., and Murray, R. M. (2017). The role of genes, stress, and dopamine in the development of schizophrenia. *Biol. Psychiatry* 81, 9–20. doi: 10.1016/j.biopsych.2016.07.014

Jaaro-Peled, H. (2009). Gene models of schizophrenia: DISC1 mouse models. *Prog. Brain Res.* 179, 75–86. doi: 10.1016/s0079-6123(09)17909-8

Johnstone, M., Thomson, P. A., Hall, J., McIntosh, A. M., Lawrie, S. M., and Porteous, D. J. (2011). DISC1 in schizophrenia: genetic mouse models and human genomic imaging. *Schizophr. Bull.* 37, 14–20. doi: 10.1093/schbul/sbq135

Jones, C. A., Watson, D. J. G., and Fone, K. C. F. (2011). Animal models of schizophrenia. *Br. J. Pharmacol.* 164, 1162–1194. doi: 10.1111/j.1476-5381.2011.01386.x

Kaidanovich-Beilin, O., Lipina, T., Vukobradovic, I., Roder, J., and Woodgett, J. R. (2011). Assessment of social interaction behaviors. *J. Vis. Exp.* 48:2473. doi: 10.3791/2473

Karl, T., and Arnold, J. C. (2014). Schizophrenia: a consequence of gene-environment interactions? *Front. Behav. Neurosci.* 8:435. doi: 10.3389/fnbeh.2014.00435

Knutson, B., Burgdorf, J., and Panksepp, J. (2002). Ultrasonic vocalizations as indices of affective states in rats. *Psychol. Bull.* 128, 961–977. doi: 10.1037/0033-2909.128.6.961

Koike, H., Arguello, P. A., Kvajo, M., Karayiorgou, M., and Gogos, J. (2006). Disc1 is mutated in the 129S6/SvEv strain and modulates working memory in mice. *Proc. Natl. Acad. Sci. U S A* 103, 3693–3697. doi: 10.1073/pnas.0511189103

Krystal, J. H., Karper, L. P., Seibyl, J. P., Freeman, G. K., Delaney, R., Bremner, J. D., et al. (1994). Subanesthetic effects of the noncompetitive nmda antagonist, ketamine, in humans: psychotomimetic, perceptual, cognitive, and neuroendocrine responses. *Arch. Gen. Psychiatry* 51, 199–214. doi: 10.1001/archpsyc.1994.03950030035004

Lee, F. H. F., Fadel, M. P., Preston-Maher, K., Cordes, S. P., Clapcote, S. J., Price, D. J., et al. (2011). Disc1 point mutations in mice affect development

of the cerebral cortex. *J. Neurosci.* 31, 3197–3206. doi: 10.1523/JNEUROSCI.4219-10.2011

Lehmann, J., Logeay, C., and Feldon, J. (2000). Long-term effects of a single 24-hour maternal separation on three different latent inhibition paradigms. *Psychobiology* 28, 411–419.

Mäki, P., Veijola, J., Joukamaa, M., Läärä, E., Hakko, H., Jones, P. B., et al. (2003). Maternal separation at birth and schizophrenia—a long-term follow-up of the Finnish Christmas Seal Home Children. *Schizophr. Res.* 60, 13–19. doi: 10.1016/s0920-9964(02)00203-7

Malhotra, A. K., Pinals, D. A., Adler, C. M., Elman, I., Clifton, A., Pickar, D., et al. (1997). Ketamine-induced exacerbation of psychotic symptoms and cognitive impairment in neuroleptic-free schizophrenics. *Neuropsychopharmacology* 17, 141–150. doi: 10.1016/s0893-133x(97)00036-5

Maynard, T. M., Sikich, L., Lieberman, J. A., and LaMantia, A.-S. (2001). Neural development, cell-cell signaling, and the 'two-hit' hypothesis of schizophrenia. *Schizophr. Bull.* 27, 457–476. doi: 10.1093/oxfordjournals.schbul.a006887

McClellan, J. M., Susser, E., and King, M.-C. (2007). Schizophrenia: a common disease caused by multiple rare alleles. *Br. J. Psychiatry* 190, 194–199. doi: 10.1192/bjp.bp.106.025585

McGrath, J. J., Féron, F. P., Burne, T. H. J., Mackay-Sim, A., and Eyles, D. W. (2003). The neurodevelopmental hypothesis of schizophrenia: a review of recent developments. *Ann. Med.* 35, 86–93. doi: 10.1080/07853890310010005

Millstein, R. A., and Holmes, A. (2007). Effects of repeated maternal separation on anxiety- and depression-related phenotypes in different mouse strains. *Neurosci. Biobehav. Rev.* 31, 3–17. doi: 10.1016/j.neubiorev.2006.05.003

Morgan, C., and Fisher, H. (2006). Environment and schizophrenia: environmental factors in schizophrenia: childhood trauma—a critical review. *Schizophr. Bull.* 33, 3–10. doi: 10.1093/schbul/sbl053

Mun, H.-S., Lipina, T. V., and Roder, J. C. (2015). Ultrasonic vocalizations in mice during exploratory behavior are context-dependent. *Front. Behav. Neurosci.* 9:316. doi: 10.3389/fnbeh.2015.00316

Munafò, M. R., Bowes, L., Clark, T. G., and Flint, J. (2005). Lack of association of the COMT (Val158/108 Met) gene and schizophrenia: a meta-analysis of case-control studies. *Mol. Psychiatry* 10, 765–770. doi: 10.1038/sj.mp.4001664

Niwa, M., Kamiya, A., Murai, R., Kubo, K.-I., Gruber, A. J., Tomita, K., et al. (2010). Knockdown of DISC1 by in utero gene transfer disturbs postnatal dopaminergic maturation in the frontal cortex and leads to adult behavioral deficits. *Neuron* 65, 480–489. doi: 10.1016/j.neuron.2010.01.019

Ogundele, O. M., and Lee, C. C. (2018). CaMKIIα expression in a mouse model of NMDAR hypofunction schizophrenia: putative roles for IGF-1R and TLR4. *Brain Res. Bull.* 137, 53–70. doi: 10.1016/j.brainresbull.2017.11.007

Paksarian, D., Eaton, W. W., Mortensen, P. B., Merikangas, K. R., and Pedersen, C. B. (2015). A population-based study of the risk of schizophrenia and bipolar disorder associated with parent-child separation during development. *Psychol. Med.* 45, 2825–2837. doi: 10.1017/s0033291715000781

Paspalas, C. D., Wang, M., and Arnsten, A. F. T. (2013). Constellation of HCN channels and cAMP regulating proteins in dendritic spines of the primate prefrontal cortex: potential substrate for working memory deficits in schizophrenia. *Cereb. Cortex* 23, 1643–1654. doi: 10.1093/cercor/bhs152

Porteous, D., and Millar, K. (2009). How DISC1 regulates postnatal brain development: girdin gets in on the AKT. *Neuron* 63, 711–713. doi: 10.1016/j.neuron.2009.09.017

Powell, C. M., and Miyakawa, T. (2006). Schizophrenia-relevant behavioral testing in rodent models: a uniquely human disorder? *Biol. Psychiatry* 59, 1198–1207. doi: 10.1016/j.biopsych.2006.05.008

Ramsey, A. J., Milenkovic, M., Oliveira, A. F., Escobedo-Lozoya, Y., Seshadri, S., Salahpour, A., et al. (2011). Impaired NMDA receptor transmission alters striatal synapses and DISC1 protein in an age-dependent manner. *Proc. Natl. Acad. Sci. U S A* 108, 5795–5800. doi: 10.1073/pnas.1012621108

Rice, D., and Barone, S. Jr. (2000). Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environ. Health Perspect.* 108, 511–533. doi: 10.1289/ehp.00108s3511

Roceri, M., Hendriks, W., Racagni, G., Ellenbroek, B. A., and Riva, M. A. (2002). Early maternal deprivation reduces the expression of BDNF and NMDA receptor subunits in rat hippocampus. *Mol. Psychiatry* 7, 609–616. doi: 10.1038/sj.mp.4001036

Roth, T. L., Lubin, F. D., Sodhi, M., and Kleinman, J. E. (2009). Epigenetic mechanisms in schizophrenia. *Biochim. Biophys. Acta* 1790, 869–877. doi: 10.1016/j.bbagen.2009.06.009

Sachs, N. A., Sawa, A., Holmes, S. E., Ross, C. A., DeLisi, L. E., and Margolis, R. L. (2005). A frameshift mutation in disrupted in schizophrenia 1 in an american family with schizophrenia and schizoaffective disorder. *Mol. Psychiatry* 10, 758–764. doi: 10.1038/sj.mp.4001667

Salleh, M. R. (2004). The genetics of schizophrenia. *Malays. J. Med. Sci.* 11, 3–11.

Schwarting, R. K. W., and Wöhr, M. (2012). On the relationships between ultrasonic calling and anxiety-related behavior in rats. *Braz. J. Med. Biol. Res.* 45, 337–348. doi: 10.1590/s0100-879x2012007500038

Seibenhener, M. L., and Wooten, M. C. (2015). Use of the Open Field Maze to measure locomotor and anxiety-like behavior in mice. *J. Vis. Exp.* 96:e52434. doi: 10.3791/52434

Simon, P., Dupuis, R., and Costentin, J. (1994). Thigmotaxis as an index of anxiety in mice. Influence of dopaminergic transmissions. *Behav. Brain Res.* 61, 59–64. doi: 10.1016/0166-4328(94)90008-6

Snyder, M. A., and Gao, W.-J. (2013). NMDA hypofunction as a convergence point for progression and symptoms of schizophrenia. *Front. Cell. Neurosci.* 7:31. doi: 10.3389/fncel.2013.00031

Soares, D. C., Carlyle, B. C., Bradshaw, N. J., and Porteous, D. J. (2011). DISC1: structure, function, and therapeutic potential for major mental illness. *ACS Chem. Neurosci.* 2, 609–632. doi: 10.1021/cn200062k

Song, W., Li, W., Feng, J., Heston, L. L., Scaringe, W. A., and Sommer, S. S. (2008). Identification of high risk DISC1 structural variants with a 2% attributable risk for schizophrenia. *Biochem. Biophys. Res. Commun.* 367, 700–706. doi: 10.1016/j.bbrc.2007.12.117

Sullivan, P. F., Kendler, K. S., and Neale, M. C. (2003). Schizophrenia as a complex trait: evidence from a meta-analysis of twin studies. *Arch. Gen. Psychiatry* 60, 1187–1192. doi: 10.1001/archpsyc.60.12.1187

Sultana, R., Ghandi, T., Davila, A. M., Lee, C. C., and Ogundele, O. M. (2018). Upregulated SK2 expression and impaired CaMKII phosphorylation are shared synaptic defects between 16p11.2del and 129S: Δdisc1 mutant mice. *ASN Neuro* 10, 1–14. doi: 10.1177/1759091419847891

Sultana, R., Ogundele, O. M., and Lee, C. C. (2019). Contrasting characteristic behaviours among common laboratory mouse strains. *R. Soc. Open Sci.* 6:190574. doi: 10.1098/rsos.190574

Swerdlow, N. R., Light, G. A., Cadenhead, K. S., Sprock, J., Hsieh, M. H., and Braff, D. L. (2006). Startle gating deficits in a large cohort of patients with schizophrenia: relationship to medications, symptoms, neurocognition, and level of function. *JAMA Psychiatry* 63, 1325–1335. doi: 10.1001/archpsyc.63.12.1325

Temmingh, H., and Stein, D. J. (2015). Anxiety in patients with schizophrenia: epidemiology and management. *CNS Drugs* 29, 819–832. doi: 10.1007/s40263-015-0282-7

Teng, S., Thomson, P. A., McCarthy, S., Kramer, M., Muller, S., Lihm, J., et al. (2018). Rare disruptive variants in the DISC1 interactome and regulome: association with cognitive ability and schizophrenia. *Mol. Psychiatry* 23, 1270–1277. doi: 10.1038/mp.2017.115

Tomoda, T., Dzirasa, K., Saudou, F., Sawa, A., and Jaaro-Peled, H. (2017). 439. Role for DISC1 in sensorimotor gating: its impact on corticostriatal transport of BDNF. *Biol. Psychiatry* 81, S179–S180. doi: 10.1016/j.biopsych.2017.02.923

Tomoda, T., Sumitomo, A., Jaaro-Peled, H. A. S., and Sawa, A. (2016). Utility and validity of DISC1 mouse models in biological psychiatry. *Neuroscience* 321, 99–107. doi: 10.1016/j.neuroscience.2015.12.061

Tsuang, M. (2000). Schizophrenia: genes and environment. *Biol. Psychiatry* 47, 210–220. doi: 10.1016/s0006-3223(99)00289-9

Tsuang, M. T., Stone, W. S., and Faraone, S. V. (2001). Genes, environment and schizophrenia. *Br. J. Psychiatry* 178, s18–s24. doi: 10.1192/bjp.178.40.s18

Uher, R. (2014). Gene-environment interactions in severe mental illness. *Front. Psychiatry* 5:48. doi: 10.3389/fpsyt.2014.00048

Valsamis, B., and Schmid, S. (2011). Habituation and prepulse inhibition of acoustic startle in rodents. *J. Vis. Exp.* 55, e3446. doi: 10.3791/3446

van de Leemput, J., Hess, J. L., Glatt, S. J., and Tsuang, M. T. (2016). Genetics of schizophrenia: historical insights and prevailing evidence. *Adv. Genet.* 96, 99–141. doi: 10.1016/bs.adgen.2016.08.001

Van Winkel, R., Esquivel, G., Kenis, G., Wichers, M., Collip, D., Peerbooms, O., et al. (2010). REVIEW: genome-wide findings in schizophrenia and the role of gene-environment interplay. *CNS Neurosci. Ther.* 16, e185–e192. doi: 10.1111/j.1755-5949.2010.00155.x

Walz, N., Mühlberger, A., and Pauli, P. (2016). A human open field test reveals thigmotaxis related to agoraphobic fear. *Biol. Psychiatry* 80, 390–397. doi: 10.1016/j.biopsych.2015.12.016

Wang, G., and Zhu, J. J. (2014). DISC1 dynamically regulates synaptic n-methyl-d-aspartate responses in excitatory neurons. *Biol. Psychiatry* 75, 348–350. doi: 10.1016/j.biopsych.2013.12.003

Wei, J., Graziane, N. M., Wang, H., Zhong, P., Wang, Q., Liu, W., et al. (2014). Regulation of N-methyl-D-aspartate receptors by disrupted-in-schizophrenia-1. *Biol. Psychiatry* 75, 414–424. doi: 10.1016/j.biopsych.2013.06.009

Weng, Y.-T., Chien, T., Kuan, I. I., and Chern, Y. (2018). The TRAX, DISC1, and GSK3 complex in mental disorders and therapeutic interventions. *J. Biomed. Sci.* 25:71. doi: 10.1186/s12929-018-0473-x

Williams, J. M., Beck, T. F., Pearson, D. M., Proud, M. B., Cheung, S. W., and Scott, D. A. (2009). A 1q42 deletion involving DISC1, DISC2, and TSNAX in an autism spectrum disorder. *Am. J. Med. Genet. A* 149A, 1758–1762. doi: 10.1002/ajmg.a.32941

Yin, X., Chen, L., Xia, Y., Cheng, Q., Yuan, J., Yang, Y., et al. (2016). Maternal deprivation influences pup ultrasonic vocalizations of C57BL/6J mice. *PLoS One* 11:e0160409. doi: 10.1371/journal.pone.0160409

Zhang, F., Sarginson, J., Crombie, C., Walker, N., StClair, D., and Shaw, D. (2006). Genetic association between schizophrenia and the DISC1 gene in the Scottish population. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 141B, 155–159. doi: 10.1002/ajmg.b.30274

Zimmerberg, B., Kim, J. H., Davidson, A. N., and Rosenthal, A. J. (2003). Early deprivation alters the vocalization behavior of neonates directing maternal attention in a rat model of child neglect. *Ann. N Y Acad. Sci.* 1008, 308–313. doi: 10.1196/annals.1301.039

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MINI REVIEW
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Gender Differences in the Outcome of Offspring Prenatally Exposed to Drugs of Abuse

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Despite great efforts to warn pregnant women that drugs of abuse impact development of the embryo and the fetus, the use of legal and illegal drugs by childbearing women is still a major public health concern. In parallel with well-established teratogenic effects elicited by some drugs of abuse, epidemiological studies show that certain psychoactive substances do not induce birth defects but lead to subtle neurobehavioral alterations in the offspring that manifest as early as during infancy. Although gender differences in offspring susceptibility have not been fully investigated, a number of longitudinal studies indicate that male and female progeny exposed *in utero* to drugs of abuse show different vulnerabilities to deleterious effects of these substances in cognitive, executive, and behavioral domains. Here, we briefly review the existing literature focusing on gender differences in the neurobehavioral consequences of maternal exposure to drugs of abuse. Overall, the data strongly indicate that male exposed progeny are more susceptible than female to dysfunctions in cognitive processing and emotional regulation. However, insights into the mechanisms determining this natural phenomenon are not currently available. Our analysis prompts future investigations to implement clinical studies including the influence of gender/sex as a biological variable in the outcome of offspring prenatally exposed to drugs of abuse.

Keywords: development, drugs of abuse, gender, neuropsychiatric, prenatal, sex, vulnerability

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INTRODUCTION

As a rule, drugs should not be used during pregnancy unless prescribed, because many can be toxic to the placenta or the developing fetus. Yet, the use of drugs, including prescription or non-prescription drugs, medicinal herbs, and licit (tobacco and alcohol) or illicit drugs, during pregnancy keeps increasing (SAMHSA, 2011). Indeed, objective measurements of xenobiotics in meconium, amniotic fluid, and cord blood indicate widespread fetal exposure to such agents during their intrauterine life (for an excellent review see Barr et al., 2007). Such exposure may induce developmental adaptations that can be interpreted as derangements from normal development, which not only interfere with the immediate viability of the fetus but may also result in the individual's adverse health outcome in the short and long term (Hales and Barker, 2001; Barker, 2007). Hence, the "developmental origin of health and disease" hypothesis (Barker, 2007) stems from epidemiological studies showing that malnutrition, exposure to xenobiotics (e.g., environmental chemicals and prescription, legal, and illegal drugs), infective diseases, or stress during specific periods of development might increase the risk of disorders later in life. This hypothesis also stresses the importance of investigating the mechanisms of fetal exposure to xenobiotics and further in general to adverse intrauterine and perinatal factors.

In this minireview, we will provide an up-to-date analysis of the evidence for a sex differential in the susceptibility to the consequences of maternal drug use on neurocognitive and behavioral development of the offspring. Research has pointed to gender differences in these sequelae, since exposed males often appear more vulnerable than exposed females. Insights into the neurobiological mechanisms underlying the sex bias observed in certain neurobehavioral outcomes remain unidentified. At this stage, we could only make inferences from animal studies, although they do not allow for a precise understanding of the underpinnings, especially in the context of sex differences. In particular, many factors might moderate the reported sex dichotomy, including individual (e.g., species, strain, age) and experimental (e.g., design, drug, dosage, route, regimen) variables, and objective endpoints (e.g., behavioral paradigm, experimental technique). Here, we attempt to integrate the gender difference results across drugs used by pregnant women. Such integration could be useful for physicians and healthcare providers when caring for a pregnant substance abusing woman. Interspecies extrapolations will be carefully avoided to ensure sound conclusions. The authors refer to excellent preclinical studies' reviews (Bruin et al., 2010; Schneider et al., 2011; Ross et al., 2015; Gkioka et al., 2016; Comasco et al., 2018; Scheyer et al., 2019).

SUBSTANCE USE IN WOMEN

The historical gap in substance use prevalence between men and women has gradually narrowed in the past decade, particularly among adolescents (Keyes et al., 2008; Seedat et al., 2009; Steingrimsson et al., 2012; EMCDDA, 2019). While women still exhibit lower rates of drug use disorder than men, prevalence rates indicate that the number of female drug abusers is on the rise. A recent snapshot of the European drug use situation shows that women account for one-quarter of the general population with drug issues and around one-fifth of all first-time drug abuse treatment seekers (EMCDDA, 2019). Gender differences are clear in the pattern of use at each stage of the addiction cycle. Women typically begin to use substances later in life (Greenfield et al., 2010; Keyes et al., 2010), misuse prescription drugs (e.g., opioids) (McHugh et al., 2013), and their rate of consumption increases more rapidly than that of men (Greenfield et al., 2010; Keyes et al., 2010). Women also exhibit higher prevalence rates of comorbidity with other psychiatric disorders as well as of relapse (Wilcox and Yates, 1993; Conway et al., 2006; Back et al., 2011; Khan et al., 2013).

DRUG USE DURING PREGNANCY AND BREASTFEEDING: EFFECTS ON MALE AND FEMALE OFFSPRING

The consumption of drugs in childbearing women has been progressively increasing. Women abusing recreational drugs before pregnancy tend to continue the use even during gestation (Forray, 2016), and this use is not limited to illegal drugs but includes prescription and over-the-counter drugs. Approximately 60% of pregnant women take prescription drugs and about 13% of them use herbal supplements. Furthermore, the infographics based on the National Survey on Drug Use and Health (SAMHSA, 2018) show that 5.4% of pregnant women have used illicit drugs in the past 30 days, while 9.9 and 11.6% reported past-month alcohol or cigarette smoking use, respectively. To complicate this issue, many women take drugs when they are not aware of being pregnant.

Regardless of their legal status, all drugs cross and/or alter the placental barrier, reach the fetus, and affect infant development. Additionally, multiple drugs also pass into mother's breast milk, thus resulting in prolonged drug exposure of the newborn. According to the United States Centers for Disease Control and Prevention, almost 3% of newborns have birth defects because of genetic, environmental, or other unknown causes (Parker et al., 2010). Among environmental factors, drug use is the major cause leading to birth defects ranging from fetal growth reductions to medical complications such as preterm birth and infections. Furthermore, the progeny prenatally exposed to drugs of abuse develop neurobehavioral phenotypes that manifest during infancy and persist to adolescence and young adulthood. Research on the effects of prenatal alcohol, tobacco, opioids, stimulants, and cannabis indicates an association between fetal exposure to these substances and deficits in cognitive and behavioral domains. However, in humans, the role of fetal sex on functional consequences of prenatal exposure to drugs of abuse remains grossly understudied. Here we present data on illicit psychostimulants, opioids, cannabis, nicotine, and alcohol in an attempt to provide a clear picture of neurobehavioral outcomes in male and female progeny. When gender differences have not been examined, our interpretation is limited to the overall outcome.

Effects of *in utero* Exposure to Psychostimulants

Psychostimulants, including cocaine and methamphetamine, are the illicit drugs most commonly used by childbearing women, though no recent estimate of their consumption during pregnancy is known. Despite their well-described neurotoxic effects on central nervous system (CNS) development, only very few studies have addressed the negative neurobehavioral sequalae on human offspring, particularly when gender is included as an additional biological variable (**Table 1** and **Figure 1**).

Longitudinal studies of long-term consequences of cocaine use during pregnancy on the offspring focusing on emotional regulation, behavior, and cognition suggest that female gender is a protective factor (Singer et al., 2004; Dennis et al., 2006; Accornero et al., 2007; Bennett et al., 2008; Ackerman et al., 2010; Bridgett and Mayes, 2011). Male progeny exhibit stronger impairment in inhibitory response, whereas females exhibit only mild alterations that disappear with age (Carmody et al., 2011). Accordingly, male offspring exhibit greater emotion regulation problems and externalizing symptoms (e.g., aggressive and risky behaviors); lower intellectual capabilities; and deficits in attention, short-term memory, and problem solving compared

TABLE 1 | Detailed information on the studies covered in this minireview examining gender as a variable.

Author year	Substance	Type of study	Experimental groups	Neuro developmental outcome	Prenatal polysubstance exposure	Socioeconomic status	Age	Performed tasks	Gender results
Prenatal psychostimulant exposure									
Lu et al., 2009	Meth amphetamine	Cross-sectional study	Methamphetamine exposed vs alcohol exposed vs control	Cognitive abilities	Yes (methamphetamine, alcohol)	Matched for socioeconomic status	7–15 year	Wechsler Intelligence Scale for Children, 4th Edition (WISC-4), California Verbal Learning Test for Children (CVLT-C)	Impaired verbal learning capacities in methamphetamine and alcohol exposed
Diaz et al., 2014	Meth amphetamine	Longitudinal study	Exposed vs control	Cognitive abilities	Yes (methamphetamine, alcohol, cannabis, tobacco)	Matched for socioeconomic status	7.5 year	Conners' Parent Rating Scale–Revised: Short Form (CPRS-R:S)	Significantly higher cognitive problems scores in exposed children
Piper et al., 2011	Meth amphetamine	Cross-sectional study	Methamphetamine and polysubstance exposed vs Unexposed	Cognitive Abilities	Yes (methamphetamine, alcohol, tobacco, cannabis)	Matched for socioeconomic status	7–9 year	Wechsler Abbreviated Scale of Intelligence, Conners' Continuous Performance Test II, Behavioral Rating Inventory of Executive Function, the CMS Family Pictures and Dot Location tests, the Spatial Span test from WISC-IV-Integrated, and a recently developed spatial learning and memory measure (Memory Island)	Exposed children show deficit in executive functions (e.g., behavioral regulation and metacognition) and spatial memory
Kiblawi et al., 2013	Meth amphetamine	Longitudinal	Exposed vs unexposed	ADHD risk	Yes (methamphetamine, alcohol, cannabis, tobacco)	Controlled for low socioeconomic status	5 year	Conners' Kiddie Continuous Performance Test (K-CPT)	KCPT scores suggest higher ADHD risk for exposed children
LaGasse et al., 2012	Meth amphetamine	Longitudinal study	Exposed vs unexposed	ADHD risk	Yes (methadone, alcohol and tobacco, cannabis)	Adjusted for low socioeconomic status	3–5 year	Child Behavior Checklist	Higher prevalence of ADHD symptoms in exposed males than girls
LaGasse et al., 2012	Meth amphetamine	Longitudinal study	Exposed vs unexposed	Behavioral problems	Yes (methadone, alcohol and tobacco, cannabis)	Adjusted for low socioeconomic status	3–5 year	Child Behavior Checklist	More externalizing problems and aggressive behavior in exposed males than girls
Bennett et al., 2008	Cocaine	Longitudinal study	Exposed vs unexposed	Cognitive abilities	Yes (cocaine, alcohol, tobacco, cannabis)	Measured as environmental risk	4, 6, 9 year	Stanford-Binet IV intelligence test	Lower composite IQ score (abstract/visual and verbal reasoning, short-term memory) in exposed boys but not girls

(Continued)

TABLE 1 | Continued

Author year	Substance	Type of study	Experimental groups	Neuro developmental outcome	Prenatal polysubstance exposure	Socioeconomic status	Age	Performed tasks	Gender results
Dennis et al., 2006	Cocaine	Longitudinal study	Exposed vs unexposed	Cognitive abilities	Yes (cocaine, alcohol, tobacco, cannabis)	Measured as environmental risk	5 year	The impossible pulley task	More difficulties in problem solving and altered reactivity/ regulating behavior in exposed males than females
Singer et al., 2004	Cocaine	Longitudinal study	Exposed vs non-exposed	Cognitive abilities	Yes (cocaine, alcohol, tobacco, cannabis)	Measured as caregiving environmental risk	0–4 year	Wechsler Preschool and Primary Scales of Intelligence-Revised	Mild but significant difficulties in cognitive abilities (visual-spatial and arithmetic skills) in exposed males
Mayes et al., 2003	Cocaine	Longitudinal study	Exposed vs non-drug and non-cocaine-exposed	Cognitive abilities	Yes (cocaine, alcohol, tobacco, cannabis)	Measured as environmental risk	0–3 year	Bayley Scales of Infant Development (BSID-II)	Lower BSID-II mental performance in cocaine exposed children compared to both non-drug and non-cocaine-exposed children
Accornero et al., 2007	Cocaine	Longitudinal study	Exposed vs unexposed	Deficit in attention and inhibition response	Yes	Matched for socioeconomic status	5–7 year	Continuous performance tests (CPTs)	Deficits in attention processing in exposed offspring
Karmel and Gardner, 1996	Cocaine	Longitudinal study	Exposed vs unexposed	Attention and arousal	Yes (cocaine, alcohol, tobacco)	ND	0–1 year	Visual looking preferences	Arousal-modulated attention deficit in exposed male and female infants
Bennett et al., 2007	Cocaine	Longitudinal study	Exposed vs unexposed	Neurobehavioral problems	Yes (cocaine, alcohol, tobacco, cannabis)	Adjusted for low socioeconomic status	10 year	Youth Risk Behavior Survey	Highest scores for aggression, substance use, high-risk behavior in exposed males
Nordstrom Bailey et al., 2005	Cocaine	Longitudinal study	Exposed vs unexposed	Neurobehavioral problems (aggressive behavior)	Yes (cocaine, alcohol)	Controlled for socioeconomic status	6–7 year	Achenbach Teacher Report Form (TRF)	Delinquent behavior and clinically significant externalizing behavior scores in exposed boys
Sood et al., 2005	Cocaine	Historical prospective study	Alcohol exposed vs cocaine and/or alcohol exposed	Neurobehavioral problems	Yes (cocaine, alcohol)	Controlled for socioeconomic status	6–7 year	Caregiver reported Achenbach Child Behavior Checklist (CBCL)	Higher aggressive (in females) and delinquent behaviors (in males) scores in exposed offspring
Bendersky et al., 2006	Cocaine	Longitudinal study	Exposed vs unexposed	Neurobehavioral problems	Yes (cocaine, alcohol, tobacco, cannabis)	Measured as environmental risk	5 year	ACHENBACHChild Behavior Checklist (CBCL), LRRH Reinisch Revision, Teacher Rating of Aggression (TRA)	Aggressive behavior in exposed male offspring

(Continued)

TABLE 1 | Continued

Author year	Substance	Type of study	Experimental groups	Neuro developmental outcome	Prenatal polysubstance exposure	Socioeconomic status	Age	Performed tasks	Gender results
Prenatal opioid exposure									
Nygaard et al., 2015	Heroin	Longitudinal study	Heroin exposed vs polysubstance exposed	Cognitive abilities	Yes (heroin, benzodiazepines, alcohol)	Controlled for low socioeconomic status	1–3 year	Bayley Scales II (Mental Development Index, MDI)	Significantly and stably lower levels of cognitive functioning in male progeny
Nygaard et al., 2015	Heroin	Longitudinal study	Heroin exposed vs polysubstance exposed	Cognitive abilities	Yes (heroin, benzodiazepines, alcohol)	Controlled for low socioeconomic status	4 year	McCarthy Scales of Children's Abilities	Significantly and stably lower levels of cognitive functioning in male progeny
Nygaard et al., 2015	Heroin	Longitudinal study	Heroin exposed vs polysubstance exposed	Cognitive abilities	Yes (heroin, benzodiazepines, alcohol)	Controlled for low socioeconomic status	8 year	The Wechsler Intelligence Scale for Children—Revised	Significantly lower cognitive scores in both exposed males and females
Nygaard et al., 2017	Heroin	Longitudinal study	Heroin exposed vs polysubstance exposed	Cognitive abilities	Yes (heroin, tobacco, benzodiazepines, alcohol, psychopharmaca)	Matched for socioeconomic status	17–21 year	Wechsler Abbreviated Scale of Intelligence (WASI), The Rey Complex Figure Test (RCFT), The California Verbal Learning Test – 2nd Ed (CVLT-II), Wechsler Adult Intelligence Scale 3rd Ed(WAIS-III)	Significantly worse cognitive performances in male and female exposed offspring compared to controls
Suffet and Brotman, 1984	Methadone	Longitudinal study	Exposed males vs exposed females	Cognitive abilities	ND	ND	0–2 year	Bayley Scales (Mental Development Index, MDI)	Significantly lower cognitive scores in both male and female exposed offspring
Ornoy et al., 2001	Heroin	Longitudinal study	Exposed vs unexposed	Inattention/ hyperactivity phenotype or risk for ADHD	Yes (heroin, methadone, benzodiazepines and other psychoactive drugs)	Compared for socioeconomic status	8 year (5–12 year)	The Conners and Achenbach questionnaires and the Pollack Taper test	Highest rate of ADHD in both heroin exposed boys and girls
Prenatal tobacco exposure									
Moe and Slinning, 2001	Tobacco	Longitudinal study	Exposed vs unexposed	Cognitive abilities	Yes (tobacco, opioids, alcohol, cannabis, psychostimulants, and more)	Compared for socioeconomic status	1–3 year	Bayley Scales II (Mental Development Index, MDI)	Lower Mental Developmental Scores in exposed male infants
Kotimaa et al., 2003	Tobacco	Longitudinal study	Exposed vs control	Hyperactivity phenotype and ADHD risk	Yes (tobacco and alcohol)	Adjusted for socioeconomic status	8 year	Children's Behavior Questionnaire (Rutter B2)	Hyperactivity in males and females prenatally exposed to nicotine
Willoughby et al., 2007	Tobacco	Epidemiological Study	Exposed vs control	Attention, reactivity, irritability	Yes (tobacco, alcohol)	Adjusted for socioeconomic status	0–1 year	Infant Behavior Record (IBR)	Significantly lower cognitive performances in exposed males

(Continued)

TABLE 1 | Continued

Author year	Substance	Type of study	Experimental groups	Neuro developmental outcome	Prenatal polysubstance exposure	Socioeconomic status	Age	Performed tasks	Gender results
Cornelius et al., 2007	Tobacco	Longitudinal study	Exposed vs unexposed offspring from teenager mothers	Inattention/ hyperactivity phenotype	Yes (tobacco, cannabis, alcohol)	Controlled for socioeconomic status	6 year	Child Behavior Checklist, RouthActivity Scale, and the SNAP	Increased activity and attention problems in both male and female exposedoffspring
Gatzke-Kopp and Beauchaine, 2007	Tobacco	Longitudinal study	Exposed vs unexposed	Neurobehavioral problems, ADHD and cognitive abilities	Yes (tobacco, alcohol, cannabis, amphetamines, heroin)	Controlled for socioeconomic status	7–15 year	Child Behavior Checklist (CBCL; Achenbach, 1991), Child Symptom Inventory (CSI)	Exposed offspring shows more severe ADHD symptoms and cognitive behavioral problems
Langley et al., 2007	Tobacco	Cross-sectional study	Exposed with ADHD vs unexposed with ADHD	ADHD diagnosis	ND	Measured as environmental risk	7–8 year	Clinical diagnosis	Maternal smoking in pregnancy and high environmental risk, independently influence the clinical presentation of the ADHDphenotype without sex-vulnerability
Hutchinson et al., 2010	Tobacco	Longitudinal study	Exposed vs unexposed	ADHD risk and neurobehavioral problems	ND	Confounding factor	3 year	SDQs	Higher risk for conduct and hyperactivity–inattention problems in males whose mothers persistently smoked throughout pregnancy
Wakschlag and Hans, 2002	Tobacco	Longitudinal study	Exposed vs unexposed	Neurobehavioral problems (conduct disorder)	Yes (tobacco, alcohol, opioids, cannabis)	Controlled for socioeconomic status	10 year	The Diagnostic Interview for Children and Adolescents (DICA)	Exposed boys, but not girls, are significantly more likely to develop conduct disorder symptoms
Fergusson et al., 1998	Tobacco	Longitudinal study	Exposed vs unexposed	Neurobehavioral problems (conduct disorder)	Yes (tobacco, alcohol, illicit drugs)	Adjusted for socioeconomic status	16–18 year	Composite International Diagnostic Interview and the Self-Report Delinquency Inventory	More severe conduct disorders symptoms in male adolescents than in females prenatally exposed to tobacco
Prenatal alcohol exposure									
Richardson et al., 2002	Alcohol	Longitudinal study	Exposed vs control	Cognitive abilities	Yes (alcohol, cannabis, tobacco, cocaine)	Controlled for low socioeconomic status	10 year	Wisconsin Card Sorting Test, Wide Range Assessment of Memory and Learning (WRAML), Trail Making	Significantly lower cognitive scores (learning and memory) in both male and female exposed offspring
Howell et al., 2006	Alcohol	Longitudinal study	Exposed vs control	Cognitive abilities	Yes (alcohol, cannabis, tobacco, cocaine)	Controlled for low socioeconomic status	15 year	Wechsler Intelligence Scale for Children (WISC-III), Wechsler Individual Achievement Test (WIAT)	Significantly lower IQ score and mathematical abilities in both male and female exposed offspring

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TABLE 1 | Continued

Author year	Substance	Type of study	Experimental groups	Neuro developmental outcome	Prenatal polysubstance exposure	Socioeconomic status	Age	Performed tasks	Gender results
Kelly et al., 2009	Alcohol	Longitudinal study	Exposed vs control	Cognitive abilities	Yes (alcohol, tobacco)	Adjusted for low socioeconomic status	3 year	British Ability Scale (BAS), Bracken School Readiness Assessment (BSRA)	Significantly lower cognitive scores in males born to heavy-drinking mothers compared to exposed females
Willford et al., 2004	Alcohol	Longitudinal study	Exposed vs control	Cognitive abilities	Yes (alcohol, cannabis, tobacco, cocaine)	Controlled for low socioeconomic status	14 year	Children's Memory Scale	Deficits in learning, short-term and long-term memory, specifically in the verbal domain, in both exposed males and females
Coles et al., 2002	Alcohol	Longitudinal study	Exposed vs control	Inattention/ hyperactivity phenotype	Yes (alcohol, cannabis, tobacco)	Controlled for low socioeconomic status	15 year	Continuous performance task (CPT)	Deficits in sustained attention, processing in the visual and auditory modality in exposed progeny
Herman et al., 2008	Alcohol	Cross-sectional study	FASD offspring with ADHD vs FASD offspring	ADHD diagnosis	ND	Controlled for low socioeconomic status	6–16 year	ADHD diagnosis	Higher prevalence of ADHD diagnosis in exposed males than females
Kelly et al., 2009	Alcohol	Longitudinal study	Exposed vs control	Behavior problems (hyperactivity, conduct, peer problems)	Yes (alcohol, tobacco)	Adjusted for low socioeconomic status	3 year	Parent-report version of the Strengths and Difficulties Questionnaire (SDQ)	Exposed males were more likely to have clinically relevant high total difficulties, hyperactivity, conduct and peer problems compared to girls
Prenatal cannabis Exposure									
Noland et al., 2005	Cannabis	Longitudinal study	Exposed vs control	Cognitive abilities	Yes (cannabis, tobacco, alcohol, cocaine)	Controlled for low socioeconomic status	4 year	Picture deletion task (PDT), continuous performance task (CPT), Wechsler Preschool and Primary Scales of Intelligence-Revised (WPPSI-R)	Higher omission error rates in exposed offspring
El Marroun et al., 2011	Cannabis	Longitudinal study	Exposed vs control	Attention problems	Yes (cannabis, tobacco, alcohol)	ND	1–2 year	Child Behavior Checklist	Prenatal cannabis is associated with attention problems specifically in exposed girls
Richardson et al., 2002	Cannabis	Longitudinal study	Exposed vs control	Cognitive abilities, attention and impulsivity	Yes (alcohol, cannabis, tobacco, cocaine)	Controlled for low socioeconomic status	10 year	Continuous performance test	Deficit in memory and learning, together with higher impulsivity score in both males and females

(Continued)

TABLE 1 | Continued

Author year	Substance	Type of study	Experimental groups	Neuro developmental outcome	Prenatal polysubstance exposure	Socioeconomic status	Age	Performed tasks	Gender results
El Marroun et al., 2011	Cannabis	Longitudinal study	Exposed vs control	Behavioral problems (aggressive behavior)	Yes (cannabis, tobacco, alcohol)	ND	1–2 year	Child Behavior Checklist	Externalizing problems (aggressive behavior) in girls during early childhood
Day et al., 2011	Cannabis	Longitudinal study	Exposed vs control	Behavioral problems (delinquent behavior)	Yes (cannabis, tobacco, alcohol)	Matched	14 year	Self-Report Delinquency scale and Child Behavior Checklist	Delinquent behavior in exposed offspring of heavy marijuana users

A thorough description of each study is presented with designed study type, experimental groups enrolled, outcomes, occurrence of poly-drug use, socioeconomic status, age at assessment, type of task used, and gender results. Gender effect is displayed as pink and blue for female and male progeny, respectively. Gender differences not observed are white.

to female offspring (Bennett et al., 2002, 2007, 2008; Delaney-Black et al., 2004; Nordstrom Bailey et al., 2005; Sood et al., 2005; Bendersky et al., 2006; Dennis et al., 2006; Carmody et al., 2011). In contrast, no gender dichotomy was found in the occurrence of attention deficit/hyperactivity disorder (ADHD) phenotypes from infancy to preadolescence (Karmel and Gardner, 1996; Mayes, 1996; Mayes et al., 1998, 2003; Accornero et al., 2007).

With regard to methamphetamine, the most frequent outcomes reported in newborns occur during the first year of life and include motor dysfunctions (e.g., disorganized behaviors with poor quality of movement), which tend to disappear with development in boys (LaGasse et al., 2012; Shah et al., 2012; Zabaneh et al., 2012; Kiblawi et al., 2014), whereas they persist throughout adolescence in girls (Eriksson and Zetterström, 1994; Cernerud et al., 1996). In contrast, other neurobehavioral problems (e.g., anxious/depressive phenotypes, emotional problems) appear during late infancy and childhood and do not exhibit sex bias (LaGasse et al., 2012). Similarly, impairments in cognitive skills occur equally in both female and male offspring (Lu et al., 2009; Piper et al., 2011; Diaz et al., 2014). However, deficits in inhibitory control and ADHD-like symptoms are prevalent in boys (LaGasse et al., 2012; Kiblawi et al., 2013).

Effects of Prenatal Exposure to Opioids

Regardless of the efforts aimed at discouraging opioid use, prevalence rates show an increasing trend in pregnant women (Haight et al., 2018). However, gender was not considered in most of the human studies on the effects of heroin, methadone, and other prescription opioids.

Children born to mothers who use opioids during gestation suffer from the so-called neonatal opioid withdrawal syndrome (NOWS) (Gomez-Pomar and Finnegan, 2018), characterized by several signs and symptoms (e.g., tremors, sleep problems, hyperactive reflexes, vomiting, dehydration, and respiratory problems), which are more severe in boys than in girls (Jansson et al., 2007, 2010). Maternal consumption of methadone—the gold standard for opioid maintenance therapy—is associated with poorer cognitive performance and lower IQ scores in exposed males when compared to females during infancy, an age-dependent effect (Suffet and Brotman, 1984; Nygaard et al., 2015). However, no gender difference is found in symptoms related to ADHD and aggressive behavior up to preadolescence (Ornoy et al., 2001).

Effects of Maternal Tobacco

Nicotine and its related tobacco products are the most studied substances in relation to long-term neurobehavioral outcomes in offspring exposed to tobacco during pregnancy. Despite the limitations due to several environmental confounding factors, a high degree of consistency exists for the association of maternal smoking and cognitive and behavioral problems (for an exhaustive review, see England et al., 2017). From these studies emerge a male bias toward diverse behavioral and cognitive domains, depending on age: at 6–8 months, males appear more vulnerable to deficits in cognitive and executive functions (e.g., inattention) and in motor functions and to alterations in

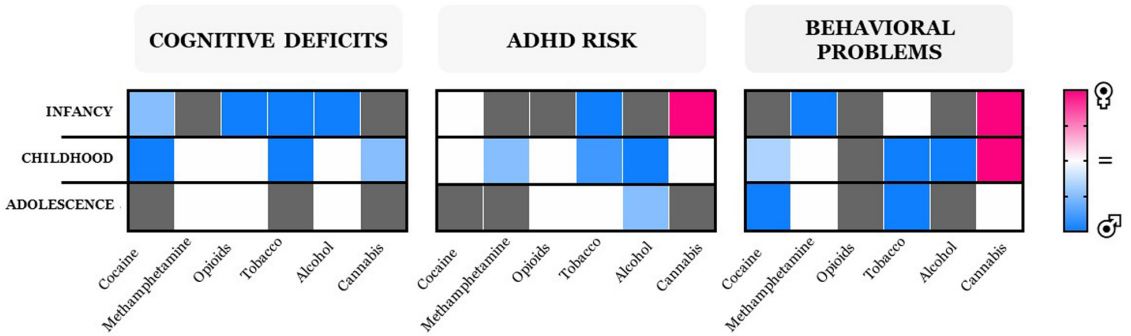

FIGURE 1 | Heatmaps of neurobehavioral outcomes collectively identified as cognitive deficits (including executive function), ADHD risk, and behavioral problems, with missing information on gender differences shown in gray. Outcome intensities are displayed as colors ranging from pink (female) to blue (male) shown in the key. Gender differences were not observed in executive functions during childhood in offspring prenatally exposed to cocaine in one study only (Accornero et al., 2007); therefore, the heatmap displays blue as a result of the larger number of consistent studies reporting that boys are more susceptible to exhibit deficits in cognitive abilities when compared to girls (see text and also **Table 1**).

reactivity (Moe and Slinning, 2001; Wakschlag and Hans, 2002; Willoughby et al., 2007). From infancy through childhood, boys appear at risk for ADHD (Kotimaa et al., 2003; Cornelius et al., 2007; Willoughby et al., 2007; Agrawal et al., 2010; Hutchinson et al., 2010); however, only during infancy do they display less positive mood (Pickett et al., 2008) than females; during childhood and adolescence, males present more externalizing and disruptive behaviors (e.g., conduct disorders, antisocial behavior) than females (Wakschlag et al., 1997; Fergusson et al., 1998; Hutchinson et al., 2010). Conversely, parental tobacco exposure is associated with nicotine dependence and high consumption of tobacco only in adolescent girls (Rydell et al., 2012). Although the risk of developing ADHD symptoms in nicotine-exposed progeny is high during adolescence, no gender differences were found (Gatzke-Kopp and Beauchaine, 2007; Agrawal et al., 2010; Sourander et al., 2019).

Effects of Maternal Alcohol

Despite the widely described dose-dependent teratogenic effect of alcohol (Kodituwakku, 2007; Ornoy and Ergaz, 2010), approximately 10% of women aged between 15 and 44 years consume alcohol during pregnancy, with 3% exhibiting a binge-drinking pattern (SAMHSA, 2011). Irrespective of the amount and pattern of consumption, a wealth of clinical evidence describes that prenatal alcohol exposure markedly impairs cognitive, behavioral, and motor functions of offspring (Mattson et al., 1998; Coles et al., 2002; Richardson et al., 2002; Willford et al., 2004; Riley and McGee, 2005; Howell et al., 2006). Maternal moderate to heavy drinking produces a group of pathological conditions termed fetal alcohol spectrum disorder (FASD). Epidemiological studies report sexual dichotomy in FASD, with prevalence rates and severity being higher in male than in female patients (May et al., 2007; Astley, 2010; Thanh et al., 2014; but see May et al., 2014; Fox et al., 2015). A sex bias is also described for other psychopathological traits, such as elevated rates of ADHD in 6- to 16-year-old boys but not girls (Coles et al., 2002; Herman et al., 2008). Boys also exhibit altered responses to stress, measured as larger changes in cortisol levels induced by stress-related cues (Haley et al., 2006). In contrast, neuroimaging studies do not reveal sex differences in long-term abnormalities of brain morphology because the reduction in both size and volume of frontal, temporal, cingulate, and striatal regions of offspring prenatally exposed to alcohol did not differ between genders (Eckstrand et al., 2012; Treit et al., 2013; De Guio et al., 2014). These findings suggest that such psychopathological traits cannot be attributed to these structural changes.

Effects of *in utero* Cannabis Exposure

In line with the data on general population, the rates of cannabis use among pregnant women have markedly increased, with prevalence rates reaching 75% between 2002 and 2016 (Brown et al., 2017). Despite this alarming scenario, a few studies have assessed the long-term neurobehavioral repercussions of maternal cannabis use on the offspring, though gender differences were not consistently examined: the *Ottawa Prenatal Prospective Study* (OPPS), the *Maternal Health Practices and Child Development Project* (MHPCDP), the *Generation R* study, and *Adolescent Brain Cognitive Development* (ABCD) study. The OPPS study included gender as a confounding factor, and it described a number of long-lasting neurobehavioral alterations, ranging from heightened tremors and startle responsiveness to deficits in executive function (e.g., attention, cognitive flexibility, problem solving, impulse control) (Fried and Makin, 1987; Fried and Smith, 2001). Similarly, gender was not examined when assessing performance in memory, verbal, and perceptual processes as well as the first clinical signs of impulsivity at childhood (Smith et al., 2006). However, when the same authors subsequently included the gender factor on clinical signs that persisted at young adulthood, such as deficits in executive function tasks that require impulse control, they found no gender differences (Smith et al., 2004). In the MHPCD study, the authors seldom included "gender" in their analysis. However, they reported (1) significant sleep disturbances and deficits in mental development as well as in short-term memory and verbal reasoning at both 9 months and 3 years of age; (2) deficits in attention and memory, increased anxiety/depressive

symptoms, impulsivity, hyperactivity, and aggression at 6 and 14 years of age (Richardson et al., 1989, 2002; Dahl et al., 1995; Leech et al., 2006; Day et al., 2011). Gender at 10 years of age did not affect cognitive deficits (Richardson et al., 2002). In contrast, the Generation R study showed that girls but not boys at 18 months of age exhibited increased scores on an aggressive behavior scale that persisted through childhood (El Marroun et al., 2011). Notably, this sex bias disappears during adolescence. Also, during infancy girls appear to be at risk for the development of ADHD, a susceptibility that is age dependent (**Table 1** and **Figure 1**). Remarkably, although from Generation R and ABCD studies maternal cannabis use has been associated to proneness to psychosis in middle to late childhood, significantly earlier than the typical onset of first psychotic episode (Bolhuis et al., 2018; Fine et al., 2019; Paul et al., 2019), again gender was not considered. Importantly, an independent investigation showed that prenatal marijuana exposure has an equally negative effect on sustained attention of the offspring from childhood to adolescence (Noland et al., 2005).

CONCLUSION

The literature here examined reveals gender differences in immediate and long-term negative consequences of maternal drug use on both cognition and behavior. When gender was included as a variable, irrespective of the drug used, male progeny appear more vulnerable to cognitive deficits and at risk of ADHD from infancy through childhood (**Table 1** and **Figure 1**). Notably, these gender differences tend to disappear with age. However, we cannot depict a clear picture for internalizing problems, drug use, and motor function deficits due to the paucity of data. Regarding the problems in the behavioral domain (i.e., externalizing problems), the current scenario is clearer: girls exposed *in utero* to cannabis are more vulnerable than boys up until adolescence, but this conclusion cannot be extended to other drugs. Remarkably, this is in contrast to what is often reported in rodent studies (Fernandez-Ruiz et al., 1998; Hurd et al., 2019; Scheyer et al., 2019; de Salas-Quiroga et al., 2020), where female sex often acts as a protective factor. Nevertheless, the advantage of animal studies is to dissect the effects of genetic, biological, and/or environmental risk factors. The establishment of a biological causality between prenatal drug exposure and repercussions on the progeny from animal investigations is pivotal. These mechanistic insights along with the observations reported in human studies may help in developing therapeutic interventions, on a gender-specific basis, which would ultimately result in more effective treatment outcome.

The longitudinal studies examined have often considered different factors that might have contributed to gender differences, including socioeconomic status, lifestyle indicators, stressful life events, social support (or lack thereof), and psychiatric comorbidity. In this regard, an additional degree of complexity arises from the evidence that single drug use is virtually non-existent. At this stage, we cannot certainly resolve this issue in human studies, as it deserves as much attention as neuroimaging and omics analyses to reveal neurobiological underpinnings of drug-exposed phenotypes. Of similar importance is the need to study the association between the perturbations of *in utero*–placental exchange and adverse mental health outcome later in life. Indeed, increasing evidence points to the role of the placenta in fetal programming, which is altered in response to prenatal insults and contributes to psychopathology (Burton et al., 2010; Khalife et al., 2012; Roescher et al., 2014; Park et al., 2018; Kratimenos and Penn, 2019). Notably, the placenta influences in a sex-dependent manner the outcome for offspring who were exposed to perinatal malnutrition and stressors (Walsh et al., 2019). However, research into whether the gender bias results from sex differences in placental structure and functions or its genes, proteins, and steroids is surprisingly lacking. Hence, future research should aim at disentangling how sex impacts neurobiology from the transfer of maternal drug concentrations across the placenta to the effect on placental gene transcription or expression of discrete transporters (e.g., ATP-binding cassette carriers) in the cord. In fact, to date, such investigations have been performed only to relate maternal drug use and placental perturbations to fetal growth and other morphological abnormalities (Janssen et al., 2015).

Substance (ab)use screening protocols, including questionnaires and urine toxicology testing, should be established worldwide as routine to identify pregnant women using drugs. Public health interventions regarding the awareness of the harm associated with maternal drug use, and special programs to enter treatment and/or increase spontaneous quit rates, should be implemented (Jantzen et al., 1998; Forray, 2016 and references therein; Patrick et al., 2017). Progress on tailored, safe, and acceptable pharmacotherapies to restore proper neurodevelopmental trajectories of the progeny should be incentivized. Additional preventative outreach programs should be implemented to raise community awareness and support and to provide access to treatment for the children who are prenatally exposed to drugs. Finally, future investigations should be implemented to include the influence of sex as a biological variable (for guidelines please refer to (Clayton, 2018; Mannon et al., 2020) in the outcome of offspring prenatally exposed to drugs of abuse.

AUTHOR CONTRIBUTIONS

All authors participated in the conceptualization, design, and preparation of the manuscript.

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REFERENCES

Accornero, V. H., Amado, A. J., Morrow, C. E., Xue, L., Anthony, J. C., and Bandstra, E. S. (2007). Impact of prenatal cocaine exposure on attention and response inhibition as assessed by continuous performance tests. *J. Dev. Behav. Pediatr.* 28, 195–205. doi: 10.1097/01.DBP.0000268560.72580.f9

Ackerman, J. P., Riggins, T., and Black, M. M. (2010). A review of the effects of prenatal cocaine exposure among school-aged children. *Pediatrics* 125, 554–565. doi: 10.1542/peds.2009-0637

Agrawal, A., Scherrer, J. F., Grant, J. D., Sartor, C. E., Pergadia, M. L., Duncan, A. E., et al. (2010). The effects of maternal smoking during pregnancy on offspring outcomes. *Prev. Med.* 50, 13–18. doi: 10.1016/j.ypmed.2009.12.009

Astley, S. J. (2010). Profile of the first 1,400 patients receiving diagnostic evaluations for fetal alcohol spectrum disorder at the Washington State Fetal Alcohol Syndrome Diagnostic & Prevention Network. *Can. J. Clin. Pharmacol.* 17, 132–164.

Back, S. E., Payne, R. L., Wahlquist, A. H., Carter, R. E., Stroud, Z., Haynes, L., et al. (2011). Comparative profiles of men and women with opioid dependence: results from a national multisite effectiveness trial. *Am. J. Drug Alcohol Abuse* 37, 313–323. doi: 10.3109/00952990.2011.596982

Barker, D. J. (2007). The origins of the developmental origins theory. *J. Intern. Med.* 261, 412–417. doi: 10.1111/j.1365-2796.2007.01809.x

Barr, D. B., Bishop, A., and Needham, L. L. (2007). Concentrations of xenobiotic chemicals in the maternal-fetal unit. *Reprod. Toxicol.* 23, 260–266. doi: 10.1016/j.reprotox.2007.03.003

Bendersky, M., Bennett, D., and Lewis, M. (2006). Aggression at age 5 as a function of prenatal exposure to cocaine, gender, and environmental risk. *J. Pediatr. Psychol.* 31, 71–84. doi: 10.1093/jpepsy/jsj025

Bennett, D., Bendersky, M., and Lewis, M. (2007). Preadolescent health risk behavior as a function of prenatal cocaine exposure and gender. *J. Dev. Behav. Pediatr.* 28, 467–472. doi: 10.1097/DBP.0b013e31811320d8

Bennett, D. S., Bendersky, M., and And Lewis, M. (2008). Children's cognitive ability from 4 to 9 years old as a function of prenatal cocaine exposure, environmental risk, and maternal verbal intelligence. *Dev. Psychol.* 44, 919–928. doi: 10.1037/0012-1649.44.4.919

Bennett, D. S., Bendersky, M., and Lewis, M. (2002). Children's intellectual and emotional-behavioral adjustment at 4 years as a function of cocaine exposure, maternal characteristics, and environmental risk. *Dev. Psychol.* 38:648. doi: 10.1037/0012-1649.38.5.648

Bolhuis, K., Kushner, S. A., Yalniz, S., Hillegers, M. H., Jaddoe, V. W., Tiemeier, H., et al. (2018). Maternal and paternal cannabis use during pregnancy and the risk of psychotic-like experiences in the offspring. *Schizophr. Res.* 202, 322–327. doi: 10.1016/j.schres.2018.06.067

Bridgett, D. J., and Mayes, L. C. (2011). Development of inhibitory control among prenatally cocaine exposed and non-cocaine exposed youths from late childhood to early adolescence: the effects of gender and risk and subsequent aggressive behavior. *Neurotoxicol. Teratol.* 33, 47–60. doi: 10.1016/j.ntt.2010.08.002

Brown, Q. L., Sarvet, A. L., Shmulewitz, D., Martins, S. S., Wall, M. M., and Hasin, D. S. (2017). Trends in marijuana use among pregnant and nonpregnant reproductive-aged women, 2002-2014. *JAMA* 317, 207–209. doi: 10.1001/jama.2016.17383

Bruin, J. E., Gerstein, H. C., and Holloway, A. C. (2010). Long-term consequences of fetal and neonatal nicotine exposure: a critical review. *Toxicol. Sci.* 116, 364–374. doi: 10.1093/toxsci/kfq103

Burton, G. J., Jauniaux, E., and Charnock-Jones, D. S. (2010). The influence of the intrauterine environment on human placental development. *Int. J. Dev. Biol.* 54, 303–311.

Carmody, D. P., Bennett, D. S., and Lewis, M. (2011). The effects of prenatal cocaine exposure and gender on inhibitory control and attention. *Neurotoxicol. Teratol.* 33, 61–68. doi: 10.1016/j.ntt.2010.07.004

Cernerud, L. A. R. S., Eriksson, M., Jonsson, B., Steneroth, G., and Zetterstrom, R. (1996). Amphetamine addiction during pregnancy: 14-year follow-up of growth and school performance. *Acta Paediatr.* 85, 204–208. doi: 10.1111/j.1651-2227.1996.tb13993.x

Clayton, J. A. (2018). Applying the new SABV (sex as a biological variable) policy to research and clinical care. *Physiol. Behav.* 187, 2–5. doi: 10.1016/j.physbeh.2017.08.012

Coles, C. D., Platzman, K. A., Lynch, M. E., and Freides, D. (2002). Auditory and visual sustained attention in adolescents prenatally exposed to alcohol. *Alcohol. Clin. Exp. Res.* 26, 263–271. doi: 10.1111/j.1530-0277.2002.tb02533.x

Comasco, E., Rangmar, J., Eriksson, U. J., and Oreland, L. (2018). Neurological and neuropsychological effects of low and moderate prenatal alcohol exposure. *Acta Physiol.* 222:e12892. doi: 10.1111/apha.12892

Conway, K. P., Compton, W., Stinson, F. S., and Grant, B. F. (2006). Lifetime comorbidity of DSM-IV mood and anxiety disorders and specific drug use disorders: results from the national epidemiologic survey on alcohol and related conditions. *J. Clin. Psychiatry* 67, 247–257. doi: 10.4088/jcp.v67n0211

Cornelius, M. D., Goldschmidt, L., DeGenna, N., and Day, N. L. (2007). Smoking during teenage pregnancies: effects on behavioral problems in offspring. *Nicotine Tob. Res.* 9, 739–750. doi: 10.1080/14622200701416971

Dahl, R. E., Scher, M. S., Williamson, D. E., Robles, N., and Day, N. (1995). A longitudinal study of prenatal marijuana use: Effects on sleep and arousal at age 3 years. *Arch. Pediatr. Adolesc. Med.* 149, 145–150.

Day, N. L., Leech, S. L., and Goldschmidt, L. (2011). The effects of prenatal marijuana exposure on delinquent behaviors are mediated by measures of neurocognitive functioning. *Neurotoxicol. Teratol.* 33, 129–136. doi: 10.1016/j.ntt.2010.07.006

De Guio, F., Mangin, J. F., Rivière, D., Perrot, M., Molteno, C. D., Jacobson, S. W., et al. (2014). A study of cortical morphology in children with fetal alcohol spectrum disorders. *Hum. Brain Mapp.* 35, 2285–2296. doi: 10.1002/hbm.22327

de Salas-Quiroga, A., García-Rincón, D., Gómez-Domínguez, D., Valero, M., Simón-Sánchez, S., Paraíso-Luna, J., et al. (2020). Long-term hippocampal interneuronopathy drives sex-dimorphic spatial memory impairment induced by prenatal THC exposure. *Neuropsychopharmacology* 45, 877–886. doi: 10.1038/s41386-020-0621-3

Delaney-Black, V., Covington, C., Nordstrom, B., Ager, J., Janisse, J., Hannigan, J. H., et al. (2004). Prenatal cocaine: quantity of exposure and gender moderation. *J. Dev. Behav. Pediatr.* 25, 254–263. doi: 10.1097/00004703-200408000-00005

Dennis, T., Bendersky, M., Ramsay, D., and Lewis, M. (2006). Reactivity and regulation in children prenatally exposed to cocaine. *Dev. Psychol.* 42, 688–697. doi: 10.1037/0012-1649.42.4.688

Diaz, S. D., Smith, L. M., LaGasse, L. L., Derauf, C., Newman, E., Shah, R., et al. (2014). Effects of prenatal methamphetamine exposure on behavioral and cognitive findings at 7.5 years of age. *J. Pediatr.* 164, 1333–1338. doi: 10.1016/j.jpeds.2014.01.053

Eckstrand, K. L., Ding, Z., Dodge, N. C., Cowan, R. L., Jacobson, J. L., Jacobson, S. W., et al. (2012). Persistent dose-dependent changes in brain structure in young adults with low-to-moderate alcohol exposure in utero. *Alcohol. Clin. Exp. Res.* 36, 1892–1902. doi: 10.1111/j.1530-0277.2012.01819.x

El Marroun, H., Hudziak, J. J., Tiemeier, H., Creemers, H., Steegers, E. A., Jaddoe, V. W., et al. (2011). Intrauterine cannabis exposure leads to more aggressive behavior and attention problems in 18-month-old girls. *Drug Alcohol. Dep.* 118, 470–474. doi: 10.1016/j.drugalcdep.2011.03.004

EMCDDA (2019). *European Drug Report. Trends and Developments. European Monitoring Centre for Drugs and Drug Addiction* [preprint]. Available online at: http://www.emcdda.europa.eu/system/files/publications/11364/20191724_TDAT19001ENN_PDF.pdf (accessed December 12, 2019).

England, L. J., Aagaard, K., Bloch, M., Conway, K., Cosgrove, K., Grana, R., et al. (2017). Developmental toxicity of nicotine: a transdisciplinary synthesis and implications for emerging tobacco products. *Neurosci. Biobehav. Rev.* 72, 176–189. doi: 10.1016/j.neubiorev.2016.11.013

Eriksson, M., and Zetterström, R. (1994). Amphetamine addiction during pregnancy: 10-year follow-up. *Acta Paediatr.* 83, 27–31. doi: 10.1111/j.1651-2227.1994.tb13380.x

Fergusson, D. M., Woodward, L. J., and Horwood, L. J. (1998). Maternal smoking during pregnancy and psychiatric adjustment in late adolescence. *Arch. Gen. Psychiatry* 55, 721–727. doi: 10.1001/archpsyc.55.8.721

Fernandez-Ruiz, J., Bonnin, A., De Miguel, R., Castro, J. G., and Ramos, J. A. (1998). Peinatal exposure to marihuana or its main psychotive constituent, delta9-tetrahydrocannabinol, affects the development of brain dopaminergic nerons. *Arq. Med.* 12, 67–77.

Fine, J. D., Moreau, A. L., Karcher, N. R., Agrawal, A., Rogers, C. E., Barch, D. M., et al. (2019). Association of prenatal cannabis exposure with psychosis proneness among children in the adolescent brain cognitive development

(ABCD) study. *JAMA Psychiatry* 76, 762–764. doi: 10.1001/jamapsychiatry.2019.0076

Forray, A. (2016). Substance use during pregnancy. *F1000Res* 5:F1000 Faculty Rev-887.

Fox, D. J., Pettygrove, S., Cunniff, C., O'Leary, L. A., Gilboa, S. M., Bertrand, J., et al. (2015). Fetal alcohol syndrome among children aged 7–9 years—Arizona. Colorado, and New York, 2010. *MMWR* 64, 54–57.

Fried, P. A., and Makin, J. E. (1987). Neonatal behavioural correlates of prenatal exposure to marihuana, cigarettes and alcohol in a low risk population. *Neurotoxicol. Teratol.* 9, 1–7. doi: 10.1016/0892-0362(87)90062-6

Fried, P. A., and Smith, A. M. (2001). A literature review of the consequences of prenatal marihuana exposure: an emerging theme of a deficiency in aspects of executive function. *Neurotoxicol. Teratol.* 23, 1–11. doi: 10.1016/s0892-0362(00)00119-7

Gatzke-Kopp, L. M., and Beauchaine, T. P. (2007). Direct and passive prenatal nicotine exposure and the development of externalizing psychopathology. *Child. Psychiatry Hum. Dev.* 38, 255–269. doi: 10.1007/s10578-007-0059-4

Gkioka, E., Korou, L. M., Daskalopoulou, A., Misitzi, A., Batsidis, E., Bakoyiannis, I., et al. (2016). Prenatal cocaine exposure and its impact on cognitive functions of offspring: a pathophysiological insight. *Rev. Neurosci.* 27, 523–534. doi: 10.1515/revneuro-2015-0064

Gomez-Pomar, E., and Finnegan, L. P. (2018). The epidemic of neonatal abstinence syndrome, historical references of Its'. Origins, assessment, and management. *Front. Pediatr.* 6:33. doi: 10.3389/fped.2018.00033

Greenfield, S. F., Back, S. E., Lawson, K., and Brady, K. T. (2010). Substance abuse in women. *Psychiatr. Clin. North Am.* 33, 339–355. doi: 10.1016/j.psc.2010.01.004

Haight, S. C., Ko, J. Y., Tong, V. T., Bohm, M. K., and Callaghan, W. M. (2018). Opioid use disorder documented at delivery hospitalization - United States, 1999-2014. *MMWR* 67, 845–849. doi: 10.15585/mmwr.mm6731a1

Hales, C. N., and Barker, D. J. (2001). The thrifty phenotype hypothesis. *Br. Med. Bull.* 60, 5–20. doi: 10.1093/bmb/60.1.5

Haley, D. W., Handmaker, N. S., and Lowe, J. (2006). Infant stress reactivity and prenatal alcohol exposure. *Alcohol. Clin. Exp. Res.* 30, 2055–2064. doi: 10.1111/j.1530-0277.2006.00251.x

Herman, L. E., Acosta, M. C., and Chang, P. N. (2008). Gender and attention deficits in children diagnosed with a fetal alcohol spectrum disorder. *Can. J. Clin. Pharmacol.* 15, 411–419.

Howell, K. K., Lynch, M. E., Platzman, K. A., Smith, G. H., and Coles, C. D. (2006). Prenatal alcohol exposure and ability, academic achievement, and school functioning in adolescence: a longitudinal follow-up. *J. Pediatr. Psychol.* 31, 116–126. doi: 10.1093/jpepsy/jsj029

Hurd, Y. L., Manzoni, O. J., Pletnikov, M. V., Lee, F. S., Bhattacharyya, S., and Melis, M. (2019). Cannabis and the developing brain: Insights into its long-lasting effects. *J. Neurosci.* 39, 8250–8258. doi: 10.1523/jneurosci.1165-19.2019

Hutchinson, J., Pickett, K. E., Green, J., and Wakschlag, L. S. (2010). Smoking in pregnancy and disruptive behaviour in 3-year-old boys and girls: an analysis of the UK Millennium Cohort Study. *J. Epidemiol. Community Health* 64, 82–88. doi: 10.1136/jech.2009.089334

Janssen, B. G., Byun, H.-M., Gyselaers, W., Lefebvre, W., Baccarelli, A. A., and Nawrot, T. S. (2015). Placental mitochondrial methylation and exposure to airborne particulate matter in the early life environment: an ENVIR ON AGE birth cohort study. *Epigenetics* 10, 536–544. doi: 10.1080/15592294.2015.1048412

Jansson, L. M., Dipietro, J. A., Elko, A., and Velez, M. (2007). Maternal vagal tone change in response to methadone is associated with neonatal abstinence syndrome severity in exposed neonates. *J. Matern. Fetal Neonatal Med.* 20, 677–685. doi: 10.1080/14767050701490327

Jansson, L. M., Dipietro, J. A., Elko, A., and Velez, M. (2010). Infant autonomic functioning and neonatal abstinence syndrome. *Drug Alcohol Depend.* 109, 198–204. doi: 10.1016/j.drugalcdep.2010.01.004

Jantzen, K., Ball, S. A., Leventhal, J. M., and Schottenfeld, R. S. (1998). Types of abuse and cocaine use in pregnant women. *J. Subst. Abuse Treat.* 15, 319–323. doi: 10.1016/s0740-5472(97)00198-0

Karmel, B. Z., and Gardner, J. M. (1996). Prenatal cocaine exposure effects on arousal-modulated attention during the neonatal period. *Dev. Psychobiol.* 29, 463–480. doi: 10.1002/(sici)1098-2302(199607)29:5<463::aid-dev5>3.0.co;2-m

Kelly, Y., Sacker, A., Gray, R., Kelly, J., Wolke, D., and Quigley, M. A. (2009). Light drinking in pregnancy, a risk for behavioural problems and cognitive deficits at 3 years of age? *Int. J. Epidemiol.* 38, 129–140. doi: 10.1093/ije/dyn230

Keyes, K. M., Hatzenbuehler, M. L., McLaughlin, K. A., Link, B., Olfson, M., Grant, B. F., et al. (2010). Stigma and treatment for alcohol disorders in the United States. *Am. J. Epidemiol.* 172, 1364–1372. doi: 10.1093/aje/kwq304

Keyes, K. M., Martins, S. S., and Hasin, D. S. (2008). Past 12-month and lifetime comorbidity and poly-drug use of ecstasy users among young adults in the United States: results from the National Epidemiologic Survey on Alcohol and Related Conditions. *Drug Alcohol Depend.* 97, 139–149. doi: 10.1016/j.drugalcdep.2008.04.001

Khalife, N., Glover, V., Hartikainen, A.-L., Taanila, A., Ebeling, H., Järvelin, M.-R., et al. (2012). Placental size is associated with mental health in children and adolescents. *PLoS One* 7:e40534. doi: 10.1371/journal.pone.0040534

Khan, A., Faucett, J., Morrison, S., and Brown, W. A. (2013). Comparative mortality risk in adult patients with schizophrenia, depression, bipolar disorder, anxiety disorders, and attention-deficit/hyperactivity disorder participating in psychopharmacology clinical trials. *JAMA Psychiatry* 70, 1091–1099. doi: 10.1001/jamapsychiatry.2013.149

Kiblawi, Z. N., Smith, L. M., Diaz, S. D., LaGasse, L. L., Derauf, C., Newman, E., et al. (2014). Prenatal methamphetamine exposure and neonatal and infant neurobehavioral outcome: results from the IDEAL study. *Subst. Abus.* 35, 68–73. doi: 10.1080/08897077.2013.814614

Kiblawi, Z. N., Smith, L. M., LaGasse, L. L., Derauf, C., Newman, E., Shah, R., et al. (2013). The effect of prenatal methamphetamine exposure on attention as assessed by continuous performance tests: Results from the infant development, environment, and lifestyle (IDEAL) study. *J. Dev. Behav. Pediatr.* 34, 31–37. doi: 10.1097/DBP.0b013e318277a1c5

Kodituwakku, P. W. (2007). Defining the behavioral phenotype in children with fetal alcohol spectrum disorders: a review. *Neurosci. Biobehav. Rev.* 31, 192–201. doi: 10.1016/j.neubiorev.2006.06.020

Kotimaa, A. J., Moilanen, I., Taanila, A., Ebeling, H., Smalley, S. L., McGough, J. J., et al. (2003). Maternal smoking and hyperactivity in 8-year-old children. *J. Am. Acad. Child Adolesc. Psychiatry* 42, 826–833. doi: 10.1097/01.chi.0000046866.56865.a2

Kratimenos, P., and Penn, A. A. (2019). Placental programming of neuropsychiatric disease. *Pediatr. Res.* 86, 157–164. doi: 10.1038/s41390-019-0405-9

LaGasse, L. L., Derauf, C., Smith, L. M., Newman, E., Shah, R., Neal, C., et al. (2012). Prenatal methamphetamine exposure and childhood behavior problems at 3 and 5 years of age. *Pediatrics* 129, 681–688. doi: 10.1542/peds.2011-2209

Langley, K., Holmans, P. A., van den Bree, M. B., and Thapar, A. (2007). Effects of low birth weight, maternal smoking in pregnancy and social class on the phenotypic manifestation of Attention Deficit Hyperactivity Disorder and associated antisocial behaviour: investigation in a clinical sample. *BMC Psychiatry* 7:26. doi: 10.1186/1471-244x-7-26

Leech, S. L., Larkby, C. A., Day, R., and Day, N. L. (2006). Predictors and correlates of high levels of depression and anxiety symptoms among children at age 10. *J. Am. Acad. Child. Adolesc. Psychiatry* 45, 223–230. doi: 10.1097/01.chi.0000184930.18552.4d

Lu, L. H., Johnson, A., O'Hare, E. D., Bookheimer, S. Y., Smith, L. M., O'Connor, M. J., et al. (2009). Effects of prenatal methamphetamine exposure on verbal memory revealed with fMRI. *J. Dev. Behav. Pediatr.* 30, 185–192. doi: 10.1097/DBP.0b013e3181a7ee6b

Mannon, E. C., Ray, S. C., Ryan, M. J., and Sullivan, J. C. (2020). Does sex matter: an update on the implementation of sex as a biological variable in research. *Am. J. Physiol. Renal Physiol.* 318, F329–F331.

Mattson, S. N., Riley, E. P., Gramling, L., Delis, D. C., and Jones, K. L. (1998). Neuropsychological comparison of alcohol-exposed children with or without physical features of fetal alcohol syndrome. *Neuropsychology* 12, 146–153. doi: 10.1037/0894-4105.12.1.146

May, P. A., Baete, A., Russo, J., Elliott, A. J., Blankenship, J., Kalberg, W. O., et al. (2014). Prevalence and characteristics of fetal alcohol spectrum disorders. *Pediatrics* 134, 855–866. doi: 10.1542/peds.2013-3319

May, P. A., Gossage, J. P., Marais, A. S., Adnams, C. M., Hoyme, H. E., Jones, K. L., et al. (2007). The epidemiology of fetal alcohol syndrome and partial FAS in a South African community. *Drug Alcohol. Depend.* 88, 259–271. doi: 10.1016/j.drugalcdep.2006.11.007

Mayes, L. C. (1996). Exposure to cocaine: behavioral outcomes in preschool and school-age children. *NIDA Res. Monogr.* 164, 211–229.

Mayes, L. C., Cicchetti, D., Acharyya, S., and Zhang, H. (2003). Developmental trajectories of cocaine-and-other-drug-exposed and non-cocaine-exposed children. *J. Dev. Behav. Pediatr.* 24, 323–335. doi: 10.1097/00004703-200310000-00003

Mayes, L. C., Grillon, C., Granger, R., and Schottenfeld, R. (1998). Regulation of arousal and attention in preschool children exposed to cocaine prenatally. *Ann. N. Y. Acad. Sci.* 846, 126–143. doi: 10.1111/j.1749-6632.1998.tb09731.x

McHugh, R. K., DeVito, E. E., Dodd, D., Carroll, K. M., Potter, J. S., Greenfield, S. F., et al. (2013). Gender differences in a clinical trial for prescription opioid dependence. *J. Subst. Abuse Treat.* 45, 38–43. doi: 10.1016/j.jsat.2012.12.007

Moe, V., and Slinning, K. (2001). Children prenatally exposed to substances: Gender-related differences in outcome from infancy to 3 years of age. *Infant Mental Health J.* 22, 334–350. doi: 10.1002/imhj.1005

Noland, J. S., Singer, L. T., Short, E. J., Minnes, S., Arendt, R. E., Kirchner, H. L., et al. (2005). Prenatal drug exposure and selective attention in preschoolers. *Neurotoxicol. Teratol.* 27, 429–438. doi: 10.1016/j.ntt.2005.02.001

Nordstrom Bailey, B., Sood, B. G., Sokol, R. J., Ager, J., Janisse, J., Hannigan, J. H., et al. (2005). Gender and alcohol moderate prenatal cocaine effects on teacher-report of child behavior. *Neurotoxicol. Teratol.* 27, 181–189. doi: 10.1016/j.ntt.2004.10.004

Nygaard, E., Moe, V., Slinning, K., and Walhovd, K. B. (2015). Longitudinal cognitive development of children born to mothers with opioid and polysubstance use. *Pediatr. Res.* 78, 330–335. doi: 10.1038/pr.2015.95

Nygaard, E., Slinning, K., Moe, V., and Walhovd, K. B. (2017). Cognitive function of youths born to mothers with opioid and poly-substance abuse problems during pregnancy. *Child Neuropsychol.* 23, 159–187. doi: 10.1080/09297049.2015.1092509

Ornoy, A., and Ergaz, Z. (2010). Alcohol abuse in pregnant women: effects on the fetus and newborn, mode of action and maternal treatment. *Int. J. Environ. Res. Public Health* 7, 364–379. doi: 10.3390/ijerph7020364

Ornoy, A., Segal, J., Bar-Hamburger, R., and Greenbaum, C. (2001). Developmental outcome of school-age children born to mothers with heroin dependency: importance of environmental factors. *Dev. Med. Child. Neurol.* 43, 668–675. doi: 10.1017/s0012162201001219

Park, B. Y., Misra, D. P., Moye, J., Miller, R. K., Croen, L., Fallin, M. D., et al. (2018). Placental gross shape differences in a high autism risk cohort and the general population. *PLoS One* 13:e0191276. doi: 10.1371/journal.pone.0191276

Parker, S. E., Mai, C. T., Canfield, M. A., Rickard, R., Wang, Y., Meyer, R. E., et al. (2010). Updated National Birth Prevalence estimates for selected birth defects in the United States, 2004-2006. *Birth Defects Res. A Clin. Mol. Teratol.* 88, 1008–1016. doi: 10.1002/bdra.20735

Patrick, S. W., Cooper, W. O., and Davis, M. M. (2017). Prescribing opioids and psychotropic drugs in pregnancy. *BMJ* 358:3616.

Paul, S. E., Hatoum, A. S., Fine, J. D., Johnson, E. C., Hansen, I., Karcher, N. R., et al. (2019). Prenatal cannabis exposure and childhood outcomes: Results from the ABCD study. *medRxiv* [Preprint]. doi: 10.1101/2019.12.18.19015164

Pickett, K. E., Wood, C., Adamson, J., D'Souza, L., and Wakschlag, L. S. (2008). Meaningful differences in maternal smoking behaviour during pregnancy: implications for infant behavioural vulnerability. *J. Epidemiol. Community Health* 62, 318–324. doi: 10.1136/jech.2006.058768

Piper, B. J., Acevedo, S. F., Kolchugina, G. K., Butler, R. W., Corbett, S. M., Honeycutt, E. B., et al. (2011). Abnormalities in parentally rated executive function in methamphetamine/polysubstance exposed children. *Pharmacol. Biochem. Behav.* 98, 432–439. doi: 10.1016/j.pbb.2011.02.013

Richardson, G. A., Day, N. L., and Taylor, P. M. (1989). The effect of prenatal alcohol, marijuana, and tobacco exposure on neonatal behavior. *Infant Behav. Dev.* 12, 199–209. doi: 10.1016/0163-6383(89)90006-4

Richardson, G. A., Ryan, C., Willford, J., Day, N. L., and Goldschmidt, L. (2002). Prenatal alcohol and marijuana exposure: effects on neuropsychological outcomes at 10 years. *Neurotoxicol. Teratol.* 24, 309–320. doi: 10.1016/s0892-0362(02)00193-9

Riley, E. P., and McGee, C. L. (2005). Fetal alcohol spectrum disorders: an overview with emphasis on changes in brain and behavior. *Exp. Biol. Med.* 230, 357–365. doi: 10.1177/15353702-0323006-03

Roescher, A. M., Timmer, A., Erwich, J. J. H., and Bos, A. F. (2014). Placental pathology, perinatal death, neonatal outcome, and neurological development: a systematic review. *PLoS One* 9:e89419. doi: 10.1371/journal.pone.0089419

Ross, E. J., Graham, D. L., Money, K. M., and Stanwood, G. D. (2015). Developmental consequences of fetal exposure to drugs: what we know and what we still must learn. *Neuropsychopharmacology* 40, 61–87. doi: 10.1038/npp.2014.147

Rydell, M., Cnattingius, S., Granath, F., Magnusson, C., and Galanti, M. R. (2012). Prenatal exposure to tobacco and future nicotine dependence: population-based cohort study. *Br. J. Psychiatry* 200, 202–209. doi: 10.1192/bjp.bp.111.100123

SAMHSA (2011). *Results from the 2010 National Survey on Drug Use and Health: Summary of National Findings. U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES, Substance Abuse and Mental Health Services Administration, Center for Behavioral Health Statistics and Quality [Preprint]*. Available online at: https://www.samhsa.gov/data/sites/default/files/NSDUHNationalFindingsResults2010-web/2k10ResultsRev/NSDUHresultsRev2010.pdf (accessed March 8, 2020).

SAMHSA (2018). *Key Substance Use and Mental Health Indicators in the United States: Results from the 2017 National Survey on Drug Use and Health. U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES, Substance Abuse and Mental Health Services Administration, Center for Behavioral Health Statistics and Quality [Preprint]*. Available online at: http://www.samhsa.gov/data/sites/default/files/cbhsq-reports/NSDUHFFR2017/NSDUHFFR2017.pdf (accessed March 8, 2020).

Scheyer, A. F., Melis, M., Trezza, V., and Manzoni, O. J. (2019). Consequences of perinatal cannabis exposure. *Trends Neurosci.* 42, 871–884. doi: 10.1016/j.tins.2019.08.010

Schneider, M. L., Moore, C. F., and Adkins, M. M. (2011). The effects of prenatal alcohol exposure on behavior: rodent and primate studies. *Neuropsychol. Rev.* 21, 186–203. doi: 10.1007/s11065-011-9168-8

Seedat, S., Scott, K. M., Angermeyer, M. C., Berglund, P., Bromet, E. J., Brugha, T. S., et al. (2009). Cross-national associations between gender and mental disorders in the World Health Organization World Mental Health Surveys. *Arch. Gen. Psychiatry* 66, 785–795. doi: 10.1001/archgenpsychiatry.2009.36

Shah, R., Diaz, S. D., Arria, A., LaGasse, L. L., Derauf, C., Newman, E., et al. (2012). Prenatal methamphetamine exposure and short-term maternal and infant medical outcomes. *Am. J. Perinatol.* 29, 391–400. doi: 10.1055/s-0032-1304818

Singer, L. T., Minnes, S., Short, E., Arendt, R., Farkas, K., Lewis, B., et al. (2004). Cognitive outcomes of preschool children with prenatal cocaine exposure. *JAMA* 291, 2448–2456.

Smith, A. M., Fried, P. A., Hogan, M. J., and Cameron, I. (2004). Effects of prenatal marijuana on response inhibition: an fMRI study of young adults. *Neurotoxicol. Teratol.* 26, 533–542. doi: 10.1016/j.ntt.2004.04.004

Smith, A. M., Fried, P. A., Hogan, M. J., and Cameron, I. (2006). Effects of prenatal marijuana on visuospatial working memory: an fMRI study in young adults. *Neurotoxicol. Teratol.* 28, 286–295. doi: 10.1016/j.ntt.2005.12.008

Sood, B. G., Nordstrom Bailey, B., Covington, C., Sokol, R. J., Ager, J., Janisse, J., et al. (2005). Gender and alcohol moderate caregiver reported child behavior after prenatal cocaine. *Neurotoxicol. Teratol.* 27, 191–201. doi: 10.1016/j.ntt.2004.10.005

Sourander, A., Sucksdorff, M., Chudal, R., Surcel, H.-M., Hinkka-Yli-Salomäki, S., Gyllenberg, D., et al. (2019). Prenatal cotinine levels and ADHD among offspring. *Pediatrics* 143:e20183144. doi: 10.1542/peds.2018-3144

Steingrimsson, S., Carlsen, H. K., Sigfusson, S., and Magnusson, A. (2012). The changing gender gap in substance use disorder: a total population-based study of psychiatric in-patients. *Addiction* 107, 1957–1962. doi: 10.1111/j.1360-0443.2012.03954.x

Suffet, F., and Brotman, R. (1984). A comprehensive care program for pregnant addicts: obstetrical, neonatal, and child development outcomes. *Int. J. Addict.* 19, 199–219. doi: 10.3109/10826088409057176

Thanh, N. X., Jonsson, E., Salmon, A., and Sebastianski, M. (2014). Incidence and prevalence of fetal alcohol spectrum disorder by sex and age group in Alberta. Canada. *J. Popul. Ther. Clin. Pharmacol.* 21, 395–404.

Treit, S., Lebel, C., Baugh, L., Rasmussen, C., Andrew, G., and Beaulieu, C. (2013). Longitudinal MRI reveals altered trajectory of brain development during childhood and adolescence in fetal alcohol spectrum disorders. *J. Neurosci.* 33, 10098–10109. doi: 10.1523/JNEUROSCI.5004-12.2013

Wakschlag, L. S., and Hans, S. L. (2002). Maternal smoking during pregnancy and conduct problems in high-risk youth: a developmental framework. *Dev. Psychopathol.* 14, 351–369. doi: 10.1017/s0954579402002092

Wakschlag, L. S., Lahey, B. B., Loeber, R., Green, S. M., Gordon, R. A., and Leventhal, B. L. (1997). Maternal smoking during pregnancy and the risk of conduct disorder in boys. *Arch. Gen. Psychiatry* 54, 670–676. doi: 10.1001/archpsyc.1997.01830190098010

Walsh, K., McCormack, C. A., Webster, R., Pinto, A., Lee, S., Feng, T., et al. (2019). Maternal prenatal stress phenotypes associate with fetal neurodevelopment and birth outcomes. *Proc. Natl. Acad. Sci. U.S.A.* 116, 23996–24005. doi: 10.1073/pnas.1905890116

Wilcox, J. A., and Yates, W. R. (1993). Gender and psychiatric comorbidity in substance-abusing individuals. *Am. J. Addict.* 2, 202–206. doi: 10.3109/10550499309113939

Willford, J. A., Richardson, G. A., Leech, S. L., and Day, N. L. (2004). Verbal and visuospatial learning and memory function in children with moderate prenatal alcohol exposure. *Alcohol. Clin. Exp. Res.* 28, 497–507. doi: 10.1097/01.alc.0000117868.97486.2d

Willoughby, M., Greenberg, M., Blair, C., and Stifter, C. (2007). Neurobehavioral consequences of prenatal exposure to smoking at 6 to 8 months of age. *Infancy* 12, 273–301. doi: 10.1111/j.1532-7078.2007.tb00244.x

Zabaneh, R., Smith, L. M., LaGasse, L. L., Derauf, C., Newman, E., Shah, R., et al. (2012). The effects of prenatal methamphetamine exposure on childhood growth patterns from birth to 3 years of age. *Am. J. of Perinatal.* 29, 203–210. doi: 10.1055/s-0031-1285094

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Environmental Enrichment During Adolescence Mitigates Cognitive Deficits and Alcohol Vulnerability due to Continuous and Intermittent Perinatal Alcohol Exposure in Adult Rats

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Perinatal alcohol exposure affects ontogenic neurodevelopment, causing physical and functional long-term abnormalities with limited treatment options. This study investigated long-term consequences of continuous and intermittent maternal alcohol drinking on behavioral readouts of cognitive function and alcohol vulnerability in the offspring. The effects of environmental enrichment (EE) during adolescence were also evaluated. Female rats underwent continuous alcohol drinking (CAD)—or intermittent alcohol drinking paradigm (IAD), along pregestation, gestation, and lactation periods—equivalent to the whole gestational period in humans. Male offspring were reared in standard conditions or EE until adulthood and were then assessed for declarative memory in the novel object recognition test; spatial learning, cognitive flexibility, and reference memory in the Morris water maze (MWM); alcohol consumption and relapse by a two-bottle choice paradigm. Our data show that perinatal CAD decreased locomotor activity, exploratory behavior, and declarative memory with respect to controls, whereas perinatal IAD displayed impaired declarative memory and spatial learning and memory. Moreover, both perinatal alcohol-exposed offspring showed higher vulnerability to alcohol consummatory behavior than controls, albeit perinatal IAD rats showed a greater alcohol consumption and relapse behavior with respect to perinatal-CAD progeny. EE ameliorated declarative memory in perinatal CAD, while it mitigated spatial learning and reference memory impairment in perinatal-IAD progeny. In addition, EE decreased vulnerability to alcohol in both control and perinatal alcohol-exposed rats. Maternal alcohol consumption produces drinking pattern-related

long-term consequences on cognition and vulnerability to alcohol in the offspring. However, increased positive environmental stimuli during adolescence may curtail the detrimental effects of developmental alcohol exposure.

Keywords: alcohol, perinatal binge alcohol drinking, perinatal continuous alcohol drinking, declarative memory, spatial memory, alcohol vulnerability, environmental enrichment

INTRODUCTION

Perinatal exposure to alcohol can affect *in utero* neurodevelopment, causing both physical and functional long-term alterations (Dejong et al., 2019). Despite pre-conceptional alcohol cessation is recommended, alcohol drinking during pregnancy is prevalent worldwide, especially in Europe (Popova et al., 2017). One of the best predictors of alcohol use throughout the perinatal period is the pattern of alcohol use before pregnancy; indeed, women who report binge or heavy drinking prior to pregnancy likely maintain it during pregnancy and throughout lactation (Davidson et al., 1981; Ethen et al., 2009; Mallard et al., 2013; Anderson et al., 2014; Kitsantas et al., 2014), increasing the risk for growth deficits, facial dysmorphology, and behavioral and neurocognitive abnormalities in the progeny (Viljoen et al., 2005; May et al., 2007; Urban et al., 2008). Aside from the more severe fetal alcohol syndrome (FAS), "fetal alcohol spectrum disorders" (FASD) have been recently characterized as a broad range of deficits observed in the child when exposed to alcohol at any time prenatally (Dejong et al., 2019). Those alterations involve memory, attention, affective and social behavior, abnormal responses to stress and natural rewards (American Psychiatric Association, 2013), and susceptibility to drug and alcohol abuse later in life (Baer et al., 2003; Alati et al., 2006; Glantz and Chambers, 2006).

While the consequences related to heavy prenatal alcohol exposure are generally acknowledged, the assessment of the neurobehavioral alterations potentially produces by low-to-moderate alcohol exposure in humans displays mixed results (Kelly et al., 2013; Flak et al., 2014; Kilburn et al., 2015). This may be due to a number of methodological issues—most of the studies focus on physical malformations—and confounding variables, such as the unreliable self-reports about the degree of alcohol exposure (number of drinks per week rather than amount at one session) and the underestimation of subtle neurobehavioral deficits which may appear later in life (Conover and Jones, 2012).

Preclinical models of maternal alcohol drinking can enhance our understanding of the adverse outcomes secondary to developmental alcohol exposure. Indeed, fetal alcohol exposure in humans can be modeled by perinatal alcohol exposure in rats, since the full gestational period in rodents is equivalent to the first and second trimesters in humans, while the first 10 postnatal days in rats correspond to the third trimester in humans (Patten et al., 2014). Besides, high levels of alcohol consumption can be induced in Sardinian alcohol-preferring and Wistar female rats by manipulating the schedule of alcohol access (Loi et al., 2014; Brancato et al., 2016). First developed and characterized in male rats (Wise, 1973; Simms et al., 2008), the intermittent access procedure in the two-bottle choice paradigm, consisting of cycles of drinking and abstinence, leads to a rapid increase in voluntary alcohol consumption, in comparison with continuous access to alcohol (Carnicella et al., 2014). Rats exposed to this procedure consume the most abundant amount of their daily total intake within the first hour of availability of the alcohol bottle, reaching intoxicating blood alcohol levels in a short period of time (about mg/dl after the first 30 min–1 h, Simms et al., 2008; Carnicella et al., 2009; Loi et al., 2014). This procedure models a voluntary binge-like drinking pattern (Crabbe et al., 2011; Sprow and Thiele, 2012; Sabino et al., 2013; Carnicella et al., 2014; Spear, 2018; Jeanblanc et al., 2019) and, as such, may represent a valuable tool to model drinking trajectories during pregnancy and lactation. Interestingly, when female rats are exposed to a long-term binge-like intermittent alcohol drinking (IAD) paradigm, they display a significant decrease in alcohol consumption during pregnancy and resume excessive alcohol consumption during the lactation period (Brancato et al., 2016).

Thus, in the present study, we aimed at investigating whether the binge-like IAD paradigm, resulting in higher and irregular peaks of blood alcohol levels in the dams, could lead to distinct long-term consequences on cognition and vulnerability to alcohol abuse in the offspring, with respect to continuous alcohol drinking (CAD), which produces steady lower peaks of blood alcohol levels, even in the face of overall high levels of exposure. On the other hand, even the exposure to low to moderate blood alcohol concentrations can cause significant neuronal damage, when it occurs during the neurodevelopmental window such as throughout gestation (Patten et al., 2014). Therefore, it follows that according to time, dosage, and duration of perinatal alcohol exposure, different developmental alterations thus, may occur.

The long-term cognitive effects of perinatal alcohol exposure, either continuous or intermittent, were assessed in the adult offspring, through a multidimensional behavioral battery, including declarative memory in the novel object recognition test, spatial learning, cognitive flexibility, and reference memory in the Morris water maze (MWM). Vulnerability to excessive alcohol drinking, in terms of rate of voluntary alcohol consumption and relapse behavior after a period of forced abstinence, was assessed using a two-bottle "alcohol vs. water" choice drinking paradigm.

It is worth noting that treatment strategies to prevent or mitigate perinatal alcohol-related deficits are currently very limited (Murawski et al., 2015). In this regard, growing evidence supports a beneficial role of the exposure to positive stimuli during sensitive time windows of brain development. Indeed, the environmental-enrichment (EE), experimental paradigm consisting of housing conditions that include novelty, social

interaction and exercise, enhances sensory, cognitive, and motor stimulation, which, in turn, translates into increased neuroplasticity in brain regions critical for emotional regulation, cognitive functions and reward sensitivity (Bayat et al., 2015; Crofton et al., 2015; Morera-Herreras et al., 2019). However, conflicting evidence is reported when the effect of EE was evaluated toward motivational effects of drugs of abuse, including alcohol (Nithianantharajah and Hannan, 2006; Solinas et al., 2009; Pautassi et al., 2017; Rae et al., 2018). Thus, while it is critical to identify maternal alcohol consumption as a primary target to prevent fetal consequences, we investigated whether EE during adolescence could prevent or mitigate the effects of perinatal alcohol exposure on behavioral readouts of cognitive function and alcohol vulnerability.

MATERIALS AND METHODS

Animals, Perinatal Alcohol Exposure, and Rearing Conditions

The methods used for perinatal alcohol exposure and breeding have been previously reported in detail (Brancato et al., 2018).

Briefly, adult female Wistar rats (200–220 g, Envigo, Italy) were housed individually in standard rat cages (40 × 60 cm, 20 cm in height), with *ad libitum* access to water and food, in a temperature- (22 ± 2°C) and humidity- (55 ± 5%) controlled room, on a 12-h light/dark cycle (08:00–20:00).

Rats were gently handled for 3 min per day for a week before the experimental procedures, when they were randomly assigned to one of the three experimental groups, according to the two-bottle choice self-administration paradigm: water drinking controls (CTRL), CAD, and IAD. Female rats underwent the self-administration procedure during pre-gestation (12 weeks), gestation (3 weeks), and post-gestation (3 weeks) periods, accordingly to the respective home-cage two-bottle "alcohol vs. water"-choice-drinking paradigm.

Indeed, CTRL rats were given two bottles of tap water. CAD rats were given a 24-h free choice between one bottle of alcohol (20% v/v) and one of tap water, 7 days per week; IAD rats were given 24-h alcohol (20% v/v) access during 3 days per week, i.e., on Monday, Wednesday, and Friday, while they received two bottles of tap water on the intervening days.

Plastic bottles (120 ml; metal cap 0.8-mm-diameter hole, Tecniplast, Italy) were filled every day with 100 ml of 20% alcohol (daily prepared from alcohol 96° (Carlo Erba Reagents, Italy) diluted with tap water) and presented at lights-off in an alternative left–right position in order, to avoid side preference. Rats were weighed daily, and alcohol and water intake was measured 1 h after lights-off and the day after, immediately before lights-off, by weighing the bottles. Possible fluid spillage was monitored by using multiple bottles filled with water and alcohol 20%, allocated in empty cages interspersed in the racks (Loi et al., 2014).

At the end of the 12-week two-bottle choice drinking paradigm, each female rat was housed with a single breeder. The day when pregnancy was confirmed by vaginal smear (Cannizzaro et al., 2008; Plescia et al., 2014b), designed as gestational day 1 (GD1), eight female rats were randomly selected from each experimental group (n = 12), housed in standard maternity cages, filled with wood shavings. Dams were inspected twice daily for delivery until the day of parturition, considered as postnatal day 0 (PND 0); dams and litters were kept in a nursery (24 ± 2°C) and not separated until weaning, in order to model the human condition and avoid confounding factors (Subramanian, 1992; Wilson et al., 1996; Santangeli et al., 2016). Mean alcohol consumption at 1 h and 24 h by CAD and IAD rat dams during pre-conception period, gestation, and lactation was recorded and reported as g/kg ± SEM. After weaning, two male rats from each litter of the three drinking groups were randomly assigned to either the standard (SE) or enriched (EE) rearing environment, so that the experimental groups of rat offspring were perinatal water-exposed controls (p-CTRL SE, n = 8); perinatal continuous alcohol-exposed rats (p-CAD SE, n = 8); perinatal intermittent alcohol-exposed rats (p-IAD SE, n = 8); perinatal water-exposed controls + EE (p-CTRL EE, n = 8); perinatal continuous alcohol-exposed rats + EE (p-CAD EE, n = 8); and perinatal intermittent alcohol-exposed rats + EE (p-IAD EE, n = 8). In detail, from PND 21 onward, the rats reared in SE conditions were housed in pair in standard rat cages and left undisturbed by the experimenters except for weekly cage change, whereas the EE rats were group-housed (8/cage) in large cages (60 × 45 × 76 cm) with pet toys, pots, hideouts, ropes, running wheel, ladder, tunnel and plastic boxes, etc., which were relocated or changed daily to create novelty (Griva et al., 2017).

Experiments were approved by the Committee for the Protection and Use of Animals of the University of Palermo, in accordance with the current Italian legislation on animal experimentation (D.L. 26/2014) and the European directive (2010/63/EU) on care and use of laboratory animals. Every effort was made to minimize the number of animals used and their sorrow.

Behavioral Procedures

The offspring were tested for behavioral reactivity in the open-field test at PND 55, for declarative memory in the novel object recognition test at PND 56–58, and for spatial learning, memory, and cognitive flexibility in MWM from PND 60 to PND 65. Afterward, they were assessed for alcohol vulnerability, in terms of rate of voluntary alcohol consumption in the induction and relapse-like phases of the two-bottle choice drinking paradigm, from PND 66 to PND 143.

The experiments were carried out in a sound-isolated room between 9:00 and 14:00. On the test days, rats were acclimatized to the testing room for 60 min before the experimental session. Rats' performance was recorded and monitored in an adjacent room. The equipment was thoroughly cleaned in between each test, to avoid that rats' behavior was affected by the detection of other rats' scent.

Open-Field Test

Behavioral reactivity in a novel environment was tested in the open-field test. The open-field arena is a Plexiglas square box (44 × 44 × 44 cm where locomotor activity

and explorative behavior were measured) by employing an automatic video-tracking system (AnyMaze, Ugo Basile, Italy), in a mean light- (100 lx) illuminated chamber. Each experimental session lasted 5 min (Cannizzaro et al., 2016). The video-tracking system produces a quali-quantitative mapping of the motor pattern, measuring total distance traveled (TDT, m), as a measure of locomotor activity in a novel environment.

Novel Object Recognition Test

Declarative learning and memory were tested in the novel object recognition test, as previously described (Brancato et al., 2020). On day 1, a 5-min habituation session was performed at 10.00 a.m., in order to let the animals freely explore the arena (44 × 44 × 44 cm) in a dim light-illuminated room. Twenty-four hours after the habituation session, rats underwent a 5-min training session when they were presented with two identical, nontoxic objects (i.e., two red metal cans) which were placed against a wall in the open-field arena. To prevent coercion to explore the objects, rats were released against the center of the opposite wall with its back to the objects. The time spent on exploring each object was recorded by using the AnyMaze video-tracking system (Stoelting Europe); a 2-cm^2 area surrounding the objects was defined such that nose entries were recorded as time exploring the object. After the training session, animals were placed in their home cage for the retention interval. Then, animals were returned to the arena for the test session, 24 h after the training session. During the 5-min test session, the arena was equipped with two objects, one was identical to the one presented in the training session (i.e., familiar); the other was a novel object (a yellow hard plastic cup/ a green hard plastic pepper). Objects were randomized and counterbalanced across animals. Objects and arena were thoroughly cleaned at the end of each experimental session. Time spent on exploring familiar and novel objects was recorded during both training and test sessions. The recognition index (RI%), i.e., the percentage of time spent on investigating the novel object, out of the total object investigation time [RI % = Time novel object /(Time novel object + Time familiar object)%], is a measure of novel object recognition and the main index of recognition memory. If RI% is higher than 50%, it indicates that the rat spent more time investigating the novel object, thus recalling the memory of the familiar one.

Morris Water Maze

Spatial learning, cognitive flexibility, and reference memory were assessed in the MWM, by employing place learning, new place learning, and probe tasks (Cacace et al., 2011, 2012; Plescia et al., 2015) as described in detail below.

Apparatus

The MWM apparatus consisted of a circular, light-blue swimming pool with a diameter of 160 cm, and walls 70 cm high. It was filled with tap water to a depth of 50 cm. The water temperature was carefully maintained at 23 ± 2°C, and no agent was added to make the water opaque. The pool was divided into four quadrants of equal size by two imaginary diagonal lines running through the center, designated NW, NE, SW, and SE. A removable transparent escape platform (10 cm × 10 cm) was positioned in the middle of the quadrant, with the center 30 cm away from the wall and 1.5 cm below the water level, and not visible to the swimming rat. The pool was placed in an experimental room, decorated with several extra-maze cues (e.g., bookshelves and posters), and not modified throughout the entire experimental period. The experimental room was illuminated by a white light (60 W). The paths taken by the animals in the pool were monitored by a video camera mounted in the ceiling and recorded by the automatic video-tracking system (ANY MAZE, Ugo Basile, Italy).

Experimental Design

Place Learning (Days 1–3)

The Place learning task was employed to assess spatial learning and consisted of training the rats to escape from the water and reach the hidden platform placed in the SE zone, where it was maintained throughout the experimental session. The rat was introduced into the pool facing the wall of each quadrant, in the following order of starting points: NE, SW, NW, SE. Each rat underwent four trials a day, along 3 days, and was allowed to swim until the escape on the platform for a maximum of 90 s; escape latency was recorded as a measure of spatial learning and memory and reported as mean value of the four trials performed on each day of the experiment.

If the escape platform was reached, the rat was allowed to remain 15 s on it to reinforce the information on the visual–spatial cues in the environment. If the rat did not find the escape platform within 90 s, the experimenter guided gently the rat to the platform and allowed it to stay on it for 15 s. During the 5-min intertrial interval, rats were placed into their home cages and warmed under a heating lamp.

New Place Learning (Days 4–5)

The new place learning task was aimed at assessing rats' cognitive flexibility. On the first day of task, the position of the escape platform was moved to the opposite quadrant (NW) compared to the place learning session. In this task, the rat was required to learn the new location of the platform during four trials, and escape latency was recorded as a measure of new spatial information acquisition, i.e., reversal learning. On the second day, the position of the platform was maintained in the same quadrant as in the first day of the new place learning task. The escape latency was recorded as a measure of acquisition and retrieval of the spatial information necessary to reach the platform location. Starting points, trial duration, inter-trial interval, reinforcement time on the platform, and any other experimental condition were the same as in the previous days.

Probe Test (Day 6)

Twenty-four hours after the last place learning session, rats were returned to the water maze for the probe test, aiming at assessing reference memory at the end of learning. The hidden platform was removed from the pool, and rats were allowed to swim freely for 90 s. The amount of time spent in the quadrant where the platform was previously located (target quadrant) was used as an index of the rat's spatial reference memory.

Two-Bottle "Alcohol vs. Water" Choice Drinking Paradigm

The offspring underwent the two-bottle "alcohol vs. water"-choice drinking paradigm (modified from Cacace et al., 2011) and were given 24-h free choice between one bottle of alcohol (10% v/v) and one of tap water, 7 days per week, for 8 weeks (induction period), followed by a 2-week relapse period, after 7 days of alcohol deprivation. 10% alcohol was daily prepared by diluting alcohol 96° (Carlo Erba Reagents, Italy) with tap water.

Plastic bottles (120 ml; metal cap 0.8 mm diameter hole, Tecniplast, Italy) were filled with 100 ml solution every day and presented at lights-off in an alternative left–right position, to avoid side preference. Alcohol and water intake were measured by weighing the bottles. Possible fluid spillage was monitored by using multiple bottles filled with water and 10% alcohol, positioned in empty cages interspersed in the cage racks (Loi et al., 2014). Rats' body weight was daily monitored, and rats' consummatory behavior was measured, in terms of g/kg of alcohol consumed along the drinking paradigm.

Statistical Analysis

Statistical analysis was performed using Prism 8, GraphPad Software, LLC, and IBM Statistical Package for the Social Sciences (SPSS) Statistics software (IBM, Armonk, NY, USA). Data were assessed for variance and normality by employing the Brown–Forsythe test and D'Agostino–Pearson omnibus K2 test, respectively, and for sphericity, by the Mauchly test. When data showed equal variance and normal distribution, the analysis included two-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test to assess simple effects of the two different perinatal alcohol exposures, and repeated-measure ANOVA using the generalized linear model, with Bonferroni correction for pairwise comparisons. When data did not show normal distribution or sphericity, log-transformation and Geisser–Greenhouse correction were employed. Data are reported as mean ± SEM. Statistical significance was set at $p < 0.05$.

RESULTS

Perinatal Alcohol Exposure and Developmental Data

Alcohol intake of CAD and IAD dams is reported in **Table 1**. Alcohol consumption did not affect maternal weight gain, litter size or pup birth weight, compared to controls.

TABLE 1 | Mean alcohol consumption (g/kg) of continuous alcohol drinking (CAD) rats and intermittent alcohol drinking (IAD) rats at pre-conception, gestation, and lactation time.

	CAD		IAD	
Period	1 h	24 h	1 h	24 h
Pre-conception	0.8 ± 0.2	3.5 ± 0.1	3.4 ± 0.2	8.1 ± 0.3
Gestation	2.1 ± 0.2	3.4 ± 0.4	2.6 ± 0.3	5.4 ± 0.6
Lactation	3.1 ± 0.3	5.6 ± 0.6	3.6 ± 0.4	8.5 ± 0.4

Data refer to mean ± SEM of n = 8 female rats along the alcohol drinking paradigm.

EE Prevents Alcohol-Induced Alteration in Behavioral Reactivity in p-CAD Offspring

Two-way ANOVA on log-transformed TDT data, including perinatal alcohol exposure and rearing conditions as statistical factors, highlights a significant main effect of perinatal alcohol exposure ($F_{(2,42)} = 0.6.412$, $p = 0.01742$). The Tukey multiple-comparison test indicates that p-CAD SE offspring showed a significant decrease in locomotor activity with respect to p-CTRL SE rats ($q = 5.184$, $df = 42$, $p = 0.0019$) and p-IAD SE rats ($q = 4.752$, $df = 42$, $p = 0.0047$). No significant difference was observed among EE offspring (**Figure 1A**).

EE Rescues Recognition Memory in p-CAD Offspring

The results of the two-way ANOVA on the total time spent on the exploration of the two identical objects during the sample phase reveal a significant main effect of perinatal alcohol exposure ($F_{(2,42)} = 11.21$, $p = 0.0001$) and EE ($F_{(1,42)} = 9.658$, $p = 0.0034$). Tukey's *post hoc* test shows that p-CAD SE offspring spent significantly less time exploring the objects than p-CTRL SE rats ($q = 4.378$, $df = 42$, $p = 0.0382$) and p-IAD SE rats ($q = 4.797$, $df = 42$, $p = 0.0178$). EE rats showed increased total exploration than SE offspring, with no significant pattern influence (**Figure 1B**).

Data analysis from familiar- and novel-object exploration during the test session included perinatal alcohol exposure and environmental rearing conditions as the between-subject factors and object as the within-subject factor. The results indicate a significant main effect of object ($F_{(1,42)} = 62.673$, $p < 0.001$), perinatal alcohol exposure ($F_{(2,42)} = 8.501$, $p < 0.001$), and rearing environment ($F_{(1,42)} = 13.489$, $p < 0.001$) and a significant interaction between perinatal alcohol exposure and rearing environment ($F_{(2,42)} = 4.796$, $p = 0.013$), object and perinatal alcohol exposure ($F_{(2,42)}$ 13.506, $p < 0.001$), and object, perinatal alcohol exposure, and rearing environment ($F_{(2,42)} = 14.367$, $p < 0.001$). Pairwise comparisons with Bonferroni correction show that both p-CAD SE and p-IAD SE rats displayed a significant decrease in the exploration of the novel object, when compared to p-CTRL SE offspring ($p < 0.001$; $p < 0.001$; **Figure 1C**). In addition, while p-CTRL EE rats showed decreased exploration of the novel object, with respect to p-CTRL SE offspring ($p < 0.001$), p-CAD EE rats increased the exploration of the novel object with respect to their SE counterparts ($p < 0.001$; **Figure 1C**).

When RI% values from the test session were analyzed, two-way ANOVA, considering perinatal alcohol exposure and EE as statistical factors, showed a significant main effect of perinatal alcohol exposure ($F_{(2,42)} = 6.812$, $p = 0.0027$) and EE ($F_{(1,42)} = 4.577$, $p = 0.0383$) and a significant interaction ($F_{(2,42)} = 6.348$, $p = 0.0039$). In detail, Tukey's *post hoc* test indicates a significant decrease in RI% of p-CAD SE- ($q = 6.188$, $df = 42$, $p = 0.0010$) and p-IAD SE rats ($q = 4.835$, $df = 42$, $p = 0.0165$), with respect to p-CTRL SE rats. EE rescued the RI% deficit in p-CAD rats ($q = 5.581$, $df = 42$, $p = 0.0038$), whereas no significant difference was observed between SE and EE p-IAD progeny ($q = 1.121$, $df = 42$, $p = 0.9673$; **Figure 1D**).

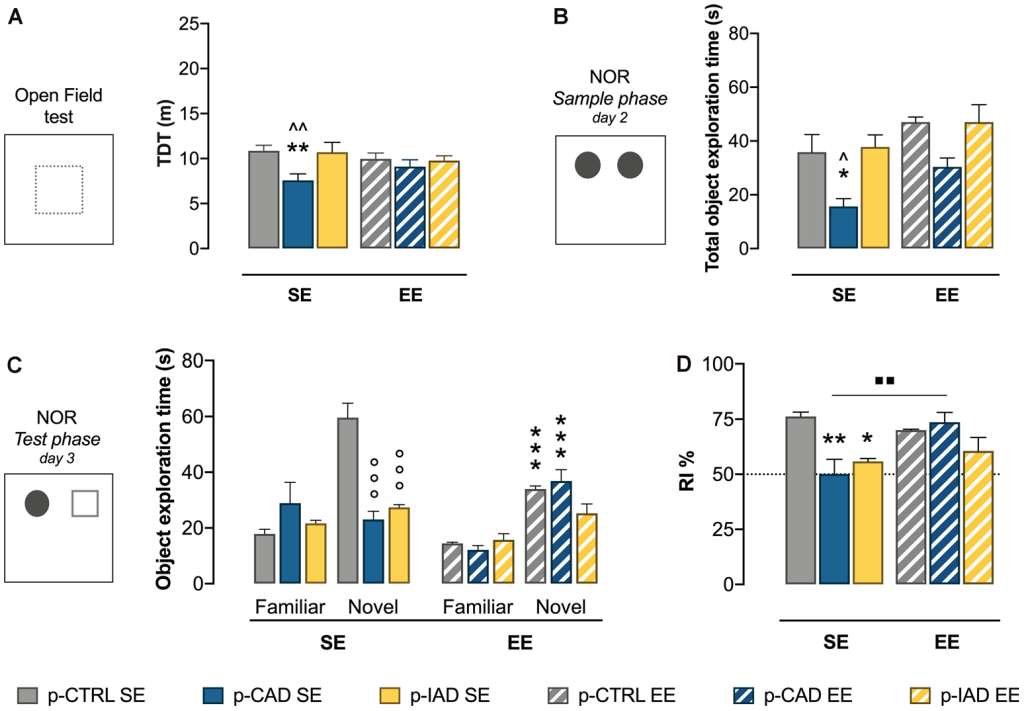

FIGURE 1 | Effects of perinatal alcohol exposure and rearing conditions on locomotor activity and declarative memory. **(A)** In the open-field test, p-CAD SE offspring showed a significant decrease in locomotor activity (**$p < 0.01$ vs. p-CTRL SE, ^^$p < 0.01$ vs. p-IAD SE). **(B)** In the sample phase of the NOR test, p-CAD SE progeny showed a significant decrease in total object exploration (*$p < 0.05$ vs. p-CTRL SE; ^$p < 0.05$ vs. p-IAD SE). **(C)** During the test phase of the NOR test, p-CAD SE and p-IAD SE rats displayed decreased exploration of the novel object, which was increased by environmental enrichment (EE) only in p-CTRL and p-CAD rats (°°°$p > 0.001$ vs. novel–p-CTRL SE; ***$p > 0.001$ vs. respective SE groups). **(D)** p-CAD SE and p-IAD SE rats showed decreased object discrimination in terms of recognition index. EE during adolescence was able to ameliorate the declarative memory performance in p-CAD offspring (*$p < 0.05$; **$p < 0.01$ vs. p-CTRL SE, ■■$p < 0.01$ vs. p-CAD SE). Each bar represents the mean ± SEM of $n = 8$ rats. p-CTRL, perinatal control; p-CAD, perinatal continuous alcohol drinking; p-IAD, perinatal intermittent alcohol drinking; SE, standard rearing environment; EE, enriched rearing environment. TDT, total distance travelled; NOR, novel object recognition.

EE Mitigates Spatial Learning and Memory Deficits in p-IAD Offspring

Spatial Learning in the Place Learning Task

Data analysis performed on escape latency during the place learning task, when the offspring were trained to find the hidden platform over 3 days, considered perinatal alcohol exposure and rearing environment as the between-subject factors, and days as the repeated-measure factor. The results indicate a significant main effect of days ($F_{(2,84)} = 80.256$, $p < 0.0001$) and rearing environment ($F_{(1,42)} = 5.636$, $p = 0.022$) and a significant interaction between days and rearing environment ($F_{(2,84)} = 12.319$, $p < 0.001$), perinatal alcohol exposure and rearing environment ($F_{(2,42)} = 5.048$, $p = 0.011$), and among day, perinatal alcohol exposure, and rearing environment ($F_{(4,84)} = 4.54$, $p = 0.002$). Pairwise comparisons with Bonferroni correction show that p-IAD SE rats displayed increased escape latency with respect to p-CTRL SE ($p = 0.003$) and p-CAD SE ($p = 0.004$) offspring on day 1; in addition, p-CAD EE rats showed a significant decrease in escape latency with respect to p-CAD SE ($p = 0.005$) on day 3, whereas p-IAD EE offspring displayed a significantly decreased latency with respect to p-IAD SE rats ($p < 0.001$) on day 1 (**Figures 2A–D**).

Cognitive Flexibility in the New Place Learning Task

Statistical analysis on escape latency during the new place learning task, when the platform was moved to the NW quadrant, included perinatal alcohol exposure and rearing environment as the between-subject factors and days as the repeated-measure factor. The results reveal a significant main effect of days ($F_{(1,42)} = 14.541$, $p < 0.001$); perinatal alcohol exposure, rearing environment and their interactions displayed no significant effect (**Figures 2A–D**).

Spatial Reference Memory in the Probe Task

Data analysis performed on time spent in each of the MWM quadrants during the probe task included perinatal alcohol exposure and rearing environment as the between-subject factors and quadrant as the within-subject factor. The results show a significant main effect of quadrant ($F_{(2.250,94.499)} = 85.652$, $p < 0.001$) and a significant interaction between quadrant and perinatal alcohol exposure ($F_{(4.5,94.499)} = 3.889$, $p = 0.004$), quadrant and rearing environment ($F_{(2.25,94.499)} = 3.051$, $p = 0.046$), and perinatal alcohol exposure and rearing environment ($F_{(2,42)} = 4.667$, $p = 0.015$). Pairwise comparisons with Bonferroni correction indicate that p-IAD SE offspring spent significantly less time in the NW quadrant ($p = 0.003$), and longer time in the NE quadrant ($p = 0.042$) than p-CTRL

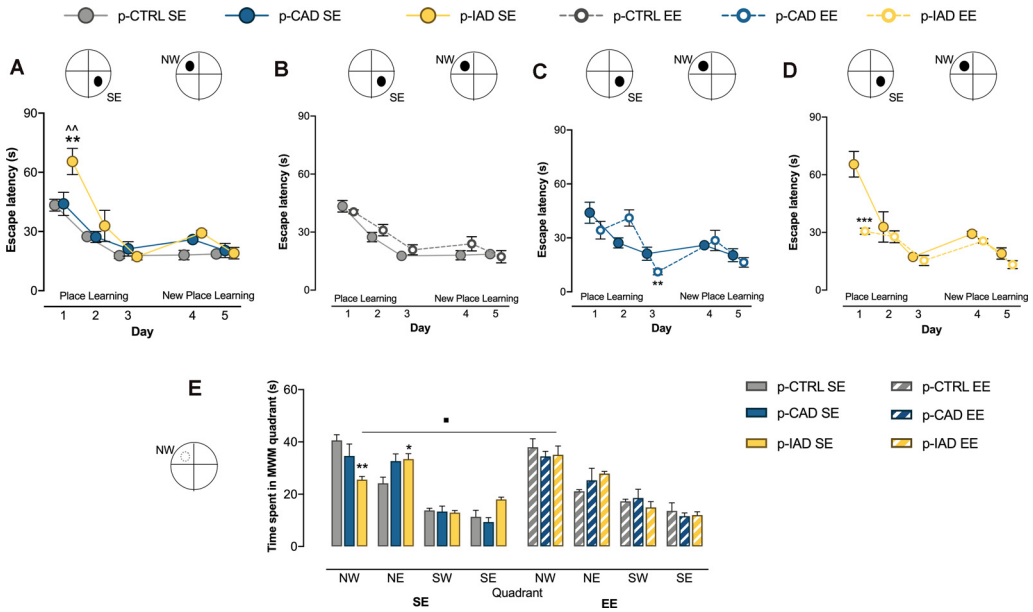

FIGURE 2 | Effects of perinatal alcohol exposure and rearing conditions on spatial learning, cognitive flexibility, and reference memory. **(A)** p-IAD SE offspring showed a significant impairment in spatial learning (**$p > 0.01$ vs. p-CTRL SE; ^^$p < 0.01$ vs. p-CAD SE). **(B)** p-CTRL rats exposed to EE during adolescence did not differ from their SE counterparts. EE ameliorated the spatial learning performance in **(C)** p-CAD and **(D)** p-IAD offspring (**$p < 0.01$; ***$p < 0.001$ vs. respective SE counterparts). In addition, **(E)** p-IAD SE rats showed a reference memory deficit in the probe task, which was rescued by EE (*$p < 0.05$; **$p < 0.01$ vs. p-CTRL SE; ■$p < 0.05$ vs. p-IAD SE). Each dot and each bar represent the mean ± SEM of $n = 8$ rats. p-CTRL, perinatal control; p-CAD, perinatal continuous alcohol drinking; p-IAD, perinatal intermittent alcohol drinking; SE, standard rearing environment; EE, enriched rearing environment. MWM, Morris water maze; NW, nord—west quadrant; NE, nord—east quadrant; SW, sud—west quadrant; SE, sud—east quadrant.

SE rats. On the other hand, p-IAD EE rats spent increased time in the NW quadrant with respect to p-IAD SE rats ($p = 0.028$; **Figure 2E**).

EE in Adolescence Blunts Long-Time Alcohol Vulnerability in p-CAD and p-IAD Offspring

Induction Period

Data analysis performed on mean alcohol intake along the 8 weeks of the two-bottle choice paradigm included perinatal alcohol exposure and rearing environment as the between-subject factors and weeks as the repeated-measure factor. The results show a significant main effect of weeks ($F_{(2.37,99.521)} = 14.091$, $p < 0.001$), rearing environment ($F_{(1,42)} = 18.554$, $p < 0.001$), and perinatal alcohol exposure ($F_{(2,42)} = 13.807$, $p < 0.001$) and a significant interaction between perinatal alcohol exposure and rearing environment ($F_{(2,42)} = 10.225$, $p < 0.001$), weeks and perinatal alcohol exposure ($F_{(4.739,99.521)} = 6.668$, $p < 0.001$), weeks and rearing environment ($F_{(2.37,99.521)} = 9.576$, $p < 0.0001$), and among weeks, perinatal alcohol exposure, and rearing environment ($F_{(4.739,99.521)} = 7.559$, $p < 0.001$). Pairwise comparisons with Bonferroni correction indicate that p-CAD SE offspring displayed decreased alcohol intake on week 1 ($p = 0.012$) and increased alcohol consumption on week 6 ($p < 0.001$), 7 ($p = 0.003$) and 8 ($p = 0.011$) with respect to p-CTRL SE rats. Moreover, p-IAD SE rats showed increased alcohol consumption on weeks 2 ($p < 0.001$), 3 ($p < 0.001$), 5 ($p < 0.001$), 6 ($p < 0.001$) 7 ($p < 0.001$), and 8 ($p < 0.001$) with respect to p-CTRL SE rats, along with increased alcohol intake on weeks 1 ($p < 0.001$), 2 ($p < 0.001$), and 8 ($p < 0.001$) with respect to p-CAD SE rats (**Figure 3A**).

EE modified alcohol consumption in p-CTRL rats, with a significant increase on week 2 ($p = 0.001$) and a decrease on week 4 ($p = 0.001$), when compared with the SE rearing condition (**Figure 3B**). Similarly, the enriched rearing environment increased alcohol intake in p-CAD offspring on week 2 ($p = 0.007$) and significantly decreased it afterward, on weeks 3 ($p = 0.005$), 4 ($p = 0.016$), 6 ($p < 0.001$), and 7 ($p = 0.001$) with respect to p-CAD SE rats (**Figure 3C**).

On the other hand, EE decreased alcohol intake in p-IAD progeny on weeks 1 ($p = 0.021$), 5 ($p = 0.010$), 6 ($p < 0.001$), 7 ($p < 0.001$), and 8 ($p < 0.001$) when compared with p-IAD SE counterparts (**Figure 3D**).

Relapse Period

The analysis of data from mean alcohol intake over the 2 weeks of the relapse paradigm included perinatal alcohol exposure and rearing environment as the between-subject factors and weeks as the repeated-measure factor. The results indicate a significant main effect of weeks ($F_{(1,42)} = 76.57$, $p < 0.001$), rearing environment ($F_{(1,42)} = 17.112$, $p < 0.001$), and perinatal alcohol exposure ($F_{(2,42)} = 12.215$, $p < 0.001$) and a significant

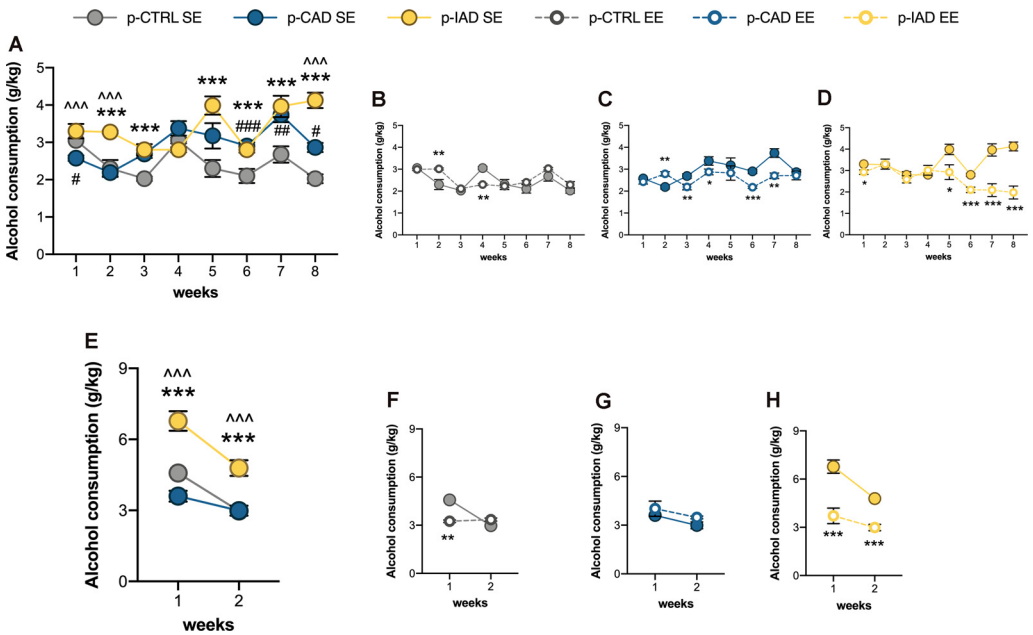

FIGURE 3 | Effects of perinatal alcohol exposure and rearing conditions on alcohol consummatory behavior. **(A)** Apart from the first week of the two-bottle choice paradigm, both p-CAD- and p-IAD SE offspring showed increased alcohol intake with respect to p-CTRL SE rats; moreover, p-IAD SE rats displayed increased alcohol consumption with respect to p-CAD SE offspring ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ p-CAD SE vs. p-CTRL SE; $^{***}p < 0.001$ p-IAD SE vs. p-CTRL SE; $^{\wedge\wedge\wedge}p < 0.001$ p-IAD SE vs. p-CAD SE). EE exposure during adolescence. **(B)** Altered alcohol consumption in p-CTRL rats in **(C)** p-CAD rats and **(D)** decreased alcohol consumption p-IAD offspring ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. respective SE). **(E)** When offspring were assessed for alcohol deprivation effect during the relapse-like weeks, p-IAD-SE offspring showed higher alcohol intake with respect to p-CTRL SE and p-CAD SE rats; $^{***}p < 0.001$ vs. p-CTRL SE; $^{\wedge\wedge\wedge}$ vs. p-CAD SE). EE exposure during adolescence **(F)** decreased alcohol deprivation effect in p-CTRL offspring, **(G)** did not alter alcohol-related behavior in p-CAD rats, and **(H)** prevented alcohol deprivation effect in p-IAD offspring ($^{**}p < 0.01$; $^{***}p < 0.001$ vs. respective SE). Each dot represents the mean ± SEM of $n = 8$ rats. p-CTRL, perinatal control; p-CAD, perinatal continuous alcohol drinking; p-IAD, perinatal intermittent alcohol drinking; SE, standard rearing environment; EE, enriched rearing environment.

interaction between perinatal alcohol exposure and rearing environment ($F_{(2,42)} = 18.4513$, $p < 0.001$), weeks and perinatal alcohol exposure ($F_{(2,42)} = 5.371$, $p = 0.008$), weeks and rearing environment ($F_{(1,42)} = 24.567$, $p < 0.001$), and among weeks, perinatal alcohol exposure, and rearing environment ($F_{(2,42)} = 5.648$, $p = 0.007$). Pairwise comparisons with Bonferroni correction indicate that p-IAD SE offspring showed higher alcohol intake than p-CTRL SE and p-CAD SE rats on week 1 ($p < 0.001$; $p < 0.001$) and week 2 ($p < 0.001$; $p < 0.001$; **Figure 3E**).

EE significantly decreased alcohol consumption in p-CTRL rats on week 1 ($p = 0.008$) with respect to their SE counterparts (**Figure 3F**) whereas no difference was observed between p-CAD SE and EE offspring (**Figure 3G**). On the other hand, p-IAD EE offspring displayed significantly lower alcohol intake on both week 1 and 2 ($p < 0.001$; $p < 0.001$), when compared to p-IAD SE rats (**Figure 3H**).

DISCUSSION

The present study aimed at evaluating the long-term consequences of maternal continuous- and binge-like intermittent alcohol drinking, from pre-conceptional time to lactation, on the adult male offspring's cognitive behavioral readouts, including behavioral reactivity, declarative and spatial learning and memory, and alcohol vulnerability.

Moreover, we also exposed the offspring to an enriched rearing environment during adolescence, in order to evaluate whether sensorimotor stimulation and social interaction at that age could result in a rescue strategy able to mitigate or prevent perinatal alcohol-induced adverse effects.

In human studies, records on maternal blood alcohol levels are generally not available; however, estimates suggest that blood alcohol levels of over 200 mg/dl may be responsible for the severe FAS phenotype, while lower levels (80 mg/dl) may produce milder forms of FASD (Maier and West, 2001). In addition, high peaks of blood alcohol concentrations, rather than steady levels, as a result of both dose and pattern of alcohol exposure (i.e., binge-drinking vs. daily) during the brain developmental time-window, are associated with increased neurotoxicity (Ieraci and Herrera, 2007; Parnell et al., 2009).

In our experimental conditions, female rats were trained to voluntarily consume 20% alcohol in the drinking water prior to pregnancy (Patten et al., 2014) and consumed relevant amounts throughout pregnancy and lactation. In particular, CAD dams showed a mean daily alcohol consumption of 3.4 ± 0.4 g/kg during pregnancy, and 5.6 ± 0.6 g/kg during lactation, resulting in a daily low-to-moderate perinatal exposure for p-CAD offspring (Marquardt and Brigman, 2016). On the

other hand, IAD dams engaged in a binge-like drinking pattern by every-other-day intermittent alcohol access to 20% alcohol, which resulted in mean alcohol consumption of 5.4 ± 0.6 g/kg during pregnancy and 8.5 ± 0.4 during the postpartum period. In particular, the IAD dams' mean alcohol intake during the lactation period, measured after the first hour following alcohol presentation, is suggestive of an intermittent exposure to intoxicating blood alcohol concentrations for p-IAD offspring (>80 mg/dl, Loi et al., 2014). This evidence is particularly relevant since the intermittent pattern of exposure causes high peaks of blood alcohol concentrations during lactation in the rat dams, and this time window corresponds to the third developmental trimester in humans (Patten et al., 2014).

Our first data on the behavioral sequelae of perinatal alcohol exposure show pattern-related consequences on behavioral reactivity. In detail, p-CAD rats displayed a decrease in locomotor activity in the novel environment of the open field, with respect to p-CTRL and p-IAD rats, whereas p-IAD rats showed no alteration in total distance traveled, in comparison to p-CTRL. These results confirm early findings from this laboratory showing that perinatal long-term continuous exposure to alcohol decreased behavioral reactivity in the adolescent male offspring (Brancato et al., 2018). While moderate- and heavy-alcohol exposure during early-middle pregnancy either increased behavioral reactivity (Riley et al., 1993; Abel and Berman, 1994; Thomas et al., 2004; Kim et al., 2013) or did not affect locomotion (Dursun et al., 2006; Hellemans et al., 2010; Brady et al., 2012), the exposure to moderate alcohol concentration throughout gestation and the early postnatal period decreased locomotion in mice (Kleiber et al., 2011). Our data further suggest that the developmental effects of alcohol on locomotion and behavioral reactivity are affected not only by the dose and timing but also by the pattern of alcohol exposure.

In accordance with our first evidence, the analysis of the behavior in the sample phase of the novel object recognition test revealed that p-CAD rats showed a significant decrease in the exploration of the two identical objects when compared to both p-CTRL and p-IAD, while p-IAD rats explored the objects at the same extent as the control group did. Similarly, a decrease in exploration during the sample phase of the novel object recognition test was reported in Sardinian alcohol-preferring rats exposed to 3% alcohol from day 15 of gestation to day 7 after parturition (Tattoli et al., 2001) and interpreted as an altered responsiveness to situations requiring adaptation to novel environmental stimuli (Colombo et al., 1995).

On the other hand, the analysis of the test phase of the novel object recognition test suggested a deficit in declarative explicit memory, since both prenatal alcohol-exposed groups displayed a significant decrease in discrimination of the novel object: indeed, they spent the same time in the exploration of the familiar and the novel object, and this led to a significant decrease in the recognition index with respect to control offspring.

Preclinical findings have provided inconsistent evidence on the consequences of perinatal alcohol exposure on object discrimination, and the discrepancies are likely dependent on different times of exposure and blood concentrations.

In detail, alcohol exposure (dose range from 4.00 to 5.25 g/kg) during the developmental equivalent of the second and/or third trimesters in humans did not impair recognition memory in rats (Jablonski et al., 2013; Tattoli et al., 2001; MacIlvane et al., 2016), but when Sprague–Dawley female rats were given continuous unlimited access to alcohol from pre-conceptional period until weaning time, the offspring failed to discriminate the novel object in the object recognition test (Dandekar et al., 2019; Sanchez et al., 2019). Interestingly, maternal binge-like drinking during both gestation and lactation was reported to decrease recognition memory along with the expression of brain-derived neurotrophic factor (BDNF), the main neurotrophin involved in learning and memory (Montagud-Romero et al., 2019). Notably, even low levels of alcohol administered by oral gavage from GD 10–16 are able to exert a disruption in object recognition in the NOR, but not in object-place location; accordingly, this was associated with alterations in BDNF expression in the perirhinal cortex—a brain area which plays a crucial role in object discrimination-—rather than in the hippocampus, which is more involved in place location (Plescia et al., 2014c; Terasaki and Schwarz, 2017).

In our experimental conditions, perinatal alcohol exposure induced memory deficits regardless of the drinking pattern, suggesting an impairment in the regional circuitries underpinning declarative memory and that deserve attention from a translational point of view. However, it should not be overlooked that the recognition memory performance displayed by p-CAD rats could have been affected by their low behavioral reactivity and object exploration, rather than a pure deficit in declarative memory formation.

On the other hand, when offspring were tested for spatial learning and memory in the MWM, spatial navigation of p-CAD rats did not differ from control offspring, with no difference in spatial learning, in terms of latency to find the hidden platform over the 3 days of place learning, and in cognitive flexibility, along the 2 days of new place learning task. The evidence of no impairment in spatial reference memory supports the presence of regular spatial learning abilities in p-CAD progeny, since they searched the platform in the target quadrant during the probe trial, 24 h after the last new place learning session.

Taken together, our data are in line with previous reports demonstrating that chronic prenatal exposure to low-to-moderate doses of alcohol is sufficient to induce decreased behavioral reactivity in the open field (Kleiber et al., 2011) and declarative memory deficits in the novel object recognition test (Dandekar et al., 2019), together with a detrimental impact on the neuroimmune function of the perirhinal cortex (Terasaki and Schwarz, 2017). On the other hand, repeated low-dose prenatal alcohol exposure does not produce detrimental effects on pyramidal cells within the dorsal hippocampus or does not impair spatial learning and memory performance in the MWM (Cullen et al., 2014).

When interpreting these data, the stressful nature of the MWM task needs to be taken into account. The training in the MWM task increases the neuroendocrine stress response in rats, inducing high serum corticosterone concentrations that may affect the cognitive response in accordance with the positive role of glucocorticoids on learning and memory consolidation

(Aguilar-Valles et al., 2005). It is reported that chronic alcohol exposure during pregnancy induces a reduction in ACTH basal levels while corticosterone secretion is not modified; besides, the exposure to some stressors induces an increase in corticosterone and CRH secretion, more than in controls (Lu et al., 2018). It is therefore reasonable to hypothesize that the stressful contingency of the MWM may boost the coping strategies of p-CAD progeny, by an "*ad hoc*" compensatory response of the HPA axis that makes p-CAD performance as "fair" as controls' (Franks et al., 2020).

On the contrary, p-IAD offspring displayed a spatial learning impairment, in terms of increased latency to reach the hidden platform in the place learning test on day 1, compared to control offspring. In addition, p-IAD rats showed reference memory deficits, since they spent less time in the target quadrant in the probe trial, compared to p-CTRL groups. The impairment in spatial learning and reference memory in the water maze tasks is suggestive of hippocampal dysfunction likely resulting from the perinatal exposure to the binge-like alcohol drinking in the intermittent access. Indeed, despite some inconsistencies about alcohol-induced developmental effects on BDNF expression in the rat hippocampus (Feng et al., 2005; Ceccanti et al., 2012), binge-like alcohol exposure from one-to-third trimester-equivalent causes significant deficits in hippocampal and cortical neuroplasticity, resulting in alterations in dendritic arborization, adult, neurogenesis, neuroimmune activation in the hippocampus, and spatial learning impairment (Blanchard et al., 1987; Christie et al., 2005; An and Zhang, 2013; Harvey et al., 2019). Thus, due to the strong correlation between BDNF, hippocampal function and HPA axis reactivity, it is possible to interpret the current data on the basis of a pattern-specific effect exerted by the perinatal exposure to IAD on the stress axis response.

To our knowledge, the study by Wieczorek et al. (2015) is the only one focusing on HPA axis and behavioral sequelae of prenatal binge-like alcohol exposure. According to their findings, male mice exposed to an early binge-like dose of alcohol on gestational day 7 showed no difference in corticosterone levels with respect to controls, whereas they observed a blunted ACTH response to an acute stressor. Thus, it is reasonable to hypothesize that the exposure to intermittent alcohol drinking, which produces the cycling repetition of intoxications and withdrawals (Plescia et al., 2014a), when "brain growth spurt" and synaptogenesis occur (Patten et al., 2014), may impair spatial learning and memory in the MWM through a pronounced alteration in the neurodevelopmental programming of corticosteroid signaling in the hippocampus (Conrad et al., 1999).

The complex relationship between stress and alcohol is bidirectional, and the dysregulation of the stress response is a well-known risk factor for alcohol abuse vulnerability (Lee et al., 2018). The present data extend our previous findings and show that perinatal alcohol exposure is able to produce an alcohol-prone phenotype in adult rats in a pattern-related fashion. While p-CAD offspring increased their alcohol intake with respect to controls in the long-term, p-IAD rats showed a higher vulnerability to alcohol consummatory behavior starting from the first weeks of the two-bottle choice paradigm. In addition, while p-CAD-rats did not show higher consumption of alcohol after a week of deprivation with respect to control offspring, p-IAD progeny displayed a pronounced relapse behavior, when compared to both p-CAD and p-CTRL progenies. The alcohol deprivation effect is a reliable proxy of increased motivation to seek and consume alcohol, loss of control, and relapse (Spanagel and Hölter, 2000; Martin-Fardon and Weiss, 2013), and our data indicate that the perinatal exposure to a drinking pattern that promotes high peaks of blood alcohol level is discretely crucial in conferring a permanent vulnerability to alcohol abuse, whose occurrence can be detected since adolescence (Brancato et al., 2018).

This evidence supports clinical data showing that prenatally alcohol-exposed offspring display increased vulnerability to the rewarding properties of alcohol (Barbier et al., 2008) and risk for alcohol abuse and drug dependence later in life (Baer et al., 2003; Alati et al., 2006). This phenotype may result from morpho-functional alterations in the ventral tegmental area, such as decreased number of dopamine neurons and spontaneous action potentials, reduced size of their cell bodies, increased activated microglia (Shen et al., 1999; Aghaie et al., 2020), and persistent expression of immature excitatory synapses onto dopaminergic neurons (Wang et al., 2006). As far as the pattern-related behavioral abnormalities observed in this study concern, we could speculate that a dysregulation in the HPA axis may critically impact memory performance especially in the stressful setting of the MWM and may predispose to alcohol vulnerability (Brancato et al., 2014; Maniaci et al., 2015; Lee et al., 2018).

Notably, enriched rearing conditions ameliorated the behavioral performance of p-CAD rats in the novel object recognition test, likely remodeling p-CAD rats' behavioral reactivity, decreasing emotionality and restoring those perceptive and attentive skills that make them able to overcome the cognitive impairment resulting from the perinatal continuous alcohol exposure. Accordingly, previous evidence showed that EE in early adulthood can recover cognitive impairment due to alcohol exposure during adolescence (Rico-Barrio et al., 2019). On the other hand, in our experimental conditions, declarative memory performance of p-IAD EE rats was not different from their SE counterparts' one, suggesting that the abnormalities in declarative memory formation due to the perinatal intermittent exposure to alcohol are not rescued by the EE. Besides, the repeated exposure to environmental stimuli has been reported to decrease the incentive value of novelty (Cain et al., 2006; Garcia et al., 2017), suggesting that a lower interest in the novel object may explain its lower exploration by the EE offspring. Interestingly, our data show that EE mitigated the spatial learning and reference memory deficits induced by the perinatal intermittent alcohol paradigm. These findings are in agreement with previous reports, indicating that enriched environment attenuates hippocampal-dependent memory impairment induced by prenatal alcohol exposure, *via* an increase in hippocampal BDNF (Tipyasang et al., 2014; Di Liberto et al., 2017). The interpretation of the effects of EE upon alcohol vulnerability in the first weeks of the two-bottle-choice paradigm is not univocal since we observed

mixed effects in the control offspring. In this regard, EE has been reported to promote the formation of conditioned place preference to alcohol in adolescent mice, likely recruiting the oxytocin signaling (Pautassi et al., 2017; Rae et al., 2018). However, the postweaning exposure to EE substantially rescued the increased vulnerability induced by perinatal alcohol exposure in p-CAD and p-IAD offspring. Indeed, EE decreased alcohol consumption in p-CAD and p-IAD rats, with respect to standard housing, during the last weeks of the self-administration paradigm. Thus, the increase in alcohol consumption, as time goes by, is a hallmark feature of early stages of the addiction cycle and represents a substantial risk factor predicting the development of alcohol addiction (Crabbe et al., 2011).

In addition, our data show that the enriched rearing environment decreases the deprivation effect after a week of forced abstinence, in p-IAD offspring and in p-CTRL rats. These observations are consistent with previous reports showing that exposure to enriched environmental conditions mitigates VTA dopamine neurons' dysfunction due to perinatal alcohol exposure (Wang et al., 2018; Aghaie et al., 2020) and, overall, decreases the occurrence of an addictive-like phenotype (Galaj et al., 2020). Notably, the effect of the EE against the development of excessive alcohol intake seems to be protective when the exposure occurs during adolescence, while its protective role is limited when EE occurs during adulthood (Rodríguez-Ortega et al., 2018). To date, circumstantial evidence suggests that its protective effect against alcohol drinking is due to decreased CRH signaling in the amygdala and its downstream target (Sztainberg et al., 2010). Whether CRH abnormalities may be the *primum movens* for the occurrence of the dysfunctional phenotype consequent to perinatal alcohol exposure observed in this study, and at what extent alteration in maternal care can contribute to alcohol developmental effects, are interesting questions to address in further cross-fostering experiments. Moreover, studies including female offspring are needed to explore sex differences in the developmental effects of alcohol, and their underlying mechanisms. Overall, subsequent developmental periods, such as adolescence, provide a window of opportunity for inducing positive experience-based neuroplasticity in brain regions critical for emotional regulation, cognitive functions, and reward sensitivity, which allow curtailing the lifetime consequences of developmental alcohol exposure.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee for the Protection and Use of Animals of the University of Palermo.

AUTHOR CONTRIBUTIONS

AB: experimental procedures and data analysis and contribution to writing. VC and GL: experimental procedures and contribution to writing. CC: experimental design, data interpretation, and writing. All authors contributed to the article and approved the submitted version.

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REFERENCES

Abel, E. L., and Berman, R. F. (1994). Long-term behavioral effects of prenatal alcohol exposure in rats. *Neurotoxicol. Teratol.* 16, 467–470. doi: 10.1016/0892-0362(94)90124-4

Aghaie, C. I., Hausknecht, K. A., Wang, R., Dezfuli, P. H., Haj-Dahmane, S., Kane, C. J. M., et al. (2020). Prenatal ethanol exposure and postnatal environmental intervention alter dopaminergic neuron and microglia morphology in the ventral tegmental area during adulthood. *Alcohol. Clin. Exp. Res.* 44, 435–444. doi: 10.1111/acer.14275

Aguilar-Valles, A., Sánchez, E., de Gortari, P., Balderas, I., Ramírez-Amaya, V., Bermúdez-Rattoni, F., et al. (2005). Analysis of the stress response in rats trained in the water-maze: differential expression of corticotropin-releasing hormone, CRH-R1, glucocorticoid receptors and brain-derived neurotrophic factor in limbic regions. *Neuroendocrinology* 82, 306–319. doi: 10.1159/000093129

Alati, R., Al Mamun, A., Williams, G. M., O'Callaghan, M., Najman, J. M., and Bor, W. (2006). *In utero* alcohol exposure and prediction of alcohol disorders in early adulthood: a birth cohort study. *Arch. Gen. Psychiatry* 63, 1009–1016. doi: 10.1001/archpsyc.63.9.1009

American Psychiatric Association. (2013). *Diagnostic and Statistical Manual.* 5th Edn. Washington, DC: American Psychiatric Publishing, USA.

An, L., and Zhang, T. (2013). Spatial cognition and sexually dimorphic synaptic plasticity balance impairment in rats with chronic prenatal ethanol exposure. *Behav. Brain Res.* 256, 564–574. doi: 10.1016/j.bbr.2013.09.017

Anderson, A. E., Hure, A. J., Forder, P. M., Powers, J., Kay-Lambkin, F. J., and Loxton, D. J. (2014). Risky drinking patterns are being continued into pregnancy: a prospective cohort study. *PLoS One* 9:e86171. doi: 10.1371/journal.pone.0086171

Baer, J. S., Sampson, P. D., Barr, H. M., Connor, P. D., and Streissguth, A. P. (2003). A 21-year longitudinal analysis of the effects of prenatal alcohol exposure on young adult drinking. *Arch. Gen. Psychiatry* 60, 377–385. doi: 10.1001/archpsyc.60.4.377

Barbier, E., Pierrefiche, O., Vaudry, D., Vaudry, H., Daoust, M., and Naassila, M. (2008). Long-term alterations in vulnerability to addiction to drugs of abuse and in brain gene expression after early life ethanol exposure. *Neuropharmacology* 55, 1199–1211. doi: 10.1016/j.neuropharm.2008.07.030

Bayat, M., Sharifi, M. D., Haghani, M., and Shabani, M. (2015). Enriched environment improves synaptic plasticity and cognitive deficiency in chronic cerebral hypoperfused rats. *Brain Res. Bull.* 119, 34–40. doi: 10.1016/j.brainresbull.2015.10.001

Blanchard, B. A., Riley, E. P., and Hannigan, J. H. (1987). Deficits on a spatial navigation task following prenatal exposure to ethanol. *Neurotoxicol. Teratol.* 9, 253–258. doi: 10.1016/0892-0362(87)90010-9

Brady, M. L., Allan, A. M., and Caldwell, K. K. (2012). A limited access mouse model of prenatal alcohol exposure that produces long-lasting deficits in hippocampal-dependent learning and memory. *Alcohol. Clin. Exp. Res.* 36, 457–466. doi: 10.1111/j.1530-0277.2011.01644.x

Brancato, A., Plescia, F., Marino, R. A., Maniaci, G., Navarra, M., and Cannizzaro, C. (2014). Involvement of dopamine D2 receptors in addictive-like

behaviour for acetaldehyde. *PLoS One* 9:e99454. doi: 10.1371/journal.pone.0099454

Brancato, A., Castelli, V., Cavallaro, A., Lavanco, G., Plescia, F., and Cannizzaro, C. (2018). Pre-conceptional and peri-gestational maternal binge alcohol drinking produces inheritance of mood disturbances and alcohol vulnerability in the adolescent offspring. *Front. Psychiatry* 9:150. doi: 10.3389/fpsyt.2018.00150

Brancato, A., Castelli, V., Lavanco, G., Marino, R. A. M., and Cannizzaro, C. (2020). *In utero* Δ9-tetrahydrocannabinol exposure confers vulnerability towards cognitive impairments and alcohol drinking in the adolescent offspring: is there a role for neuropeptide Y? *J. Psychopharmacol.* 34, 663–679. doi: 10.1177/0269881120916135

Brancato, A., Plescia, F., Lavanco, G., Cavallaro, A., and Cannizzaro, C. (2016). Continuous and Intermittent alcohol free-choice from pre-gestational time to lactation: focus on drinking trajectories and maternal behavior. *Front. Behav. Neurosci.* 10:31. doi: 10.3389/fnbeh.2016.00031

Cacace, S., Plescia, F., La Barbera, M., and Cannizzaro, C. (2011). Evaluation of chronic alcohol self-administration by a 3-bottle choice paradigm in adult male rats. Effects on behavioural reactivity, spatial learning and reference memory. *Behav Brain Res.* 219, 213–220. doi: 10.1016/j.bbr.2011.01.004

Cacace, S., Plescia, F., Sardo, P., and Cannizzaro, C. (2012). Alcohol preference, behavioural reactivity and cognitive functioning in female rats exposed to a three-bottle choice paradigm. *Behav. Brain Res.* 234, 11–19. doi: 10.1016/j.bbr.2012.05.018

Cain, M. E., Green, T. A., and Bardo, M. T. (2006). Environmental enrichment decreases responding for visual novelty. *Behav. Processes.* 73, 360–366. doi: 10.1016/j.beproc.2006.08.007

Cannizzaro, C., Malta, G., Argo, A., Brancato, A., Roda, G., Casagni, E., et al. (2016). Behavioural and pharmacological characterization of a novel cannabinomimetic adamantane-derived indole, APICA and considerations on the possible misuse as a psychotropic spice abuse, in C57bl/6J mice. *Forensic Sci. Int.* 265, 6–12. doi: 10.1016/j.forsciint.2015.12.035

Cannizzaro, C., Plescia, F., Gagliano, M., Cannizzaro, G., Mantia, G., La Barbera, M., et al. (2008). Perinatal exposure to 5-metoxytryptamine, behavioural-stress reactivity and functional response of 5-HT1A receptors in the adolescent rat. *Behav. Brain Res.* 186, 98–106. doi: 10.1016/j.bbr.2007.07.036

Carnicella, S., Amamoto, R., and Ron, D. (2009). Excessive alcohol consumption is blocked by glial cell line-derived neurotrophic factor. *Alcohol* 43, 35–43. doi: 10.1016/j.alcohol.2008.12.001

Carnicella, S., Ron, D., and Barak, S. (2014). Intermittent ethanol access schedule in rats as a preclinical model of alcohol abuse. *Alcohol* 48, 243–252. doi: 10.1016/j.alcohol.2014.01.006

Ceccanti, M., Mancinelli, R., Tirassa, P., Laviola, G., Rossi, S., Romeo, M., et al. (2012). Early exposure to ethanol or red wine and long-lasting effects in aged mice. A study on nerve growth factor, brain-derived neurotrophic factor, hepatocyte growth factor and vascular endothelial growth factor. *Neurobiol. Aging* 33, 359–367. doi: 10.1016/j.neurobiolaging.2010.03.005

Christie, B. R., Swann, S. E., Fox, C. J., Froc, D., Lieblich, S. E., Redila, V., et al. (2005). Voluntary exercise rescues deficits in spatial memory and long-term potentiation in prenatal ethanol-exposed male rats. *Eur. J. Neurosci.* 21, 1719–1726. doi: 10.1111/j.1460-9568.2005.04004.x

Colombo, G., Agabio, R., Lobina, C., Reali, R., Zocchi, A., Fadda, F., et al. (1995). Sardinian alcohol-preferring rats: a genetic animal model of anxiety. *Physiol. Behav.* 57, 1181–1185. doi: 10.1016/0031-9384(94)00382-f

Conover, E. A., and Jones, K. L. (2012). Safety concerns regarding binge drinking in pregnancy: a review. *Birth Defects Res. A Clin. Mol. Teratol.* 94, 570–575. doi: 10.1002/bdra.23034

Conrad, C. D., Lupien, S. J., and McEwen, B. S. (1999). Support for a Bimodal Role for Type II Adrenal Steroid Receptors in Spatial Memory. *Neurobiol. Learn. Mem.* 72, 39–46. doi: 10.1006/nlme.1998.3898

Crabbe, J. C., Harris, R. A., and Koob, G. F. (2011). Preclinical studies of alcohol binge drinking. *Ann. N Y Acad. Sci.* 1216, 24–40. doi: 10.1111/j.1749-6632.2010.05895.x

Crofton, E. J., Zhang, Y., and Green, T. A. (2015). Inoculation stress hypothesis of environmental enrichment. *Neurosci. Biobehav. Rev.* 49, 19–31. doi: 10.1016/j.neubiorev.2014.11.017

Cullen, C. L., Burne, T. H. J., Lavidis, N. A., and Moritz, K. M. (2014). Low dose prenatal alcohol exposure does not impair spatial learning and memory in two tests in adult and aged rats. *PLoS One* 9:e101482. doi: 10.1371/journal.pone.0101482

Dandekar, M. P., Bharne, A. P., Borkar, P. D., Subhedar, N. K., and Kokare, D. M. (2019). Maternal ethanol exposure reshapes CART system in the rat brain: correlation with development of anxiety, depression and memory deficits. *Neuroscience* 406, 126–139. doi: 10.1016/j.neuroscience.2019.02.010

Davidson, S., Alden, L., and Davidson, P. (1981). Changes in alcohol consumption after childbirth. *J. Adv. Nurs.* 6, 195–198. doi: 10.1111/j.1365-2648.1981.tb03212.x

Dejong, K., Olyaei, A., and Lo, J. O. (2019). Alcohol use in pregnancy. *Clin. Obstet. Gynecol.* 62, 142–155. doi: 10.1097/GRF.0000000000000414

Di Liberto, V., Frinchi, M., Verdi, V., Vitale, A., Plescia, F., Cannizzaro, C., et al. (2017). Anxiolytic effects of muscarinic acetylcholine receptors agonist oxotremorine in chronically stressed rats and related changes in BDNF and FGF2 levels in the hippocampus and prefrontal cortex. *Psychopharmacology* 234, 559–573. doi: 10.1007/s00213-016-4498-0

Dursun, I., Jakubowska-Dogru, E., and Uzbay, T. (2006). Effects of prenatal exposure to alcohol on activity, anxiety, motor coordination and memory in young adult Wistar rats. *Pharmacol. Biochem. Behav.* 85, 345–355. doi: 10.1016/j.pbb.2006.09.001

Ethen, M., Ramadhani, T., Scheuerle, A., Canfield, M., Wyszynski, D., Druschel, C. M., et al. (2009). Alcohol consumption by women before and during pregnancy. *Matern Child Health J.* 13, 274–285. doi: 10.1007/s10995-008-0328-2

Feng, M. J., Yan, S. E., and Yan, Q. S. (2005). Effects of prenatal alcohol exposure on brain-derived neurotrophic factor and its receptor tyrosine kinase B in offspring. *Brain Res.* 1042, 125–132. doi: 10.1016/j.brainres.2005.02.017

Flak, A. L., Su, S., Bertrand, J., Denny, C. H., Kesmodel, U. S., and Cogswell, M. E. (2014). The association of mild, moderate and binge prenatal alcohol exposure and child neuropsychological outcomes: a meta-analysis. *Alcohol. Clin. Exp. Res.* 38, 214–226. doi: 10.1111/acer.12214

Franks, A. L., Berry, K. J., and DeFranco, D. B. (2020). Prenatal drug exposure and neurodevelopmental programming of glucocorticoid signalling. *J. Neuroendocrinol.* 32:e12786. doi: 10.1111/jne.12786

Galaj, E., Barrera, E. D., and Ranaldi, R. (2020). Therapeutic efficacy of environmental enrichment for substance use disorders. *Pharmacol. Biochem. Behav.* 188:172829. doi: 10.1016/j.pbb.2019.172829

Garcia, E. J., Haddon, T. N., Saucier, D. A., and Cain, M. E. (2017). Differential housing and novelty response: Protection and risk from locomotor sensitization. *Pharmacol. Biochem. Behav.* 154, 20–30. doi: 10.1016/j.pbb.2017.01.004

Glantz, M. D., and Chambers, J. C. (2006). Prenatal drug exposure effects on subsequent vulnerability to drug abuse. *Dev. Psychopathol.* 18, 893–922. doi: 10.1017/s0954579406060445

Griva, M., Lagoudaki, R., Touloumi, O., Nousiopoulou, E., Karalis, F., Georgiou, T., et al. (2017). Long-term effects of enriched environment following neonatal hypoxia-ischemia on behavior, BDNF and synaptophysin levels in rat hippocampus: effect of combined treatment with G-CSF. *Brain Res.* 1667, 55–67. doi: 10.1016/j.brainres.2017.05.004

Harvey, R. E., Berkowitz, L. E., Hamilton, D. A., and Clark, B. J. (2019). The effects of developmental alcohol exposure on the neurobiology of spatial processing. *Neurosci. Biobehav. Rev.* 107, 775–794. doi: 10.1016/j.neubiorev.2019.09.018

Hellemans, K. G., Verma, P., Yoon, E., Yu, W. K., Young, A. H., and Weinberg, J. (2010). Prenatal alcohol exposure and chronic mild stress differentially alter depressive- and anxiety-like behaviors in male and female offspring. *Alcohol. Clin. Exp. Res.* 34, 633–645. doi: 10.1111/j.1530-0277.2009.01132.x

Ieraci, A., and Herrera, D. G. (2007). Single alcohol exposure in early life damages hippocampal stem/progenitor cells and reduces adult neurogenesis. *Neurobiol. Dis.* 26, 597–605. doi: 10.1016/j.nbd.2007.02.011

Jablonski, S. A., Schreiber, W. B., Westbrook, S. R., Brennan, L. E., and Stanton, M. E. (2013). Determinants of novel object and location recognition during development. *Behav. Brain Res.* 256, 140–150. doi: 10.1016/j.bbr.2013.07.055

Jeanblanc, J., Rolland, B., Gierski, F., Martinetti, M. P., and Naassila, M. (2019). Animal models of binge drinking, current challenges to improve face validity. *Neurosci. Biobehav. Rev.* 106, 112–121. doi: 10.1016/j.neubiorev.2018.05.002

Kelly, Y., Iacovou, M., Quigley, M. A., Gray, R., Wolke, D., Kelly, J., et al. (2013). Light drinking versus abstinence in pregnancy—behavioural and cognitive

outcomes in 7-year-old children: a longitudinal cohort study. *BJOG* 120, 1340–1347. doi: 10.1111/1471-0528.12246

Kilburn, T. R., Eriksen, H. L., Underbjerg, M., Thorsen, P., Lykke Mortensen, E., Inge Landro, N., et al. (2015). Low to moderate average alcohol consumption and binge drinking in early pregnancy: effects on choice reaction time and information processing time in five-year-old children. *PLoS One* 10:e0138611. doi: 10.1371/journal.pone.0138611

Kim, P., Park, J. H., Choi, C. S., Choi, I., Joo, S. H., Kim, M. K., et al. (2013). Effects of ethanol exposure during early pregnancy in hyperactive, inattentive and impulsive behaviors and MeCP2 expression in rodent offspring. *Neurochem. Res.* 38, 620–631. doi: 10.1007/s11064-012-0960-5

Kitsantas, P., Gaffney, K. F., Wu, H., and Kastello, J. C. (2014). Determinants of alcohol cessation, reduction and no reduction during pregnancy. *Arch. Gynecol. Obstet.* 289, 771–779. doi: 10.1007/s00404-013-3056-9

Kleiber, M. L., Wright, E., and Singh, S. M. (2011). Maternal voluntary drinking in C57BL/6J mice: advancing a model for fetal alcohol spectrum disorders. *Behav. Brain Res.* 223, 376–387. doi: 10.1016/j.bbr.2011.05.005

Lee, R. S., Oswald, L. M., and Wand, G. S. (2018). Early life stress as a predictor of co-occurring alcohol use disorder and post-traumatic stress disorder. *Alcohol Res.* 39, 147–159.

Loi, B., Colombo, G., Maccioni, P., Carai, M. A., Franconi, F., and Gessa, G. L. (2014). High alcohol intake in female Sardinian alcohol-preferring rats. *Alcohol* 48, 345–351. doi: 10.1016/j.alcohol.2014.01.001

Lu, J., Jiao, Z., Yu, Y., Zhang, C., He, X., Li, Q., et al. (2018). Programming for increased expression of hippocampal GAD67 mediated the hypersensitivity of the hypothalamic-pituitary-adrenal axis in male offspring rats with prenatal ethanol exposure. *Cell Death Dis.* 9:659. doi: 10.1038/s41419-018-0663-1

MacIlvane, N. M., Pochiro, J. M., Hurwitz, N. R., Goodfellow, M. J., and Lindquist, D. H. (2016). Recognition memory is selectively impaired in adult rats exposed to binge-like doses of ethanol during early postnatal life. *Alcohol* 57, 55–63. doi: 10.1016/j.alcohol.2016.09.027

Maier, S. E., and West, J. R. (2001). Drinking patterns and alcohol-related birth defects. *Alcohol Res. Health* 25, 168–174. doi: 10.1016/S2214-109X(17)30021-9

Mallard, S. R., Connor, J. L., and Houghton, L. A. (2013). Maternal factors associated with heavy periconceptional alcohol intake and drinking following pregnancy recognition: a post-partum survey of new zealand women. *Drug Alcohol Rev.* 32, 389–397. doi: 10.1111/dar.12024

Maniaci, G., Picone, F., and Dimarco, T. (2015). Psychodiagnostic assessment of pathological gamblers: a focus on personality disorders, clinical syndromes and alexithymia. *Int. J. Ment Health Addiction* 13, 728–739. doi: 10.1007/s11469-015-9550-5

Marquardt, K., and Brigman, J. L. (2016). The impact of prenatal alcohol exposure on social, cognitive and affective behavioral domains: insights from rodent models. *Alcohol* 51, 1–15. doi: 10.1016/j.alcohol.2015.12.002

Martin-Fardon, R., and Weiss, F. (2013). Modeling relapse in animals. *Curr. Top. Behav. Neurosci.* 13, 403–432. doi: 10.1007/7854_2012_202

May, P. A., Gossage, J. P., Marais, A. S., Adnams, C. M., Hoyme, H. E., Jones, K. L., et al. (2007). The epidemiology of fetal alcohol syndrome and partial FAS in a South African community. *Drug Alcohol Depend.* 88, 259–271. doi: 10.1016/j.drugalcdep.2006.11.007

Montagud-Romero, S., Cantacorps, L., and Valverde, O. (2019). Histone deacetylases inhibitor trichostatin a reverses anxiety-like symptoms and memory impairments induced by maternal binge alcohol drinking in mice. *J. Psychopharmacol.* 33, 1573–1587. doi: 10.1177/0269881119857208

Morera-Herreras, T., Gioanni, Y., Perez, S., Vignoud, G., and Venance, L. (2019). Environmental enrichment shapes striatal spike-timing-dependent plasticity *in vivo*. *Sci. Rep.* 9:19451. doi: 10.1038/s41598-019-55842-z

Murawski, N. J., Moore, E. M., Thomas, J. D., and Riley, E. P. (2015). Advances in diagnosis and treatment of fetal alcohol spectrum disorders: from animal models to human studies. *Alcohol Res.* 37, 97–108.

Nithianantharajah, J., and Hannan, A. J. (2006). Enriched environments, experience-dependent plasticity and disorders of the nervous system. *Nat. Rev. Neurosci.* 7, 697–709. doi: 10.1038/nrn1970

Parnell, S. E., O'Leary-Moore, S. K., Godin, E. A., Dehart, D. B., Johnson, B. W., Allan Johnson, G., et al. (2009). Magnetic resonance microscopy defines ethanol-induced brain abnormalities in prenatal mice: effects of acute insult on gestational day 8. *Alcohol. Clin. Exp. Res.* 33, 1001–1011. doi: 10.1111/j.1530-0277.2009.00921.x

Patten, A. R., Fontaine, C. J., and Christie, B. R. (2014). A comparison of the different animal models of fetal alcohol spectrum disorders and their use in studying complex behaviors. *Front. Pediatr.* 2:93. doi: 10.3389/fped.2014.00093

Pautassi, R. M., Suárez, A. B., Hoffmann, L. B., Rueda, A. V., Rae, M., Marianno, P., et al. (2017). Effects of environmental enrichment upon ethanol-induced conditioned place preference and pre-frontal BDNF levels in adolescent and adult mice. *Sci. Rep.* 7:8574. doi: 10.1038/s41598-017-08795-0

Plescia, F., Brancato, A., Marino, R. A., Vita, C., Navarra, M., and Cannizzaro, C. (2014a). Effect of acetaldehyde intoxication and withdrawal on NPY expression: focus on endocannabinoidergic system involvement. *Front. Psychiatry* 5:138. doi: 10.3389/fpsyt.2014.00138

Plescia, F., Marino, R. A. M., Navarra, M., Gambino, G., Brancato, A., Sardo, P., et al. (2014b). Early handling effect on female rat spatial and non-spatial learning and memory. *Behav. Processes.* 103, 9–16. doi: 10.1016/j.beproc.2013.10.011

Plescia, F., Sardo, P., Rizzo, V., Cacace, S., Marino, R. A. M., Brancato, A., et al. (2014c). Pregnenolone sulphate enhances spatial orientation and object discrimination in adult male rats: evidence from a behavioural and electrophysiological study. *Behav. Brain Res.* 258, 193–201. doi: 10.1016/j.bbr.2013.10.026

Plescia, F., Brancato, A., Venniro, M., Maniaci, G., Cannizzaro, E., Sutera, F. M., et al. (2015). Acetaldehyde self-administration by a two-bottle choice paradigm: consequences on emotional reactivity, spatial learning and memory. *Alcohol* 49, 139–148. doi: 10.1016/j.alcohol.2015.01.002

Popova, S., Lange, S., Probst, C., Gmel, G., and Rehm, J. (2017). Estimation of national, regional and global prevalence of alcohol use during pregnancy and fetal alcohol syndrome: a systematic review and meta-analysis. *Lancet Glob. Health* 5, e290–e299. doi: 10.1016/S2214-109X(17)30021-9

Rae, M., Zanos, P., Georgiou, P., Chivers, P., Bailey, A., and Camarini, R. (2018). Environmental enrichment enhances conditioned place preference to ethanol *via* an oxytocinergic-dependent mechanism in male mice. *Neuropharmacology* 138, 267–274. doi: 10.1016/j.neuropharm.2018.06.013

Rico-Barrio, I., Peñasco, S., Puente, N., Ramos, A., Fontaine, C. J., Reguero, L., et al. (2019). Cognitive and neurobehavioral benefits of an enriched environment on young adult mice after chronic ethanol consumption during adolescence. *Addict Biol.* 24, 969–980. doi: 10.1111/adb.12667

Riley, E. P., Barron, S., Melcer, T., and Gonzalez, D. (1993). Alterations in activity following alcohol administration during the third trimester equivalent in P and NP rats. *Alcohol. Clin. Exp. Res.* 17, 1240–1246. doi: 10.1111/j.1530-0277.1993.tb05236.x

Rodríguez-Ortega, E., de la Fuente, L., de Amo, E., and Cubero, I. (2018). Environmental enrichment during adolescence acts as a protective and therapeutic tool for ethanol binge-drinking, anxiety-like, novelty seeking and compulsive-like behaviors in C57BL/6J mice during adulthood. *Front. Behav. Neurosci.* 12:177. doi: 10.3389/fnbeh.2018.00177

Sabino, V., Kwak, J., Rice, K. C., and Cottone, P. (2013). Pharmacological characterization of the 20% alcohol intermittent access model in Sardinian alcohol-preferring rats: a model of binge-like drinking. *Alcohol. Clin. Exp. Res.* 37, 635–643. doi: 10.1111/acer.12008

Sanchez, L. M., Goss, J., Wagner, J., Davies, S., Savage, D. D., Hamilton, D. A., et al. (2019). Moderate prenatal alcohol exposure impairs performance by adult male rats in an object-place paired-associate task. *Behav. Brain Res.* 360, 228–234. doi: 10.1016/j.bbr.2018.12.014

Santangeli, O., Lehtikuja, H., Palomäki, E., Wigren, H. K., Paunio, T., and Heiskanen, T. P. (2016). Sleep and behavior in cross-fostering rats: developmental and sex aspects. *Sleep* 39, 2211–2221. doi: 10.5665/sleep.6328

Shen, R. Y., Hannigan, J. H., and Kapatos, G. (1999). Prenatal ethanol reduces the activity of adult midbrain dopamine neurons. *Alcohol. Clin. Exp. Res.* 23, 1801–1807. doi: 10.1111/j.1530-0277.1999.tb04076.x

Simms, J. A., Steensland, P., Medina, B., Abernathy, K. E., Chandler, L. J., Wise, R., et al. (2008). Intermittent access to 20% ethanol induces high ethanol consumption in Long-Evans and Wistar rats. *Alcohol. Clin. Exp. Res.* 32, 1816–1823. doi: 10.1111/j.1530-0277.2008.00753.x

Solinas, M., Thiriet, N., El Rawas, R., Lardeux, V., and Jaber, M. (2009). Environmental enrichment during early stages of life reduces the behavioral, neurochemical and molecular effects of cocaine. *Neuropsychopharmacology* 34, 1102–1111. doi: 10.1038/npp.2008.51

Spanagel, R., and Hölter, S. M. (2000). Pharmacological validation of a new animal model of alcoholism. *J. Neural Transm.* 107, 669–680. doi: 10.1007/s007020070068

Spear, L. P. (2018). Effects of adolescent alcohol consumption on the brain and behaviour. *Nat. Rev. Neurosci.* 19, 197–214. doi: 10.1038/nrn.2018.10

Sprow, G. M., and Thiele, T. E. (2012). The neurobiology of binge-like ethanol drinking: evidence from rodent models. *Physiol. Behav.* 106, 325–331. doi: 10.1016/j.physbeh.2011.12.026

Subramanian, M. G. (1992). Lactation and prolactin release in foster dams suckling prenatally ethanol exposed pups. *Alcohol. Clin. Exp. Res.* 16, 891–894. doi: 10.1111/j.1530-0277.1992.tb01888.x

Sztainberg, Y., Kuperman, Y., Tsoory, M., Lebow, M., and Chen, A. (2010). The anxiolytic effect of environmental enrichment is mediated *via* amygdalar CRF receptor type 1. *Mol. Psychiatry* 15, 905–917. doi: 10.1038/mp.2009.151

Tattoli, M., Cagiano, R., Gaetani, S., Ghiglieri, V., Giustino, A., Mereu, G., et al. (2001). Neurofunctional effects of developmental alcohol exposure in alcohol-preferring and alcohol-nonpreferring rats. *Neuropsychopharmacology* 24, 691–705. doi: 10.1016/s0893-133x(00)00225-6

Terasaki, L. S., and Schwarz, J. M. (2017). Impact of prenatal and subsequent adult alcohol exposure on pro-inflammatory cytokine expression in brain regions necessary for simple recognition memory. *Brain Sci.* 7:125. doi: 10.3390/brainsci7100125

Thomas, J. D., Garrison, M., and O'Neill, T. M. (2004). Perinatal choline supplementation attenuates behavioral alterations associated with neonatal alcohol exposure in rats. *Neurotoxicol. Teratol.* 26, 35–45. doi: 10.1016/j.ntt.2003.10.002

Tipyasang, R., Kunwittaya, S., Mukda, S., Kotchabhakdi, N. J., and Kotchabhakdi, N. (2014). Enriched environment attenuates changes in water-maze performance and BDNF level caused by prenatal alcohol exposure. *EXCLI J.* 13, 536–547.

Urban, M., Chersich, M. F., Fourie, L. A., Chetty, C., Olivier, L., and Viljoen, D. (2008). Fetal alcohol syndrome among grade 1 schoolchildren in Northern Cape Province: prevalence and risk factors. *S. Afr. Med. J.* 98, 877–882.

Viljoen, D. L., Gossage, J. P., Brooke, L., Adnams, C. M., Jones, K. L., Robinson, L. K., et al. (2005). Fetal alcohol syndrome epidemiology in a South African community: a second study of a very high prevalence area. *J. Stud. Alcohol.* 66, 593–604. doi: 10.15288/jsa.2005.66.593

Wang, J., Haj-Dahmane, S., and Shen, R. Y. (2006). Effects of prenatal ethanol exposure on the excitability of ventral tegmental area dopamine neurons *in vitro*. *J. Pharmacol. Exp. Ther.* 319, 857–863. doi: 10.1124/jpet.106.109041

Wang, R., Hausknecht, K. A., Shen, Y. L., Haj-Dahmane, S., Vezina, P., and Shen, R. Y. (2018). Environmental enrichment reverses increased addiction risk caused by prenatal ethanol exposure. *Drug Alcohol Depend.* 191, 343–347. doi: 10.1016/j.drugalcdep.2018.07.013

Wieczorek, L., Fish, E. W., O'Leary-Moore, S. K., Parnell, S. E., and Sulik, K. K. (2015). Hypothalamic-pituitary-adrenal axis and behavioral dysfunction following early binge-like prenatal alcohol exposure in mice. *Alcohol* 49, 207–217. doi: 10.1016/j.alcohol.2015.01.005

Wilson, J. H., Kelly, S. J., and Wilson, M. A. (1996). Early postnatal alcohol exposure in rats: maternal behavior and estradiol levels. *Physiol. Behav.* 59, 287–293. doi: 10.1016/0031-9384(95)02094-2

Wise, R. A. (1973). Voluntary ethanol intake in rats following exposure to ethanol on various schedules. *Psychopharmacology* 29, 203–210. doi: 10.1007/bf00414034

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Hypothalamic Gene Expression and Postpartum Behavior in a Genetic Rat Model of Depression

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Postpartum depression is a complex illness that often occurs in genetically predisposed individuals. Closely related inbred rat strains are a great resource to identify novel causative genes and mechanisms underlying complex traits such as postpartum behavior. We report differences in these behaviors between the inbred depression model, Wistar Kyoto (WKY) More Immobile (WMI), and the isogenic control Wistar Kyoto Less Immobile (WLI) dams. WMI dams showed significantly lower litter survival rate and frequency of arched back and blanket nursing, but increased pup-directed licking, grooming, and retrieval during postpartum days (PPD) 1–10, compared to control WLIs. This increased pup-directed behavior and the frequency of self-directed behaviors segregated during selective breeding of the progenitor strain of WKY, which is also a depression model. These behaviors are manifested in the WMIs in contrast to those of WLIs. Furthermore, habitual differences in the self-directed behavior between light and dark cycles present in WLIs were missing in WMI dams. Hypothalamic transcript levels of the circadian rhythm-related gene Lysine Demethylase 5A (*Kdm5a*), period 2 (*Per2*), and the maternal behavior-related oxytocin receptor (*Oxtr*), vasopressin (*Avp*), and vasopressin receptor 1a (*Avpr1a*) were significantly greater in the post-weaning WMI dams at PPD 24 compared to those of WLIs, and also to those of WMI dams whose litter died before PPD 5. Expression correlation amongst genes differed in WLI and WMI dams and between the two time-points postpartum, suggesting genetic and litter-survival differences between these strains affect transcript levels. These data demonstrate that the genetically close, but behaviorally disparate WMI and WLI strains would be suitable for investigating the underlying genetic basis of postpartum behavior.

Keywords: Wistar Kyoto More Immobile, oxytocin receptor, vasopressin, vasopressin receptor 1a, lysine demethylase 5A, period 2

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INTRODUCTION

Maternal behavior has long-term effects on the brain development of offspring, and depressive disorders impair maternal behaviors. One of the largest risk factors for depressive episodes in the perinatal period is depression before pregnancy (Rich-Edwards et al., 2006; Grant et al., 2008; Topiwala et al., 2012; Perani and Slattery, 2014). While most animal models

of postpartum depression focus on mirroring the group of women who are experiencing depression for the first time in their life during postpartum (Perani and Slattery, 2014; Putnam et al., 2017; Eid et al., 2019), the present study employs a genetic model of depression-like behavior and its isogenic control strain to begin to investigate characteristics of these dams during postpartum, modeling the risk factor of depression before pregnancy.

The genetic rat model of depression-like behaviors, the Wistar Kyoto (WKY) More Immobile (WMI) rat strain, was bi-directionally selectively bred from the parental WKY strain. The WKY rat strain is a well-established model for depression as its behavior mirrors symptoms of human major depression and anxiety, including despair-like behavior, excessive anxiety, learned helplessness, disturbed sleep patterns, and hypoactivity (Paré and Redei, 1993; Paré, 1994a,b; Dugovic et al., 2000; Redei et al., 2001; Solberg et al., 2001, 2004; Malkesman et al., 2005; Baum et al., 2006). Chronic treatments with antidepressants, electroshock administration (model for electroconvulsive therapy), and deep-brain stimulation can all reverse these depression-like behaviors (Jeannotte et al., 2008; Falowski et al., 2011; Kyeremanteng et al., 2012). The WKY strain was developed as the normotensive control for the Spontaneously Hypertensive Rat (SHR) strain. Louis and Howes (Louis and Howes, 1990) demonstrated that the WKY strain was distributed to different vendors and universities between F12 and F17 generations of inbreeding.

The fact that the WKY rats showed genetic and behavioral differences (Kurtz et al., 1989; Paré and Kluczynski, 1997) motivated the bi-directional selective breeding using FST immobility as a functional selector (Will et al., 2003). The WMIs, now at their 44th generation and completely inbred after >35 generations of full-sib breeding, show despair-like behaviors and greater sensitivity to stress compared to their isogenic control counterparts, the WKY Less Immobile (WLI) rats (Will et al., 2003; Andrus et al., 2012; Lim et al., 2018b). While the WMIs show higher immobility behavior in the forced swim test, which was the original functional selector for this strain, WLI males and females present immobility behavior comparable to that of other control strains. In our lab, Sprague–Dawley and Fischer 344 male rats show immobility very similar to that of WLI males (Solberg et al., 2003; Wilcoxon et al., 2005; Andrus et al., 2012; Mehta et al., 2013; Mehta-Raghavan et al., 2016). WLI female immobility is similar to that of Wistar and F344 females (Kokras et al., 2018). Through the WMI genetic model of depression and its WLI control strain, any observed behavioral and transcriptomic differences may be directly or indirectly related to the identified <5,000 sequence variations between the two strains (Chen et al., 2017; Bryant et al., 2020).

During the early postnatal period, adaptive changes occur in the mothers' (dam) neuroendocrine system, which enables them to provide appropriate maternal care. These adaptive changes are pivotal and brought about by the hormonal alterations due to parturition (Levy, 2016). The decrease in estrogen and/or progesterone at parturition may directly affect maternal behaviors (Hauser and Gandelman, 1985; Glynn et al., 2016; Murakami, 2016). Changes in estrogen levels alter the expression of its receptors with subsequent effects on the transcription of their target genes and their receptors. Clinical trials involving estrogen as a potential treatment for negative maternal behaviors are ongoing; for example, one study investigated the potential use of transdermal estradiol as a treatment for postpartum depression (Wisner et al., 2015). In addition to hormonal regulation, several genes and their pathways have been shown to influence maternal behavior, including those related to oxytocin (*Oxt*) and vasopressin (*Avp*). Oxytocin is released within various brain regions including the paraventricular nucleus (PVN), supraoptic nucleus, septum, hippocampus, and olfactory bulb (Bosch and Neumann, 2012). In humans, mothers with higher oxytocin expression show increased maternal touch and contact with their children (Pratt et al., 2015). In rats, Oxt release leads to mothers fostering more positive interactions with their offspring (Leng et al., 2008), and central administration of Oxt receptor antagonist can block the onset of maternal care, and reduce pup-directed behaviors (Champagne et al., 2001; Pedersen and Boccia, 2003). The medial preoptic area seems to be a key brain region of vasopressin (Avp) actions on maternal care. It receives vasopressinergic input from the suprachiasmatic nucleus, and diurnal changes in local Avp release have been described (Kalsbeek and Buijs, 2002). Vasopressin regulates maternal care and it seems to occur *via* vasopressin receptor 1a (Avpr1a; Pedersen et al., 1994; Bosch et al., 2007, 2010). As many of the Oxt- and Avp-relevant brain regions are within the hypothalamus, which is also intimately involved in affecting maternal behaviors (Fang et al., 2018), we focused on the expression of these neuropeptides and their receptors in the whole hypothalamus in this study.

The purpose of this study is to determine whether a genetic model of depression, the WMI rat, shows alterations in maternal functioning compared to its isogenic control strain. Here, we explore postpartum behaviors and expression of relevant hypothalamic genes in these strains of inbred rats.

MATERIALS AND METHODS

Animals and Behavioral Assessments

All animal procedures were approved by the Institutional Animal Care and Use Committee of Northwestern University. The 40th generation WLI and WMI inbred strains were housed under temperature and humidity-control with food and water *ad libitum* on a 12:12 LD cycle, lights on at 06:00 h. During the experiment, red lights were on between 18:00 and 23:00 h to allow video recording during the dark phase.

Females of both strains (18 for WLI and 28 for WMI) were mated for this study. Maternal behavior of dams (six WLI and six WMI) that birthed litters that survived to wean (postpartum day 24: PPD 24) was monitored and recorded daily. Maternal behavior was observed for 10 days PPD 1–PPD 10, as described before (Ahmadiyeh et al., 2004), which was a modification of previous studies (Myers et al., 1989; Francis et al., 2003). Behaviors were automatically recorded for an hour under light (11:00–14:00 h) and dark (18:00–23:00 h) conditions, starting at PPD 1, the day after birth. Behavioral analyses were scored

manually. Blind scoring was not feasible as the WMI strain had many more mating pairs (to account for litter loss) and, therefore, the experimenter was aware of which strain was giving birth at any given time. The following behaviors were scored every 3 min: arched-back nursing; blanket nursing (mother lies over pups); proximity to pups, which is either passive nursing (mother is on the side or back with pups feeding) or just resting with pups very close by; licking/grooming of pups and pup retrieval; no contact (mother leaves pup alone more than half a cage length away); or self-directed behaviors (eating and drinking; Ahmadiyeh et al., 2004). Arched back nursing and blanket nursing categories were combined, and also licking/grooming of pups and pup retrieval behaviors. Behavioral measures were shown as a frequency of observations for each hour of monitoring.

Litter deaths were observed during a previous 8-month mating period, and results from this period prompted the investigation of maternal behavior in this study. Litter death was also recorded in the current study. Although we have not made a quantitative assessment, most pups that died before weaning either died from cannibalism by the mother or showed signs of undernourishment with no milk in their stomachs.

Dams were euthanized, either before PPD 5 after their litters died (four WLI and six WMI) or at PPD 24 (six WLI and six WMI) after the pups were weaned, during lights on at 11:00–14:00 h by fast decapitation.

Brain Dissection and RNA Isolation

Hypothalami were dissected with a brain matrix according to Paxinos coordinates (anterior-posterior, −0.30 to −4.16; medial-lateral, 0–2.2; dorsal-ventral, −0.40 to −2.8) and were temporarily stored in RNAlater (Ambion, Austin, TX, USA) at −80°C. Tissue samples were homogenized using a TRIzol reagent (Ambion, Austin, TX, USA) and total RNA from each hypothalamic sample was isolated with Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's protocol. Once isolated, 1 μg of the total RNA was reverse transcribed using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). All of these methods have been described previously (Raghavan et al., 2017; Lim et al., 2018a,b; Meckes et al., 2018).

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-qPCR)

For each experimental group, RT-qPCR was performed to compare the hypothalamic target gene expression levels between strains (WLI vs. WMI). Primers for each target gene were designed using Applied Biosystems Primer Express software (version 3.0, PE Applied Biosystems, Foster City, CA, USA); the primer sequences can be found in **Supplementary Table 1**. Five ng of cDNA was amplified in a 20 μl reaction using SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) in the QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Triplicates of reactions were performed and reached threshold amplification within 34 cycles. Target transcript levels were quantified relative to Gapdh, a housekeeping gene previously demonstrated to show similar expression across strains and conditions, and to a general WLI male hypothalamic calibrator using the $2^{(-\Delta\Delta CT)}$ method.

Statistical Analysis

Data are presented as mean ± standard error of the mean. All statistical analyses were performed using GraphPad Prism v8.0 (GraphPad Software, La Jolla, CA, USA). Behavioral observations were analyzed across postpartum days for the light and the dark phase by two-way ANOVA with repeated measures or mixed effect models, followed by Sidak's *post hoc* analysis for multiple comparisons. Cohen's *d* values were calculated by using the Cohen's $d = (M2 - M1)/SD_{pooled}$ equation, where M1 and M2 are means of the groups to be compared. Gene expression differences were analyzed by two-way ANOVA followed by Tukey's multiple comparison test. Pearson correlation of the gene expression data was corrected for multiple comparisons. Technical outliers, when multiple days of behavioral observations were lost or when RT-qPCR data were marked by the program as technical outliers, were omitted from the analysis.

ANOVAs, mixed effect analyses, and Cohen's *d* effect sizes are described in the results, while *post hoc* analyses are shown on the figures.

RESULTS

Litter Statistics

Body weights of adult female WLIs were significantly higher compared to those of same age WMI females before mating (175.3 ± 2.3 g vs. 134.7 ± 1.6 g; $p = 1.9e\text{-}15$).

WLI and WMI litters differed significantly in their rate of survival to weaning ($p = 0.044$; **Table 1**). While the WLI litters had a survival rate of 50%, their WMI counterparts had a survival rate of less than half, 21.4%. This survival rate is in agreement with the one observed during a previous 8-month mating period (13 WLI litters survived out of 22 total litters for a 59% rate compared to 10 WMI litters survived from a total of 37 litters, 27% survival rate; $p = 0.014$). Additionally, WMI litters seemed to be smaller at birth compared to WLI litters, based on the counts without disturbing the cage. Due to the high pup mortality, the exact number of pups at birth was not determined since we did not want to disturb the dams and litters after birth. WMI litters

TABLE 1 | Litter characteristics.

Strain	Litters survived/total; % survival	Days survived by litters not weaned	Number of pups at weaning	Male : female ratio at weaning
WLI	9/18, 50%	5.22 ± 2.17	8.50 ± 0.68	4.41 ± 0.55 : 4.08 ± 0.41
WMI	6/28*, 21.4%	3.375 ± 0.63	5.92 ± 0.47**	2.83 ± 0.44*: 3.08 ± 0.35

*Values are mean ± SEM; *p < 0.05, **p < 0.01 strain differences.*

had significantly fewer pups than the WLI litters at weaning ($t_{(22)} = 3.13$, $p = 0.005$; Cohen's $d = 1.28$), and the numbers were uneven between the sexes with less male WMI pups survived compared to WLI males (males, $t_{(22)} = 2.29$, $p = 0.036$; Cohen's $d = 0.91$; females, $t_{(22)} = 1.82$, $p = 0.082$; Cohen's $d = 0.74$; **Table 1**).

Maternal Behavior

Maternal and self-directed behaviors were assessed for 10 days postpartum and their distribution is shown in **Figure 1A**. The figure shows the percentage of different behaviors in both strains, measured as the average frequency observed during the 1-h observation period in the light and the dark phase. A significant strain difference was observed in arched-back and blanket nursing (strain, $F_{(1,18)} = 7.77$, $p = 0.012$), with no differences between light and dark phases (**Figure 1B**). Large effect sizes were detected for the strain comparisons (Cohen's *d*, light: 1.23; dark: 1.28). Similarly, there were strain differences in licking, grooming, and pup retrieval ($F_{(1,19)} = 4.61$, $p = 0.045$; **Figure 1B**), with WMIs showing the greater of these pup-directed behaviors. The effect sizes revealed that this strain difference originated more from the dark phase comparison (Cohen's *d*, light: 0.76; dark: 1.24). Both strains of dams showed a lower frequency of these pup-directed behaviors during the dark phase (time of day, $F_{(1,19)} = 8.79$, $p = 0.008$). Self-directed behaviors such as eating, drinking, and self-grooming, also differed by strain and time of day (strain, $F_{(1,19)} = 6.12$, $p = 0.023$; time of day $F_{(1,19)} = 10.29$, $p = 0.005$; **Figure 1B**). Interestingly, the diurnal change in this behavior was only seen in the WLI dams (strain × time of day, $F_{(1,19)} = 6.19$, $p = 0.022$). This is confirmed by the large effect size in strain comparisons at the dark phase (Cohen's *d*, light: 0.02; dark: 1.59).

Figure 1C shows self-directed behaviors across the 10 days observation period for both WLI and WMI dams. WLI dams showed day by day differences in self-directed behaviors across the observation period, and also between the light and dark phases (time of day, $F_{(1,3)} = 23.42$, $p = 0.017$; days × time of day, $F_{(9,27)} = 2.45$, $p = 0.035$). Specifically, self-directed behaviors were significantly greater in the dark phase than the light phase on PPD 5–7 and 10 (PPD 5, $t_{(30)} = 3.56$, $p = 0.013$; PPD 6, $t_{(30)} = 4.07$, $p = 0.003$; PPD 7, $t_{(30)} = 3.56$, $p = 0.013$, and PPD 10, $t_{(30)} = 4.07$, $p = 0.003$). In contrast, there were no significant differences in self-directed behaviors across the days of observation in WMIs, but light, dark phase differences tended to occur postpartum day 5 (days × time of day, $F_{(9,35)} = 2.12$, $p = 0.054$; PPD 5, $t_{(39)} = 3.07$, $p = 0.038$).

Hypothalamic Target Gene Expression

The observation of the lack of circadian rhythm in the self-directed behaviors of WMI dams prompted us to examine any potential causative genetic differences in circadian rhythm-regulating genes between WLI and WMI. The Lysine Demethylase 5A (*Kdm5a* or Jumonji/ARID domain-containing protein1A: *JARID1A*) is connected to the circadian epigenome (Masri and Sassone-Corsi, 2013). We first identified the Arg745Cys variation in the *Kdm5a* gene between WMI and WLI strains from whole-genome sequencing data. This was validated using Sanger Sequencing (**Supplementary Figure 1**). Therefore, the hypothalamic expression of *Kdm5a* and the transcribed clock genes period 1 and 2 (*Per1*, *Per2*) was measured. Transcript levels of *Kdm5a* differed significantly between WLI and WMI females, regardless of postpartum days (*Kdm5a*, $F_{(1,15)} = 68.77$, $p < 0.0001$), with WMI dams showing higher expression (**Figure 2**). Transcript levels of *Per1* and *Per2* also differed significantly between WLI and WMI females (*Per1*, $F_{(1,15)} = 4.63$, $p = 0.048$; *Per2*, $F_{(1,15)} = 8.05$, $p = 0.013$), with WMI dams showing higher expression (**Figure 2**).

Hypothalamic transcript levels of target genes are shown in **Figure 3**. Interestingly, while *Oxt* expression tended to differ significantly between WLI and WMI dams only by litter survival (litter survival by strain, $F_{(1,11)} = 7$, 15, $p = 0.022$), expression of *Oxtr* showed both a clear strain and a strain by litter survival effect (strain, $F_{(1,13)} = 4.80$, $p = 0.047$; litter survival by strain, $F_{(1,13)} = 5.38$, $p = 0.037$). The *Avp* system showed major differences between the strains and litter survival. Specifically, expression of both *Avp* and *Avpr1a* showed strain differences (*Avp*, $F_{(1,12)} = 12.54$, $p = 0.004$; *Avpr1a*, $F_{(1,15)} = 7.01$, $p = 0.018$). However, while there were no litter survival and strain by litter survival effects for *Avp* expression, *Avpr1a* expression was greater in PPD 24 WMI hypothalamus compared to all other groups (litter survival, $F_{(1,15)} = 8.64$, $p = 0.010$; strain by litter survival, $F_{(1,15)} = 5.62$, $p = 0.032$). In contrast, hypothalamic transcript levels of *Avpr1b* did not differ between the strains but showed a significant association with litter survival ($F_{(1,13)} = 21.56$, $p = 0.0005$). Expression of *Esr1* differed significantly between the WLI and WMI hypothalamus ($F_{(1,12)} = 5.24$, $p = 0.041$), but the expression of *Esr2* only showed a difference by litter survival ($F_{(1,15)} = 7.97$, $p < 0.013$).

Heatmaps of the correlations between hypothalamic gene expressions are shown in **Supplementary Figure 2** for WLIs and WMIs. The heatmaps illustrate the differential pattern of correlations between the strains and between the days postpartum. Unique strain differences in a significant correlation are present in WLIs regardless of PPD, such as the correlation between hypothalamic expression of *Oxtr* and *Avpr1a* and *Kdm5a* and *Per2*. These correlations are not present in WMIs, while the *Avp*, *Per1*, *Kdm5a*, and *Avpr1b* correlations are unique to PPD 24 WMI hypothalamus. Correlations unique to postpartum days or litter survival regardless of strain indicate that in the PPD <5 groups, hypothalamic expression of *Oxt* correlated with *Avp*, while at PPD 24, *Avpr1a* correlated with *Esr2* expression.

DISCUSSION

The WMI genetic animal model of depression has shown major differences during postpartum compared to their isogenic controls, the WLI strain that does not indicate depression-like behavior. Many of these characteristics segregated from the WKY parent phenotype during selective breeding with most behavioral phenotypes of WMIs being the same as the WKYs, while WLIs being different. Particularly, WMI dams have reduced

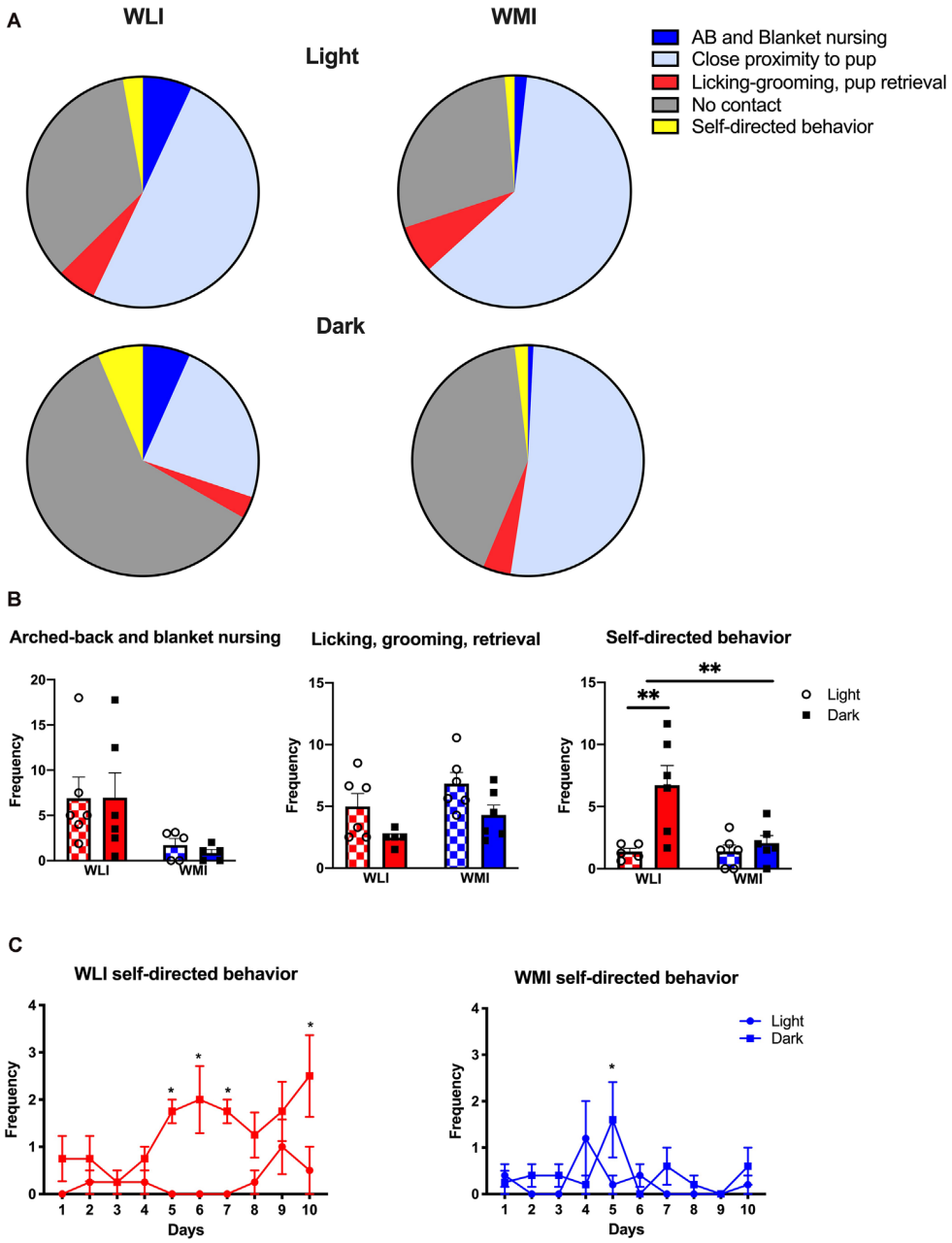

FIGURE 1 | Maternal and self-directed behaviors of Wistar Kyoto Less Immobile (WLI) and Wistar Kyoto More Immobile (WMI) dams as observed during postpartum day 1–10. **(A)** Percentage of average frequency/hr of each category of maternal behaviors in the light and the dark phases of the day are shown. **(B)** Frequency per hour of observation of arched-back and blanket nursing is greater in WLI dams compared to WMIs, regardless of the time of day. Pup-directed behaviors, such as licking, grooming, and retrieval are greater in WMI than WLI dams and differ between the light and the dark phases of the day, the latter being less. Self-directed behaviors do not differ between WLI and WMI dams during the light phase, but during the dark phase (when nocturnal animals are more active), WMI dams spend significantly less time eating and drinking compared to WLIs. **(C)** Self-directed behaviors are greater in the dark phase in WLIs across the 10 days observation, with only one time of day difference in WMIs. Data are presented as mean ± standard error of the mean. $^{*}p < 0.05$, $^{**}p < 0.01$.

litter survival, smaller litter size and decreased arched-back and blanket nursing, but increased licking, grooming retrieval behaviors toward their pups than WLI dams over the first 10 days postpartum. WMIs self-directed behaviors are decreased and WMIs show no diurnal rhythm in these behaviors while WLI dams do. This is similar to the biological rhythm disturbances observed in patients with depression compared to healthy controls (Mondin et al., 2017; Ozcelik and Sahbaz, 2020). Hypothalamic expression of *Kdm5a*, *Per1*, and *Per2* is greater in WMIs than WLIs, both in dams whose litter died before postpartum day 5 and in those whose litter survived to wean. Hypothalamic transcript levels

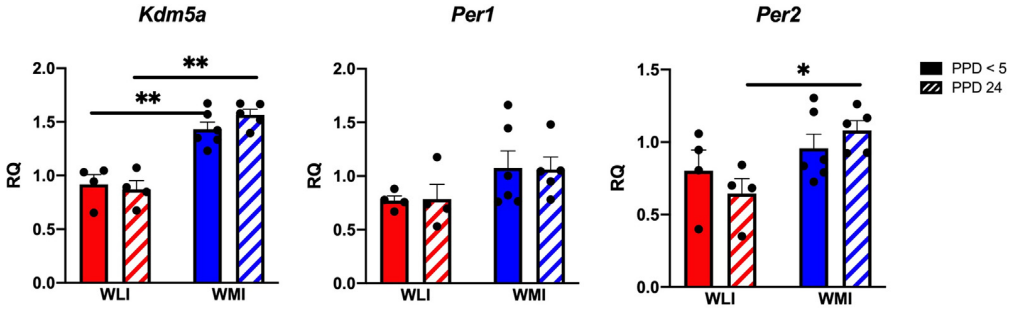

FIGURE 2 | Hypothalamic gene expression of *Kdm5a*, *Per1*, and *Per2* in WLI and WMI dams who lost their litter before postpartum day 5 (PPD < 5) and in those whose litter were weaned at PPD 24. Hypothalamic transcript levels were measured by quantitative RT-PCR and shown as Relative Quantification (RQ). Data are presented as mean ± standard error of the mean. N = 4–6 per group. $^{*}P < 0.05$ and $^{**}P < 0.01$ represent *post hoc* comparisons.

of *Oxtr*, *Avp*, and *Avpr1a* are also increased in the WMI dams compared to their isogenic control WLIs, but only in dams whose litter survived to wean. Another parallel to the human condition is that these gene expression increases are similar to what was found in the hypothalamus of depressed patients (Meynen et al., 2006; Wang et al., 2008). Since WMI dams show characteristics similar to depressed patients and decreased nursing, but increased pup-directed behaviors, we propose that the dams of the WMI genetic animal model of depression are a potential animal model of postpartum depression.

In prior studies, differences in maternal care were not related to basic measures of reproductive success, such as litter survival to weaning (Champagne et al., 2003). The parental strain of the WLI and WMI, the WKY rat, has an average litter size of 8.16 pups which is similar to litter sizes of the control WLI dams and other rat strains (Gill Iii et al., 1979). However, after the establishment of these new inbred strains, the litter size for the WMI dams became close to half of that of the WLI dams. Furthermore, although WMI litters were birthed at the same rate as WLI litters, they showed a significantly lower survival rate than WLI litters. One of the potential causes of these findings could be aberrant maternal behavior of the WMI dam, as more nourishment-directed maternal behaviors are thought to increase litter survival (Weber et al., 2016). While dams of both strains show limited arched-back and blanket nursing behaviors during the observation period, WMI dams spent significantly less time with arched-back and blanket nursing of the pups than WLIs. In contrast, WMI dams spent more time licking, grooming, and retrieval of the pups than WLIs. Although arched-back, blanket nursing and licking, grooming and retrieval of the pups often co-occur, previous studies have found them to vary independently. For example, undernourished pups elicit increased licking, grooming from dams regardless of the dam's ability or frequency of nursing (Lynch, 1976). It has been suggested that these two behaviors have distinct developmental roles with distinct effects on offspring biobehavioral outcomes, and also that arched-back nursing is typically not a significant predictor of offspring outcomes (Jensen Peña and Champagne, 2013).

Altered maternal-child interactions are reported when women suffer from postpartum depression and comorbid anxiety. Decreased breastfeeding, or premature cessation of it, has been observed in women with depression before or during pregnancy (Wallenborn et al., 2018; Jordan et al., 2019). Genetic rodent models with depression-like behavior show some of the characteristics of these altered interactions, as seen with the decreased nursing of WMIs compared to WLIs. Although maternal behavior has been examined in many inbred and outbred strains of rats and mice (Perani and Slattery, 2014), findings from the Flinders Sensitive Line (FSL) and the WKY rats, two different genetic animal models of depression (Lavi-Avnon et al., 2005; Braw et al., 2009), are particularly relevant to the current study. FSL dams do not differ from control Sprague Dawley dams in their maternal behavior, while WKY dams perform more pup-directed activities and fewer self-directed activities compared to Wistar controls (Braw et al., 2009). This again implies that the WKY parental phenotype of pup-directed and self-directed activities are segregated together and present in the WMI strain, differently from that of the litter size phenotype. Interestingly, increased pup-directed behavior could represent the "helicopter parenting" maternal phenotype observed in mothers with postpartum depression and anxiety (Perani and Slattery, 2014). Even more specifically, maternal anxiety is associated with higher maternal control and intrusiveness in the mother-infant interaction (Stein et al., 2012; Parfitt et al., 2013; Hakanen et al., 2019). Since both WKY (Solberg et al., 2003; Baum et al., 2006) and WMI females show concurrent increased depression and anxiety-like phenotypes (Mehta et al., 2013) and more pup-directed activities compared to their respective controls (Wistar and WLI; Braw et al., 2009), it is feasible that their behavior is also associated with maternal anxiety. This is similar to findings in which women with postpartum depression very often show comorbid anxiety (Farr et al., 2014). The main predictor for depressive, anxiety, or psychotic diseases after delivery is an antenatal episode of the illness (Perani and Slattery, 2014). Thus, WKY and WMI females do have risk factors for showing characteristics of postpartum depression after delivery.

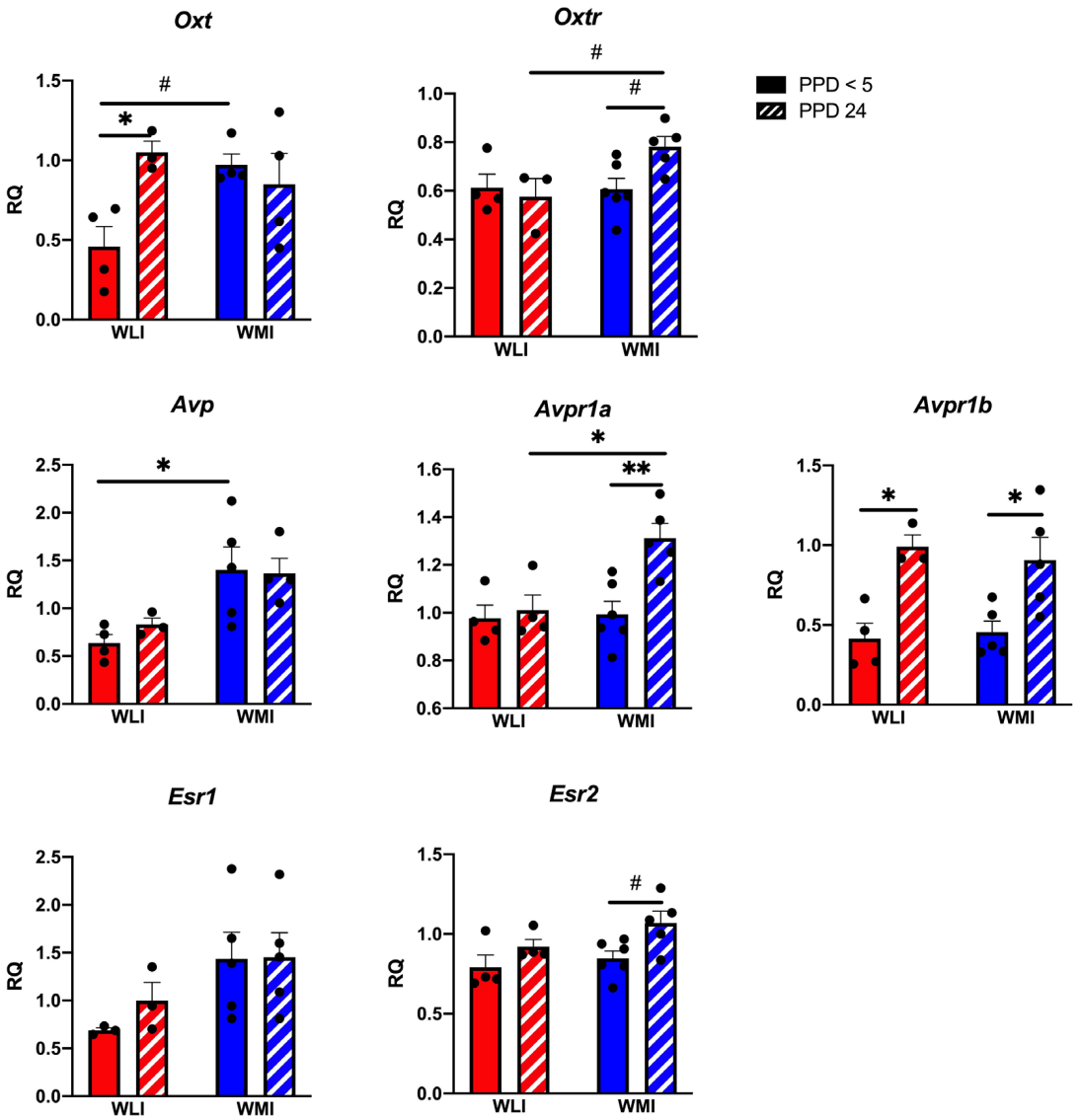

FIGURE 3 | Hypothalamic gene expression of *Oxt, Oxtr, Avp, Avpr1a and Avpr1b, and Esr1 and Esr2* in WLI and WMI dams who lost their litter before postpartum day 5 (PPD < 5) and in those whose litter were weaned at PPD 24. Hypothalamic transcript levels were measured by quantitative RT-PCR and shown as Relative Quantification (RQ). Data are presented as mean ± standard error of the mean. N = 4–6 per group. $^{\#} < 0.10$, $^{*}P < 0.05$, and $^{**}P < 0.01$ represent *post hoc* comparisons.

The decreased self-directed behavior of the WMI dams became very apparent in this study because we observed maternal behavior during both the light and the dark phase for 10 days postpartum. This is in contrast to some other studies in which spot check observations were conducted on PPD 4 and 9 only (Braw et al., 2009), or behaviors were examined on PPD 3–4 and 17–18 only in the light phase (Lavi-Avnon et al., 2005). Nocturnal animals eat and drink more during the dark, just as the WLI dams did, but the disturbed rhythm of WMIs recalls a similar phenomenon in the WKYs. The WKY rats were found to be less responsive to light, which may cause possible alterations in daily rhythm patterns of this strain (Rosenwasser, 1993; Solberg et al., 2001). Interestingly, the FSL has shown a similar phenotype, suggesting that it may be a common phenotype in animal models of depression (Shiromani and Overstreet, 1994). Some data demonstrate that patients with depression may have an altered sensitivity to light (Duncan, 1996). Additionally, greater biological rhythm disturbances have been observed in patients with depression compared to healthy controls (Mondin et al., 2017; Ozcelik and Sahbaz, 2020). Sleep disturbances have also been observed in WKYs (Dugovic et al., 2000; Dasilva et al., 2011), very similar to those described in depressed patients (Rosenwasser and Wirz-Justice, 1997). Thus, the lack of diurnal rhythm of self-directed behaviors in the WMI dams suggests that this phenotype segregated with the depression-like behavior.

Because the WLI and WMI strains are isogenic, we could peruse the sequences for possible causative sequence variations that may be associated with the lack of diurnal rhythm observed in the WMI dams. The confirmed nonsynonymous sequence variation in the coding region of lysine-specific demethylase 5A (*Kdm5a*/*Jarid1a*) elevated this gene to a possible causative gene, although the single nucleotide polymorphism (SNP) was in the WLI strain. Kdm5a forms a complex with transcription factors Clock and Bmal1, which results in transcriptional activation of the Period genes and maintenance of circadian oscillations (DiTacchio et al., 2011). Interestingly, *Kdm5a* and *Per2* expression were increased in the WMI compared to WLI, regardless of litter survival or postpartum days. Increased hypothalamic expression of *Kdm5a* and *Per2* may contribute to the lack of circadian rhythm seen in the WMI's behavior. Constitutive expression of *Per2* abolishes diurnal rhythm (Chen et al., 2009), and *Kdm5a* is known to activate *Per2* expression (DiTacchio et al., 2011). Thus, the lack of diurnal variation in self-directed behaviors of the WMI dam could be related to the increased expression of *Per2* in the hypothalamus. Whether the increased *Per2* is also a molecular characterization of the WMI's depressive behavior is not known, but antidepressants are known to reduce *Per2* expression in experimental models (Orozco-Solis et al., 2017).

Alternatively, the SNP in the *Kdm5a* gene in the WLIs could have a loss or gain of function effect. The gain of function could enhance the circadian rhythm of WLI in their self-directed behavior, and the Kdm5a-induced negative regulation of transcription by RNA polymerase II could suppress the expression of the target genes in WLIs, and not in WMIs. However, there were no correlations between the expression of *Kdm5a* and other genes except for *Per2* in the WLIs. In contrast, *Kdm5a* expression correlated with *Avp*, but only in the WMIs, suggesting a loss of function effect of the WLI SNP. These proposed mechanisms do not explain the lack of diurnal rhythm in the WMIs self-directed behavior, as no single regulator can. However, they implicate that genetic manipulations in these strains could, potentially, identify causative variations affecting maternal behavior.

The observed increases in the hypothalamic expression of *Oxtr*, *Avp*, and *Avp1ra* in the WMI dams compared to WLIs and also to those WMIs whose litter died are difficult to interpret in the context of their accepted role in maternal behavior. A large body of literature supports the role of neuroendocrine processes in the induction and regulation of maternal behavior (Bridges, 2015). Both neuropeptides, Oxt and Avp, have been known to have an impact on maternal behavior (Pedersen and Prange, 1979; Pedersen et al., 1982; Bosch and Neumann, 2008; Bayerl and Bosch, 2019). Their receptors, Avpr1a and Oxtr, have also been implicated in maternal behaviors (Donaldson and Young, 2008) with complementary expression patterns in the ventromedial hypothalamus (Raggenbass, 2008). Several rat and mice studies showed a correlation between *Oxtr* and *Avpr* expression, similar to what is seen in the WLI dams' hypothalami. However, more maternal care has been associated with higher levels of oxytocin binding in relevant brain regions (Champagne et al., 2001; Curley et al., 2012), including the paraventricular nucleus of the hypothalamus (Bayerl et al., 2016). Furthermore, the central administration of an Oxtr antagonist reduces high levels of pup licking, grooming, and arched-back nursing in dams (Champagne et al., 2001). Thus, increased pup licking, grooming behavior of the WMI dam is in concordance with their increased hypothalamic expression of *Oxtr*, but not with their decreased arched-back and blanket nursing compared to that of WLIs. Administration of Avp increases pup grooming in rats (Caldwell et al., 1986; Elkabir et al., 1990), while antagonism of Avpr1a does not affect arched back nursing and pup retrieval, but decreases other types of nursing (Bayerl et al., 2016). Thus again, the increased *Avp* expression in the WMI hypothalamus is as per a component of the licking, grooming behavior of WMI dams, but not with the rest of the observed behavior. These seeming contradictions suggest that discrete components of maternal behavior are influenced by different neuropeptidergic mechanisms or differing neurocircuitry (Bosch and Neumann, 2012). The complexity of the association between maternal behavioral differences and these neuropeptidergic mechanisms is exaggerated by the findings that the administration of an Avpr1a antagonist reduces anxiety/depression-like behavior in preclinical studies (Wigger et al., 2004). Furthermore, *AVP* and *AVPR1A* expressions are higher in the human hypothalamus of depressed patients compared to that of controls (Meynen et al., 2006; Wang et al., 2008). Therefore, the increased hypothalamic expression of *Avp* and *Avpr1a* could be the manifestation of the depression-like behavior of the WMI dams.

The most thought-provoking findings of the present study, such as the increased licking, grooming, and retrieval but decreased arched-back and blanket nursing and self-directed behaviors of the WMI dams, seem to parallel postpartum behavior of its progenitor, the WKY strain, which is considered an animal model of depression. In contrast, these behaviors are very different in the WLIs, where the inbred strain does not show depression-like behavior. Thus, some postpartum behaviors segregated together with depression-like behavior between the two strains during selective breeding. Genetic studies using the WKY and another strain with phenotypic differences are possible, but less likely to produce causative genes due to the nature of these quantitative trait loci studies (Solberg et al., 2004). In contrast, exploring the underlying genetic basis of these behaviors in the isogenic WLI and WMI strains using the reduced complexity cross approach would be more meaningful, as we described it recently (Bryant et al., 2020). The loss of diurnal patterns in self-directed behaviors and increased hypothalamic *Avp* and *Avpr1a* expression are resonant to findings in human depressed patients. Future studies could investigate whether attenuating the WMI's depression-like behavior before gestation, by antidepressant treatment (Will et al., 2003) or by environmental enrichment (Mehta-Raghavan et al., 2016), would equalize litter survival, maternal and self-directed behaviors, and neuropeptide receptor expression in the hypothalamus of WMI and WLI dams. Since the

greatest risk factor for postpartum depression is a history of depression before pregnancy (Putnam et al., 2017), the WLI and WMI rat strains could be valuable tools to investigate the molecular and genetic underpinning of this disorder.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Northwestern University Institutional ACUC.

AUTHOR CONTRIBUTIONS

The study was designed by EER and WL. Experimental work was carried out by WL, PHL, SLW, SAG, and HC. Data analysis was carried out by WL and EER. The manuscript was drafted by WL, EER, and HC. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2020.589967/full#supplementary-material.

REFERENCES

Ahmadiyeh, N., Slone-Wilcoxon, J. L., Takahashi, J. S., and Redei, E. E. (2004). Maternal behavior modulates X-linked inheritance of behavioral coping in the defensive burying test. *Biol. Psychiatry* 55, 1069–1074. doi: 10.1016/j.biopsych.2004.02.014

Andrus, B. M., Blizinsky, K., Vedell, P. T., Dennis, K., Shukla, P. K., Schaffer, D. J., et al. (2012). Gene expression patterns in the hippocampus and amygdala of endogenous depression and chronic stress models. *Mol. Psychiatry* 17, 49–61. doi: 10.1038/mp.2010.119

Baum, A. E., Solberg, L. C., Churchill, G. A., Ahmadiyeh, N., Takahashi, J. S., and Redei, E. E. (2006). Test- and behavior-specific genetic factors affect WKY hypoactivity in tests of emotionality. *Behav. Brain Res.* 169, 220–230. doi: 10.1016/j.bbr.2006.01.007

Bayerl, D. S., and Bosch, O. J. (2019). Brain vasopressin signaling modulates aspects of maternal behavior in lactating rats. *Genes Brain Behav.* 18:e12517. doi: 10.1111/gbb.12517

Bayerl, D. S., Hönig, J. N., and Bosch, O. J. (2016). Vasopressin V1a, but not V1b, receptors within the PVN of lactating rats mediate maternal care and anxiety-related behaviour. *Behav. Brain Res.* 305, 18–22. doi: 10.1016/j.bbr.2016.02.020

Bosch, O. J., Müsch, W., Bredewold, R., Slattery, D. A., and Neumann, I. D. (2007). Prenatal stress increases HPA axis activity and impairs maternal care in lactating female offspring: implications for postpartum mood disorder. *Psychoneuroendocrinology* 32, 267–278. doi: 10.1016/j.psyneuen.2006.12.012

Bosch, O. J., and Neumann, I. D. (2008). Brain vasopressin is an important regulator of maternal behavior independent of dams' trait anxiety. *Proc. Natl. Acad. Sci. U S A* 105, 17139–17144. doi: 10.1073/pnas.0807412105

Bosch, O. J., and Neumann, I. D. (2012). Both oxytocin and vasopressin are mediators of maternal care and aggression in rodents: from central release to sites of action. *Horm. Behav.* 61, 293–303. doi: 10.1016/j.yhbeh.2011.11.002

Bosch, O. J., Pförtsch, J., Beiderbeck, D. I., Landgraf, R., and Neumann, I. D. (2010). Maternal behaviour is associated with vasopressin release in the medial preoptic area and bed nucleus of the stria terminalis in the rat. *J. Neuroendocrinol.* 22, 420–429. doi: 10.1111/j.1365-2826.2010.01984.x

Braw, Y., Malkesman, O., Merenlender, A., Dagan, M., Bercovich, A., Lavi-Avnon, Y., et al. (2009). Divergent maternal behavioral patterns in two genetic animal models of depression. *Physiol. Behav.* 96, 209–217. doi: 10.1016/j.physbeh.2008.10.002

Bridges, R. S. (2015). Neuroendocrine regulation of maternal behavior. *Front. Neuroendocrinol.* 36, 178–196. doi: 10.1016/j.yfrne.2014.11.007

Bryant, C. D., Smith, D. J., Kantak, K. M., Nowak, T. S. Jr., Williams, R. W., Damaj, M. I., et al. (2020). Facilitating complex trait analysis *via* reduced complexity crosses. *Trends Genet.* 36, 549–562. doi: 10.1016/j.tig.2020.05.003

Caldwell, J. D., Drago, F., Prange, A. J. Jr., and Pedersen, C. A. (1986). A comparison of grooming behavior potencies of neurohypophyseal nonapeptides. *Regul. Pept.* 14, 261–271. doi: 10.1016/0167-0115(86)90009-1

Champagne, F., Diorio, J., Sharma, S., and Meaney, M. J. (2001). Naturally occurring variations in maternal behavior in the rat are associated with differences in estrogen-inducible central oxytocin receptors. *Proc. Natl. Acad. Sci. U S A* 98, 12736–12741. doi: 10.1073/pnas.221224598

Champagne, F. A., Francis, D. D., Mar, A., and Meaney, M. J. (2003). Variations in maternal care in the rat as a mediating influence for the effects of environment on development. *Physiol. Behav.* 79, 359–371. doi: 10.1016/s0031-9384(03)00149-5

Chen, H., Guryev, V., Mulligan, M., Redei, E., and Williams, R. (2017). "Sequence variations between a genetic rat model of depression and its control strain," in *Proceedings of the 15th Annual Meeting of the Complex Trait Community in Collaboration with the 10th Annual Meeting of Rat Genomics and Models*, Memphis, TN, USA.

Chen, R., Schirmer, A., Lee, Y., Lee, H., Kumar, V., Yoo, S.-H., et al. (2009). Rhythmic PER abundance defines a critical nodal point for negative feedback within the circadian clock mechanism. *Mol. Cell* 36, 417–430. doi: 10.1016/j.molcel.2009.10.012

Curley, J. P., Jensen, C. L., Franks, B., and Champagne, F. A. (2012). Variation in maternal and anxiety-like behavior associated with discrete patterns of oxytocin and vasopressin 1a receptor density in the lateral septum. *Horm. Behav.* 61, 454–461. doi: 10.1016/j.yhbeh.2012.01.013

Dasilva, J. K., Lei, Y., Madan, V., Mann, G. L., Ross, R. J., Tejani-Butt, S., et al. (2011). Fear conditioning fragments REM sleep in stress-sensitive Wistar-Kyoto, but not Wistar, rats. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 35, 67–73. doi: 10.1016/j.pnpbp.2010.08.023

DiTacchio, L., Le, H. D., Vollmers, C., Hatori, M., Witcher, M., Secombe, J., et al. (2011). Histone lysine demethylase JARID1a activates CLOCK-BMAL1 and influences the circadian clock. *Science* 333, 1881–1885. doi: 10.1126/science.1206022

Donaldson, Z. R., and Young, L. J. (2008). Oxytocin, vasopressin and the neurogenetics of sociality. *Science* 322, 900–904. doi: 10.1126/science.1158668

Dugovic, C., Solberg, L. C., Redei, E., Reeth, O. V., and Turek, F. W. (2000). Sleep in the Wistar-Kyoto rat, a putative genetic animal model for depression. *Neuroreport* 11, 627–631. doi: 10.1097/00001756-200002280-00038

Duncan, W. C. Jr. (1996). Circadian rhythms and the pharmacology of affective illness. *Pharmacol. Ther.* 71, 253–312. doi: 10.1016/s0163-7258(96)00092-7

Eid, R. S., Gobinath, A. R., and Galea, L. A. M. (2019). Sex differences in depression: insights from clinical and preclinical studies. *Prog. Neurobiol.* 176, 86–102. doi: 10.1016/j.pneurobio.2019.01.006

Elkabir, D. R., Wyatt, M. E., Vellucci, S. V., and Herbert, J. (1990). The effects of separate or combined infusions of corticotrophin-releasing factor and vasopressin either intraventricularly or into the amygdala on aggressive and investigative behaviour in the rat. *Regul. Pept.* 28, 199–214. doi: 10.1023/a:1012360809183

Falowski, S. M., Sharan, A., Reyes, B. A., Sikkema, C., Szot, P., and Van Bockstaele, E. J. (2011). An evaluation of neuroplasticity and behavior after deep brain stimulation of the nucleus accumbens in an animal model of depression. *Neurosurgery* 69, 1281–1290. doi: 10.1227/NEU.0b013e3182237346

Fang, Y. Y., Yamaguchi, T., Song, S. C., Tritsch, N. X., and Lin, D. (2018). A hypothalamic midbrain pathway essential for driving maternal behaviors. *Neuron* 98, 192.e10–207.e10.doi: 10.1016/j.neuron.2018.02.019

Farr, S. L., Dietz, P. M., O'Hara, M. W., Burley, K., and Ko, J. Y. (2014). Postpartum anxiety and comorbid depression in a population-based sample of women. *J. Womens Health* 23, 120–128. doi: 10.1089/jwh.2013.4438

Francis, D. D., Szegda, K., Campbell, G., Martin, W. D., and Insel, T. R. (2003). Epigenetic sources of behavioral differences in mice. *Nat. Neurosci.* 6, 445–446. doi: 10.1038/nn1038

Gill Iii, T., Kunz, H., and Hansen, C. (1979). Litter sizes in inbred strains of rats (Rattus norvegicus). *Int. J. Immunogenet.* 6, 461–463. doi: 10.1111/j.1744-313x.1979.tb00701.x

Glynn, L. M., Davis, E. P., Sandman, C. A., and Goldberg, W. A. (2016). Gestational hormone profiles predict human maternal behavior at 1-year postpartum. *Horm. Behav.* 85, 19–25. doi: 10.1016/j.yhbeh.2016.07.002

Grant, K. A., Mcmahon, C., and Austin, M. P. (2008). Maternal anxiety during the transition to parenthood: a prospective study. *J. Affect. Disord.* 108, 101–111. doi: 10.1016/j.jad.2007.10.002

Hakanen, H., Flykt, M., Sinervä, E., Nolvi, S., Kataja, E.-L., Pelto, J., et al. (2019). How maternal pre- and postnatal symptoms of depression and anxiety affect early mother-infant interaction? *J. Affect. Disord.* 257, 83–90. doi: 10.1016/j.jad.2019.06.048

Hauser, H., and Gandelman, R. (1985). Lever pressing for pups: evidence for hormonal influence upon maternal behavior of mice. *Horm. Behav.* 19, 454–468. doi: 10.1016/0018-506x(85)90041-8

Jeannotte, A. M., Mccarthy, J. G., Redei, E. E., and Sidhu, A. (2008). Desipramine modulation of α-, γ-synuclein and the norepinephrine transporter in an animal model of depression. *Neuropsychopharmacology* 34, 987–998. doi: 10.1038/npp.2008.146

Jensen Peña, C., and Champagne, F. A. (2013). Implications of temporal variation in maternal care for the prediction of neurobiological and behavioral outcomes in offspring. *Behav. Neurosci.* 127, 33–46. doi: 10.1037/a0031219

Jordan, S., Davies, G. I., Thayer, D. S., Tucker, D., and Humphreys, I. (2019). Antidepressant prescriptions, discontinuation, depression and perinatal outcomes, including breastfeeding: a population cohort analysis. *PLoS One* 14:e0225133. doi: 10.1371/journal.pone.0225133

Kalsbeek, A., and Buijs, R. M. (2002). Output pathways of the mammalian suprachiasmatic nucleus: coding circadian time by transmitter selection and specific targeting. *Cell Tissue Res.* 309, 109–118. doi: 10.1007/s00441-002-0577-0

Kokras, N., Pastromas, N., Papasava, D., De Bournonville, C., Cornil, C. A., and Dalla, C. (2018). Sex differences in behavioral and neurochemical effects of gonadectomy and aromatase inhibition in rats. *Psychoneuroendocrinology* 87, 93–107. doi: 10.1016/j.psyneuen.2017.10.007

Kurtz, T. W., Montano, M., Chan, L., and Kabra, P. (1989). Molecular evidence of genetic heterogeneity in Wistar-Kyoto rats: implications for research with the spontaneously hypertensive rat. *Hypertension* 13, 188–192. doi: 10.1161/01.hyp.13.2.188

Kyeremanteng, C., James, J., Mackay, J., and Merali, Z. (2012). A study of brain and serum brain-derived neurotrophic factor protein in Wistar and Wistar-Kyoto rat strains after electroconvulsive stimulus. *Pharmacopsychiatry* 45, 244–249. doi: 10.1055/s-0032-1306278

Lavi-Avnon, Y., Shayit, M., Yadid, G., Overstreet, H. D., and Weller, A. (2005). Immobility in the swim test and observations of maternal behavior in lactating Flinders sensitive line rats. *Behav. Brain Res.* 161, 155–163. doi: 10.1016/j.bbr.2005.02.002

Leng, G., Meddle, S. L., and Douglas, A. J. (2008). Oxytocin and the maternal brain. *Curr. Opin. Pharmacol.* 8, 731–734. doi: 10.1016/j.coph.2008.07.001

Levy, F. (2016). Neuroendocrine control of maternal behavior in non-human and human mammals. *Ann. Endocrinol.* 77, 114–125. doi: 10.1016/j.ando.2016.04.002

Lim, P. H., Shi, G., Wang, T., Jenz, S. T., Mulligan, M. K., Redei, E. E., et al. (2018a). Genetic model to study the co-morbid phenotypes of increased alcohol intake and prior stress-induced enhanced fear memory. *Front. Genet.* 9:566. doi: 10.3389/fgene.2018.00566

Lim, P. H., Wert, S. L., Tunc-Ozcan, E., Marr, R., Ferreira, A., and Redei, E. E. (2018b). Premature hippocampus-dependent memory decline in middle-aged females of a genetic rat model of depression. *Behav. Brain Res.* 353, 242–249. doi: 10.1016/j.bbr.2018.02.030

Louis, W. J., and Howes, L. G. (1990). Geneaology of the spontaneously hypertensive rat and Wistar-kyoto rat strains: implications for studies of inherited hypertension. *J. Cardiovasc. Pharmacol.* 16, S1–S5.

Lynch, A. (1976). Postnatal undernutrition: an alternative method. *Dev. Psychobiol.* 9, 39–48. doi: 10.1002/dev.420090107

Malkesman, O., Braw, Y., Zagoory-Sharon, O., Golan, O., Lavi-Avnon, Y., Schroeder, M., et al. (2005). Reward and anxiety in genetic animal models of childhood depression. *Behav. Brain Res.* 164, 1–10. doi: 10.1016/j.bbr.2005.04.023

Masri, S., and Sassone-Corsi, P. (2013). The circadian clock: a framework linking metabolism, epigenetics and neuronal function. *Nat. Rev. Neurosci.* 14:69. doi: 10.1038/nrn3393

Meckes, J. K., Lim, P. H., Wert, S. L., Luo, W., Gacek, S. A., Platt, D., et al. (2018). Brain region-specific expression of genes mapped within quantitative trait loci for behavioral responsiveness to acute stress in Fisher 344 and Wistar Kyoto male rats. *PLoS One* 13:e0194293. doi: 10.1371/journal.pone.0194293

Mehta, N. S., Wang, L., and Redei, E. E. (2013). Sex differences in depressive, anxious behaviors and hippocampal transcript levels in a genetic rat model. *Genes Brain Behav.* 12, 695–704. doi: 10.1111/gbb.12063

Mehta-Raghavan, N. S., Wert, S. L., Morley, C., Graf, E. N., and Redei, E. E. (2016). Nature and nurture: environmental influences on a genetic rat model of depression. *Transl. Psychiatry* 6:e770. doi: 10.1038/tp.2016.28

Meynen, G., Unmehopa, U. A., Van Heerikhuize, J. J., Hofman, M. A., Swaab, D. F., and Hoogendijk, W. J. (2006). Increased arginine vasopressin mRNA expression in the human hypothalamus in depression: a preliminary report. *Biol. Psychiatry* 60, 892–895. doi: 10.1016/j.biopsych.2005.12.010

Mondin, T. C., Cardoso, T. A., Souza, L. D. M., Jansen, K., Da Silva Magalhães, P. V., Kapczinski, F., et al. (2017). Mood disorders and biological rhythms in young adults: a large population-based study. *J. Psychiatr. Res.* 84, 98–104. doi: 10.1016/j.jpsychires.2016.09.030

Murakami, G. (2016). Distinct effects of estrogen on mouse maternal behavior: the contribution of estrogen synthesis in the brain. *PLoS One* 11:e0150728. doi: 10.1371/journal.pone.0150728

Myers, M. M., Brunelli, S. A., Shair, H. N., Squire, J. M., and Hofer, M. A. (1989). Relationships between maternal behavior of SHR and WKY dams and adult blood pressures of cross-fostered F1 pups. *Dev. Psychobiol.* 22, 55–67. doi: 10.1002/dev.420220105

Orozco-Solis, R., Montellier, E., Aguilar-Arnal, L., Sato, S., Vawter, M. P., Bunney, B. G., et al. (2017). A circadian genomic signature common to ketamine and sleep deprivation in the anterior cingulate cortex. *Biol. Psychiatry* 82, 351–360. doi: 10.1016/j.biopsych.2017.02.1176

Ozcelik, M., and Sahbaz, C. (2020). Clinical evaluation of biological rhythm domains in patients with major depression. *Braz. J. Psychiatry* 42, 258–263. doi: 10.1590/1516-4446-2019-0570

Paré, W. P. (1994a). Hyponeophagia in Wistar Kyoto (WKY) rats. *Physiol. Behav.* 55, 975–978. doi: 10.1016/0031-9384(94)90090-6

Paré, W. P. (1994b). Open field, learned helplessness, conditioned defensive burying and forced-swim tests in WKY rats. *Physiol. Behav.* 55, 433–439. doi: 10.1016/0031-9384(94)90097-3

Paré, W. P., and Kluczynski, J. (1997). Differences in the stress response of Wistar-Kyoto (WKY) rats from different vendors. *Physiol. Behav.* 62, 643–648. doi: 10.1016/s0031-9384(97)00191-1

Paré, W. P., and Redei, E. (1993). Depressive behavior and stress ulcer in Wistar Kyoto rats. *J. Physiol.* 87, 229–238. doi: 10.1016/0928-4257(93)90010-q

Parfitt, Y., Pike, A., and Ayers, S. (2013). The impact of parents' mental health on parent-baby interaction: a prospective study. *Infant Behav. Dev.* 36, 599–608. doi: 10.1016/j.infbeh.2013.06.003

Pedersen, C. A., Ascher, J. A., Monroe, Y. L., and Prange, A. J. Jr. (1982). Oxytocin induces maternal behavior in virgin female rats. *Science* 216, 648–650. doi: 10.1126/science.7071605

Pedersen, C. A., and Boccia, M. L. (2003). Oxytocin antagonism alters rat dams' oral grooming and upright posturing over pups. *Physiol. Behav.* 80, 233–241. doi: 10.1016/j.physbeh.2003.07.011

Pedersen, C. A., Caldwell, J. D., Walker, C., Ayers, G., and Mason, G. A. (1994). Oxytocin activates the postpartum onset of rat maternal behavior in the ventral tegmental and medial preoptic areas. *Behav. Neurosci.* 108, 1163–1171. doi: 10.1037/0735-7044.108.6.1163

Pedersen, C. A., and Prange, A. J. Jr. (1979). Induction of maternal behavior in virgin rats after intracerebroventricular administration of oxytocin. *Proc. Natl. Acad. Sci. U S A* 76, 6661–6665. doi: 10.1073/pnas.76.12.6661

Perani, C., and Slattery, D. (2014). Using animal models to study post-partum psychiatric disorders. *Br. J. Pharmacol.* 171, 4539–4555. doi: 10.1111/bph.12640

Pratt, M., Apter-Levi, Y., Vakart, A., Feldman, M., Fishman, R., Feldman, T., et al. (2015). Maternal depression and child oxytocin response; moderation by maternal oxytocin and relational behavior. *Depress Anxiety* 32, 635–646. doi: 10.1002/da.22392

Putnam, K. T., Wilcox, M., Robertson-Blackmore, E., Sharkey, K., Bergink, V., Munk-Olsen, T., et al. (2017). Clinical phenotypes of perinatal depression and time of symptom onset: analysis of data from an international consortium. *Lancet Psychiatry* 4, 477–485. doi: 10.1016/S2215-0366(17)30136-0

Raggenbass, M. (2008). Overview of cellular electrophysiological actions of vasopressin. *Eur. J. Pharmacol.* 583, 243–254. doi: 10.1016/j.ejphar.2007.11.074

Raghavan, N. S., Chen, H., Schipma, M., Luo, W., Chung, S., Wang, L., et al. (2017). Prepubertal ovariectomy exaggerates adult affective behaviors and alters the hippocampal transcriptome in a genetic rat model of depression. *Front. Endocrinol.* 8:373. doi: 10.3389/fendo.2017.00373

Redei, E. E., Solberg, L. C., Kluczynski, J. M., and Pare, W. P. (2001). Paradoxical hormonal and behavioral responses to hypothyroid and hyperthyroid states in the Wistar-Kyoto rat. *Neuropsychopharmacology* 24, 632–639. doi: 10.1016/S0893-133X(00)00229-3

Rich-Edwards, J. W., Kleinman, K., Abrams, A., Harlow, B. L., Mclaughlin, T. J., Joffe, H., et al. (2006). Sociodemographic predictors of antenatal and postpartum depressive symptoms among women in a medical group practice. *J. Epidemiol. Community Health* 60, 221–227. doi: 10.1136/jech.2005.039370

Rosenwasser, A. M. (1993). Circadian drinking rhythms in SHR and WKY rats: effects of increasing light intensity. *Physiol. Behav.* 53, 1035–1041. doi: 10.1016/0031-9384(93)90356-k

Rosenwasser, A. M., and Wirz-Justice, A. (1997). "Circadian rhythms and depression: clinical and experimental models," in *Physiology and Pharmacology of Biological Rhythms*, eds P. H. Redfern and B. Lemmer (Berlin: Springer), 457–486.

Shiromani, P. J., and Overstreet, D. (1994). Free-running period of circadian rhythms is shorter in rats with a genetically upregulated central cholinergic system. *Biol. Psychiatry* 36, 622–626. doi: 10.1016/0006-3223(94)90075-2

Solberg, L. C., Ahmadiyeh, N., Baum, A. E., Vitaterna, M. H., Takahashi, J. S., Turek, F. W., et al. (2003). Depressive-like behavior and stress reactivity are independent traits in a Wistar Kyoto × Fisher 344 cross. *Mol. Psychiatry* 8, 423–433. doi: 10.1038/sj.mp.4001255

Solberg, L. C., Baum, A. E., Ahmadiyeh, N., Shimomura, K., Li, R., Turek, F. W., et al. (2004). Sex- and lineage-specific inheritance of depression-like behavior in the rat. *Mamm. Genome* 15, 648–662. doi: 10.1007/s00335-004-2326-z

Solberg, L. C., Olson, S. L., Turek, F. W., and Redei, E. (2001). Altered hormone levels and circadian rhythm of activity in the WKY rat, a putative animal model of depression. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 281, R786–R794. doi: 10.1152/ajpregu.2001.281.3.R786

Stein, A., Craske, M. G., Lehtonen, A., Harvey, A., Savage-Mcglynn, E., Davies, B., et al. (2012). Maternal cognitions and mother-infant interaction in postnatal depression and generalized anxiety disorder. *J. Abnorm. Psychol.* 121, 795–809. doi: 10.1037/a0026847

Topiwala, A., Hothi, G., and Ebmeier, K. P. (2012). Identifying patients at risk of perinatal mood disorders. *Practitioner* 256, 15–18.

Wallenborn, J. T., Joseph, A.-C., Graves, W. C., and Masho, S. W. (2018). Prepregnancy depression and breastfeeding duration: a look at maternal age. *J. Pregnancy* 2018:4825727. doi: 10.1155/2018/4825727

Wang, S. S., Kamphuis, W., Huitinga, I., Zhou, J. N., and Swaab, D. F. (2008). Gene expression analysis in the human hypothalamus in depression by laser microdissection and real-time PCR: the presence of multiple receptor imbalances. *Mol. Psychiatry* 13, 786–799, 741.doi: 10.1038/mp.2008.38

Weber, E. M., Hultgren, J., Algers, B., and Olsson, I. A. S. (2016). Do laboratory mouse females that lose their litters behave differently around parturition? *PLoS One* 11:e0161238. doi: 10.1371/journal.pone.0161238

Wigger, A., Sánchez, M. M., Mathys, K. C., Ebner, K., Frank, E., Liu, D., et al. (2004). Alterations in central neuropeptide expression, release and receptor binding in rats bred for high anxiety: critical role of vasopressin. *Neuropsychopharmacology* 29, 1–14. doi: 10.1038/sj.npp.1300290

Wilcoxon, J. S., Kuo, A. G., Disterhoft, J. F., and Redei, E. E. (2005). Behavioral deficits associated with fetal alcohol exposure are reversed by prenatal thyroid hormone treatment: a role for maternal thyroid hormone deficiency in FAE. *Mol. Psychiatry* 10, 961–971. doi: 10.1038/sj.mp.4001694

Will, C. C., Aird, F., and Redei, E. E. (2003). Selectively bred Wistar-Kyoto rats: an animal model of depression and hyper-responsiveness to antidepressants. *Mol. Psychiatry* 8, 925–932. doi: 10.1038/sj.mp.4001345

Wisner, K. L., Sit, D. K., Moses-Kolko, E. L., Driscoll, K. E., Prairie, B., Stika, C. S., et al. (2015). Transdermal estradiol treatment for postpartum depression: a pilot randomized trial. *J. Clin. Psychopharmacol.* 35, 389–395. doi: 10.1097/JCP.0000000000000351

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Postnatal Antioxidant and Anti-inflammatory Treatments Prevent Early Ketamine-Induced Cortical Dysfunctions in Adult Mice

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Early brain insult, interfering with its maturation, may result in psychotic-like disturbances in adult life. Redox dysfunctions and neuroinflammation contribute to long-term psychiatric consequences due to neurodevelopmental abnormalities. Here, we investigated the effects of early pharmacological modulation of the redox and inflammatory states, through celastrol, and indomethacin administration, on reactive oxygen species (ROS) amount, levels of malondialdehyde (MDA) and antioxidant enzymes (superoxide dismutase 1, SOD1, glutathione, GSH, and catalase, CAT), as well as of pro-inflammatory cytokines (tumor necrosis factor-alpha, TNF-α, interleukin-6, IL-6, and interleukin-1 beta, IL-1β), in the prefrontal cortex of adult mice exposed to a neurotoxic insult, i.e. ketamine administration, in postnatal life. Early celastrol or indomethacin prevented ketamine-induced elevations in cortical ROS production. MDA levels in ketamine-treated mice, also administered with celastrol, were comparable with the control ones. Indomethacin also prevented the increase in lipid peroxidation following early ketamine administration. Whereas no significant differences were detected in SOD1, GSH, and CAT levels between ketamine and saline-administered mice, celastrol elevated the cortical amount of these antioxidant enzymes and the same effect was induced by indomethacin *per se*. Both celastrol and indomethacin prevented ketamine-induced enhancement in TNF-α and IL-1β levels, however, they had no effects on increased IL-6 amount resulting from ketamine exposure in postnatal life. In conclusion, our data suggest that an early increase in cortical ROS scavenging and reduction of lipid peroxidation, via the enhancement of antioxidant defense, together with inhibition of neuroinflammation, may represent a therapeutic opportunity against psychotic-like disturbances resulting, later in life, from the effects of a neurotoxic insult on the developing brain.

Keywords: celastrol, indomethacin, ketamine, prefrontal cortex, redox, inflammation, animal models

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INTRODUCTION

Early insults affecting the central nervous system (CNS) during crucial phases of its maturation have been reported to induce neurodevelopmental abnormalities. This has been associated with increased risk of developing psychotic-like disturbances in adult life (Hussain and Murray, 2015). In this pathological process, the prefrontal cortex (PFC), characterized by highly

vulnerable cellular populations, has been described as one of the most consistently implicated brain regions (Selemon and Zecevic, 2015).

Multiple molecular mechanisms underlying long-term psychiatric consequences of early brain insults have been proposed. Among them, dysfunctions of the antioxidant enzymes, such as superoxide dismutase 1 (SOD1), glutathione (GSH), and catalase (CAT), have been described (Cabungcal et al., 2013). The expression and activity of these enzymes physiologically occur during key neurodevelopmental phases. Indeed, SOD1, expressed primarily in cortical neurons (Peluffo et al., 2005), has been shown to reach a peak in the second postnatal week (Ceballos-Picot et al., 1992). Similarly, CAT activity in rodent developing CNS has been found to be higher than in the mature brain (Del Maestro and McDonald, 1987; Hamby-Mason et al., 1997), with a maximum observed from postnatal day (PND) 5 to PND 10 (Del Maestro and McDonald, 1987; Aspberg and Tottmar, 1992). Moreover, GSH has been reported to increase and modify the redox state of the cells toward a more reduced condition starting from PND 10 until PND 30 (Galkina et al., 2017). Altered antioxidant defense in the brain may result in increased levels of reactive oxygen species (ROS) and consequent lipid peroxidation in neurons. One of the final products of this biochemical process is malondialdehyde (MDA). Enhanced amount of this highly reactive compound has been reported in the PFC of young mice perinatally exposed to a neurotoxic insult (Del Rio et al., 2005; Tsikas, 2017). The natural compound celastrol, derived from the root of *Tripterygium wilfordii*, pharmacologically modulate ROS amount and antioxidant defense system. It has shown to be effective for a broad range of pathological conditions, including neurodegenerative disorders (Kiaei et al., 2005; Paris et al., 2010; Choi et al., 2014), cerebral ischemia (Li et al., 2012; Jiang et al., 2018) and traumatic brain injury (Eroglu et al., 2014). Moreover, it has been reported to prevent psychotic-like behavioral alterations, oxidative stress and inflammatory imbalance in adult mice exposed to a neurotoxic insult in their postnatal life (Schiavone et al., 2019).

Neuroinflammation is a crucial contributor of long-term psychiatric consequences of early neurodetrimental insults. In particular, the developing brain is characterized by increased vulnerability to proinflammatory cytokines, such as Tumor necrosis factor (TNF)-α, interleukin-6 (IL-6), and interleukin-1 beta (IL-1β) (Hagberg and Mallard, 2005). In this regard, levels of TNF-α were enhanced in the cerebellum of adult mice postnatally exposed to a neurotoxic insult (Schiavone et al., 2019). In addition, non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to exert protective effects on neurodevelopmental processes. This occurs via the inhibition of the synthesis of inflammatory mediators at systemic level and via cyclooxygenase (COX) inhibition at blood brain barrier site (Favrais et al., 2007). Among NSAIDs, indomethacin, a non-selective inhibitor of COX 1 and 2, has been shown to readily pass the blood brain barrier (Parepally et al., 2006; Novakova et al., 2014). It also exerted neuroprotective effects in newborn rodents exposed to hypoxic-ischemic insult (Tutak et al., 2005; Taskin et al., 2009). Accordingly, clinical evidence showed that indomethacin, unlike ibuprofen, might be neuroprotective against the long term effects of cerebral insults, such as ventricular hemorrhage (Favrais et al., 2014).

Postnatal administration of subanesthetic doses of ketamine, a NMDA receptor (NMDA-R) antagonist, is a reliable tool to mimic in rodents an early insult interfering with brain maturation (Frohlich and Van Horn, 2014). In this regard, NMDA-Rs reach their maximum expression in the first 2 weeks of postnatal life. Hence, inhibition of these receptors in this period is associated with increased neuronal damage (Bubenikova-Valesova et al., 2008). Indeed, ketamine exposure during CNS development has been shown to cause a down-regulation of NMDA-Rs located in the PFC resulting in psychiatric-like symptoms in rat adult offspring (Ren et al., 2019). Furthermore, early genetic ablation or ketamine-induced blockade of NMDA-Rs of cortical parvalbumin-expressing GABAergic interneurons can induce in adult animals persistent behavioral deficits, reminiscent of cognitive and negative psychotic symptoms (Jeevakumar et al., 2015). In addition, we have demonstrated that ketamine administration at PNDs 7, 9 and 11 caused psychotic-like neurochemical and behavioral alterations in adult mice (Schiavone et al., 2019, 2020).

Here, we assessed the effects of early pharmacological modulation of the redox and inflammatory states, through celastrol and indomethacin administration, on possible alteration of ROS production, lipid peroxidation, as well as SOD1, GSH, and CAT levels in the PFC, induced by the exposure to an early neurotoxic trigger, i.e., ketamine, in postnatal life. Moreover, celastrol or indomethacin effects on possible ketamine-induced changes of proinflammatory cytokines levels, i.e., TNF-α, IL-6, and IL-1β were also assessed in the same brain region.

MATERIALS AND METHODS

Animals

Mice were housed at constant room temperature (22 ± 1°C) and relative humidity (55 ± 5%), under a 12 h light/dark cycle (lights on from 7:00 AM to 7:00 PM). They had free access to food and water. Experimental procedures involving animals and their care were performed in conformity with the institutional guidelines of the Italian Ministry of Health (D.Lgs. n. 26/2014), the Guide for the Care and Use of Laboratory Animals: Eight Edition, the Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council, 2004), the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, as well as the ARRIVE guidelines. The experimental protocol was approved by the Italian Ministry of Health (approval number 679/2017-PR, protocol n. B2EF8.17) Animal welfare was daily monitored throughout the experimental phase. All efforts were made to minimize the number of animals used, as well as their suffering.

Experimental Protocol

A total of five C57/Bl6 male mice of 8–10 weeks of age, weighting 25–30 g, and ten age and weight-matched adult females (Envigo,

San Pietro al Natisone, Italy) were mated (one male and two females per cage). Male pups were divided into the following experimental groups, according to the different treatments they received at PNDs 7, 9, and 11:

(1) Saline (10 ml/kg i.p.);
(2) Ketamine (Sigma-Aldrich Corporation, Saint Louis, MO, United States; 30 mg/kg i.p., dissolved in saline) (Sorce et al., 2010; Jeevakumar et al., 2015);
(3) Celastrol (Sigma Aldrich, Milano, Italy; 1 mg/kg i.p., dissolved in 50% DMSO/PBS) (Paris et al., 2010; Schiavone et al., 2019);
(4) A 50% DMSO/PBS solution (5 ml/kg i.p.);
(5) Ketamine (30 mg/kg i.p., dissolved in saline, injected in the right side of the peritoneum) and celastrol (1 mg/kg i.p., dissolved in 50% DMSO/PBS, injected in the left side of the peritoneum) (Schiavone et al., 2019)-indicated throughout the text as "ketamine + celastrol";
(6) Indomethacin [Promedica, Parma, Italy, 10 mg/kg i.p., (La Vitola et al., 2018), dissolved in saline];
(7) Ketamine (30 mg/kg i.p., dissolved in saline, injected in the right side of the peritoneum) and indomethacin (10 mg/kg i.p., dissolved in saline, injected in the left side of the peritoneum) – indicated throughout the text as "ketamine + indomethacin".

For ethical reasons, in keeping with the pursuing of 3R requirements foreseen by the Directive 2010/63/EU of the European Parliament, as well as of the Council of 22 September 2010 on the protection of animals used for scientific purposes, the ARRIVE guidelines, and also based on our previous experience (we did not detect any differences between a double with respect to single injection of vehicles), the group consisting of double-vehicle injection was omitted from the experimental protocol.

All pups were grown until adulthood (10 weeks of age). At this time point, they were euthanized by cervical dislocation for PFC collection.

PFC Collection

The PFC of 10-weeks mice was collected by using the Mouse Brain Matrix, making coronal sections of 1 mm of thickness and dissecting it from the obtained brain slices according to the Mouse Brain in Stereotaxic Coordinates, 3rd Edition, Franklin and Paxinos (2015). Immediately after, tissues were frozen in isopentane and stored at −80°C, until biomolecular analyses were performed (Bove et al., 2018).

ROS Measurement

Reactive oxygen species measurement in PFC was performed as previously described (Baek et al., 2018; Pirozzi et al., 2020), by using the fluorogenic dye 2′,7′dichlorofluorescein diacetate (Sigma Aldrich, Milano, Italy) (Kirkland et al., 2007). Briefly, tissue was homogenized in PBS 1× (pH = 7.4) according to the following proportion: 500 µl of PBS 1× for 2,5 mg of tissue. The dye was added to the sample with a final concentration of 5 µM and incubation was performed for 15 min at 37°C. Samples were than centrifuged for 10 min at 4°C and 12,500 rpm. The pellet was resuspended in 5 ml PBS 1× and put in ice for 10 min. After a 1-h incubation at 37°C, samples were analyzed in 96-well microplate by using a fluorometer (Filter Max F5, Multi-Mode Microplate Reader, excitation length 475 nm, emission length 535 nm). Results were expressed as µmol DCF/mg of tissue.

MDA Assay

MDA assay was performed by using a commercially available kit (Sigma-Aldrich, Milano, Italy) as previously described (Fan et al., 2019), according to the manufacturer's instructions. Each sample and standard analysis was performed in duplicate to avoid intra-assay variations.

Enzyme-Linked Immunosorbent Assays

Samples were homogenized in 10 volumes of PBS with protease inhibitors, as previously described (Schiavone et al., 2019). Commercially available Enzyme-Linked Immunosorbent Assay (ELISA) kits were used for measurement of SOD1 (Wuhan Fine Biotech Co., Ltd.-FineTest, Wuhan, China), GSH (Biomatik Life Science Products and Service, Ontario, Canada), CAT (Wuhan Fine Biotech Co., Ltd.-FineTest, Wuhan, China), TNF-α (MyBiosource, San Diego, CA, United States), IL-6 (MyBiosource, San Diego, CA, United States) and IL-1ß (MyBiosource, San Diego, CA, United States) in the PFC, according to the manufacturer's instructions. All samples and standards were analyzed in duplicate to avoid intra-assay variations.

Blindness of the Study

Data analysis was performed by researchers who were blind with respect to the treatment conditions. The blindness of the study was maintained until data analysis ended.

Statistical Analysis

Statistical analysis was performed by using GraphPad 5.0 software for Windows. Data were checked for normality by using Bartlett's test and then analyzed by One Way ANOVA, followed by Tukey's *post hoc* test or Kruskal-Wallis test, followed by Dunn's multiple comparison test. For all tests, a p-value < 0.05 was considered as statistically significant. Results are expressed as means ± mean standard error (SEM). No significant differences in all the considered parameters were detected between saline and 50% DMSO/PBS-treated animals. Therefore, graphs only include results related to saline-treated animals.

RESULTS

Effects of Early Celastrol or Indomethacin Administration on ROS Production and Lipid Peroxidation in the PFC of Adult Mice Treated With Ketamine in Postnatal Life

To assess possible effects of early celastrol or indomethacin administration on ROS production and lipid peroxidation induced by ketamine exposure in postnatal life, we quantified

ROS, and MDA levels in the PFC of adult mice. No significant differences in cortical ROS production were detected between controls and celastrol-injected animals. Ketamine exposure in postnatal life resulted in increased ROS production compared to saline-treated mice which was prevented by ketamine + celastrol administration (**Figure 1A**, One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,12)} = 5.742$, $p < 0.05$). While no significant differences in MDA levels were observed between animals administered with saline and the celastrol group, adult mice that received ketamine in early postnatal life, showed significant MDA elevations in the considered brain region. Early celastrol administration was able to prevent ketamine-induced lipid peroxidation (**Figure 1B**, Kruskal-Wallis test, followed by Dunn's multiple comparison test, Kruskal-Wallis statistic = 9.573, $p < 0.05$). Early ketamine + indomethacin administration prevented ketamine-induced elevation in ROS levels (**Figure 2A**, One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,13)} = 9.642$, $p < 0.05$, and $p < 0.001$). Indomethacin treatment *per se* was able to significantly lower MDA levels with respect to both controls and ketamine-exposed mice. Lipid peroxidation in mice early receiving ketamine + indomethacin was decreased compared to ketamine-treated mice but did not reach the same levels than the ones detected in mice early exposed to indomethacin alone (**Figure 2B**, One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,18)} = 35.10$, $p < 0.01$ and $p < 0.001$).

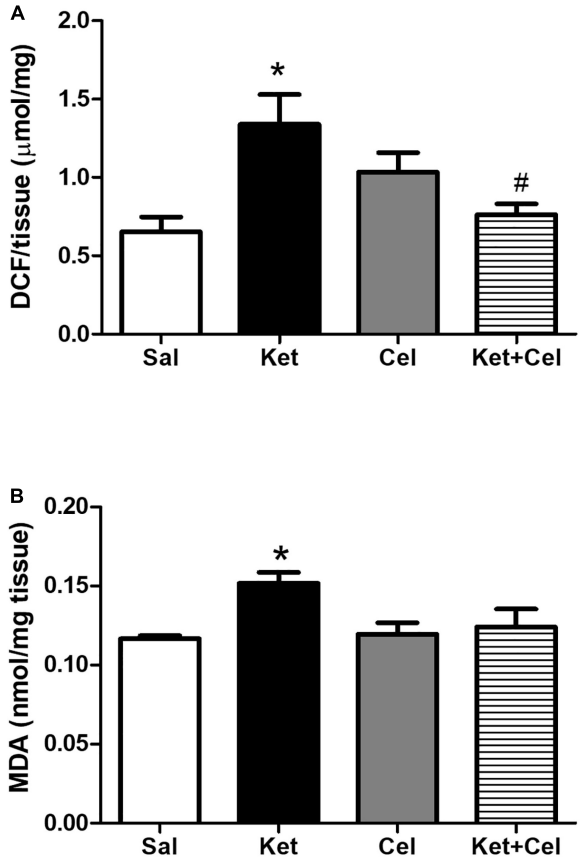

FIGURE 1 | Effects of early celastrol administration on ROS production and MDA levels in the PFC of adult mice, administered with ketamine in postnatal life. **(A)** ROS production (μmol DCF/mg of tissue) in the PFC of 10 weeks mice receiving saline (Sal, $n = 4$) or ketamine (Ket, $n = 4$) or celastrol (Cel, $n = 4$) or ketamine + celastrol (Ket + Cel, $n = 4$) at PNDs 7, 9, and 11. One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,12)} = 5.742$, $^{*}p < 0.05$ Ket vs Sal, $^{\#}p < 0.05$ Ket + Cel vs Ket. **(B)** MDA levels (nmol/mg tissue) in the PFC of 10 weeks mice receiving saline (Sal, $n = 5$) or ketamine (Ket, $n = 8$) or celastrol (Cel, $n = 5$) or ketamine + celastrol (Ket + Cel, $n = 6$) at PNDs 7, 9, and 11. Kruskal-Wallis test, followed by Dunn's multiple comparison test, Kruskal-Wallis statistic = 9.573 $^{*}p < 0.05$ Ket vs Sal.

Effects of Early Celastrol or Indomethacin Administration on Antioxidant Enzyme Expression in the PFC of Adult Mice Treated With Ketamine in Postnatal Life

To investigate the possible impact of early celastrol or indomethacin administration on antioxidant enzyme expression following ketamine exposure in postnatal life, we quantified levels of SOD1, CAT, and GSH in the PFC of adult mice. Whereas comparable SOD1 amount was detected among saline, ketamine and celastrol-treated animals, significant increased expression of this antioxidant enzyme was observed in ketamine + celastrol-treated animals with respect to both saline and ketamine groups (**Figure 3A**, One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,14)} = 5.318$, $p < 0.05$). Early indomethacin treatment *per se* resulted in an increased SOD1 expression with respect to both saline and ketamine-administered mice (**Figure 4A**, One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,12)} = 7.715$, $p < 0.05$ and $p < 0.01$).

No significant differences in GSH levels were detected between early ketamine and saline-treated animals. Increased amount of this enzyme was observed in celastrol-treated animals with respect to the saline and ketamine groups and in animals exposed to ketamine + celastrol compared to ketamine and celastrol-treated mice (**Figure 3B**, One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,16)} = 29.89$, $p < 0.01$, and $p < 0.001$). Administration of indomethacin in early postnatal life, alone or in combination with ketamine, was able to elevate GSH expression with respect to controls and ketamine-treated mice (**Figure 4B**, One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,16)} = 25.59$, $p < 0.01$, $p < 0.001$).

Whereas early ketamine exposure did not affect CAT levels in the PFC of adult mice, increased levels of this antioxidant enzyme were detected in ketamine + celastrol-treated animals compared to ketamine groups (**Figure 3C**, One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,13)} = 5.095$, $p < 0.05$). Levels of this enzyme were enhanced following early administration of indomethacin compared to saline or ketamine-treated mice and this was prevented by ketamine + indomethacin injection (**Figure 4C**, One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,15)} = 7.200$, $p < 0.05$ and $p < 0.01$).

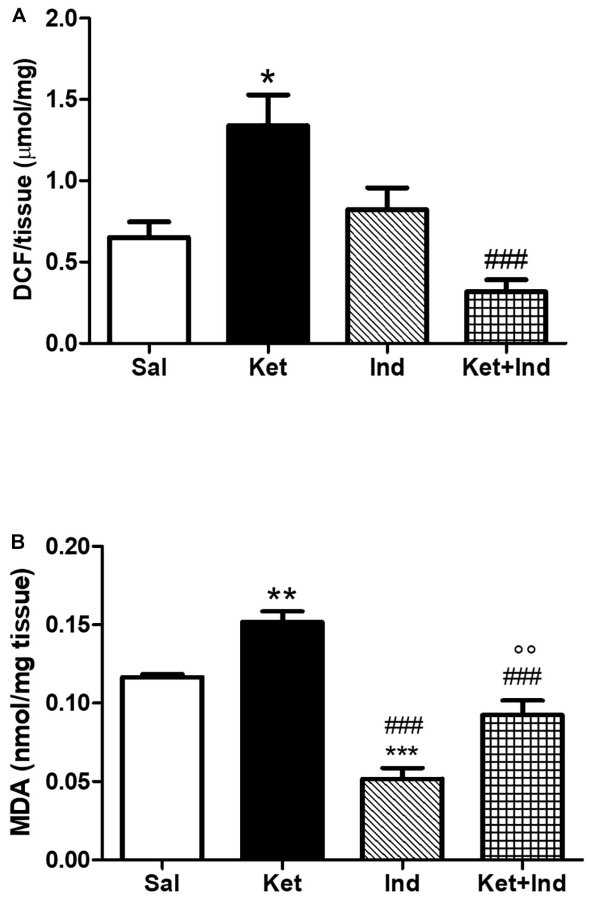

FIGURE 2 | Effects of early indomethacin administration on ROS production and MDA levels in the PFC of adult mice, administered with ketamine in postnatal life. **(A)** ROS production (μmol DCF/mg of tissue) in the PFC of 10 weeks mice receiving saline (Sal, $n = 4$) or ketamine (Ket, $n = 4$) or indomethacin (Ind, $n = 5$) or ketamine + indomethacin (Ket + Ind, $n = 4$) at PNDs 7, 9, and 11. One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,13)} = 9.642$, $^{*}p < 0.05$ Ket vs Sal, $^{\#\#\#}p < 0.001$ Ket + Ind vs Ket. **(B)** MDA levels (nmol/mg tissue) in the PFC of 10 weeks mice receiving saline (Sal, $n = 5$) or ketamine (Ket, $n = 8$) or indomethacin (Ind, $n = 4$) or ketamine + indomethacin (Ket + Ind, $n = 5$) at PNDs 7, 9, and 11. One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,18)} = 35.10$, $^{**}p < 0.01$ Ket vs Sal, $^{***}p < 0.001$ Ind vs Sal, $^{\#\#\#}p < 0.001$ Ind vs Ket and Ket + Ind vs Ket, $^{\circ\circ}p < 0.01$ Ket + Ind vs Ind.

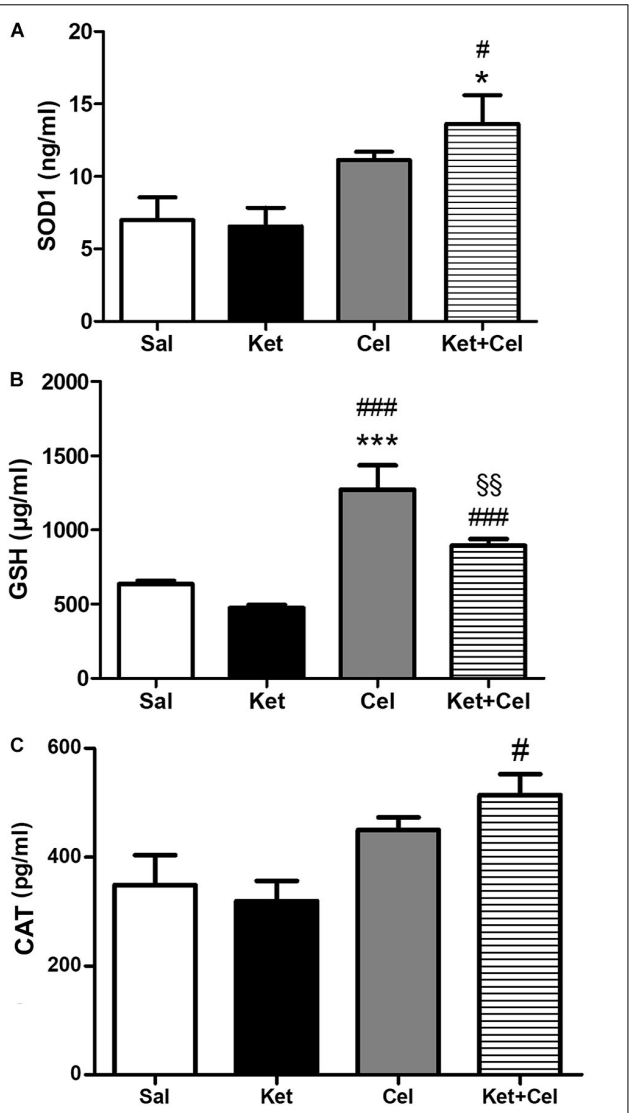

FIGURE 3 | Effects of early celastrol administration on SOD1, GSH, and CAT levels in the PFC of adult mice, administered with ketamine in postnatal life. **(A)** SOD1 levels (ng/ml) in the PFC of 10 weeks mice receiving saline (Sal, $n = 5$) or ketamine (Ket, $n = 5$) or celastrol (Cel, $n = 4$) or ketamine + celastrol (Ket + Cel, $n = 4$) at PNDs 7, 9, and 11. One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,14)} = 5.318$, $^{*}p < 0.05$ Ket + Cel vs Sal, $^{\#}p < 0.05$ Ket + Cel vs Ket. **(B)** GSH levels (μg/ml) in the PFC of 10 weeks mice receiving saline (Sal, $n = 3$) or ketamine (Ket, $n = 7$) or celastrol (Cel, $n = 3$) or ketamine + celastrol (Ket + Cel, $n = 7$) at PNDs 7, 9, and 11. One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,16)} = 29.89$, $^{***}p < 0.001$ Cel vs Sal, $^{\#\#\#}p < 0.001$ Cel vs Ket and Ket + Cel vs Ket, $^{\S\S}p < 0.01$ Ket + Cel vs Cel. **(C)** CAT levels (pg/ml) in the PFC of 10 weeks mice receiving saline (Sal, $n = 4$) or ketamine (Ket, $n = 5$) or celastrol (Cel, $n = 3$) or ketamine + celastrol (Ket + Cel, $n = 5$) at PNDs 7, 9, and 11. One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,13)} = 5.095$, $^{\#}p < 0.05$ Ket + Cel vs Ket.

Effects of Early Celastrol or Indomethacin Administration on Pro-inflammatory Cytokines in the PFC of Adult Mice Treated With Ketamine in Postnatal Life

To evaluate possible effects of early celastrol or indomethacin administration on pro-inflammatory cytokines following ketamine exposure in postnatal life, we quantified levels of TNF-α, IL-1ß, and IL-6 in the PFC of adult mice. Whereas no significant differences in TNF-α amount was detected among saline and celastrol treatments, ketamine administration at PNDs 7, 9, and 11 resulted in TNF-α elevations, which were prevented by early celastrol administration (**Figure 5A**, One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,14)} = 4.708$, $p < 0.05$). Indomethacin, both *per se* and

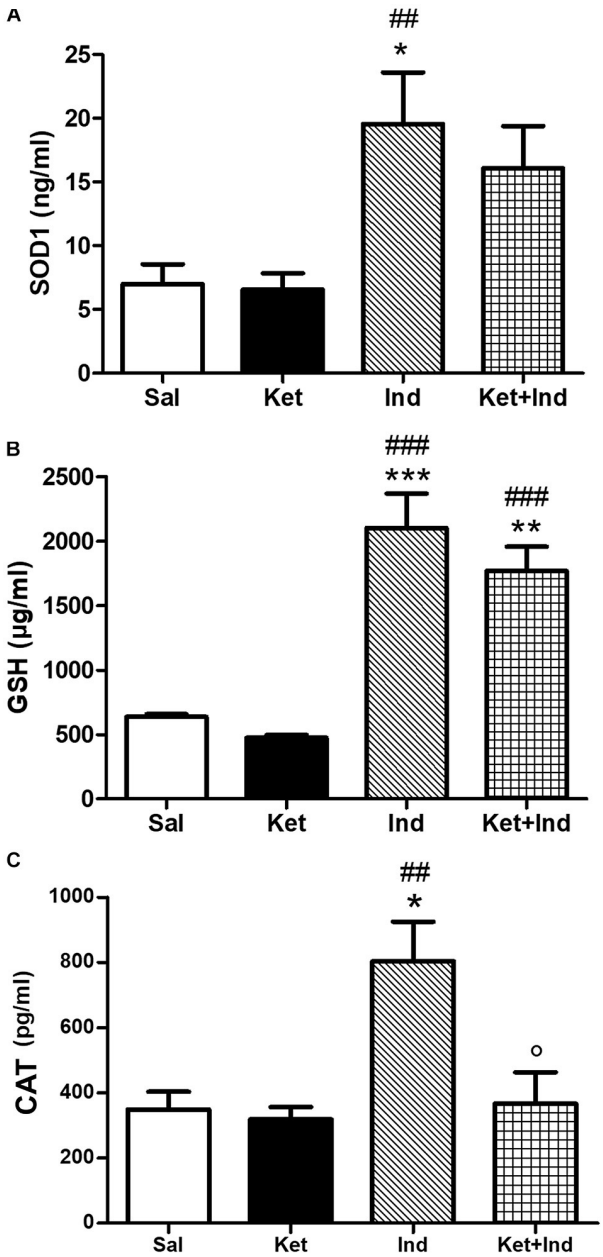

FIGURE 4 | Effects of early indomethacin administration on SOD1, GSH, and CAT levels in the PFC of adult mice, administered with ketamine in postnatal life. **(A)** SOD1 levels (ng/ml) in the PFC of 10 weeks mice receiving saline (Sal, $n = 5$) or ketamine (Ket, $n = 5$) or indomethacin (Ind, $n = 3$) or ketamine + indomethacin (Ket + Ind, $n = 3$). One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,12)} = 7.715$, $^{*}p < 0.05$ Ind vs Sal, $^{\#\#}p < 0.01$ Ind vs Ket. **(B)** GSH levels (µg/ml) in the PFC of 10 weeks mice receiving saline (Sal, $n = 3$) or ketamine (Ket, $n = 7$) or indomethacin (Ind, $n = 5$) or ketamine + indomethacin (Ket + Ind, $n = 5$). One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,16)} = 25.59$, $^{***}p < 0.001$ Ind vs Sal, $^{**}p < 0.01$ Ket + Ind vs Sal, $^{\#\#\#}p < 0.001$ Ind and Ket + Ind vs Ket. **(C)** CAT levels (pg/ml) in the PFC of 10 weeks mice receiving saline (Sal, $n = 4$) or ketamine (Ket, $n = 5$) or indomethacin (Ind, $n = 5$) or ketamine + indomethacin (Ket + Ind, $n = 5$). One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,15)} = 7.200$, $^{*}p < 0.05$ Ind vs Sal, $^{\#\#}p < 0.01$ Ind vs Ket, $p < 0.05$ Ind + Ket vs Ind.

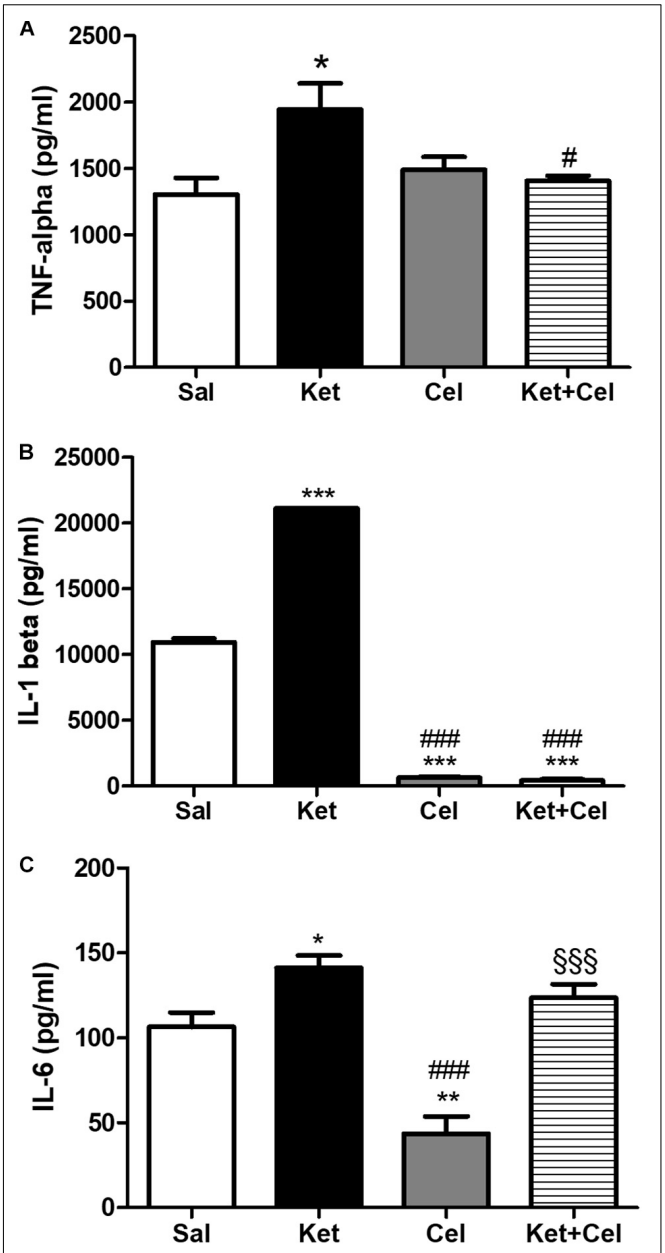

FIGURE 5 | Effects of early celastrol administration on TNF-α and IL-1ß levels in the PFC of adult mice, administered with ketamine in postnatal life. **(A)** TNF-α levels (pg/ml) in the PFC of 10 weeks mice receiving saline (Sal, $n = 4$) or ketamine (Ket, $n = 5$) or celastrol (Cel, $n = 4$) or ketamine + celastrol (Ket + Cel, $n = 5$) at PNDs 7, 9, and 11. One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,14)} = 4.708$; $^{*}p < 0.05$ Ket vs Sal; $^{\#}p < 0.05$ Ket + Cel vs Ket. **(B)** IL-1ß levels (pg/ml) in the PFC of 10 weeks mice receiving saline (Sal, $n = 3$) or ketamine (Ket, $n = 4$) or celastrol (Cel, $n = 3$) or ketamine + celastrol (Ket + Cel, $n = 4$) at PNDs 7, 9, and 11. One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,10)} = 5538$, $^{***}p < 0.001$ Ket, Cel and Ket + Cel vs Sal; $^{\#\#\#}p < 0.001$ Cel and Ket + Cel vs Ket. **(C)** IL-6 levels (pg/ml) in the PFC of 10 weeks mice receiving saline (Sal, $n = 5$) or ketamine (Ket, $n = 5$) or celastrol (Cel, $n = 3$) or ketamine + celastrol (Ket + Cel, $n = 5$) at PNDs 7, 9, and 11. One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,14)} = 20.08$, $^{*}p < 0.05$ Ket vs Sal; $^{**}p < 0.01$ Cel vs Sal, $^{\#\#\#}p < 0.001$ Cel vs Ket, $^{§§§}p < 0.001$ Ket + Cel vs Cel.

concomitantly administered with ketamine, was able to decrease TNF-α levels compared to controls and ketamine-exposed mice (**Figure 6A**, One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,13)} = 45.20$, $p < 0.05$, and $p < 0.001$).

Cortical levels of IL-1ß in adult mice were enhanced following ketamine administration in early life compared to saline-treated animals. Celastrol treatment was able to significantly decrease amount of these pro-inflammatory cytokines in the PFC and this was also observed when it was administered with ketamine (**Figure 5B**, One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,10)} = 5538$, $p < 0.001$). Despite IL-1ß amount was significantly lowered by indomethacin *per se* compared to both saline and ketamine-treated mice, levels of this cytokine following early ketamine + indomethacin administration were comparable to the ones detected in the saline group, although still significantly decreased with respect to ketamine-exposed mice (**Figure 6B**, One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,11)} = 39.92$, $p < 0.01$, and $p < 0.001$).

Early ketamine treatment resulted in increased IL-6 levels in the PFC of adult mice. Pups that received only celastrol or indomethacin showed, at adulthood, a significant decrease of this cytokine compared to both saline and ketamine-administered animals. However, in mice concomitantly injected with ketamine, no significant differences were detected compared to animals that received saline or ketamine in early life (**Figure 5C**, One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,14)} = 20.08$, $p < 0.05$, $p < 0.01$, and $p < 0.001$ and **Figure 6C**, One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,13)} = 18.41$, $p < 0.05$, $p < 0.01$, and $p < 0.001$).

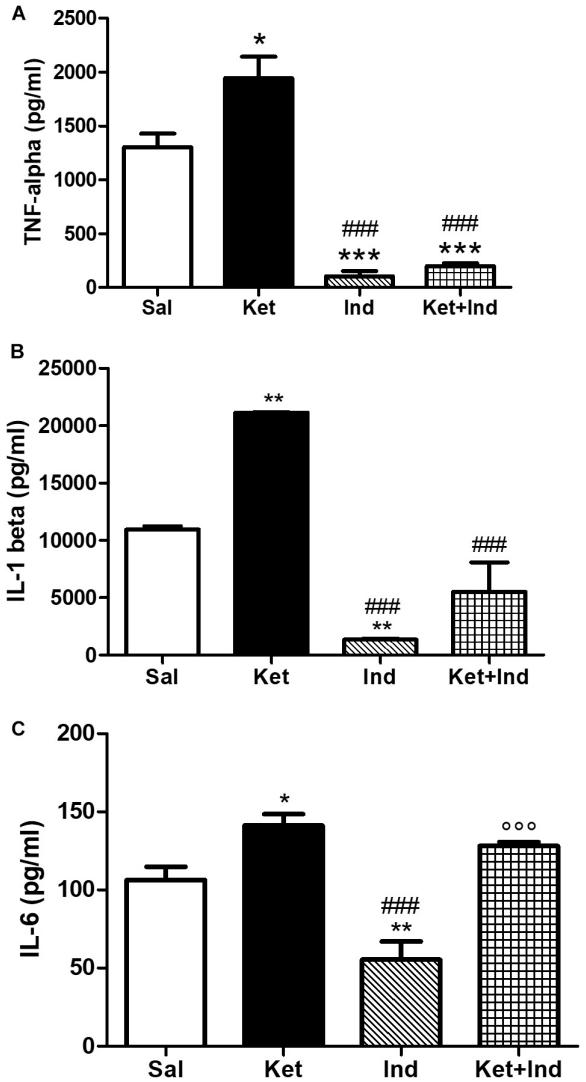

FIGURE 6 | Effects of early indomethacin administration on TNF-α and IL-1ß levels in the PFC of adult mice, administered with ketamine in postnatal life. **(A)** TNF-α levels (pg/ml) in the PFC of 10 weeks mice receiving saline (Sal, $n = 4$) or ketamine (Ket, $n = 5$) or indomethacin (Ind, $n = 4$) or ketamine + indomethacin (Ket + Ind, $n = 4$) at PNDs 7, 9, and 11. One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,13)} = 45.20$, $^{*}p < 0.05$ Ket vs Sal, $^{***}p < 0.001$ Ind and Ket + Ind vs Sal, $^{\#\#\#}p < 0.001$ Ind and Ket + Ind vs Ket. **(B)** IL-1ß levels (pg/ml) in the PFC of 10 weeks mice receiving saline (Sal, $n = 3$) or ketamine (Ket, $n = 4$) or indomethacin (Ind, $n = 4$) or ketamine + indomethacin (Ket + Ind, $n = 4$) at PNDs 7, 9, and 11. One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,11)} = 39.92$, $^{**}p < 0.01$ Ket and Ind vs Sal, $^{\#\#\#}p < 0.001$ Ind and Ket + Ind vs Ket. **(C)** IL-6 levels (pg/ml) in the PFC of 10 weeks mice receiving saline (Sal, $n = 5$) or ketamine (Ket, $n = 5$) or indomethacin (Ind, $n = 4$) or ketamine + indomethacin (Ket + Ind, $n = 3$) at PNDs 7, 9, and 11. One way ANOVA, followed by Tukey's Multiple Comparison *post hoc* test, $F_{(3,13)} = 18.41$, $^{*}p < 0.05$ Ket vs Sal, $^{**}p < 0.01$ Ind vs Sal, $^{\#\#\#}p < 0.001$ Ind vs Ket, $^{°°}p < 0.001$ Ket + Ind vs Ind.

DISCUSSION

In this work, we demonstrated that administration of subanesthetic doses of ketamine at PNDs 7, 9, and 11 caused increased ROS production in the PFC of adult mice. Supporting these findings, previous observations, obtained on the same animal model, showed both early and persistent increased levels of 8-hydroxydeoxyguanosine (8OHdG), an indirect marker of oxidative stress, and NOX2, a ROS-producing enzyme, in the same brain region (Schiavone et al., 2020). Accordingly, it has been reported that, although oxidative stress contributes to the physiological postnatal brain development in rodents, the effects of increased ROS levels on the CNS, following an external insult, might be revealed later in life (Wilhelm et al., 2016). Moreover, antioxidant treatment with N-acetyl cysteine in mice could prevent cognitive and behavioral dysfunctions at adulthood, resulting from ketamine administration at PNDs 7, 9, and 11 (Phensy et al., 2017).

Here, we also observed enhanced lipid peroxidation after early ketamine exposure. In good agreement with this finding, ketamine-induced increase in MDA content in the cortex of young rodents, associated with elevations in levels of indirect markers of oxidative stress were previously reported (Cheung and Yew, 2019). Interestingly, changes in brain lipid peroxidation have been described during early postnatal development, with a physiological decrease in adult animals (Galkina et al., 2009). Indeed, during the neonatal period, brain has been reported

to have low peroxidation potential corresponding to the rapid phase of cell proliferation (Pushpendran et al., 1994). Dysfunctions of this process, induced by an external trigger, such as the exposure to NMDA-R antagonists, have been shown to result in the persistence of high levels of lipid peroxidation at adulthood, contributing to the development of psychotic-like neuropathological and behavioral dysfunctions in rodents (de Carvalho Cartágenes et al., 2019), as well as neuropsychiatric disorders in humans (Joshi and Pratico, 2014; Romano et al., 2017).

An important finding of our study consists in the lack of significant differences in cortical amounts of antioxidant enzymes between early ketamine-treated mice and controls. This result might appear contrasting with preclinical evidence describing, instead, a decreased activity of SOD and CAT in the PFC of ketamine-treated rats (de Oliveira et al., 2009), as well as a reduction of GSH concentration (Abdel-Salam et al., 2015). However, in these previously published studies, ketamine exposure occurred in adult life. Furthermore, the lack of differences in levels of antioxidant enzymes observed in our experimental conditions might be also interpreted as a long-term dysfunction, induced by early ketamine exposure, of the physiological roles of the antioxidant system in controlling ROS damage and in regulating ROS signaling (Wang et al., 2018).

Here, we also demonstrated that early celastrol treatment prevented ketamine-induced increased lipid peroxidation and ROS production in the PFC of adult mice. Different mechanisms of action have been proposed to describe celastrol pharmacological effects. Among these, the induction of the expression of neuroprotective factors, the block of ROS-induced cellular apoptosis and the decrease of oxidative stress have been reported (Chen et al., 2018). In particular, the reduction of oxidative stress also occurs via the inhibition of the NADPH oxidase NOX enzymes with an increased potency against NOX1 and NOX2 isoforms, resulting in the lack of the functional association between the cytosolic subunits and the membrane flavocytochrome of these enzymes (Jaquet et al., 2011; Tarafdar and Pula, 2018). The effect of celastrol on ketamine-induced increase in MDA and ROS levels observed in the present study is in line with previous evidence obtained on the same animal model, where celastrol administration in postnatal life was found to prevent ketamine-induced elevations in cerebellar expression of 8OHdG, a marker of oxidative damage to DNA, and of the ROS producing enzyme NADPH oxidase NOX1 (Schiavone et al., 2019). Intriguingly, in our experimental conditions, the reduction of cortical ROS amount and lipid peroxidation induced by early celastrol administration was accompanied by increased levels of SOD1, GSH and CAT with respect to mice receiving only ketamine in postnatal life. Supporting our hypothesis, previous preclinical evidence have reported that celastrol could attenuate oxidative damage by increasing levels and activity of SOD, GSH, glutathione peroxidase, glutathione reductase, and CAT (Shaker et al., 2014; Wang et al., 2014; Boran et al., 2019; Gao et al., 2020). Although still speculative, the effects of celastrol on cortical ROS levels and lipid peroxidation, at least in our experimental conditions, might be explained by a concomitant action of this compound on ROS production and their degradation, finally resulting in the recovery of the redox balance, early altered by ketamine. Indeed, it might be hypothesized that celastrol administration may inhibit ketamine-induced increase in NOX2 expression observed in the PFC of mice pups and, consequently, the persistent elevations of this enzyme in adult life (Schiavone et al., 2020). On the other side of the redox balance, celastrol exposure in the early phases of postnatal life might result in the recovery of the physiological role of the antioxidant system in response to ketamine-induced oxidative stress elevations. Hence, ketamine-induced increase of ROS production and consequent lipid peroxidation might be prevented by the synergistic action of these two hypothesized mechanisms.

We also showed that early administration of indomethacin *per se* could decrease MDA levels in the PFC of adult mice treated with ketamine in early life. This finding might appear in contrast with a previous report showing an aggravation of lipid peroxidation, in terms of increased MDA levels, in newborn rats with hypoxic-ischemic cerebral injury following indomethacin administration (Taskin et al., 2009). However, the result obtained in our experimental conditions might be explained by both the different time point at which this parameter (adult life) was evaluated and the kind of insult impacting on the developing brain. Indeed, in this regard, previous evidence reported a beneficial effect of indomethacin in reducing peripheral and central MDA levels following rat exposure to other neurotoxic insult, such as CCl_4 (Kadiiska et al., 2005). Moreover, cortical increase of lipid peroxidation, induced in rats by an infectious insult, was also found to be reduced following intraperitoneal indomethacin administration (Guzman et al., 2018). However, we cannot totally exclude that indomethacin *per se* could induce an increase in MDA levels in the early phases of postnatal life, thus stimulating in the developing brain the activation of neuroprotective mechanisms that may result in the decreased lipid peroxidation we observed in adult mice.

Interestingly, indomethacin *per se* induced elevations in levels of all the considered antioxidant enzymes compared to both controls and early ketamine-exposed mice. Thus, it might be hypothesized that increased antioxidant defense might be a possible mechanism underlying indomethacin effects on cortical lipid peroxidation. Supporting our hypothesis, COX inhibition has been reported to significantly improve antioxidant defense both at peripheral and central levels (Kumar et al., 2011; Ahmed et al., 2014). Concomitant administration of ketamine and indomethacin could prevent the cortical increase in ROS amount and in MDA levels induced by early ketamine exposure, suggesting a neuroprotective role of this compound against the impact that a neurodetrimental insult might have on the developing brain. In support of this hypothesis, indomethacin has been reported to prevent the loss of neurogenesis markers following a neurotoxic insult, i.e., ethanol in adolescent rodents (Vetreno et al., 2018). Furthermore, indomethacin was able to regulate the peripheral expression of neurotrophins, such as BDNF and NGF (Kemi et al., 2006; Hochstrasser et al., 2013). Hence, it can be hypothesized that this might also happen at central level following an early neurotoxic insult affecting CNS development. Moreover, clinical evidence reported the use of indomethacin as neuroprotective strategy to prevent the later consequences of neonatal brain injury (Favrais et al., 2014),

via the strengthening of the immature blood-brain barrier (Sims, 2012).

In this manuscript, it is also showed that ketamine administration in early life stages caused an enhancement of proinflammatory cytokines, i.e., TNF-α, IL-1ß, and IL-6, at adulthood. In good agreement with our findings, previous preclinical and clinical reports highlighted a crucial role of early and persistent cortical neuroinflammation in the development of psychotic-like symptoms in rodents (Schiavone et al., 2017; Ben-Azu et al., 2019; Kogan et al., 2019) as well as of schizophrenia in humans (Zhang et al., 2016; Barron et al., 2017). Importantly, it has been reported that dysregulation of the redox, immune and glutamatergic systems, induced by NMDA-R antagonists, including ketamine, especially when it occurs during brain development, represents a "central hub" in schizophrenia pathophysiology (Steullet et al., 2016). In line with this concept, in the same animal model, together with increased levels of pro-inflammatory cytokines, we do observe increased cortical ROS amount and lipid peroxidation and we previously demonstrated early and persistent increase of oxidative damage to DNA as well as alterations of NADPH expression in the same brain region (Schiavone et al., 2020).

In our experimental conditions, together with an effect of celastrol *per se* on IL-1ß and of indomethacin *per se* on TNF-α and IL-1ß, we also found that early celastrol or indomethacin administrations were able to prevent ketamine-induced elevations in TNF-α and IL-1ß in PFC of adult mice, suggesting a possible protective role of these two compounds against the possible long-lasting detrimental effects exerted by early neuroinflammation on the developing brain (Franceschini and Zusso, 2019). This result should also be considered in the light of previous evidence showing a reduction of microglia activation following celastrol (Dai et al., 2019) or indomethacin (Lopes et al., 2016) administration, as well as a strict interrelation between TNF-α and IL-1ß and redox dysregulation in CNS disorders (Fischer and Maier, 2015; Schiavone and Trabace, 2017). Indeed, it might be hypothesized that ketamine administration in postnatal life may cause microglia activation, with consequent release of TNF-α and IL-1ß which, in turn, induce ROS production, further sustaining neuroinflammation and neuronal damage. In addition, the possible inhibition of ketamine-induced enhancement of microglial NADPH oxidase NOX2 by celastrol administration might also play a key role in this process. Hence, in this regard, it has been highlighted that NOX2 activation in microglia exerts neurotoxic effects via extracellular ROS production as well as the initiation of microglia redox signaling, finally resulting in the amplification of the pro-inflammatory response (Surace and Block, 2012).

Despite the decrease in cortical IL-6 levels detected following celastrol or indomethacin treatments alone compared to both controls and ketamine-exposed mice, these two compounds, administered concomitantly with ketamine, could not prevent elevations of this pro-inflammatory cytokine in postnatal life. This result should be considered in the light of the physiological expression of IL-6 and its receptor in rodent cortex during postnatal development (Gadient and Otten, 1994), as well as of the central role reported for this cytokine in the promotion of postnatal murine CNS development, most likely being perturbations in its levels the cause of long-lasting and irreversible damage (Storer et al., 2018). However, we cannot totally exclude that a possible effects of celastrol or indomethacin on ketamine-induced dysfunctions of IL-6 levels might have been detected at different time points from ketamine exposure, such as during mice adolescence or later than 10 weeks of life. Further investigations are certainly needed in this sense, also considering the physiological link existing between IL-6 and anti-inflammatory cytokines (Ropelle et al., 2010), as well as the role of the pro-inflammatory/anti-inflammatory balance in neurodevelopmental-related mental disturbances (Ratnayake et al., 2013).

With respect to a possible translation of the results of the present study to clinics, a limitation consists in the fact that, in animals, pharmacological treatments were initiated at the same time of ketamine administration. Indeed, this same therapeutic strategy cannot be directly translated into the clinical setting, because of the impossibility to identify in humans the exact time of the neurotoxic insult.

In conclusion, our data suggest that both the enhancement of antioxidant defense, reducing cerebral oxidative stress and inhibition of inflammatory pathways, may represent a suitable therapeutic approach preventing psychotic-like disturbances resulting from the impact of neurotoxic insult during crucial phases of brain maturation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Italian Ministry of Health (approval number 679/2017-PR, protocol n. B2EF8.17).

AUTHOR CONTRIBUTIONS

MB, PT, LT, SS, and MGM designed the research. MB, PT, SD, SS, and MGM performed the research. MB, PT, SS, and MGM analyzed the data. MB, PT, LT, and SS wrote the manuscript. MB, PT, SD, LT, SS, and MGM revised the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Abdel-Salam, O. M. E., Youness, E. R., Mohammed, N. A., Omara, E. A., and Sleem, A. A. (2015). Effect of ketamine on oxidative stress following lipopolysaccharide administration. *Comp. Clin. Pathol.* 24, 53–63. doi: 10.1007/s00580-013-1854-x

Ahmed, S., Gul, S., Gul, H., Zia-Ul-Haq, M., and Ercisli, S. (2014). Cyclooxygenase-2 inhibition improves antioxidative defense during experimental hypercholesterolemia. *Bosn J. Basic Med. Sci.* 14, 63–69. doi: 10.17305/bjbms.2014.2264

Aspberg, A., and Tottmar, O. (1992). Development of antioxidant enzymes in rat brain and in reaggregation culture of fetal brain cells. *Brain Res. Dev. Brain Res.* 66, 55–58. doi: 10.1016/0165-3806(92)90139-n

Baek, S. H., Cho, Y., Lee, J., Choi, B. Y., Choi, Y., Park, J. S., et al. (2018). Intracellular and mitochondrial reactive oxygen species measurement in primary cultured neurons. *Bio Protoc.* 8:e2871. doi: 10.21769/BioProtoc.2871

Barron, H., Hafizi, S., Andreazza, A. C., and Mizrahi, R. (2017). Neuroinflammation and oxidative stress in psychosis and psychosis risk. *Int. J. Mol. Sci.* 18:651. doi: 10.3390/ijms18030651

Ben-Azu, B., Aderibigbe, A. O., Ajayi, A. M., Eneni, A. O., Omogbiya, I. A., Owoeye, O., et al. (2019). Morin decreases cortical pyramidal neuron degeneration via inhibition of neuroinflammation in mouse model of schizophrenia. *Int. Immunopharmacol.* 70, 338–353. doi: 10.1016/j.intimp.2019.02.052

Boran, T., Gunaydin, A., Jannuzzi, A. T., Ozcagli, E., and Alpertunga, B. (2019). Celastrol pretreatment as a therapeutic option against cisplatin-induced nephrotoxicity. *Toxicol. Res. (Camb)* 8, 723–730. doi: 10.1039/c9tx00141g

Bove, M., Ike, K., Eldering, A., Buwalda, B., de Boer, S. F., Morgese, M. G., et al. (2018). The visible burrow system: a behavioral paradigm to assess sociability and social withdrawal in BTBR and C57BL/6J mice strains. *Behav. Brain Res.* 344, 9–19. doi: 10.1016/j.bbr.2018.02.003

Bubenikova-Valesova, V., Horacek, J., Vrajova, M., and Hoschl, C. (2008). Models of schizophrenia in humans and animals based on inhibition of NMDA receptors. *Neurosci. Biobehav. Rev.* 32, 1014–1023. doi: 10.1016/j.neubiorev.2008.03.012

Cabungcal, J. H., Steullet, P., Kraftsik, R., Cuenod, M., and Do, K. Q. (2013). Early-life insults impair parvalbumin interneurons via oxidative stress: reversal by N-acetylcysteine. *Biol. Psychiatry* 73, 574–582. doi: 10.1016/j.biopsych.2012.09.020

Ceballos-Picot, I., Nicole, A., Clement, M., Bourre, J. M., and Sinet, P. M. (1992). Age-related changes in antioxidant enzymes and lipid peroxidation in brains of control and transgenic mice overexpressing copper-zinc superoxide dismutase. *Mutat Res.* 275, 281–293. doi: 10.1016/0921-8734(92)90032-k

Chen, S. R., Dai, Y., Zhao, J., Lin, L., Wang, Y., and Wang, Y. (2018). A Mechanistic overview of triptolide and celastrol, natural products from *Tripterygium wilfordii* Hook F. *Front. Pharmacol.* 9:104. doi: 10.3389/fphar.2018.00104

Cheung, H. M., and Yew, D. T. W. (2019). Effects of perinatal exposure to ketamine on the developing brain. *Front. Neurosci.* 13:138. doi: 10.3389/fnins.2019.00138

Choi, B. S., Kim, H., Lee, H. J., Sapkota, K., Park, S. E., Kim, S., et al. (2014). Celastrol from 'Thunder God Vine' protects SH-SY5Y cells through the preservation of mitochondrial function and inhibition of p38 MAPK in a rotenone model of Parkinson's disease. *Neurochem. Res.* 39, 84–96. doi: 10.1007/s11064-013-1193-y

Dai, W., Wang, X., Teng, H., Li, C., Wang, B., and Wang, J. (2019). Celastrol inhibits microglial pyroptosis and attenuates inflammatory reaction in acute spinal cord injury rats. *Int. Immunopharmacol.* 66, 215–223. doi: 10.1016/j.intimp.2018.11.029

de Carvalho Cartágenes, S., Fernandes, L. M. P., Carvalheiro, T. C. V. S., de Sousa, T. M., Gomes, A. R. Q., Monteiro, M. C., et al. (2019). "Special K" drug on adolescent rats: oxidative damage and neurobehavioral impairments. *Oxidat. Med. Cell. Longev.* 2019:5452727. doi: 10.1155/2019/5452727

de Oliveira, L., Spiazzi, C. M., Bortolin, T., Canever, L., Petronilho, F., Mina, F. G., et al. (2009). Different sub-anesthetic doses of ketamine increase oxidative stress in the brain of rats. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 33, 1003–1008. doi: 10.1016/j.pnpbp.2009.05.010

Del Maestro, R., and McDonald, W. (1987). Distribution of superoxide dismutase, glutathione peroxidase and catalase in developing rat brain. *Mech. Ageing Dev.* 41, 29–38. doi: 10.1016/0047-6374(87)90051-0

Del Rio, D., Stewart, A. J., and Pellegrini, N. (2005). A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr. Metab. Cardiovasc. Dis.* 15, 316–328. doi: 10.1016/j.numecd.2005.05.003

Eroglu, B., Kimbler, D. E., Pang, J., Choi, J., Moskophidis, D., Yanasak, N., et al. (2014). Therapeutic inducers of the HSP70/HSP110 protect mice against traumatic brain injury. *J. Neurochem.* 130, 626–641. doi: 10.1111/jnc.12781

Fan, L. M., Geng, L., Cahill-Smith, S., Liu, F., Douglas, G., McKenzie, C. A., et al. (2019). Nox2 contributes to age-related oxidative damage to neurons and the cerebral vasculature. *J. Clin. Invest.* 129, 3374–3386. doi: 10.1172/JCI125173

Favrais, G., Schwendimann, L., Gressens, P., and Lelievre, V. (2007). Cyclooxygenase-2 mediates the sensitizing effects of systemic IL-1-beta on excitotoxic brain lesions in newborn mice. *Neurobiol. Dis.* 25, 496–505. doi: 10.1016/j.nbd.2006.10.012

Favrais, G., Tourneux, P., Lopez, E., Durrmeyer, X., Gascoin, G., Ramful, D., et al. (2014). Impact of common treatments given in the perinatal period on the developing brain. *Neonatology* 106, 163–172. doi: 10.1159/000363492

Fischer, R., and Maier, O. (2015). Interrelation of oxidative stress and inflammation in neurodegenerative disease: role of TNF. *Oxid. Med. Cell Longev.* 2015:610813. doi: 10.1155/2015/610813

Franceschini, D., and Zusso, M. (2019). Editorial: neuroinflammation in the developing brain. *Int. J. Dev. Neurosci.* 77, 1–2. doi: 10.1016/j.ijdevneu.2019.05.007

Frohlich, J., and Van Horn, J. D. (2014). Reviewing the ketamine model for schizophrenia. *J. Psychopharmacol.* 28, 287–302. doi: 10.1177/0269881113512909

Gadient, R. A., and Otten, U. (1994). Expression of interleukin-6 (IL-6) and interleukin-6 receptor (IL-6R) mRNAs in rat brain during postnatal development. *Brain Res.* 637, 10–14. doi: 10.1016/0006-8993(94)91211-4

Galkina, O. V., Bakhtyukov, A. A., Akhmetshin, M. O., Prokopenko, V. M., and Eshchenko, N. D. (2017). The glutathione system in the subcellular fractions of developing rat brain and liver. *Neurochem. J.* 11, 266–271. doi: 10.1134/s1819712417030047

Galkina, O. V., Putilina, F. E., Romanova, A. A., and Eshchenko, N. D. (2009). Changes in lipid peroxidation and antioxidant system of the brain during early postnatal development in rats. *Neurochem. J.* 3, 93–97. doi: 10.1134/S1819712409020032

Gao, Q., Qin, H., Zhu, L., Li, D., and Hao, X. (2020). Celastrol attenuates collagen-induced arthritis via inhibiting oxidative stress in rats. *Int. Immunopharmacol.* 84:106527. doi: 10.1016/j.intimp.2020.106527

Guzman, D. C., Herrera, M. O., Brizuela, N. O., Mejia, G. B., Garcia, E. H., Olguin, H. J., et al. (2018). Oseltamivir and indomethacin reduce the oxidative stress in brain and stomach of infected rats. *APMIS* 126, 128–134. doi: 10.1111/apm.12794

Franklin, K. B. J., and Paxinos, G. (2015). *The Mouse Brain in Stereotaxic Coordinates*, 3rd Edn. Cambridge, MA: Academic Press.

Hagberg, H., and Mallard, C. (2005). Effect of inflammation on central nervous system development and vulnerability. *Curr. Opin. Neurol.* 18, 117–123. doi: 10.1097/01.wco.0000162851.44897.8f

Hamby-Mason, R., Chen, J. J., Schenker, S., Perez, A., and Henderson, G. I. (1997). Catalase mediates acetaldehyde formation from ethanol in fetal and neonatal rat brain. *Alcohol Clin. Exp. Res.* 21, 1063–1072. doi: 10.1111/j.1530-0277.1997.tb04255.x

Hochstrasser, T., Ehrlich, D., Sperner-Unterweger, B., and Humpel, C. (2013). Antidepressants and anti-inflammatory drugs differentially reduce the release of NGF and BDNF from rat platelets. *Pharmacopsychiatry* 46, 29–34. doi: 10.1055/s-0032-1314843

Hussain, H., and Murray, G. K. (2015). Common childhood neurodevelopmental disorders are associated with increased risk of psychotic experiences in early adolescence. *Evid. Based Ment. Health* 18:51. doi: 10.1136/eb-2014-102044

Jaquet, V., Marcoux, J., Forest, E., Leidal, K. G., McCormick, S., Westermaier, Y., et al. (2011). NADPH oxidase (NOX) isoforms are inhibited by celastrol with a dual mode of action. *Br. J. Pharmacol.* 164, 507–520. doi: 10.1111/j.1476-5381.2011.01439.x

Jeevakumar, V., Driskill, C., Paine, A., Sobhanian, M., Vakil, H., Morris, B., et al. (2015). Ketamine administration during the second postnatal week induces enduring schizophrenia-like behavioral symptoms and reduces parvalbumin expression in the medial prefrontal cortex of adult mice. *Behav. Brain Res.* 282, 165–175. doi: 10.1016/j.bbr.2015.01.010

Jiang, M., Liu, X., Zhang, D., Wang, Y., Hu, X., Xu, F., et al. (2018). Celastrol treatment protects against acute ischemic stroke-induced brain injury by promoting an IL-33/ST2 axis-mediated microglia/macrophage M2 polarization. *J. Neuroinflamm.* 15:78. doi: 10.1186/s12974-018-1124-6

Joshi, Y. B., and Pratico, D. (2014). Lipid peroxidation in psychiatric illness: overview of clinical evidence. *Oxid. Med. Cell Longev.* 2014:828702. doi: 10.1155/2014/828702

Kadiiska, M. B., Gladen, B. C., Baird, D. D., Graham, L. B., Parker, C. E., Ames, B. N., et al. (2005). Biomarkers of oxidative stress study III. Effects of the nonsteroidal anti-inflammatory agents indomethacin and meclofenamic acid on measurements of oxidative products of lipids in CCl4 poisoning. *Free Radic. Biol. Med.* 38, 711–718. doi: 10.1016/j.freeradbiomed.2004.10.024

Kemi, C., Grunewald, J., Eklund, A., and Hoglund, C. O. (2006). Differential regulation of neurotrophin expression in human bronchial smooth muscle cells. *Respir Res.* 7:18. doi: 10.1186/1465-9921-7-18

Kiaei, M., Kipiani, K., Petri, S., Chen, J., Calingasan, N. Y., and Beal, M. F. (2005). Celastrol blocks neuronal cell death and extends life in transgenic mouse model of amyotrophic lateral sclerosis. *Neurodegener. Dis.* 2, 246–254. doi: 10.1159/000090364

Kirkland, R. A., Saavedra, G. M., Franklin, J. L. (2007). Rapid activation of antioxidant defenses by nerve growth factor suppresses reactive oxygen species during neuronal apoptosis: evidence for a role in cytochrome c redistribution. *J. Neurosci.* 27, 11315–11326. doi: 10.1523/JNEUROSCI.3590-07.2007

Kogan, S., Ospina, L. H., Mittal, V. A., and Kimhy, D. (2019). The impact of inflammation on neurocognition and risk for psychosis: a critical review. *Eur. Arch. Psychiatry Clin. Neurosci.* 270, 793–802. doi: 10.1007/s00406-019-01073-2

Kumar, P., Kalonia, H., and Kumar, A. (2011). Role of LOX/COX pathways in 3-nitropropionic acid-induced Huntington's disease-like symptoms in rats: protective effect of licofelone. *Br. J. Pharmacol.* 164, 644–654. doi: 10.1111/j.1476-5381.2011.01418.x

La Vitola, P., Balducci, C., Cerovic, M., Santamaria, G., Brandi, E., Grandi, F., et al. (2018). Alpha-synuclein oligomers impair memory through glial cell activation and via Toll-like receptor 2. *Brain Behav. Immun.* 69, 591–602. doi: 10.1016/j.bbi.2018.02.012

Li, Y., He, D., Zhang, X., Liu, Z., Zhang, X., Dong, L., et al. (2012). Protective effect of celastrol in rat cerebral ischemia model: down-regulating p-JNK, p-c-Jun and NF-kappaB. *Brain Res.* 1464, 8–13. doi: 10.1016/j.brainres.2012.04.054

Lopes, R. S., Cardoso, M. M., Sampaio, A. O., Barbosa, M. S. Jr., Souza, C. C., Mc, D. A. S., et al. (2016). Indomethacin treatment reduces microglia activation and increases numbers of neuroblasts in the subventricular zone and ischaemic striatum after focal ischaemia. *J. Biosci.* 41, 381–394. doi: 10.1007/s12038-016-9621-1

National Research Council (2004). *National Research Council (US) Committee on Guidelines for the use of Animals in Neuroscience and Behavioral Research*. Washington, DC: National Academies Press.

Novakova, I., Subileau, E. A., Toegel, S., Gruber, D., Lachmann, B., Urban, E., et al. (2014). Transport rankings of non-steroidal antiinflammatory drugs across blood-brain barrier in vitro models. *PLoS One* 9:e86806. doi: 10.1371/journal.pone.0086806

Parepally, J. M., Mandula, H., and Smith, Q. R. (2006). Brain uptake of nonsteroidal anti-inflammatory drugs: ibuprofen, flurbiprofen, and indomethacin. *Pharm. Res.* 23, 873–881. doi: 10.1007/s11095-006-9905-5

Paris, D., Ganey, N. J., Laporte, V., Patel, N. S., Beaulieu-Abdelahad, D., Bachmeier, C., et al. (2010). Reduction of beta-amyloid pathology by celastrol in a transgenic mouse model of Alzheimer's disease. *J. Neuroinflammation* 7:17. doi: 10.1186/1742-2094-7-17

Peluffo, H., Acarin, L., Faiz, M., Castellano, B., and Gonzalez, B. (2005). Cu/Zn superoxide dismutase expression in the postnatal rat brain following an excitotoxic injury. *J. Neuroinflammation* 2:12. doi: 10.1186/1742-2094-2-12

Phensy, A., Duzdabanian, H. E., Brewer, S., Panjabi, A., Driskill, C., Berz, A., et al. (2017). Antioxidant treatment with N-acetyl cysteine prevents the development of cognitive and social behavioral deficits that result from perinatal ketamine treatment. *Front. Behav. Neurosci.* 11:106. doi: 10.3389/fnbeh.2017.00106

Pirozzi, C., Lama, A., Annunziata, C., Cavaliere, G., De Caro, C., Citraro, R., et al. (2020). Butyrate prevents valproate-induced liver injury: in vitro and in vivo evidence. *FASEB J.* 34, 676–690. doi: 10.1096/fj.201900927RR

Pushpendran, C. K., Subramanian, M., and Devasagayam, T. P. (1994). Developmental changes in the peroxidation potential of rat brain homogenate and mitochondria. *Mech. Ageing Dev.* 73, 197–208. doi: 10.1016/0047-6374(94)90052-3

Ratnayake, U., Quinn, T., Walker, D. W., and Dickinson, H. (2013). Cytokines and the neurodevelopmental basis of mental illness. *Front. Neurosci.* 7:180. doi: 10.3389/fnins.2013.00180

Ren, W., Liu, X., Cheng, L., Wang, G., Liu, X., Peng, L., et al. (2019). Embryonic ketamine produces a downregulation of prefrontal cortex NMDA receptors and anxiety-like behavior in adult offspring. *Neuroscience* 415, 18–30. doi: 10.1016/j.neuroscience.2019.07.018

Romano, A., Serviddio, G., Calcagnini, S., Villani, R., Giudetti, A. M., Cassano, T., et al. (2017). Linking lipid peroxidation and neuropsychiatric disorders: focus on 4-hydroxy-2-nonenal. *Free Radic. Biol. Med.* 111, 281–293. doi: 10.1016/j.freeradbiomed.2016.12.046

Ropelle, E. R., Flores, M. B., Cintra, D. E., Rocha, G. Z., Pauli, J. R., Morari, J., et al. (2010). IL-6 and IL-10 anti-inflammatory activity links exercise to hypothalamic insulin and leptin sensitivity through IKKbeta and ER stress inhibition. *PLoS Biol.* 8:e1000465. doi: 10.1371/journal.pbio.1000465

Schiavone, S., Mhillaj, E., Neri, M., Morgese, M. G., Tucci, P., Bove, M., et al. (2017). Early loss of blood-brain barrier integrity precedes NOX2 elevation in the prefrontal cortex of an animal model of psychosis. *Mol. Neurobiol.* 54, 2031–2044. doi: 10.1007/s12035-016-9791-8

Schiavone, S., Morgese, M. G., Bove, M., Colia, A. L., Maffione, A. B., Tucci, P., et al. (2020). Ketamine administration induces early and persistent neurochemical imbalance and altered NADPH oxidase in mice. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 96:109750. doi: 10.1016/j.pnpbp.2019.109750

Schiavone, S., and Trabace, L. (2017). Inflammation, stress response, and redox dysregulation biomarkers: clinical outcomes and pharmacological implications for psychosis. *Front. Psychiatry* 8:203. doi: 10.3389/fpsyt.2017.00203

Schiavone, S., Tucci, P., Trabace, L., and Morgese, M. G. (2019). Early celastrol administration prevents ketamine-induced psychotic-like behavioral dysfunctions, oxidative stress and IL-10 reduction in the cerebellum of adult mice. *Molecules* 24:3993. doi: 10.3390/molecules24213993

Selemon, L. D., and Zecevic, N. (2015). Schizophrenia: a tale of two critical periods for prefrontal cortical development. *Transl. Psychiatry* 5:e623. doi: 10.1038/tp.2015.115

Shaker, M. E., Ashamallah, S. A., and Houssen, M. E. (2014). Celastrol ameliorates murine colitis via modulating oxidative stress, inflammatory cytokines and intestinal homeostasis. *Chem. Biol. Interact.* 210, 26–33. doi: 10.1016/j.cbi.2013.12.007

Sims, B. (2012). Neuroprotective strategies in neonatal brain injury. *Pediatr. Ther.* 2:e117. doi: 10.4172/2161-0665.1000e117

Sorce, S., Schiavone, S., Tucci, P., Colaianna, M., Jaquet, V., Cuomo, V., et al. (2010). The NADPH oxidase NOX2 controls glutamate release: a novel mechanism involved in psychosis-like ketamine responses. *J. Neurosci.* 30, 11317–11325. doi: 10.1523/JNEUROSCI.1491-10.2010

Steullet, P., Cabungcal, J. H., Monin, A., Dwir, D., O'Donnell, P., Cuenod, M., et al. (2016). Redox dysregulation, neuroinflammation, and NMDA receptor hypofunction: a "central hub" in schizophrenia pathophysiology? *Schizophr. Res.* 176, 41–51. doi: 10.1016/j.schres.2014.06.021

Storer, M. A., Gallagher, D., Fatt, M. P., Simonetta, J. V., Kaplan, D. R., and Miller, F. D. (2018). Interleukin-6 regulates adult neural stem cell numbers during normal and abnormal post-natal development. *Stem Cell Reports* 10, 1464–1480. doi: 10.1016/j.stemcr.2018.03.008

Surace, M. J., and Block, M. L. (2012). Targeting microglia-mediated neurotoxicity: the potential of NOX2 inhibitors. *Cell Mol. Life Sci.* 69, 2409–2427. doi: 10.1007/s00018-012-1015-4

Tarafdar, A., and Pula, G. (2018). The role of NADPH oxidases and oxidative stress in neurodegenerative disorders. *Int. J. Mol. Sci.* 19:3824. doi: 10.3390/ijms19123824

Taskin, E., Ozcan, K., Canacankatan, N., Satar, M., Yapicioglu, H. Y., and Erdogan, S. (2009). The effects of indomethacin on caspases, glutathione level and lipid peroxidation in the newborn rats with hypoxic-ischemic cerebral injury. *Brain Res.* 1289, 118–123. doi: 10.1016/j.brainres.2009.07.010

Tsikas, D. (2017). Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: analytical and biological challenges. *Anal. Biochem.* 524, 13–30. doi: 10.1016/j.ab.2016.10.021

Tutak, E., Satar, M., Zorludemir, S., Erdogan, S., Yapicioglu, H., and Narli, N. (2005). Neuroprotective effects of indomethacin and aminoguanidine in the

newborn rats with hypoxic-ischemic cerebral injury. *Neurochem. Res.* 30, 937–942. doi: 10.1007/s11064-005-5978-5

Vetreno, R. P., Lawrimore, C. J., Rowsey, P. J., and Crews, F. T. (2018). Persistent adult neuroimmune activation and loss of hippocampal neurogenesis following adolescent ethanol exposure: blockade by exercise and the anti-inflammatory drug indomethacin. *Front. Neurosci.* 12:200. doi: 10.3389/fnins.2018.00200

Wang, C., Shi, C., Yang, X., Yang, M., Sun, H., and Wang, C. (2014). Celastrol suppresses obesity process via increasing antioxidant capacity and improving lipid metabolism. *Eur. J. Pharmacol.* 744, 52–58. doi: 10.1016/j.ejphar.2014.09.043

Wang, Y., Branicky, R., Noe, A., and Hekimi, S. (2018). Superoxide dismutases: dual roles in controlling ROS damage and regulating ROS signaling. *J. Cell Biol.* 217, 1915–1928. doi: 10.1083/jcb.201708007

Wilhelm, J., Vytasek, R., Uhlik, J., and Vajner, L. (2016). Oxidative stress in the developing rat brain due to production of reactive oxygen and nitrogen species. *Oxid. Med. Cell Longev.* 2016:5057610. doi: 10.1155/2016/5057610

Zhang, Y., Catts, V. S., Sheedy, D., McCrossin, T., Kril, J. J., and Shannon Weickert, C. (2016). Cortical grey matter volume reduction in people with schizophrenia is associated with neuro-inflammation. *Transl. Psychiatry* 6:e982. doi: 10.1038/tp.2016.238

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Oculomotor Behavior as a Biomarker for Differentiating Pediatric Patients With Mild Traumatic Brain Injury and Age Matched Controls

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Importance: Children have the highest incidence of mild traumatic brain injury (mTBI) in the United States. However, mTBI, specifically pediatric patients with mTBI, are notoriously difficult to detect, and with a reliance on traditional, subjective measurements of eye movements, the subtle but key oculomotor deficits are often missed.

Objective: The purpose of this project is to determine if the combined measurement of saccades, smooth pursuit, fixations and reaction time represent a biomarker for differentiating pediatric patients with mild traumatic brain injury compared to age matched controls.

Design: This study used cross-sectional design. Each participant took part in a suite of tests collectively labeled the "Brain Health EyeQ" to measure saccades, smooth pursuit, fixations and reaction time.

Participants: The present study recruited 231 participants – 91 clinically diagnosed with a single incident mTBI in the last 2 days as assessed by both the Glasgow Coma Scale (GCS) and Graded Symptoms Checklist (GSC), and 140 age and gender-matched controls ($n = 165$ male, $n = 66$ female, M age $= 14.20$, $SD = 2.78$).

Results: One-way univariate analyses of variance examined the differences in performance on the tests between participants with mTBI and controls. ROC curve analysis examined the sensitivity and specificity of the tests. Results indicated that together, the "Brain Health EyeQ" tests were successfully able to identify participants with mTBI 75.3% of the time, providing further validation to a growing body of literature supporting the use of eye tracking technology for mTBI identification and diagnosis.

Keywords: eye-tracking, oculomotor, mTBI, concussion, pediateric case

INTRODUCTION

Mild traumatic brain injury (mTBI) occurs about once every 15 s, and the excessive frequency of these injuries costs the United States more than $77 billion dollars annually (Langlois et al., 2004; Prins et al., 2013). Ninety percent of TBI's are classified as mild (Langlois et al., 2004; Howell et al., 2018). Clinical diagnosis of mTBI is determined by the American Congress of Rehabilitation Medicine (ACRM) definition in which "a patient with a mTBI is a person who has had a traumatically induced physiological disruption of brain function, as induced by one of the following: a loss of consciousness, any memory loss, any alteration of mental state, and/or focal neurological deficits (Bazarian et al., 2005)."

Pediatric head injury is extremely common (Schunk and Schutzman, 2012). mTBI is the most common form of head injury accounting for 75–85% of these injuries (Goldstein and Levin, 1987). Children have the highest incidence of mTBI. In the United States, mTBI occurs in 692 of 100,000 children younger than 15 years of age (Guerrero et al., 2000). Identification of pediatric mTBI differs from adult mTBI due to age-related anatomical and physiological differences, pattern of injuries based on the physical ability of the child, and difficulty in neurological evaluation in children (Araki et al., 2017). Evidence suggests that children exhibit a specific pathological response to TBI with distinct accompanying neurological symptoms (Araki et al., 2017).

An important factor contributing to this epidemic is the fact that concussions are often hard to diagnose and therefore treat (Howell et al., 2018). Most symptoms are relatively subjective and easily attributed to other conditions (Howell et al., 2018). Therefore, it is essential to build on established means of mTBI detection that are both objective and reliable (Howell et al., 2018). Currently, there are three accepted branches to mTBI diagnosis: neurological, vestibular, and oculomotor (Sussman et al., 2016). In the past, most of the oculomotor assessment was carried out subjectively through examination by clinicians, with objective measurements of symptoms, rare (Bedell and Stevenson, 2013). Research suggests that subjective measurements of eye movements are more likely to miss subtle deficits, which makes the need for reliable, objective symptom detection increasingly important. One uniquely powerful method of objectively measuring eye movements can be achieved through eye-tracking technology (Bedell and Stevenson, 2013). Eye-tracking can be used to study neurological function, oculomotor assessment and can detect abnormalities in neurocircuitry and map oculomotor dysfunction to damaged sites (Bedell and Stevenson, 2013; Lai et al., 2013; Johnson et al., 2015).

Oculomotor assessment can be further divided into the measurement of four specific types of eye movements. These include saccades, smooth pursuits, fixations, and reaction time (Land and Tatler, 2009; Leigh and Zee, 2015; Lange et al., 2018). Saccades are short and fast eye movements between fixed points; smooth pursuits use predictive tracking to stabilize moving targets, fixations are even smaller movements that focus an image on the fovea, and reaction time is the time elapsed between a sensory stimulus and the response to it (Land and Tatler, 2009; Leigh and Zee, 2015; Lange et al., 2018). Each of these different eye movements activates different parts of the brain (Wong, 2007; Møllenbach et al., 2013).

The Saccadic system focuses on the rapid movements of the fovea between fixation points (Wong, 2007). Several different brain structures are involved in the regulation of saccades, including the brain stem, pons, midbrain, and cerebral cortex (Wong, 2007). Burst neuron circuits in the brainstem are responsible for the motor signals that control the extraocular muscles in the eyes that generate saccades (Wong, 2007). There is a division of labor between the pons and the midbrain, with the pons primarily involved in generating horizontal saccades and the midbrain primarily involved in generating vertical saccades (Wong, 2007). In addition, because eye movements are closely related to cognitive behaviors in higher mammals, the cerebral cortex also plays an important role in the function of saccades both directly through the burst neuron circuit, and via the superior colliculus (Wong, 2007).

The smooth pursuit system is what allows humans to predictively track moving objects (Wong, 2007; Møllenbach et al., 2013). Because the complete smooth pursuit pathway is so complex, it is not yet completely understood (Wong, 2007). First, visual information is relayed from the striate cortex to the extrastriate areas, which contain specialized neurons that encode both eye and object movement (Wong, 2007). These extrastriate areas have connections to the brain stem, which communicates information to the cerebellum. This explains why researchers have recently found functional similarity between the saccadic and smooth pursuit systems (Wong, 2007). Pursuits are controlled primarily by a network of cortical areas, including the frontal eye field and other structures such as the superior colliculus and basal ganglia (Wong, 2007). Vertical smooth pursuits and horizon pursuits have similar pathways differing only at a spot in the pons, the y-group, and the cerebellum (Wong, 2007).

Fixations hold a stationary object on the fovea while the head is not moving and prevent the image from fading (Wong, 2007; Leigh and Zee, 2015). This process is active and involves a network of brain regions, including the parietal eye field, V5 and V5A areas, supplemental eye field, and dorsolateral prefrontal cortex (Wong, 2007). The brain stem and part of the basal ganglia and the superior colliculus are involved, although specific functions are not localized to one area. Instead, they are distributed across several (Munoz, 2002; Wong, 2007). Fixations operate like a simple negative feedback loop in which the drifting movements of the eye (not the actual target) trigger the tracking mechanism to return the eye to the target (Leigh and Zee, 2015). This behavior explains the constant microsaccades characteristic of fixations; it's simply the gaze repeatedly returning to the target (Leigh and Zee, 2015).

Reaction time (RT) is a measure of attention (Zomeren and Brouwer, 1994). However, the applications of RT assessment are much more numerous than just measuring attention. RT has been found in numerous studies to be a marker of CNS damage and neuropathology, including mTBI (Knopman, 1991; Murtha et al., 2002; Lange et al., 2018). RT can also be used to evaluate

a person's motor skill or to determine how well they interact with their environment. RT itself is the time elapsed between the presentation of stimuli and the behavioral response (Shelton and Kumar, 2010). RT assessments can be split up into simple reaction time (SRT), choice reaction time (CRT) and discriminate reaction time (DRT) (Lange et al., 2018). SRT is a single response to a single stimulus, CRT is multiple responses to multiple stimuli and DRT is a single response to one of the multiple stimuli (Lange et al., 2018). Traditional measurements of RT often fail to account for eye-specific RT metrics, including saccadic latency, visual speed, and visual processing speed (Lange et al., 2018). Eye-tracking does measure these values, and this greater level of detail provides valuable information during RT assessment (Lange et al., 2018).

Currently, pediatric mTBIs are diagnosed using a variety of measures such as level of consciousness and length of post-traumatic amnesia (Maruta et al., 2010; Levin and Diaz-Arrastia, 2015). The Glasgow Coma Score (GCS) is commonly used to evaluate consciousness on a 13–15 scale for mTBI that accounts for a motor response, verbal response, and eye-opening ability (Arbour et al., 2016). However, the GCS is widely used but not necessarily the best measure of pediatric mTBI (Ghaffarpasand et al., 2013). Furthermore, clinicians do not usually use imagining for pediatric mTBI cases (Oakes, 2018). Therefore, The Graded Symptoms Checklist (GSC) in the Standardized Assessment of Concussion (SAC) was also used as a secondary clinical tool for measurement of mTBI as recommended by the Journal of the American Medical Association Pediatrics clinical guidelines (Adjorlolo, 2018; Lumba-Brown et al., 2018a,b). Though numerous, current methods of concussion detection are often subjective or lacking in their oculomotor components (Ventura et al., 2015). Eye tracking is capable of delivering precise and objective measurements to assist in mTBI diagnosis, and this is why it is so important to consider (Komogortsev and Karpov, 2013).

Compromised saccades, smooth pursuits, fixations, and reaction time have all been linked to mTBI. Numerous studies have found compromised saccades in patients with mTBI such as prolonged latencies and directional errors on memory-guided and antisaccades tasks and impaired self-paced saccades (Williams et al., 1997; Heitger et al., 2002; Johnson et al., 2015; DiCesare et al., 2017). Both vertical and horizontal saccades have been shown to differ in patients with mTBI, and saccades of patients with mTBI have been found especially deficient under conditions of high cognitive load (Ettenhofer et al., 2018; Hunfalvay et al., 2019). Several studies have also found deficits in smooth pursuits in patients with mTBI (Heitger et al., 2009; Hoffer et al., 2017). Patients with mTBI have been shown to have both reduced prediction and more position errors (Suh et al., 2006a,b; Armstrong, 2018). mTBI patients have also been found to have increased error and variability in gaze position and reduced smooth pursuit velocity in tracking tests (Maruta et al., 2014). Another study found that fixational errors for mTBI patients were abnormally high with evidence of increased drift, saccadic intrusions, and nystagmus (Ciuffreda et al., 2004). Though fixations do not have as much focus in current literature, this is only further reason to continue to study them. Several studies exist that consider the impact mTBI has on reaction time (MacFlynn et al., 1984; Hetherington et al., 1996; Hugenholtz et al., 1998). mTBI patients have been found to have reduced processing speed as it relates to reaction time, along with increased reaction time overall (Suh et al., 2006b; Lange et al., 2018).

Between the four eye-movements being considered, there are a plethora of studies the look at the impact of mTBI, however, none exist that consider all these components together. Nor is there much research conducted specifically on the oculomotor behavior of pediatric patients with mTBI. Nevertheless, these metrics can distinguish between mTBI and Controls, and so it stands to reason that all together, they represent a superior method of mTBI detection. Of the four factors considered, fixations especially are in need of more research. Further investigation is also necessary to determine how the four metrics interact with each other, and how the combined ability to distinguish mTBI differs from the individual capacities. The purpose of this study was to compare Brain Health EyeQ score (a composite of saccades, smooth pursuits, fixations, and reaction time) of pediatric patients with clinically diagnosed mTBI and age matched controls. A secondary purpose was to examine the reaction time responses in a choice and discriminate reaction time task.

MATERIALS AND METHODS

Participants

Data from two-hundred and thirty-one participants were analyzed. One hundred and sixteen were clinically diagnosed as having a mTBI within 2 days of the assessment. Twenty-five of these participants were excluded (see procedure), leaving 91 total participants with mTBI. One-hundred and forty participants were age and gender matched controls. Participants were between the ages of 6–18 years ($M = 14.20$, $SD = 2.78$); 165 were males (71.4%), 66 were females (28.6%). Of the 231 participants, 68.8% were White, 3.0% were Hispanic, 0.4% were Asians, 7.4% were Black, and 20.4% opted not to report ethnicity. The groups were matched by age (see **Table 1**).

Clinical Diagnosis of mTBI for Pediatric Patients

All participants had been clinically assessed by Board Certified neurologists with at least 5 years' experience in diagnosing TBIs. Clinical diagnosis of mTBI was based on the American Congress of Rehabilitation Medicine (ACRM) definition of mTBI (Mild Traumatic Brain Injury Committee, 1993). All participants were examined using the GCS and scored between 13 and 15 on the scale. However, the GCS is widely used but not

TABLE 1 | Demographic data by age and gender.

Group (n)	Mean Age (±SD)	Females	Males
Control (140)	14.31 (2.48)	39	101
mTBI (91)	14.13 (2.97)	27	64

n, Number; SD, Standard Deviation.

necessarily the best measure of pediatric mTBI (Ghaffarpasand et al., 2013). Furthermore, clinicians do not usually use imagining for pediatric mTBI cases (Oakes, 2018). Therefore, The Graded Symptoms Checklist (GSC) in the Standardized Assessment of Concussion (SAC) was also used as a secondary clinical tool for measurement of mTBI as recommended by the Journal of the American Medical Association Pediatrics clinical guidelines (Lumba-Brown et al., 2018a,b). Using results from Grubenhoff et al. (2010) and the American Academy of Neurology concussion grading scale pediatric patients (6–18 years of age) were evaluated as having mTBI if their GSC score was between 7.7 and 19.3 (Kelly et al., 1991; Grubenhoff et al., 2010). According to Grubenhoff et al. (2010) this yielded a 95% confidence interval for case-patients with an AAN grade 1 TBI (7.7–10.7) or grade 2 TBI (11.5–19.3) (Grubenhoff et al., 2010). Therefore, participants in the mTBI group in this study scored between 13–15 on the GCS and 7.7–19.3 on the GSC.

Apparatus

The RightEye tests were presented on a Tobii I15 vision 15″ monitor fitted with a Tobii 90 Hz remote eye tracker and a Logitech (model Y-R0017) wireless keyboard and mouse. The participants were seated in a stationary (non-wheeled) chair that could not be adjusted in height. They sat in front of a desk in a quiet, private room. Participants' heads were unconstrained. The accuracy of the Tobii eye tracker was 0.4° within the desired headbox of 32 cm × 21 cm at 56 cm from the screen. For standardization of testing, participants were asked to sit in front of the eye-tracking system at a distance of 56 cm (ideal positioning within the virtual headbox range of the eye tracker).

The Brain Health EyeQ Score (BHEQ)

The Brain Health EyeQ Score (BHEQ) includes a combination of saccade, pursuit, fixation and simple reaction time oculomotor variables. A total of 58 metrics make-up the testing model. Weights range from 0.1 to 13% across metrics. More about the individual tests and metrics can be found in published papers mentioned above (Lange et al., 2018; Hunfalvay et al., 2019; Murray et al., 2019). The metrics associated with the BHEQ score all passed reliability standards (Murray et al., 2019). Extreme gradient boosting (XGB) was used for the classification task using the Rworker GitHub repository R language version 3.5.2. The efficacy of the model was evaluated using accuracy of classification. This model also outputs the importance (weights) that each variable has on the classification accuracy. These weights were then applied to the respective metrics (variables) to calculate the percentile value of a participant compared to his/her peers within the same age group. The percentiles are then aggregated over all metrics that collapse into specific tests to calculate overall scores and percentile on that test; for example, all metrics that create circular smooth pursuit (CSP), horizontal smooth pursuit (HSP), and visual smooth pursuit (VSP) tests were used to calculate overall percentile and score for the test. Results revealed pursuit test weighting 60.93% (CSP: 8.4%; HSP: 40.4%; VSP: 12.13%); self-paced saccade test weighted 24.95% (horizontal saccade (HS): 15.57%; vertical saccade (VS): 9.38%); and fixation test contributed 14.2% weighting of the model.

Reaction Time Tasks

In addition to the BHEQ, we examined separately Choice Reaction Time (CRT) and Discriminate Reaction Time (DRT; see Lange et al., 2018 for further details). In brief, the CRT test, the participant viewed three stimuli and was asked to provide one of three responses. In the DRT test, the participant viewed three stimuli and was required to respond to only one stimulus.

Procedure

Participants were recruited through RightEye clinical providers. The study was conducted in accordance with the tenets of the Declaration of Helsinki. The study protocols were approved by the Institutional Review Board of East Carolina University. The nature of the study was explained to the participants and all participants provided written consent to participate. Participants were excluded from the study they had more than a single discrete episode of mTBI ($n = 21$). Following informed consent, participants were asked to complete a prescreening questionnaire and an acuity vision screening where they were required to identify four shapes at 4 mm in diameter. If any of the prescreening questions were answered positively and any of the vision screening shapes were not correctly identified, then the participant was excluded from the study ($n = 3$). Participants were excluded from the study if they reported any of the following conditions, which may have prevented successful test calibration during the prescreening process: this included vision-related issues such as extreme tropias, phorias, static visual acuity of >20/400, nystagmus, cataracts or eyelash impediments or if they had consumed drugs or alcohol within 24 h of testing ($n = 1$) (Han et al., 2010; Holmqvist and Nystrom, 2011; Renard et al., 2015; Kooiker et al., 2016; Niehorster et al., 2017). Participants were also excluded if they were unable to pass a nine-point calibration sequence. Less than 1% of the participants fell into these categories.

Qualified participants who successfully passed the nine-point calibration sequence completed the eye-tracking tests. The calibration sequence required participants to fixate one at a time on nine points displayed on the screen. The participants had to successfully fixate on at least eight out of nine points on the screen to pass the calibration sequence. Written instructions on screen and animations were provided before each test to demonstrate appropriate behavior required in each of the tests. The testing lasted less than 5 min to complete.

Data Analysis

The differences in the groups (control, mTBI) were analyzed on clinically verified data using JMP PRO 14.0 (SAS Institute, Cary, NC, United States). The comparison was evaluated using one-way univariate ANOVAs on the Brain Health EyeQ score, Choice Reaction Time measures (saccadic latency, visual speed, processing speed, and reaction time), and Discriminate RT measures (saccadic latency, visual speed, processing speed, and reaction time). The alpha level was set at $p < 0.05$ and Omega squared (ω^2) was used to determine effect size. In addition, a series of ROC curve analysis were plotted for the Oculomotor variables. Significant area under the curve (AUC) with 95% confidence intervals ($p < 0.05$) was used to indicate the ability of each variable to differentiate concussed participants

from non-concussed. We set our criteria for a satisfactorily accurate area under the curve (AUC) to the standard of least of 0.7 (Adjorlolo, 2018). We calculated cut-off points, sensitivity, specificity, and positive and negative predictive value (PPV and NPV, respectively) for each significant AUC. Optimal cut-off points were determined by visually assessing which score combines maximum sensitivity and specificity.

RESULTS

The ANOVA results for Brain Health EyeQ Score demonstrated a significant main effect for Group [$F(1,229) = 21.906$; $p < 0.001$, $\omega^2 = 0.89$]. The data revealed a significant difference between mTBI group ($M = 53.98$, $SD = 20.75$) and the Control group ($M = 67.52$, $SD = 21.92$; **Figure 1**). Further a logistic regression analysis was conducted to evaluate how well the criterion variable BHEQ predicted mTBI status (see **Figure 2**). The mTBI status was significantly related to the BHEQ, $\chi^2 = 27.31$; $p < 0.0001$, Nagelkerke $R^2 = 0.185$.

Choice Reaction Time (CRT)

The ANOVA results for Choice Reaction Time test demonstrated a significant main effect for Saccade Latency [$F(1,229) = 19.53$; $p < 0.001$, $\omega^2 = 0.074$] and processing speed [$F(1,226) = 4.17$; $p < 0.05$, $\omega^2 = 0.44$]. Further, we examined Visual Speed [$F(1, 226) = 0.182$; $p = 0.670$, $\omega^2 = -0.003$] and Reaction Time [$F(1,224) = 0.342$; $p = 0.559$, $\omega^2 = 0.003$] which demonstrated non-significant differences between Control and mTBI groups (**Table 2**).

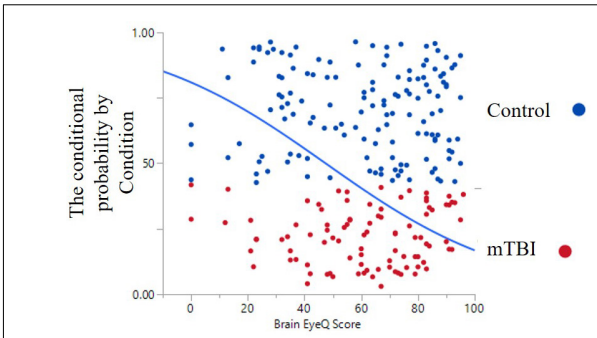

FIGURE 2 | Dot Graph and probability curve for Control group (blue) and TBI group (red).

Discriminate Reaction Time (DRT)

The ANOVA results for Discrimination Reaction Time test demonstrated a significant main effect for Saccade Latency [$F(1,226) = 9.483$; $p < 0.01$, $\omega^2 = 0.35$] and Processing Speed [$F(1,219) = 15.63$; $p < 0.001$, $\omega^2 = 0.62$]. Similar to Choice Reaction Time test, both Visual Processing Speed [$F(1,226) = 3.544$; $p = 0.061$, $\omega^2 = 0.011$] and Reaction Time [$F(1,218) = 0.164$; $p = 0.686$, $\omega^2 = 0.004$] did not differentiate between mTBI and Control groups in the Discriminate Reaction Time test (**Table 3**).

ROC Curve Analysis

Among the RightEye variables, ROC curves were significant ($p < 0.0001$) for Brain Health EyeQ score; DRT Saccade

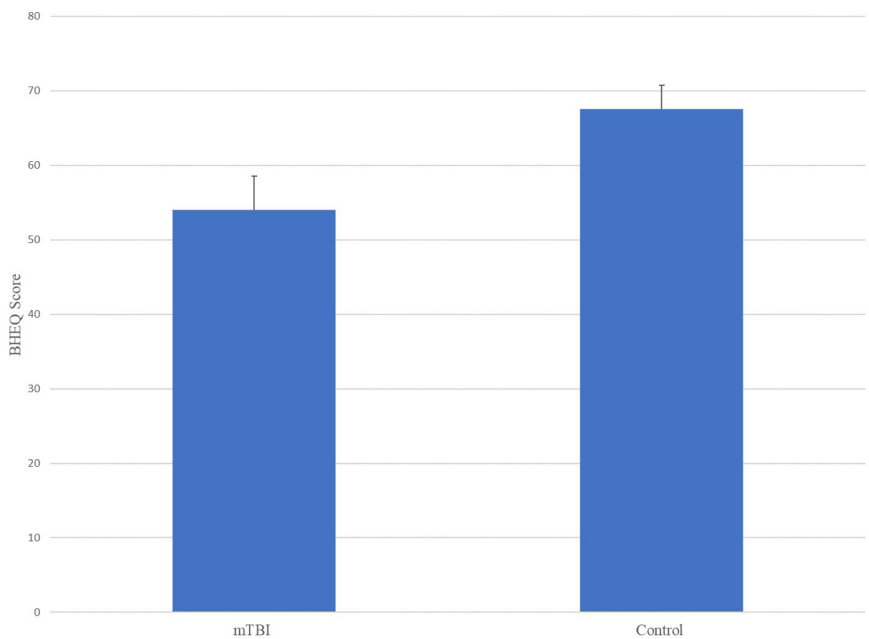

FIGURE 1 | Mean Differences (with standard error) comparing BHEQ score between mTB and Control.

TABLE 2 | Mean and standard deviation for choice reaction time variables.

Group (*n*)	Saccade latency*	Processing speed*	Visual speed	Reaction time
Control	364.95 (139.83)	609.44 (227.56)	149.01 (143.20)	1123.93 (383.98)
mTBI	288.35 (109.41)	669.91 (203.61)	141.10 (126.54)	1095.77 (304.76)

*$p < 0.05$.

TABLE 3 | Mean and standard deviation for discriminate reaction time variables.

Group (n)	Saccade latency*	Processing speed*	Visual speed	Reaction time
Control	336.81 (108.39)	379.39 (152.68)	142.32 (154.34)	856.98 (290.43)
mTBI	286.62 (136.58)	478.01 (218.24)	106.46 (117.56)	873.75 (316.35)

*$p < 0.001$.

TABLE 4 | Summarization of outcomes at the ROC curve analysis including: area under the curve (AUC) with standard error (S.E.), *p* values; cut-off points; sensitivity and specificity percentages; positive and negative predictive values (PPV and NPV), respectively.

Variables	AUC	S.E.	*p*	Cut-off	Sensitivity	Specificity	PPV	NPV
BHEQ	0.704*	0.00618	0.0001	63	75.3%	68.0%	73.7%	81.2%
BHEQ subscale analysis								
Fixation Stability	0.640	0.1346	0.0003	5.11	66.3%	67.8%	57.2%	75.4%
Horizontal Saccade Efficiency	0.560	0.0263	0.2691	7.31	59.0%	86.8%	30.6%	33.1%
Vertical Saccade Efficiency	0.597	0.210	0.0688	5.27	85.5%	81.1%	40.3%	65.1%
CSP Saccade percentage	0.68	0.273	0.0027	4.29	84.5%	71.8%	43.3%	73.3%
VSP Saccade percentage	0.55	0.018	0.2218	5.10	57.2%	11.5%	29.8%	31.5%
HSP Saccade percentage	0.42	0.17	0.698	18.45	98.6%	94.5%	40.3%	85.3%
Reaction Time Tasks								
DRT Saccade Latency	0.724*	0.00170	0.0039	259	58.8 %	86.4%	75.0%	75.2%
DRT Processing Speed	0.692*	0.00093	0.0004	365	73.2 %	60.7%	76.3%	76.6%
CRT Saccade Latency	0.716*	0.00138	0.0001	248	53.6%	91.4%	81.3%	74.0%
CRT Processing Speed	0.623	0.00062	0.045	578	64.9%	55.7%	70.4%	69.6%

**Represents an acceptable probability that the test differentiates mTBI from no TBI.*
Cut-off points (or thresholds) distinguish between a "positive" and a "negative" mTBI result and represents maximum balance between sensitivity and specificity within each test.
Sensitivity represents confidence that a person has a mTBI or the true positive rate and specificity represents the accuracy of the test or the true negatives.

Latency, DRT Processing Speed, CRT Saccade Latency, CRT Processing Speed CRT (**Table 4** and **Figure 3**). ROC curves were not significant or produced low AUC score for the remaining DRT and CRT variables (Reaction Time and Visual Speed).

DISCUSSION

The purpose of this article was to examine the oculomotor behavior of pediatric patients with clinically diagnosed mTBI versus controls. This was done using a combination of saccade, pursuit, fixation and reaction time oculomotor variables that together made up a BHEQ Score. Results revealed a significant difference between groups, with the mTBI group showing lower (poorer) oculomotor behavior than the control group. A mean difference of 13.54% (67.52–53.98) was found. This result shows that oculomotor behavior of those with mTBI is poorer, as they scored lower than those of the control group. It also shows that the BHEQ linear combination score effectively detects such differences by examining all the major oculomotor behaviors (fixations, pursuits, and saccades). Furthermore, the BHEQ score showed a significant 0.7 AUC with a sensitivity of 75.3%. These scores indicate that the BHEQ score has a balance of sensitivity and specificity and represents the ability to discriminate whether a specific condition is present or not present. It is important to note the sensitivity and specificity are based on determining appropriate cut-off points which distinguish between a "positive" and a "negative" outcome. We utilized our data to determine these appropriate cut-scores, however, with lower cut-off scores based on minimal clinically important differences would result in better sensitivity and specificity in the measure. Furthermore, BHEQ did better overall considering AUC, *p*-value, sensitivity, and specificity of the sub-measures including pursuit test, self-paced saccade test, and fixation test and the BHEQ score has more precision in distinguishing those with mTBI and without mTBI.

It is well known that independent tests, such as saccades tests show differences between those with mTBI and those without (Hunfalvay et al., 2019). The same is true for

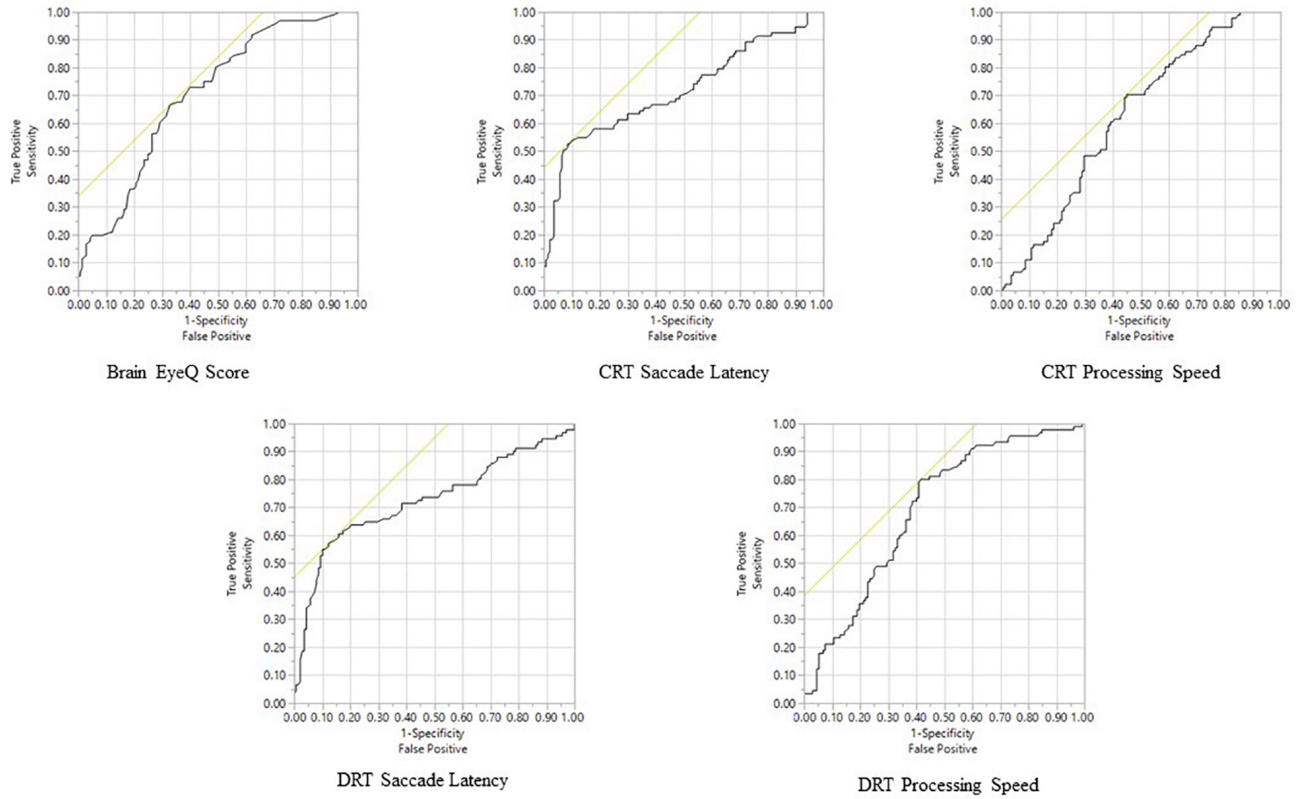

FIGURE 3 | Receiver Operator Characteristic analysis predicting mTBI Status for all significant variables.

pursuit eye movements (Suh et al., 2006b). However, to date, there has not been one combination score of all the major eye movements that a clinician can review as part of the clinical workflow to determine if there is a global oculomotor difference for a patient compared to an age matched control. One global score, one standard of reference in clinical practice, is an important benchmark for which to determine if further, more in-depth examination is required. Furthermore, the RightEye test only require 5 min to complete the test and are not impacted by acute eye fatigue during the test (Murray et al., 2019).

A secondary purpose of this article was to examine choice and discriminate reaction time tests and associated oculomotor variables between the two groups. Two variables, saccadic latency, and processing speed were found to be significantly different in both the CRT and DRT test. mTBI group had faster saccadic latency and slower processing speed than the Control group. This is consistent with past research where saccadic latency and processing speed where found to show differences between mTBI versus controls and mTBI versus athlete groups (Lange et al., 2018). Interestingly the previous research showed much larger standard deviations even with a larger sample size ($N = 651$) compared to the current research ($N = 91$). It is possible that the 10-day time limit for mTBI patients in the current study reduced the variability in results. Nevertheless, the same results were replicated. Both CRT and DRT Saccadic Latency values show a high specificity 86.4 and 91.4%, respectively. Furthermore, they showed high positive predictive values (75.0 and 81.3%). DRT and CRT Processing Speed showed high sensitivity 73.2 and 64.9%, respectively. Taken together, these metrics indicate a high predictive value, sensitivity and specificity for differentiating patients with and without mTBI. Such results further validate the use of eye movements as a biomarker for identification of mTBI. Limitations of this study include an unequal distribution of males and females in the sample populations. Past research has found conflicting evidence of gender differences in mTBI groups (Farace and Alves, 2000; Brickell et al., 2017) and future research is needed. A second limitation is that 24.7% of cases that are potentially missed. However, mTBI describes a broad term that describes a vast array of injuries and this test indicates visual motor impairment due to mTBI. Potentially, the missed cases are result from other symptoms or impairments and additional measures are needed to account for the diversity of mTBI especially in pediatric patients. A third limitation is the limited age group of pediatric patients only. Lastly, very nature of mTBI is complicated injury with completed tautology.

This study was the first to examine a combined Brain Health EyeQ score in mTBI pediatric patients. Future research should examine adults, specifically those over 65 who are the second largest group of persons who incur mTBIs and is describe as the "silent epidemic" in older adults according to Thompson et al. (2006). In conclusion, the results of this study

show that (a) oculomotor behavior differs between pediatric patients with mTBI and age matched controls; (b) the BHEQ score, that combines the major categories of oculomotor behavior, differentiates pediatric patients with mTBI from controls, and (c) the CRT and DRT tests results were replicated from past research supporting the need for RT to be part of a mTBI assessment (Lange et al., 2018).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by East Carolina University IRB. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

Adjorlolo, S. (2018). Diagnostic accuracy, sensitivity, and specificity of executive function tests in moderate traumatic brain injury in Ghana. *Assessment* 25, 498–512. doi: 10.1177/1073191116646445

Araki, T., Yokota, H., and Morita, A. (2017). Pediatric traumatic brain injury: characteristic features, diagnosis, and management. *Neurol. Med. Chir. (Tokyo)*. 57, 82–93. doi: 10.2176/nmc.ra.2016-0191

Arbour, C., Baril, A. A., Westwick, H. J., Potvin, M.-J., Gilbert, D., Giguère, J.-F., et al. (2016). Visual fixation in the ICU: a strong predictor of long-term 404 recovery after moderate-to-severe traumatic brain injury. *Crit Care Med*. 44, e1186–e1193.

Armstrong, R. A. (2018). Visual problems associated with traumatic brain injury. *Clin. Exp. Optometry* 101, 716–726. doi: 10.1111/cxo.12670

Bazarian, J. J., Mc Clung, J., and Shah, M. N. (2005). Mild traumatic brain injury in the United States1998–2000. *Brain Inj.* 19, 85–91. doi: 10.1080/02699050410001720158

Bedell, H. E., and Stevenson, S. B. (2013). Eye movement testing in clinical examination. *Vis. Res.* 90, 32–37. doi: 10.1016/j.visres.2013.02.001

Brickell, T. A., Lippa, S. M., French, L. M., Kennedy, J. E., Bailie, J. M., and Lange, R. T. (2017). Female service members and symptom reporting after combat and non-combat-related mild traumatic brain injury. *J. Neurotr.* 34, 300–312. doi: 10.1089/neu.2016.4403

Ciuffreda, K. J., Han, Y., and Kapoor, N. (2004). Oculomotor fixation and its rehabilitation in acquired brain injury. *Invest. Ophthalmol. Vis. Sci.* 45:2325.

DiCesare, C. A., Kiefer, A. W., Nalepka, P., and Myer, G. D. (2017). Quantification and analysis of saccadic and smooth pursuit eye movements and fixations to detect oculomotor deficits. *Behav. Res. Methods* 49, 258–266. doi: 10.3758/s13428-015-0693-x

Ettenhofer, M. L., Hershaw, J. N., Engle, J. R., and Hungerford, L. D. (2018). Saccadic impairment in chronic traumatic brain injury: examining the influence of cognitive load and injury severity. *Brain Injury* 32, 1740–1748. doi: 10.1080/02699052.2018.1511067

Farace, E., and Alves, W. M. (2000). Do women fare worse: a meta-analysis of gender differences in traumatic brain injury outcome. *J. Neurosurg.* 93, 167–191.

Ghaffarpasand, F., Razmkon, A., and Dehghankhalili, M. (2013). Glasgow coma scale score in pediatric patients with traumatic brain injury; limitations and reliability. *Bull. Emerg. Trauma* 1, 135–136.

Goldstein, F. C., and Levin, H. S. (1987). Epidemiology of pediatric closed head injury: incidence, clinical characteristics, and risk factors. *J. Learn. Disabil.* 20, 518–525. doi: 10.1177/002221948702000903

Grubenhoff, J. A., Kirkwood, M., Gao, D., Deakyne, S., and Wathen, J. (2010). Evaluation of the standardized assessment of concussion in a pediatric emergency department. *Pediatrics* 126, 688–695. doi: 10.1542/peds.2009-2804

Guerrero, J. L., Thurman, D. J., and Sniezek, J. E. (2000). Emergency department visits associated with traumatic brain injury: United States, 1995–1996. *Brain Inj.* 14, 181–186. doi: 10.1080/026990500120835

Han, S. J., Guo, Y., Granger-Donetti, B., Vicci, V. R., and Alvarez, T. L. (2010). Quantification of heterophoria and phoria adaptation using automated objective system compared to clinical methods. *Opthalmic. Physiol. Opt.* 30, 95–107. doi: 10.1111/j.1475-1313.2009.00681.x

Heitger, M. H., Anderson, T. J., and Jones, R. D. (2002). Saccade sequences as markers for cerebral dysfunction following mild closed head injury. *Prog. Brain Res.* 140, 433–448. doi: 10.1016/s0079-6123(02)40067-2

Heitger, M. H., Jones, R. D., Macleod, A. D., Snell, D. L., Frampton, C. M., and Anderson, T. J. (2009). Impaired eye movements in post-concussion syndrome indicate suboptimal brain function beyond the influence of depression, malingering or intellectual ability. *Brain* 132(Pt 10), 2850–2870. doi: 10.1093/brain/awp181

Hetherington, C. R., Stuss, D. T., and Finlayson, M. A. J. (1996). Reaction time and variability 5 and 10 years after traumatic brain injury. *Brain Injury* 10, 473–486. doi: 10.1080/026990596124197

Hoffer, M. E., Balaban, C., Szczupak, M., Buskirk, J., Snapp, H., Crawford, J., et al. (2017). The use of oculomotor, vestibular, and reaction time tests to assess mild traumatic brain injury (mTBI) over time. *Laryngosc. Invest. Otolaryngol.* 2, 157–165. doi: 10.1002/lio2.74

Holmqvist, K., and Nystrom, M. (2011). *Eye Tracking: A Comprehensive Guide to Methods and Measures*. Oxford: Oxford University Press.

Howell, D. R., Brilliant, A. N., Storey, E. P., Podolak, O. E., Meehan, W. P., and Master, C. L. (2018). Objective eye tracking deficits following concussion for youth seen in a sports medicine setting. *J. Child Neurol.* 33, 794–800. doi: 10.1177/0883073818789320

Hugenholtz, H., Stuss, D. T., Stethem, L., and Richard, M. T. (1998). How long does it take to recover from a mild concussion? *Neurosurgery* 22, 853–858. doi: 10.1227/00006123-198805000-00006

Hunfalvay, M., Roberts, C.-M., Murray, N., Tyagi, A., Kelly, H., and Bolte, T. (2019). Horizontal and vertical self-paced saccades as a diagnostic marker of traumatic brain injury. *Concussion* 4:CNC60. doi: 10.2217/cnc-2019-0001

Johnson, B., Zhang, K., Hallett, M., and Slobounov, S. (2015). Functional neuroimaging of acute oculomotor deficits in concussed athletes. *Brain Imaging Behav*. 9, 564–573. doi: 10.1007/s11682-014-9316-x

Kelly, J. P., Nichols, J. S., Filley, C. M., Lillenhei, K. O., Rubinstein, D., and Kleinschmidt-DeMasters, B. K. (1991). Concussion in sports: guidelines for the prevention of catastrophic outcome. *JAMA* 266, 2867–2869. doi: 10.1001/jama.1991.03470200079039

Knopman, D. (1991). Long-term retention of implicitly acquired learning in patients with Alzheimers disease. *J. Clin. Exp. Neuropsychol.* 13, 880–894. doi: 10.1080/01688639108405105

Komogortsev, O. V., and Karpov, A. (2013). Automated classification and scoring of smooth pursuit eye movements 382 in the presence of fixations and saccades. *Behav. Res. Methods* 45, 203–215. doi: 10.3758/s13428-012-0234-9

Kooiker, M. J. G., Pel, J. J. M., Verbunt, H. J. M., de Wit, G. C., van Genderen, M. M., and van der Steen, J. (2016). Quantification of visual function assessment using remote eye tracking in children: validity and applicability. *Acta Ophthal.* 94, 599–608. doi: 10.1111/aos.13038

Lai, M.-N., Tsai, M.-J., Yang, F.-Y., Hsu, C.-Y., Liu, T.-C., Lee, S. W.-Y., et al. (2013). A review of using eye- tracking technology in exploring learning from 2000 to 2012. *Educ. Res. Rev.* 10, 90–115. doi: 10.1016/j.edurev.2013.10.001

Land, M., and Tatler, B. (2009). *Looking and Acting Vision and Eye Movements in Natural Behavior*. Oxford: Oxford University Press, doi: 10.1093/acprof:oso/9780198570943.001.0001

Lange, B., Hunfalvay, M., Murray, N., Roberts, C. M., and Bolte, T. (2018). Reliability of computerized eye-tracking reaction time tests in non-athletes, athletes, and individuals with traumatic brain injury. *Optom Vis. Perf.* 6, 165–180.

Langlois, J. A., Rutland-Brown, W., and Thomas, K. (2004). *Emergency Department Visits, Hospitalizations, and Deaths*. Available online at: https://stacks.cdc.gov/view/cdc/12294 (accessed January 10, 2020).

Leigh, R. J., and Zee, D. S. (2015). *The Neurology of Eye Movements*. Oxford: Oxford University Press, doi: 10.1093/med/9780199969289.001.0001

Levin, H. S., and Diaz-Arrastia, R. R. (2015). Diagnosis, prognosis, and clinical management of mild traumatic brain injury. *Lancet Neurol.* 14, 506–517. doi: 10.1016/s1474-4422(15)00002-2

Lumba-Brown, A., Yeates, K. O., Sarmiento, K., Breiding, M. J., Haegerich, T. M., Gioia, G. A., et al. (2018a). Centers for disease control and prevention guideline on the diagnosis and management of mild traumatic brain injury among children. *JAMA Pediatr.* 172:e182853. doi: 10.1001/jamapediatrics.2018.2853

Lumba-Brown, A., Yeates, K. O., Sarmiento, K., Breiding, M. J., Haegerich, T. M., Gioia, G. A., et al. (2018b). Diagnosis and management of mild traumatic brain injury in children: a systematic review. *JAMA Pediatr.* 172:e182847. doi: 10.1001/jamapediatrics.2018.2847

MacFlynn, G., Montgomery, E. A., Fenton, G. W., and Rutherford, W. (1984). Measurement of reaction time following minor head injury. *J. Neurol.* 47, 1326. doi: 10.1136/jnnp.47.12.1326

Maruta, J., Heaton, K. J., Maule, A. L., and Ghajar, J. (2014). Predictive visual tracking: specificity in mild traumatic brain injury and sleep deprivation. *Mil. Med.* 179, 619–625. doi: 10.7205/MILMED-D-13-00420

Maruta, J., Lee, S. W., Jacobs, E. F., and Ghajar, J. (2010). A unified science of concussion. *Ann. N. Y. Acad. Sci.* 1208, 58–66. doi: 10.1111/j.1749-6632.2010.05695.x

Mild Traumatic Brain Injury Committee (1993). Definition of mild traumatic brain injury. *J. Head Trauma Rehabil.* 8, 86–87.

Møllenbach, E., Hansen, J. P., and Lillholm, M. (2013). Eye movements in gaze interaction. *J. Eye Mov. Res.* 6, 1–15. doi: 10.16910/jemr.6.2.1

Munoz, D. P. (2002). Commentary: saccadic eye movements: overview of Neural Circuitry. *Prog. Brain Res.* 140, 89–96. doi: 10.1016/s0079-6123(02)40044-1

Murray, N., Kubitz, K., Roberts, C.-M., Hunfalvay, M., Bolte, T., and Tyagi, A. (2019). An examination of the oculomotor behavior metrics within a suite of digitized eye tracking tests. *Vis. Dev. Rehabil.* 5, 269–284. doi: 10.31707/vdr2019.5.4.p269

Murtha, S., Cismaru, R., Waechter, R., and Chertkow, H. (2002). Increased variability accompanies frontal lobe damage in dementia. *J. Int. Neuropsychol. Soc.* 8, 360–372. doi: 10.1017/s1355617702813170

Niehorster, D. C., Cornelissen, T. H. W., Holmqvist, K., Hooge, I. T. C., and Hessels, R. S. (2017). What to expect from your remote eye-tracker when participants are unrestrained. *Behav. Res. Methods* 50, 1–15. doi: 10.1016/j.cps.2009.07.003

Oakes, K. (2018). *CDC Releases Guidelines for Pediatric mTBI*. Parsippany, NJ: Clinical Neurology News.

Prins, M., Greco, T., Alexander, D., and Giza, C. C. (2013). The pathophysiology of traumatic brain injury at a glance. *Dis. Models Mech.* 6, 1307–1315. doi: 10.1242/dmm.011585

Renard, D., Ferraro, A., Lorenzini, M.-C., Jeanjean, L., Portal, M. −C., Llinares, E., et al. (2015). Orthoptic and video oculographic analyses in oculopharyngeal muscular dystrophy. *Muscle Nerve* 52, 554–558. doi: 10.1002/mus.24600

Schunk, J. E., and Schutzman, S. A. (2012). Pediatric head injury. *Pediatr. Rev.* 33, 398–411. doi: 10.1542/pir.33-9-398

Shelton, J., and Kumar, G. P. (2010). Comparison between auditory and visual simple reaction times. *Neurosci. Med.* 2010, 30–32. doi: 10.4236/nm.2010.11004

Suh, M., Basu, S., Kolster, R., Sarkar, R., McCandliss, B., and Ghajar, J. (2006a). Increased oculomotor deficits during target blanking as an indicator of mild traumatic brain injury. *Neurosci. Lett.* 410, 203–207. doi: 10.1016/j.neulet.2006.10.001

Suh, M., Kolster, R., Sarkar, R., McCandliss, B., and Ghajar, J. (2006b). Deficits in predictive smooth pursuit after mild traumatic brain injury. *Neurosci. Lett.* 401, 108–113. doi: 10.1016/j.neulet.2006.02.074

Sussman, E. S., Ho, A. L., Pendharkar, A. V., and Ghajar, J. (2016). Clinical evaluation of concussion: the evolving role of oculomotor assessments. *Neurosurg. Focus* 40:E7. doi: 10.3171/2016.1.focus15610

Thompson, H. J., McCormick, W. C., and Kagan, S. H. (2006). Traumatic brain injury in older adults: epidemiology, outcomes, and future implications. *J. Am. Geriatr. Soc.* 54, 1590–1595. doi: 10.1111/j.1532-5415.2006.00894

Ventura, R. E., Jancuska, J. M., Balcer, L. J., and Galetta, S. L. (2015). Diagnostic tests for concussion: is vision part of the puzzle? *J. Neuroophthalmol.* 35, 73–81. doi: 10.1097/WNO.0000000000000223

Williams, I. M., Ponsford, J. L., Gibson, K. L., Mulhall, L. E., Curran, C. A., and Abel, L. A. (1997). Cerebral control of saccades and neuropsychological test results after head injury. *J. Clin. Neurosci.* 4, 186–196. doi: 10.3758/s13428-012-0234-9

Wong, A. M. F. (2007). *Eye Movement Disorders*. Oxford: Oxford University Press.

Zomeren, A. H., and Brouwer, W. H. (1994). *Clinical Neuropsychology of Attention*. Oxford: Oxford University Press.

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Folic Acid Fortification Prevents Morphological and Behavioral Consequences of X-Ray Exposure During Neurulation

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Previous studies suggested a causal link between pre-natal exposure to ionizing radiation and birth defects such as microphthalmos and exencephaly. In mice, these defects arise primarily after high-dose X-irradiation during early neurulation. However, the impact of sublethal (low) X-ray doses during this early developmental time window on adult behavior and morphology of central nervous system structures is not known. In addition, the efficacy of folic acid (FA) in preventing radiation-induced birth defects and persistent radiation-induced anomalies has remained unexplored. To assess the efficacy of FA in preventing radiation-induced defects, pregnant C57BL6/J mice were X-irradiated at embryonic day (E)7.5 and were fed FA-fortified food. FA partially prevented radiation-induced (1.0 Gy) anophthalmos, exencephaly and gastroschisis at E18, and reduced the number of pre-natal deaths, fetal weight loss and defects in the cervical vertebrae resulting from irradiation. Furthermore, FA food fortification counteracted radiation-induced impairments in vision and olfaction, which were evidenced after exposure to doses ≥0.1 Gy. These findings coincided with the observation of a reduction in thickness of the retinal ganglion cell and nerve fiber layer, and a decreased axial length of the eye following exposure to 0.5 Gy. Finally, MRI studies revealed a volumetric decrease of the hippocampus, striatum, thalamus, midbrain and pons following 0.5 Gy irradiation, which could be partially ameliorated after FA food fortification. Altogether, our study is the first to offer detailed insights into the long-term consequences of X-ray exposure during neurulation, and supports the use of FA as a radioprotectant and antiteratogen to counter the detrimental effects of X-ray exposure during this crucial period of gestation.

Keywords: radiation, radioprotectant, folic acid, birth defect, anophthalmos, agnathia, exencephaly, hyposmia

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INTRODUCTION

Exposure to ionizing radiation during embryonic development has been linked to an increased risk of birth defects. The type and severity of these defect are predominantly determined by the developmental stage during which exposure occurred (Craenen et al., 2017). Epidemiological studies on Ukrainian cohorts illustrated an increased prevalence of neural tube defects (NTDs) and eye defects (EDs) in regions severely contaminated with radioactive Cs-137 isotopes following the Chernobyl nuclear accident. Although there are no accurate dose estimates, uptake of radioactive isotopes is known to be particularly high in pregnant women living in these regions (Wertelecki et al., 2016). Initially, it was observed that the more recent Fukushima Daiichi nuclear power plant accident elicited no increase in birth defects and pre-natal mortality due to environmental radioisotope contamination (Fujimori et al., 2014), but subsequent papers debated this conclusion (Mangano and Sherman, 2015; Scherb et al., 2016). In contrast to these more recent observations, reports after the atomic bombings in Japan only mentioned an increased incidence of microcephaly and intellectual disability (Plummer, 1952; Neel and Schull, 1956). It is likely that the discrepancy in health effects of the nuclear accidents and atomic bombings stems from differences in dose, dose rate, exposure duration and radiation type. The above highlights the need to increase our knowledge about the effects of pre-natal irradiation on biological structures and functions.

Exposure to ionizing radiation during pregnancy most commonly occurs during clinical radiodiagnostic or therapeutic procedures (Mettler et al., 2009). Although medical practitioners advise against irradiation during pregnancy, it may be unavoidable in medical urgencies (Lazarus et al., 2009). In terms of radiation protection, conventional shielding methods are currently being used to partially mitigate the fetal dose (Chatterson et al., 2014; Moore et al., 2015; Owrangi et al., 2016). However, depending on the dose or the developmental stage during which exposure occurs, these conventional shielding strategies may not suffice. Animal studies have shown that the neurulation period in the early embryo is especially radiosensitive with regard to the pathogenesis of radiation-induced NTDs and ED (Russell, 1950, 1956; Di Majo et al., 1981; Heyer et al., 2000; Craenen et al., 2017, 2020b), but also in terms of cognitive disabilities and altered vision. Indeed, a decreased visual acuity in atomic-bomb survivors, irradiated in the first trimester, and born from mothers with acute radiation syndrome ($\leq$2 km from hypocenter) has been reported (Burrow et al., 1964). Yet, most experimental work has focused on health risks after radiation exposure during neurogenesis, coinciding with the second trimester of human pregnancy (Plummer, 1952; Neel and Schull, 1956; Verreet et al., 2015, 2016a,b). Furthermore, there are currently no anti-teratogens or radio-protectants available to prevent (congenital) morphological and functional defects that arise from irradiation during brain development.

Folic acid (FA), a synthetic vitamin, is generally known to prevent NTDs [reviewed in Imbard et al. (2013)], in addition to other defects such as heart defects and some skeletal defects (Kappen, 2013). Besides, FA has been suggested to prevent the development of age-related neurodegenerative diseases and overall cognition (Craenen et al., 2020a). Several countries enforce staple food fortification, whereas others support FA supplementation during pregnancy (Imbard et al., 2013). Of note is that FA supplementation/fortification initiatives are currently lacking in high-risk areas, such as those severely contaminated with radioisotopes from the Chernobyl disaster. Although FA food fortification can prevent some defects such as NTDs, its efficacy depends on the causative teratogens or mutations. For example, BMS-189453 (a synthetic retinoid) causes anomalies such as NTDs and heart defects that can be prevented with FA fortification (Cipollone et al., 2009), whereas arsenate-induced NTDs do not appear to be responsive (Ferm and Hanlon, 1986). Interestingly, many of the hallmark consequences of ionizing radiation exposure, including oxidative stress, DNA damage, cell cycle arrest, cell death and epigenetic alterations, might be countered by FA (Heyer et al., 2000; Martin et al., 2014; Reisz et al., 2014).

This study is the first to offer an in-depth analysis of the morphological and behavioral consequences of irradiation during neurulation in mice. To this end, we used a multidisciplinary approach, including an extensive behavioral test battery and imaging techniques such as spectral domain optical coherence tomography (SD-OCT) and magnetic resonance imaging (MRI). In addition, we assessed the efficacy of FA food fortification in preventing fetal malformations as well as adult functional and morphological defects resulting from X-ray exposure.

MATERIALS AND METHODS

Animals and FA Fortification

All animal experiments were conducted in line with the relevant guidelines and were approved by the Institutional Ethical Committees of SCK-CEN/VITO (ref. 02–012) and the Animal Welfare Committee of the KU Leuven University, and are in strict accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EU). C57BL6/J mice (Janvier, Bio Services, The Netherlands) were housed in individually ventilated cages, under standard laboratory conditions (12-h light/dark cycle) and fed *ad libitum*. One week before coupling, animals designated for the macroscopic fetal study were placed on a control Teklad (Carfil Quality, Oud-Turnhout, Belgium) diet (3.5 mg/kg FA), a FA fortified diet (8 mg/kg FA) or an extra-FA fortified diet (12 mg/kg FA). The FA concentrations within the final customized food products were investigated in compliance with ISO 17025. We selected the dose of 8 mg/kg because it was observed that this is an effective concentration to achieve antiteratogenic effects in mice (Gray and Ross, 2009; Harris, 2009). A dose of 12 mg/kg was included based on the assumption

Abbreviations: EPM, Elevated plus maze; E, embryonic day; ED, eye defect; FA, folic acid; MRI, magnetic resonance imaging; MWM, Morris water maze; NF+GCL, nerve fiber + retinal ganglionic cell layer; NTD, neural tube defect; NS, non-social odor; RARE, rapid acquisition relaxation enhancement; RM, repeated measures; S, social odor; SD-OCT, spectral domain optical coherence tomography.

TABLE 1 | Sample sizes.

	Control diet								High FA diet (8 mg/kg)								High FA diet (12 mg/kg)	
	0.0 Gy		0.1 Gy		0.5 Gy		1.0 Gy		0.0 Gy		0.1 Gy		0.5 Gy		1.0 Gy		1.0 Gy	
	n	*N*	*n*	*N*	*n*	*N*	*n*	*N*	*n*	*N*	*n*	*N*	*n*	*N*	*n*	*N*	*n*	*N*
Macroscopic	126	15	n.a.		n.a.		116	18	n.a.		n.a.		n.a.		114	19	107	16
Skeletal	9	3	n.a.		n.a.		9	3	n.a.		n.a.		n.a.		9	3	9	3
Behavior/OCT	10	4	13	6	12	6	n.a.		12	4	11	6	12	6	n.a.		n.a.	
MRI	9	4	12	6	5	3	n.a.		5	2	7	5	7	4	n.a.		n.a.	

N, number of litters; n, number of fetuses.

that some teratogens require higher doses of FA (Gray and Ross, 2009; Harris, 2009).

Animals designated for the behavioral tests and MRI were limited to the control diet or the 8 mg/kg FA diet, and were kept on their respective diets until they were euthanized. Timed couplings were performed during a 2-h period at the start of the light phase (7:30 a.m.−9:30 a.m.) to attain synchronous timing of embryonic development. The day of coupling was identified as E0. At E7.5, animals were placed in a Plexiglas holder and transported to the irradiation installation. Mice intended for the macroscopic study were either sham-irradiated or irradiated with 1.0 Gy of X-rays. Animals used for behavioral testing and MRI were sham-irradiated or received a sub-lethal dose of 0.1 or 0.5 Gy of X-rays at E7.5. Irradiation was performed using an X-strahl 320 kV (0.14 Gy/min, inherent filtration: 0.21 mmAl, additional filtration: 3.8 mm Al + 1.4 mm Cu + DAP, tube voltage: 250 kV, tube current: 12 mA,) in accordance to ISO 4037. The number of animals used for the macroscopic, skeletal, behavioral and MRI experiments is depicted in **Table 1**, unless otherwise specified.

Macroscopic Scoring and Skeletal Stainings

The dissections, macroscopic scorings and alcian blue/alizarin red skeletal stainings were performed at E18 as previously described (Craenen et al., 2017). For the skeletal analyses, E18 fetuses were randomly selected from the macroscopic study. The axial skeleton was analyzed, with a focus on the vertebrae and the ribs. A subdivision was made between atlas, cervical, thoracic, lumbar, sacral and caudal vertebrae, whilst also differentiating between true, false and floating ribs and sternum.

Behavioral Tests

Starting at week (W)5 and ending at W14, behavioral tests were performed on male mice in the order described below (**Table 2**). All experiments were performed under blinded conditions. To assess visual acuity, optokinetic tracking was performed. We included cage activity to assess global activity, during both light and dark-phase, and assessed explorative and social behavior with the open field and social exploration tests. The elevated plus maze (EPM) was included to ascertain anxiety, whereas the accelerating rotarod was used to identify issues in motility. Next, to explore olfactory performance we used the odor habituation/dis-habituation assay. Finally, two tests for memory were included: the Morris water maze (MWM) and passive avoidance, to test spatial and fear-related memory, respectively.

TABLE 2 | Overview of test order and age at time of testing.

Protocol	Age range (weeks)
Optokinetic tracking response	W5–W7
Optical coherence tomography	W5–W7
Cage activity	W7–W9
Open field	W7–W9
Social exploration	W8–W10
Elevated plus maze	W8–W10
Accelerating rotarod	W8–W10
Odor habituation/dis-habituation	W9–W11
MRI	W9–W11
Morris Water Maze	W10–W13
Passive avoidance	W12–W14

Optokinetic Tracking Response

Using a virtual-reality chamber (OptoMotry, Cerebral Mechanics, Medicine Hat, AB, Canada), the optokinetic tracking response was assessed (De Groef et al., 2016; Van Hove et al., 2016). The animal was placed on the center of an elevated platform within the optokinetic installation, where a vertical sine wave pattern was displayed on the monitors. Using a real-time camera system, visual acuity was scored manually using a staircase procedure, composed of random spatial frequencies (100% contrast, 12° per second speed).

Cage Activity

The impact of ionizing radiation exposure on ambulatory behavior was investigated over a 23 h time-period, starting at 4 p.m. until 3:30 p.m. the next day (Verreet et al., 2016a). During this period, animals were individually housed in transparent cages (20 × 26 cm) with minimal bedding, chow and water and placed in a laboratory-built activity logger with three infrared beams. Beam breaks were recorded over 30 min time bins.

Open Field and Social Exploration

To assess exploration and social interaction, a transparent Plexiglas arena (50 × 50 cm) was used (Stroobants et al., 2008; Bollen et al., 2015; Callaerts-Vegh et al., 2015). The arena was homogenously illuminated and equipped with an Any-maze (Dublin, Ireland) tracking system. For the open field test, animals were placed in the empty arena for 1 min of acclimatization, immediately followed by a 10 min test phase with active tracking. The social exploration experiment was identical to the open field test, except that in the center of the arena a small cage with two same-sex strange mice was placed.

Elevated Plus Maze

In order to investigate anxiety, animals were subjected to EPM testing as was previously described (Verreet et al., 2015). The cross-shaped EPM consisted of two perpendicular open and closed arms (21 × 5 cm). Five infrared detectors were installed on the EPM: 2 at the exits out of the closed arms and two at the entrances to the open arms (entries/exits) and one along the length of the open arms (time spent on the open section). The animal was placed in a closed arm and after 1 min of adaptation, beam breaks were recorded for 10 min.

Accelerating Rotarod

General motor function and balance following *in utero* X-ray exposure during neurulation were assessed using an accelerating rotarod (Ugo Basile, Italy), as was described previously (Verreet et al., 2015). Initially, the animals underwent two adaptation trials (2 min each), each at a constant speed of 4 rpm. In turn, the mouse was subjected to four subsequent test trials, where during each 5 min trial the rotation speed gradually increased from 4 to 40 rpm. Latency was recorded when the mouse lost its footing and fell off the rotating beam.

Odor Habituation and Dis-Habituation

To assess the interaction of mice with olfactory cues, i.e., habituation and dis-habituation, the animals were subjected to an odor discrimination test as was described previously (Yang and Crawley, 2009; Yang et al., 2012; Arbuckle et al., 2015). Animals were individually placed in a fresh cage with a small amount of bedding, followed by 30 min of acclimatization with a dry cotton swab fixed to the cover grid (tip ∼5 cm from bottom). Next, the animals were exposed to a sequence of 15 subsequent odor exposures (2 min each): Three trials with water, three trials with grape (non-social odor 1 = NS1), three trials with banana (NS2), three trials with social odor one (S1) and three trials with social odor two (S2). For the preparation of the NS odor tests, respectively 1:100 diluted grape extract (SAFC, W26820-8-K methyl anthranilate ≥98%) and 1:100 diluted banana extract (Acros Organics, AC269481000 n-Butyl propionate >99%) on cotton tips was used. For the S odors, cotton tips were dipped in water and moved in a cross-pattern through the bedding of soiled cages of same-sex mice. During each trial, sniffing-time was recorded manually whenever the subject's nose was within a 2 cm radius of the cotton swab. The inter-session interval never exceeded 2 min.

Morris Water Maze

In order to assess whether FA and sub-lethal pre-natal doses of X-rays during neurulation affected adult spatial learning, MWM was performed. Animals were tested in a circular pool (diameter 150 cm, height 30 cm), filled with opacified non-toxic water as previously described (Latif-Hernandez et al., 2016; Verreet et al., 2016a). For the acquisition trials, a see-through acrylic platform was consistently placed in the same quadrant, 1 cm below the water surface. The pool was located in the center of a homogeneously-lit room, with invariable visual cues. Acquisition training was performed over a period of 5 days, followed by a 2-day resting period, followed again by 5 days of training. During each training day, every mouse was subjected to four trials. The trial interval was approximately 15-min and the quadrant-starting positions varied in a semi-random order for every trial. If the animal was unable to find the platform within 120 s, it was placed on the platform for 10 s and subsequently removed from the basin. On day 5 of the acquisition trials and 2 days after the last acquisition trial, probe trials were performed. During these probe trials, the platform was removed from the basin and mice were subjected to a single probe trial of 100 s, where the starting position was opposite to the target quadrant. Using an automated video capture and tracking system (EthoVision, Noldus, The Netherlands), various parameters such as trajectory and swim speed were recorded. We observed floating behavior (swim velocity <5 cm/s, more than 30 s per swim) in all groups, except the control diet + 0.0 Gy group. However, for the path length analysis to determine if the animals covered the same track during learning, we included all animals due to the low animal numbers per group. Non-responders (floating >35% of test time) were excluded for the reference memory test. As such, a reduced number of animals was included for the reference memory test, as compared to **Table 1**. More specifically, for this analysis in particular we included under control diet condition 29 animals (9 for 0.0 Gy, 11 for 0.1 Gy and 9 for 0.5 Gy), and 27 under high FA condition (10 for 0.0 Gy, 9 for 0.1 Gy and 8 for 0.5 Gy).

Passive Avoidance

We investigated fear-aggravated learning and memory using a passive avoidance set-up (Lo et al., 2013). Animals were placed in a brightly lit compartment and the door leading into a dark adjacent chamber was opened after 5 s. Latency to enter the dark chamber was timed starting immediately after opening of the dark chamber and stopped when the animal had all four paws on the electric grid in the dark room. Next, the door separating the two compartments was closed and a shock (0.3 mA, 2 s) was administered. The next day, the procedure was repeated, albeit without the administration of an electric shock.

In vivo Imaging

Optical Coherence Tomography

To assess retinal development and thickness, SD-OCT was used as was previously discussed (Van Hove et al., 2016). The animal was anesthetized by intraperitoneal (ip) injection of 75 mg/kg body weight ketamine (Anesketin, Eurovet, Bladel, The Netherlands) and 1 mg/kg medetomidine (Domitor, Pfizer, NY, USA). Shortly before imaging, pupils were dilated using topical

0.5% tropicamide (0.5% Tropicol, Thea Pharma, Wetteren, Belgium). Next, SD-OCT was performed using an Envisu R2210 (Bioptigen, Morrisville, NC, USA) via 100 serial B-scan lines with each line consisting of 1,000 A-scans, in a 1.4 × 1.4 mm field. Afterwards, ip injection of atipamezol (1 mg/kg, Antisedan, Pfizer) was applied to reverse the anesthesia. Thickness of the retina was investigated using InVivoVue Diver software (Bioptigen).

Magnetic Resonance Imaging

For MRI we used female mice, which originated from the same litters as the behavioral test mice. When the female mice were on average W10, *in vivo* MR imaging of the brain was performed using a 7 T Bruker Biospec 70/30 MRI scanner (30 cm horizontal bore with actively shielded gradients (200 mT/m), Bruker Biospin, Ettlingen, Germany). All data were acquired using a quadrature volume coil (72 mm internal diameter, transmit, actively decoupled) in combination with a dedicated mouse brain surface receive coil (Bruker Biospin). To obtain high resolution 3D images of the entire mouse brain, image acquisition and animal anesthesia was performed similar to previously described experiments (Verreet et al., 2016a). In brief, after the acquisition of localizer scans morphological 3D MR imaging was performed using a rapid acquisition relaxation enhancement (RARE) T2-weighted sequence with a RARE factor of 16 and a repetition time and echo time of 1,000 ms and 67 ms, respectively. The field of view was 24 × 15 × 8.3 mm with a matrix of 256 × 160 × 88, resulting in an isotropic resolution of 94 μm. The total acquisition time was 16 min. The methodology of image post-processing and the labeled template was based on previously published work (Verreet et al., 2016a). Briefly, we first corrected for image intensity inhomogeneity using the N4 bias field correction algorithm (Tustison et al., 2010) using an in-house developed MeVislab pipeline (MeVis Medical Solutions, Germany). Images were affinely registered to the template used in Verreet et al. (2015, 2016a) to obtain brain masks for each animal, which were isotropically dilated by 2 voxels. These brain masks were applied to the raw data and the bias field correction was repeated. Finally, images were non-rigidly registered to the template using the Fast Free-Form Deformation algorithm implemented in Niftyreg (Modat et al., 2010). Template labels were propagated to the individual study images using the transformations obtained from this step, and quantified using an in-house developed Python script (Python 2.7, Python Software Foundation).

Statistics

Statistical analyses were performed with GraphPad Prism 7.02 (GraphPad Software, San Diego, CA, USA). To analyze the data on the macroscopic and skeletal defects, the Kruskal-Wallis methodology was used, in combination with Dunn's *post-hoc* testing. Data on pre-natal viability were assessed using two-way ANOVA and Dunnet testing for multiple comparisons. For most behavioral tests, MRI and SD-OCT, we used two-way ANOVA (with pairing where required) in combination with Dunnet (inter-dose comparisons) and (Holm-)Sidak (inter-diet comparisons) *post-hoc* tests. To assess dishabituation, paired *t*-testing was done, whilst two-way ANOVA + Sidak was utilized to investigate habituation. To perform inter-dose and inter-diet comparisons, one-way ANOVA + Dunnet was used in conjunction with the first trial of the different odors. For all statistical tests, a *p*-value of 0.05 was considered statistically significant. All values are represented as mean ± SEM.

RESULTS

FA Reduces the Prevalence of Radiation-Induced Anophthalmos, Exencephaly and Agnathia

First, we examined the prevalence of radiation-induced EDs and the prevention thereof with FA fortification. The prevalence of left-eye anophthalmos (**Figure 1A**), microphthalmos (**Figure 1B**) and iris anomaly (**Figure 1C**) was significantly increased following X-irradiation (respectively, 28.26 ± 4.72, 23.56 ± 4.28, and 17.92 ± 3.39 %). In contrast, X-irradiation did not affect the prevalence of the left eye open phenotype (3.03 ± 1.40%) (**Figure 1D**). Similar observations were made for the right eye (respectively 42.41 ± 5.93, 29.32 ± 3.93, and 18.59 ± 4.35%) (**Figures 1E–H**). Here, the right eye also showed an increase of the open phenotype (4.55 ± 1.89%). Of interest, we revealed a partial prevention of radiation-induced left-eye anophthalmos with both the 8 mg/kg FA (9.02 ± 3.40%) and 12 mg/kg FA (10.62 ± 2.74%) diets (**Figure 1A**). No such rescue was observed for the right eye (**Figure 1E**).

In addition, we determined the number of fetuses with exencephaly, agnathia, gastroschisis and cleft palate. X-irradiation increased the prevalence of exencephaly (15.26 ± 3.95%) and agnathia (17.88 ± 4.17%) when the animals were fed the control diet, whilst 8 and 12 mg/kg FA provided significant prevention of both exencephaly (respectively, 4.89 ± 2.10 and 4.43 ± 2.03%) (**Figure 2A**) and agnathia (respectively, 5.41 ± 1.86 and 1.56 ± 1.56%) (**Figure 2B**). Furthermore, irradiation also increased the number of fetuses affected by gastroschisis in mothers on the control diet (11.3 ± 2.83%), but here no rescue was observed with the FA fortified diets (**Figure 2C**). Finally, X-ray exposure at E7.5 did not affect the occurrence of cleft palate in the fetuses, regardless of the diet (**Figure 2D**).

FA Counteracts the Effects of X-Ray Exposure on Pre-natal Survival

In the next part of our study, we investigated the impact of X-irradiation during neurulation on the number of implants, pre-natal deaths and fetal weight. Neither X-irradiation nor FA fortification affected the total number of conceptuses per pregnant female (**Figure 3A**). In terms of late fetal deaths (E18 fetuses with no signs of life), an increase was observed after irradiation in mothers on the control diet, while this increase was prevented with 8 and 12 mg/kg FA diets (**Figure 3B**). Furthermore, we found an increase in resorptions (implantation site at E18, which holds no developed fetus, and shows evident embryonic-stage death) after 1.0 Gy irradiation, with a notable rescue after 8 mg/kg, but not after 12 mg/kg FA fortification

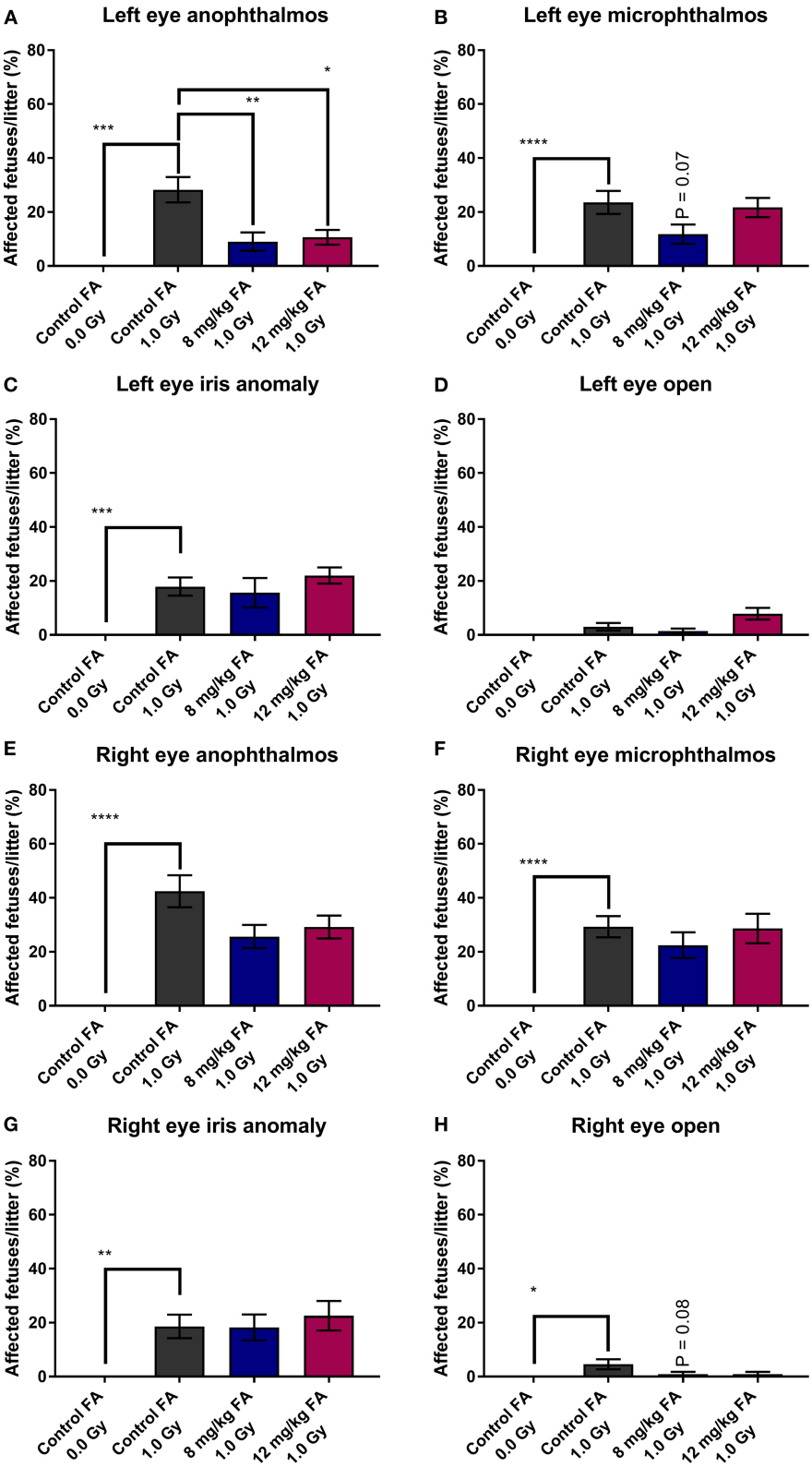

FIGURE 1 | Prevalence of various eye defects observed at E18, following 1.0 Gy X-ray exposure at E7.5, and prevention with FA fortification. Radiation significantly increased the risk for left eye anophthalmos; a defect that could in turn be prevented by both FA fortified diets (8 mg/kg and 12 mg/kg FA) **(A)**. Although both left eye

(Continued)

FIGURE 1 | microphthalmos **(B)** and iris anomaly **(C)** were induced by X-irradiation, no rescue effect of FA was observed on these phenotypes. The left eye open phenotype was not increased in prevalence following irradiation **(D)**. Although defects of the right eye, including anophthalmos **(E)**, microphthalmos **(F)**, iris anomaly **(G)** and open eye **(H)** were more prevalent following irradiation, we observed no significant prevention of these defects by FA. Data are represented as mean ± SEM, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

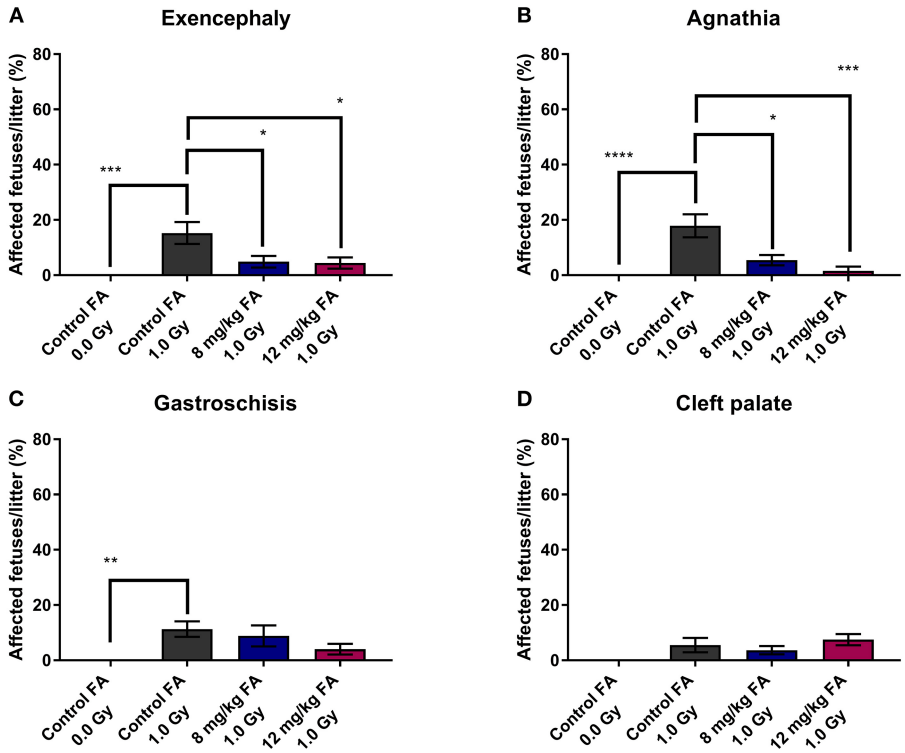

FIGURE 2 | Prevalence of radiation-induced birth defects at E18, affecting the head and abdomen, and the prevention with FA. Irradiation at E7.5 significantly increased the risk for exencephaly **(A)**, agnathia **(B)** and gastroschisis **(C)**. The first two of these radiation-induced defects could be partially prevented by FA food fortification (both 8 and 12 mg/kg) **(A,B)**. Radiation had only minimal impact on the prevalence of cleft palate **(D)**. Data are represented as mean ± SEM, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

(**Figure 3C**). Finally, irradiation resulted in a marked fetal weight loss at E18 (**Figure 3D**), which was not rescued following FA fortification. Of note, sham-irradiated fetuses gained weight when placed on the 12 mg/kg FA diet, as compared to sham-irradiated animals on the control diet (**Figure 3D**). Altogether, we were able to demonstrate a preventive role of FA for radiation-induced late fetal deaths and resorptions.

Axial Skeletal Defects and Prevention With FA

To assess general teratogenicity of X-ray exposure on the axial skeleton, alcian blue/alizarin red staining was utilized, a common methodology to assess the sub-macroscopic teratogenicity of chemical and physical agents (Young et al., 2000). X-irradiation at E7.5 increased the number of defects within the vertebrae, specifically in the atlas, the cervical vertebrae and the thoracal vertebrae when the animals were fed the control diet (**Figure 4A**, atlas, cervical and thoracal vertebrae). Within the cervical region, radiation primarily resulted in fused vertebrae and excessive cartilage (**Figures 4B,C**). At the thoracic level, the most common vertebral defects included fusions and excessive cartilage, whereas ribs were often missing (**Figures 4D,E**). Here, we also observed impaired ossification of the ribs (**Figure 4F**) and split ossification centers within the vertebrae (**Figure 4G**). Of note, irradiation also increased the incidence of a tilted sternum (**Figures 4H,I**). To a lesser extent, radiation lead to tilted vertebrae, displaced ribs, hooked (i.e., bent) ribs and short-length ribs (**Supplementary Figures 1A–C**). 8 mg/kg FA fortification prevented the occurrence of radiation-induced defects in the cervical region, whilst the 12 mg/kg diet group also showed a strong trend (henceforth defined as $P = 0.05$–0.08) toward prevention (**Figure 4**, cervical vertebrae). Surprisingly, a combination of 8 mg/kg FA and 1.0 Gy increased the number of defects within the caudal vertebrae, as compared to the 1.0 Gy irradiated animals that were fed the control diet (**Figure 4A**, caudal vertebrae). Furthermore, a trend was observed for the rescue of defects within the true and false ribs following fortification with the 8 mg/kg and 12 mg/kg diets (**Figure 4A**

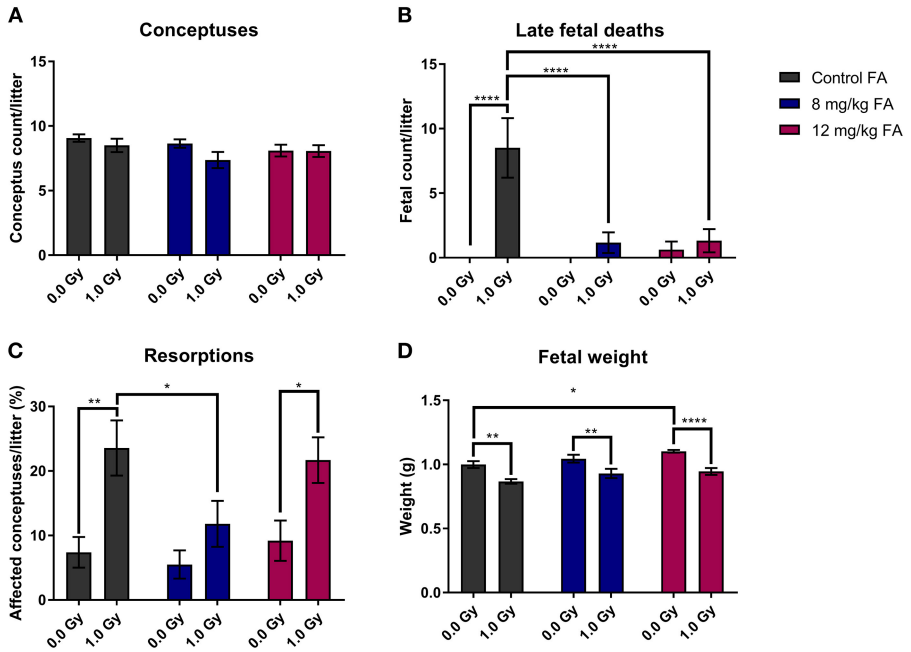

FIGURE 3 | Impact of 1.0 Gy X-ray exposure at E7.5 and FA on the number of conceptuses **(A)**, late fetal deaths **(B)**, resorptions **(C)** and fetal weight **(D)**. Neither radiation nor FA diet (8 and 12 mg/kg FA) had an impact on the number of conceptuses at E18 per litter **(A)**. Irradiation strongly increased the rate of late fetal deaths when mothers were fed the control diet, whilst FA fortification prevented this **(B)**. A significant increase in resorptions was observed for irradiated mice on the control diet, which was in turn prevented only by the 12 mg/kg FA diet **(C)**. FA fortification significantly increased fetal weight, whilst irradiation decreased fetal weight **(D)**. Data are represented as mean ± SEM, $*p \leq 0.05$, $**p \leq 0.01$, $****p \leq 0.0001$.

true and false ribs). Overall, FA fortification partially prevented skeletal defects within the cervical and thoracic vertebrae, whilst a trend toward prevention could be observed within the true and false ribs.

Abnormal Adult Brain Morphology Following Pre-natal X-Ray Exposure

We performed volumetric MRI analyses to assess whether the adult brain is structurally affected following irradiation at E7.5. Here we also assessed whether FA fortification could prevent any radiation-induced anomalies with inclusion of the 8 mg/kg FA diet, which was based on the rescue effect we observed in view of the radiation-induced fetal defects. We observed no differences in the volumes of whole brain, the olfactory system, the frontal cortex, the corpus callosum, the amygdala, the cerebellum and the corpora quadrigemina in response to radiation and/or FA (**Supplementary Table 1**). In contrast, other brain regions were affected by the radiation dose and the diet. Ventricles appeared significantly enlarged following irradiation [$F_{(2,\ 37)} = 6.125$; $P = 0.0050$] (**Figure 5A**), although no significance was reached when comparing individual radiation doses. We also found a radiation-induced reduction in volume of the hippocampus (**Figure 5B**), striatum (**Figure 5C**), thalamus (**Figure 5D**), midbrain (**Figure 5E**) and pons (**Figure 5F**) when the mothers were irradiated with 0.5 Gy. Of interest, an interaction effect between irradiation and the diet could be established for the hippocampus [$F_{(2,\ 37)} = 4.654$; $P = 0.0157$) (**Figure 5B**), midbrain [$F_{(2,\ 37)} = 4.654$; $P = 0.0157$] (**Figure 5E**) and the pons [$F_{(2,\ 37)} = 3.792$; $P = 0.0318$] (**Figure 5F**), which supports an FA-dependent rescue of radiation-induced size decrease. Furthermore, X-irradiation resulted in a trend toward a volumetric decrease of the posterior cerebral cortex [$F_{(2,\ 37)} = 2.731$; $P = 0.0783$] (**Figure 5G**) and the basal ganglia [$F_{(2,\ 37)} = 2.768$; $P = 0.0758$] (**Figure 5H**). Unexpectedly, FA food fortification reduced the size of the basal ganglia [$F_{(1,\ 37)} = 4.961$; $P = 0.0321$) (**Figure 5H**) and the striatum [$F_{(1,\ 37)} = 7.067$; $P = 0.0115$] (**Figure 5C**). A trend toward FA-induced size decrease was also observed for the anterior commissure [$F_{(1,\ 37)} = 3.796$; $P = 0.0590$] (**Figure 5I**).

Irradiation Impairs Vision and Olfaction, Which Is Ameliorated by FA Fortification

In order to determine whether X-irradiation during neurulation can affect visual acuity later in life, and whether these effects could be countered by FA, a virtual optokinetic drum was used. Here, we observed that radiation decreased visual acuity [**Figure 6A**, $F_{(2,\ 64)} = 10.02$; $P = 0.0002$], whilst FA increased visual performance as compared to animals on the control diet [**Figure 6A**, $F_{(1,\ 64)} = 6.565$; $P = 0.0128$]. Furthermore, the impairment in acuity elicited by 0.1 Gy was alleviated by FA (**Figure 6A**). SD-OCT analysis did not show any changes in total retinal thickness following X-ray exposure or FA fortification (**Figure 6B**). Yet, a more detailed investigation revealed that the nerve fiber and retinal ganglionic cell layer (NF + GCL) thickness

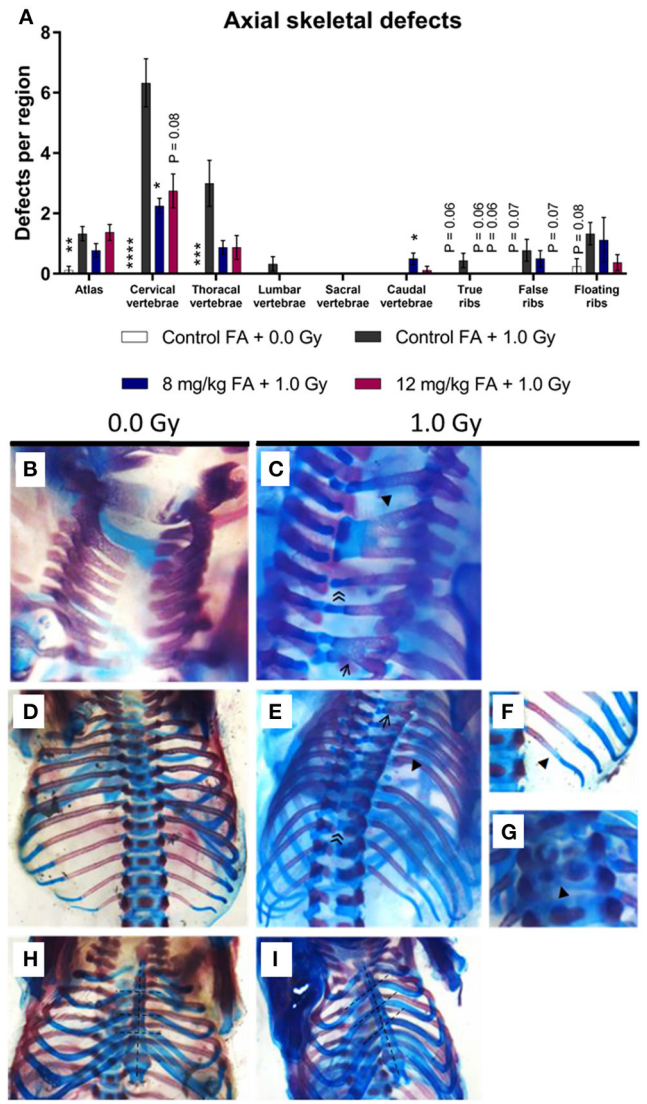

FIGURE 4 | Prevalence and categories of axial skeletal defects at E18, following 1.0 Gy at E7.5 and prevention by FA. **(A)** Radiation significantly increased the number of defects in the atlas, cervical and thoracal vertebrae, while an insignificant trend could be observed in the ribs. FA fortification with 8 mg/kg prevented defects within the cervical vertebrae, with a trend toward prevention apparent in the true and false ribs. **(B–I)** In control animals, the arches of the cervical vertebrae only sporadically demonstrated an anomaly **(B)**, whereas irradiation lead to a notable presence of vertebral fusions (affecting two or three arches, shown by an arrow ← and arrowhead ◀, respectively) and excessive cartilage ≪ **(C)**. In controls, both the thoracic vertebrae and ribs never showed any anomalies **(D)**, but irradiated fetuses often lacked ribs (arrowhead ◀) and depicted excessive cartilage (double arrowhead ≪) and fusions (arrow ←) in the vertebrae **(E)**. In addition, the ribs also showed delayed ossification (arrowhead ◀) **(F)** and the vertebral bodies showed split ossification centers (arrowhead ◀) **(G)**. Finally, radiation also promoted the presence of a tilted sternum **(H,I)**. Data are represented as mean ± SEM, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. Asterisks indicate a significant change as compared to the control FA + 1.0 Gy group of the respective skeletal region.

was decreased following 0.5 Gy irradiation [**Figure 6C**, $F_{(2, 63)} = 7.618$; $P = 0.0011$], which was not alleviated following the FA-rich diet. On the other hand, the high FA diet was shown to elicit a protective effect on the radiation-induced decrease in eye diameter (**Figure 6D**). Altogether, we showed a radiation-induced decrease in visual acuity starting from a low dose of 0.1 Gy onward, together with a reduced NF and GCL layer thickness and eye diameter following 0.5 Gy. FA prevented the decreased visual acuity elicited by 0.1 Gy, and rescued the 0.5 Gy-induced decrease in axial eye size.

To investigate whether X-ray exposure during neurulation has an impact on olfactory performance and discrimination, and whether radiation-induced differences can be rescued by FA, we performed an olfaction-dependent habituation and dis-habituation test. When presented with a novel odor, mice will show specific approach and sniffing behavior (dishabituation, see **Table 3**), which increases further in the presence of S odors, and diminishes over the three time bins of 2 min (habituation). To compare the rate of habituation and dishabituation between the groups, we calculated the difference in sniffing time between (a) within the same odor of trial 1 and trial 3 (habituation) and (b) between odors from old to new odor (dishabituation). Habituation was observed in all conditions for all odors, indicating a normal loss of interest for odors over time (**Figures 7A,B**, **Table 3**). Similarly, approach behavior to a novel odor was observed in both sham and irradiated animals and was not affected by FA enrichment (**Figures 7A,B**, **Table 3**). However, irradiation reduced the total amount of time spent sniffing NS odors under control diet conditions compared to sham-irradiated animals (**Figure 7C**). Two-way ANOVA for factor diet (control diet or FA) and dose (sham, 0.1 and 0.5 Gy) during the NS odor presentation, indicated a significant effect for diet [$F_{(1, 63)} = 4.078$; $P = 0.048$] and for dose [$F_{(2, 63)} = 3.908$; $P = 0.025$] without significant interaction. *Post-hoc* analysis revealed a significant difference between sham- and 0.5 Gy-irradiation in the control diet group, indicating a reduced approach time to NS odors after irradiation. This reduced approach was alleviated when given the high FA diet. Of note, this reduced sniffing time is not due to an inability to approach, since presentation of S odors increased the sniffing time, but is possibly due to a decrease in attractiveness or detection of the odor itself. High FA diet normalized the sniffing time and approach to novel odors to baseline levels (**Figures 7B,C**), which is indicative of a protective role for FA. A similar trend was observed for S odors, however, the two way ANOVA did not indicate a significant effect of either factors. To conclude, irradiation in conjunction with the control diet resulted in hyposmia (i.e., a decreased sense of smell) for the NS odors, or a reduced interest in NS odors. These anomalies were alleviated when the diet was fortified with FA.

No Changes in Activity and Motor Performance Following Irradiation of Animals on the Control Diet

General arousal and changes in circadian activity was assessed in the 23 h cage test. Under control diet conditions, radiation had no effect on the spontaneous activity, and all animals displayed the typical increase in night-time activity. Here, repeated measures (RM) ANOVA indicated a significant effect for time [$F_{(47, 1504)} = 38.34$; $P < 0.0001$], but not for radiation dose (**Figure 8A**).

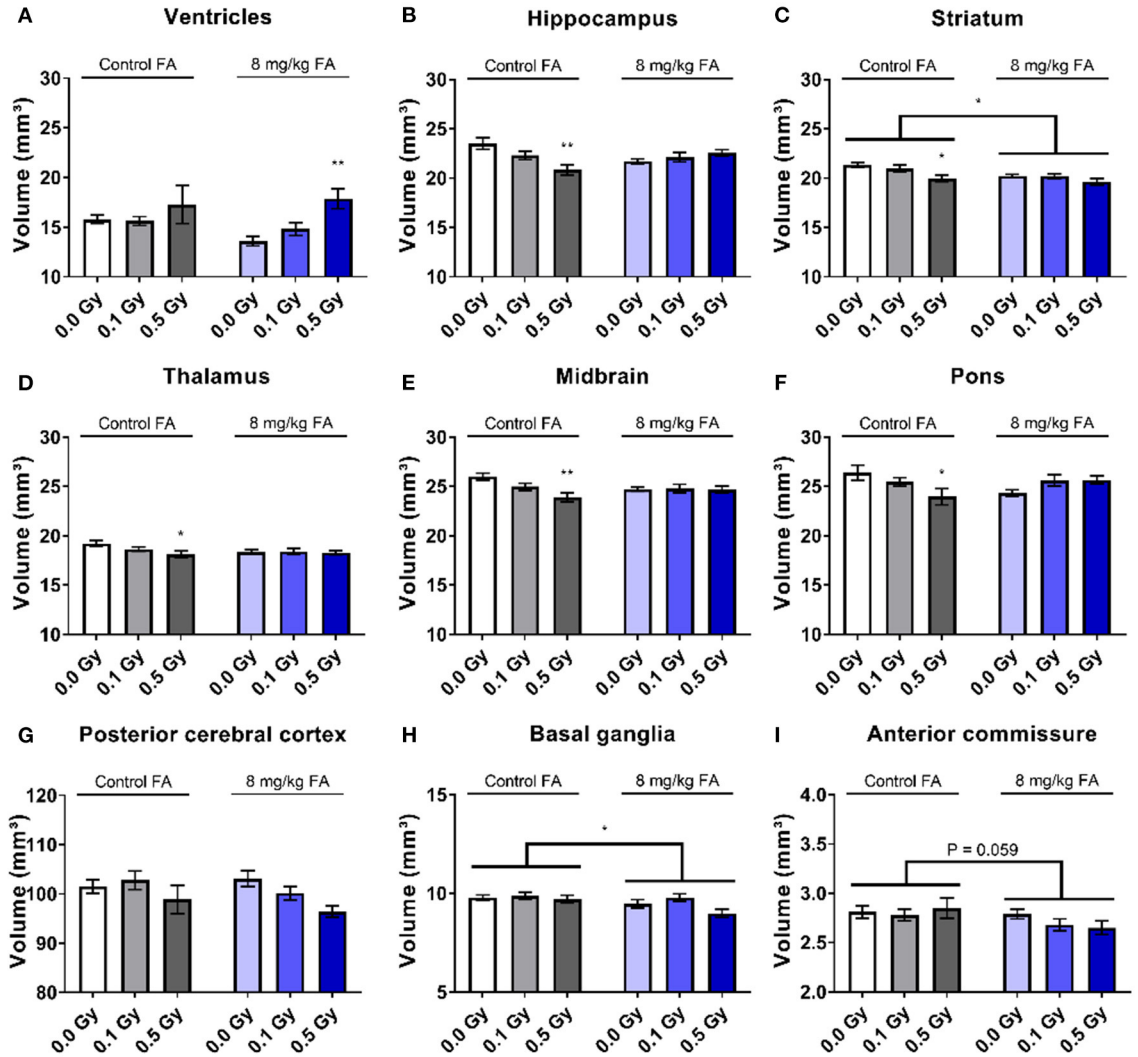

FIGURE 5 | Volumetric analyses of various brain regions after pre-natal irradiation at E7.5. Ventricles were significantly increased after irradiation **(A)**, whereas the hippocampus **(B)**, striatum **(C)**, thalamus **(D)**, midbrain **(E)** and pons **(F)** were significantly smaller following a dose of 0.5 Gy in animals on the control diet. According to two way ANOVA, the radiation factor was significant in decreasing size of the posterior cerebral cortex **(G)**. FA by itself decreased the size of the basal ganglia **(H)** and the anterior commissure **(I)**. Data are represented as mean ± SEM, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

Animals on the high FA diet also showed a typical increase in night-time activity [$F_{(47,\ 1504)} = 50.38$; $P < 0.0001$). In addition, the high FA diet increased night-time activity in mice exposed to a low dose of 0.1 Gy [$F_{(2,\ 32)} = 5.063$; $P = 0.0123$) (**Figures 8B,C**). When animals were fed the high FA diet, repeated-measures (RM) ANOVA revealed a significant interaction effect between radiation and diet during the dark period [$F_{(2,\ 64)} = 3.485$; $P = 0.0366$], and *post-hoc* analysis indicated that only 0.1 Gy was significantly different from the sham-irradiated group ($P = 0.0191$). This interaction effect was also observed in the overall duration of the experiment [$F_{(2,\ 64)} = 4.127$; $P = 0.0206$] (**Figure 8D**).

Balance and coordination was tested on the accelerating rotarod, but radiation had no effect on motor coordination, and we also saw no effect of FA diet (**Supplementary Figure 2**).

Radiation Did Not Affect Overall Cognition, but FA Adversely Altered Social Behavior

Open Field and Social Exploration

The open field test was used to assess exploratory behavior in a novel and stressful environment. The animals are dark-adapted and then placed in a brightly illuminated open field for 10 min. Anxious animals will spend their time close to the walls and will not enter the open center zone. We observed that all groups spent most of their time close to the wall, and there was no effect of radiation nor of FA enriched diet on the time spent in the periphery (**Figure 9A**). Center visits were frequent (**Figure 9B**) but overall rather short (**Figure 9C**). Furthermore, the distance the animals traveled over 10 min was similar in all groups (**Figure 9D**). Likewise, other parameters, such as latency to enter the center, walking speed or distance traveled in the

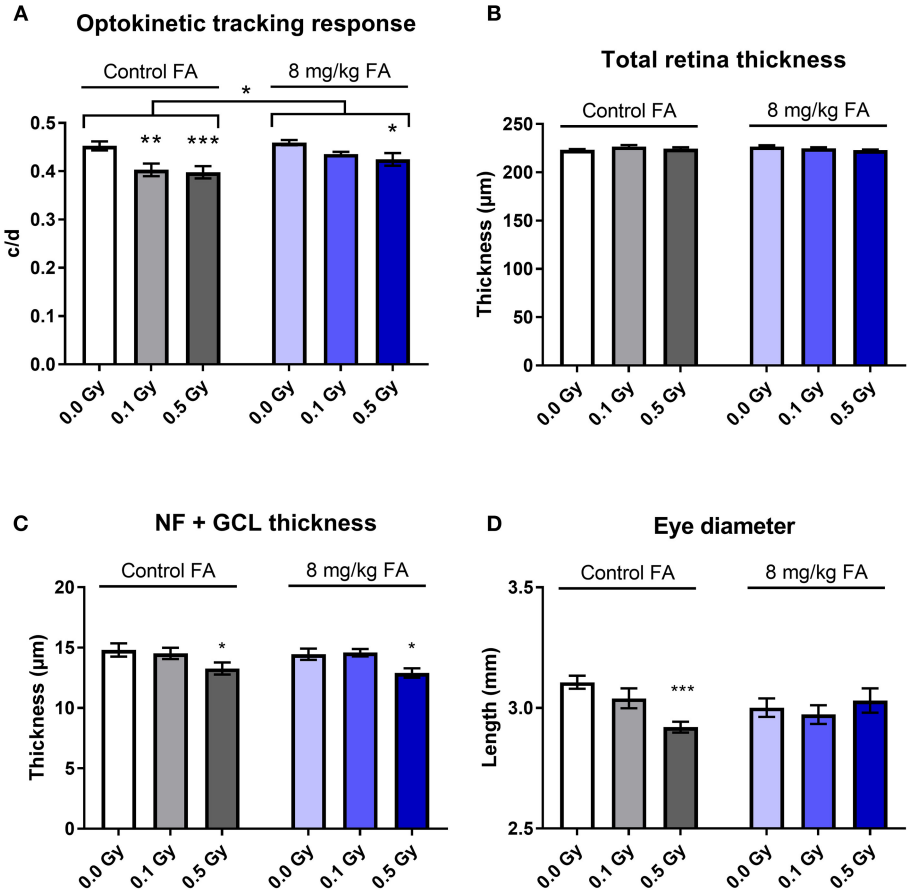

FIGURE 6 | Visual acuity, retinal morphology, eye size and the impairment thereof by X-irradiation during neurulation. Radiation decreased visual acuity in adult mice (W5-W7) starting from 0.1 Gy, according to the optomotor test **(A)**. 8 mg/kg FA increased the irradiation dose required for a significant effect on optokinetic response to 0.5 Gy **(A)**. Irradiation or FA diet did not affect total retinal thickness, according to OCT analysis **(B)**, but a more detailed analysis showed that 0.5 Gy significantly reduced thickness of the nerve fiber + ganglion cell layer **(C)**. Using the MRI images for eye-size measurements, we identified a reduced axial length following 0.5 Gy, which was in turn ameliorated by FA fortification **(D)**. Data are represented as mean ± SEM, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

TABLE 3 | Differences in sniff time used to assess habituation and dishabituation.

	Control diet			8 mg/kg FA		
Dose	0.0 Gy	0.1 Gy	0.5 Gy	0.0 Gy	0.1 Gy	0.5 Gy
Habituation: difference in sniff time within same odor						
Time (s)	−26.2 ± 3.2	−21.8 ± 3.7	−18.7 ± 2.1	−24.7 ± 2.2	−22.5 ± 3.2	−23.0 ± 3.3
Dishabituation: difference in sniff time from old to new odor						
Time (s)	30.6 ± 4.0	13.0 ± 3.9	20.4 ± 3.2	30.9 ± 4.1	25.3 ± 4.0	27.4 ± 3.2

To quantify habituation (reduction in sniffing time to the same odor) and dishabituation (changes in sniffing time from old to new odor), we calculated the difference between t_{x1}-$t_x = \Delta t$, and averaged the values across all odors for each animal. All Δt were different from 0 and there was no effect of radiation and/or FA. Data are represented as mean ± SEM.

center were also not different between the groups (respectively, **Supplementary Figures 3A–C**). We used a modified setup to evaluate social approach: two stranger mice were placed in the center of the arena and provide an attraction point for the test mouse. We observed similar distance covered in all groups (**Figure 10A**), but FA fortification decreased the time spent in the center as compared to animals on the control diet [**Figure 10B**, $F_{(1, 63)} = 4.316$; $P = 0.0418$]. Factors such as center distance, center entries, mean speed, time in periphery and latency to enter the center were unaltered (respectively, **Supplementary Figures 4A–E**).

Elevated Plus Maze

The EPM is considered the typical test to assess anxiety related exploration. We observed no significant difference in total beam breaks between the different groups. Two-way ANOVA indicated no effect of factor radiation [$F_{(2, 63)} = 0.4912$; $P = 0.6142$] nor of diet [$F_{(1, 63)} = 2.206$; $P = 0.1424$) on total beam breaks (**Figure 11A**). Open arm visits (**Figure 11B**) and open arm dwell (**Figure 11C**), both readouts for anxiety, were similar in all groups. In general, neither radiation exposure nor diet had an impact on anxiety-related activity.

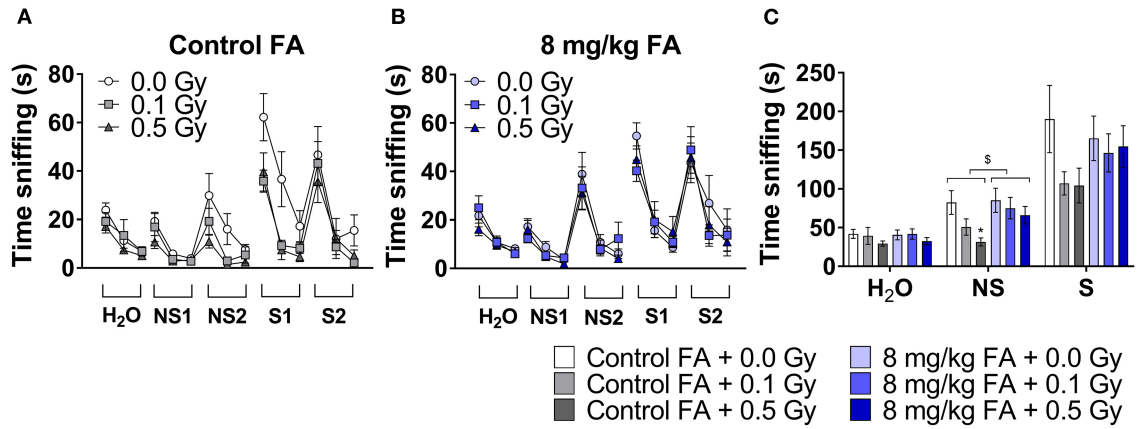

FIGURE 7 | Odor habituation and dis-habituation in adult mice (W9–W11) following pre-natal irradiation at E7.5, under control or FA fortified diet. All animals show a typical approach behavior toward novel odors (dishabituation) and reduction in sniffing over time toward the same odor (habituation) **(A,B)**. Under control diet condition, irradiated animals show reduced sniffing time to novel odors **(A)**. In contrast, under high FA diet, sniffing time is at sham level **(B)**. The total amount of sniffing time toward a non-social odor, was reduced in irradiated animals under control diet, while high FA fortification increased the sniffing time to sham levels **(C)**. Data are represented as mean ± SEM, $^*p \leq 0.05$ vs. sham (*post hoc*), $\$ p \leq 0.05$ control vs. FA diet (two-way ANOVA).

Morris Water Maze

During place learning, under control diet, all groups learned in a similar way to locate the hidden platform (**Figure 12A**). RM ANOVA indicated an effect for day [$F_{(9, 270)} = 41.4$; $P < 0.001$], but not for radiation dose and no interaction. In contrast, under FA conditions, RM ANOVA indicated an effect for day [$F_{(9, 288)} = 37.0$; $P < 0.001$], and an effect of dose [$F_{(2, 288)} = 4.34$; $P = 0.022$), without interaction (**Figure 12B**). *Post-hoc* analysis indicated, as compared to sham-irradiated animals, a significantly longer path length in 0.5 Gy-irradiated animals only on the first 2 days [$q_{(3)} = 4.0$; $P = 0.013$, and $q_{(3)} = 3.6$; $P = 0.029$] (**Figure 12B**). However, this finding was considered biologically irrelevant due to the low number of days affected. The probe trials were interspersed after day 5 and 10. Target quadrant preference was evaluated by comparing time spent in the target quadrant with chance level (25%). Under control diet conditions, 0.1 Gy-irradiated mice showed a clear lack of target quadrant preference even after 2 weeks of training, which was also true for 0.5 Gy-irradiated animals during the first probe trial (**Figure 12C**). When the diet was FA fortified, all radiation-dose groups demonstrated significant target quadrant preference during the second trial (**Figure 12C**), suggestive for a FA-induced amelioration of reference memory and supporting FA to have a role in learning and memory. Nonetheless, these results are to be interpreted with caution due to the relatively low numbers of animals being included in the analysis.

Passive Avoidance

The effect of pre-natal X-ray exposure and FA on amygdala and hippocampal dependent fear-related memory formation was tested using the passive avoidance set-up. Animals on the control diet [$F_{(1, 30)} = 86.87$; $P < 0.0001$] and on the high FA diet [$F_{(1, 30)} = 219.7$; $P < 0.0001$] demonstrated an increased latency to enter the dark chamber after the shock (**Supplementary Figure 5A**). A comparison between animals on the control diet and on the high FA diet revealed no interaction between diet and latency [**Supplementary Figure 5B**, $F_{(1, 18)} = 0.1248$; $P = 0.7280$]. These data suggest that neither radiation nor high FA diet has an impact on passive avoidance learning.

DISCUSSION AND CONCLUSION

Radiation-Induced Anophthalmos, Exencephaly and Gastroschisis Are Prevented by FA

X-irradiation at E7.5 induced various congenital eye defects, exencephaly, agnathia and gastroschisis in the offspring. Interestingly, the right eye appeared more susceptible toward radiation-induced anophthalmos as compared to the left eye. This observation is in line with our previous study (Craenen et al., 2017) and could be explained by the used mouse strain with a C57BL6/J genetic background. C57BL6/J mice have a strong natural tendency toward developing asymmetrical eye defects, with a bias toward right-eye anophthalmos/microphthalmos (Smith et al., 1994). Alternatively, in the developing embryo there are various gestational stages that demonstrate left-right asymmetry (e.g., various signaling mechanisms). Even the developing eye is known to exhibit such developmental asymmetry (Levin, 2005), hypothetically allowing potential teratogens such as ionizing radiation to interfere with the left-right axis during embryogenesis. Hence, to assess why radiation induces an asymmetric eye phenotype, it is of interest to compare our results to a different mouse strain, and to more closely explore the molecular mechanisms along the embryonic left-right axis after X-irradiation.

In this study, we demonstrated for the first time that FA fortification (8 mg/kg FA and 12 mg/kg FA) prevents radiation-induced anophthalmos, exencephaly and agnathia. Already in the 1960's a link between FA intake and the incidence of congenital

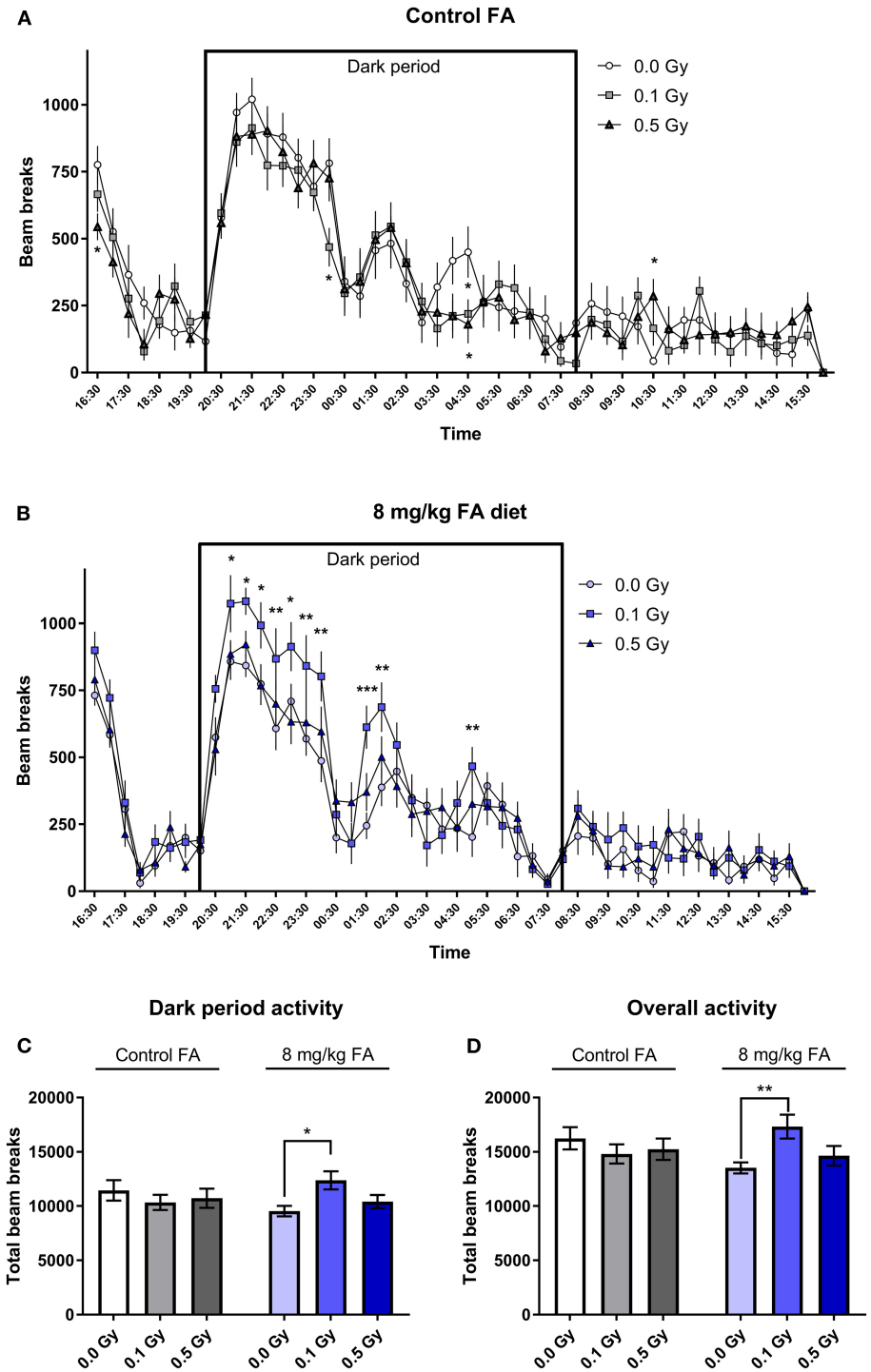

FIGURE 8 | Cage activity in adult mice (W7–W9) and the impact of X-irradiation at E7.5 and FA food fortification. When the control diet was fed, no effect of pre-natal radiation exposure on cage activity was observed **(A)**. When animals were fed the FA diet, irradiation with 0.1 Gy at E7.5 significantly increased activity during the dark period, as tested in adult 7–9 week old mice **(B)**. **(C,D)** A summarized total of beam breaks during the dark period **(C)** and the overall experiment **(D)** confirmed the observations made in **(A,B)**. Data are represented as mean ± SEM, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

EDs was suggested. For instance, maternal FA-deficiency was shown to increase the risk of EDs in rats (Armstrong and Monie, 1966). A later study supported these findings, where a FA-deficient diet in mice could lead to anomalies such as anophthalmos and microphthalmos (Maestro-de-las-Casas et al., 2013). Furthermore, ethanol-induced retinal anomalies were

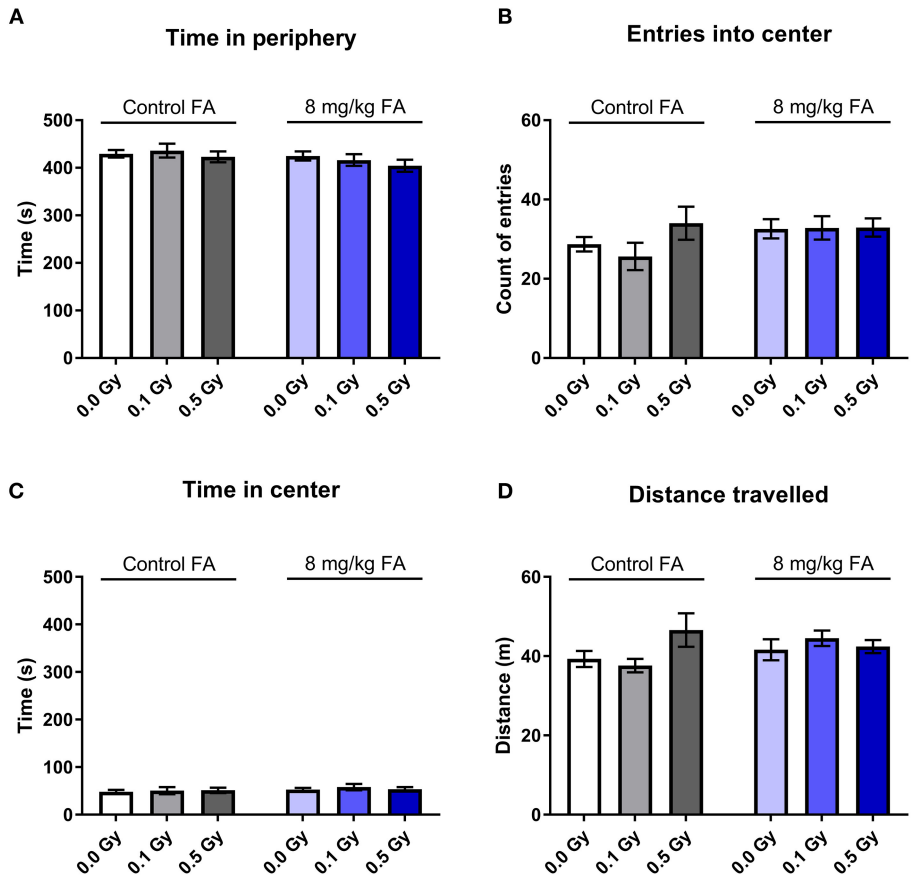

FIGURE 9 | Effect of embryonic X-irradiation and FA fortification on exploration behavior in adult mice, according to the open field test. Neither E7.5 irradiation, nor diet had an impact on the time spent in the periphery **(A)**, entries into the center **(B)**, time spent in the center **(C)** and the total distance traveled **(D)**. Data are represented as mean ± SEM.

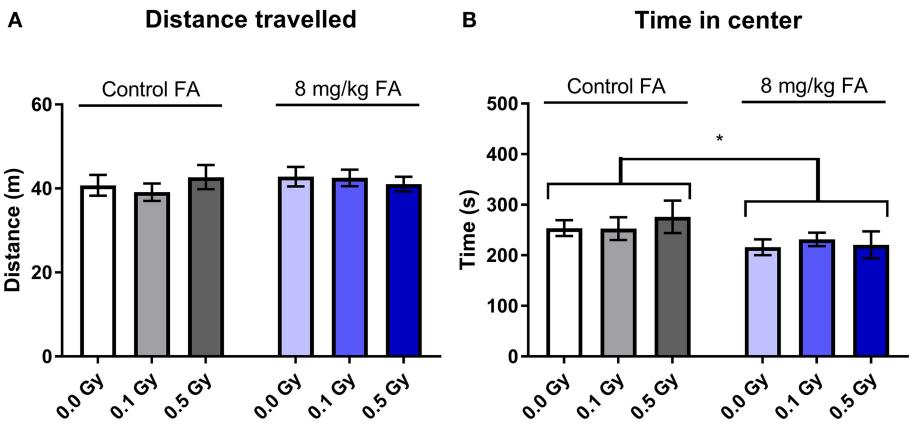

FIGURE 10 | Social exploration in an adapted open field test was unaffected by 1.0 Gy exposure at E7.5, but was impaired by FA fortification. Neither radiation, nor FA diet had an impact on exploration behavior in adult mice, according to the total distance traveled in the arena **(A)**. In contrast, FA fortification resulted in animals spending less time in the center, in close proximity to the unknown mouse **(B)**. Data are represented as mean ± SEM, $^{*}p \leq 0.05$.

rescued with FA supplementation in zebrafish (Muralidharan et al., 2015). In contrast, an epidemiological study could not determine a link between FA intake and the risk for anophthalmos and microphthalmos. Yet, the authors conceded that several caveats such as a small case population and the lack of clinical analyses of key biomarkers may have impaired proper

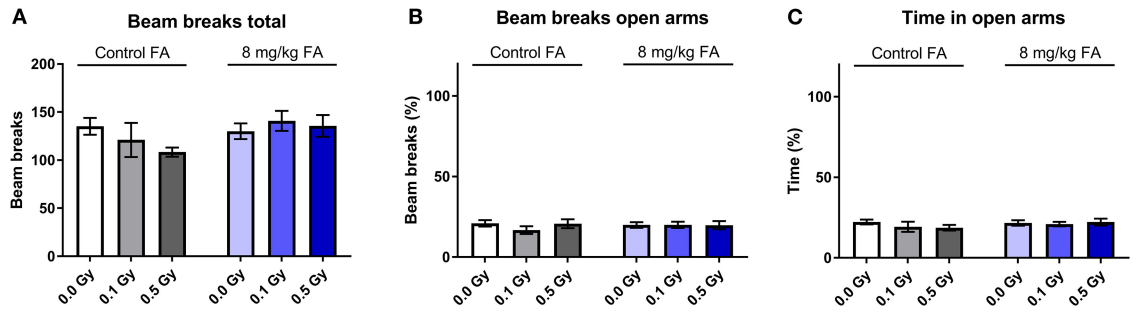

FIGURE 11 | Effect of embryonic X-irradiation and FA fortification on anxiety, tested in the elevated plus maze. Neither irradiation at E7.5 nor diet had an effect on the total number of beam breaks **(A)**, beam breaks in the open arms **(B)** or time spent in the open arms **(C)** by adult animals. Data are represented as mean ± SEM.

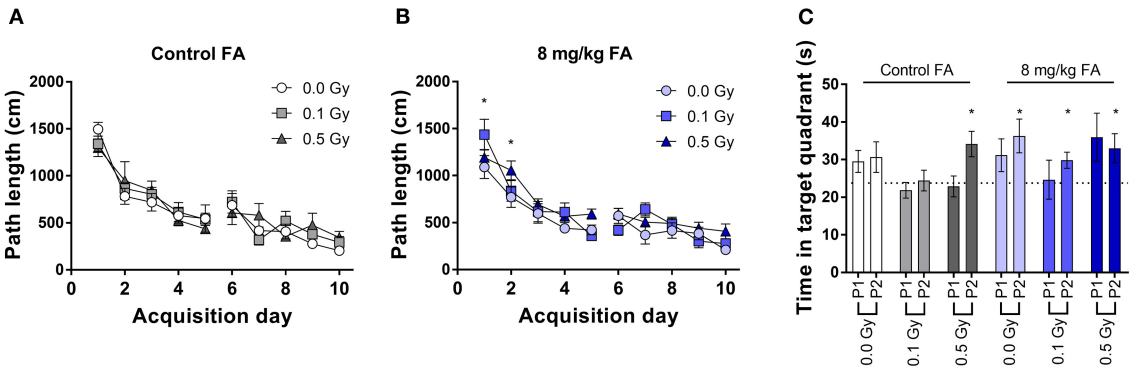

FIGURE 12 | Spatial place learning and reference memory of adult mice in the MWM, following X-ray exposure at E7.5 and/or FA food fortification (8 mg/kg FA). All groups learned to located the hidden platform under control diet **(A)** and FA fortification **(B)**. Reference memory was assessed during interspersed probe trials **(C)**. In FA enriched diet, all animals displayed significant preference to the target quadrant above chance level (25%). Data are represented as mean ± SEM, $*p \leq 0.05$ to chance level (25 %).

effect estimation (Shaw et al., 2007). The second category of radiation-induced birth defects that appears folate-responsive, is exencephaly. The prevention of exencephaly by FA is well-described in literature, albeit not with ionizing radiation as the effecting teratogen, but with e.g., ethanol (Yanaguita et al., 2007) and glucose (Wentzel and Eriksson, 2005; Oyama et al., 2009). Of note, international FA food fortification initiatives have already successfully decreased the incidence of NTDs (Blom et al., 2006). The third category of radiation-induced birth defects, that is partially preventable by FA, is agnathia. In humans, agnathia is a very rare congenital disorder, commonly classified within the otocephaly family of disorders (incidence <1/70 000 births) (Gekas et al., 2010; Herman et al., 2012; Jagtap et al., 2015; Sergouniotis et al., 2015). To our knowledge, the only published observation where FA fortification could prevent agnathia was in *Twisted gastrulation* mutant mice, which have a high penetrance of midline facial defects and jaw defects (Billington et al., 2013). Even though our study is the first to demonstrate the prevention of X-ray-induced anophthalmos, exencephaly, and agnathia, the rescue is only partial. It would be of interest to further explore the efficacy of other radioprotectant compounds, potentially in combination with FA, in preventing these defects.

In contrast to the defects discussed above, we observed no folate-responsiveness of radiation-induced iris anomalies, open eye and gastroschisis. The iris anomaly observed in our study was characterized by a strongly decreased pupil size, with the most severe cases having no apparent pupil at all (Smith et al., 1994; Craenen et al., 2017). In accordance, in a previous study on hyperthermia-induced iris anomalies, no protective effect of FA could be found (Czeizel et al., 2011). Yet, there are to our knowledge no other publications that have previously investigated the efficacy of FA in preventing open eye anomalies. Hereto, it might be worthwhile to investigate the protective effect of thyroxine supplementation on radiation-induced open eyes, as some success was already made with this hormone (Juriloff, 1985). With regard to gastroschisis, it remains severely debated whether these defects can be prevented with FA, with efficacy strongly depending upon the acting teratogen (Godwin et al., 2008; Paranjothy et al., 2012; Yang et al., 2016).

Reduced Fetal Weight and Increased Pre-natal Death Following Irradiation Are Ameliorated by FA

Aside from gross macroscopic defects, other aspects of pre-natal development were also assessed. Irradiation significantly reduced fetal weight at E18, as was also observed in a previous study (Craenen et al., 2017), which was not notably ameliorated

by FA. This stands in contrast to the teratogen ethanol, where embryotoxic weight loss can be prevented by FA (Xu et al., 2006). However, a potentially adverse outcome observed after fortification of the highest dose of FA was the increase of fetal weight at E18. Epidemiological studies already reported that increased fetal weight is a consequence of FA fortification (Tamura and Picciano, 2006; Balarajan et al., 2013; Li et al., 2016; Ramakrishnan et al., 2016), which is linked to type 2 diabetes and adult obesity (Curhan et al., 1996; Johnsson et al., 2015). Both the number of late fetal deaths and resorptions were increased following irradiation, which is consistent with previous work (Pampfer and Streffer, 1988; Kim et al., 2001; Craenen et al., 2017). These cases of pre-natal mortality were reduced by FA fortification, which is in line with epidemiological studies that focused on fetal loss (Andersen et al., 2010) and miscarriage (Byrne, 2011).

Radiation-Induced Fetal Morphological Defects, and Prevention With FA

Since the mid-twentieth century, it has already been known that exposure to (high) doses of ionizing radiation during pregnancy can result in a variety of axial and appendicular skeletal defects (Jarmonenko, 1988). These studies focused mostly on severe spinal defects such as spina bifida, which are known to result from exposure to external radiation sources such as neutrons and γ-rays (A-bombs) and internal contamination from e.g., depleted uranium (commonly used in munitions) [reviewed by Hindin et al. (2005)]. In our study, pre-natal irradiation had the most detrimental impact on the cervical and thoracic vertebrae. This was also observed in a study by Russell, who used 2.0 Gy of X-rays at E7.5 (Russell, 1956). A contrasting study described more malformations in the ribs of CRI mice than in the vertebrae, following 2.0 Gy-γ-irradiation at E7.5 (Kim et al., 2001). This difference might be mouse strain dependent, or might result from variations in radiation dose and type. In further support of our study, a dose-dependent induction of skeletal malformations after irradiation (0.5 Gy to 4.0 Gy) at E11.5 was observed (Kim et al., 2001). It would therefore be interesting to further investigate this dose-dependency and the existence of a dose-threshold in our experimental set-up. Intriguing was the presence of split spinal ossification centers after irradiation, which could lead to open vertebral arches and potentially spina bifida occulta in later life (Regnier et al., 2002). Altogether, we are the first to explore in such detail developmental defects in the axial skeleton after irradiation at E7.5, and to demonstrate that FA fortification can significantly reduce the risk for radiation-induced skeletal defects.

Although we observed an increase in axial skeletal defects after 1.0 Gy irradiation, it appears that the sub-lethal doses (Craenen et al., 2017) used for the behavioral assays ($\leq$0.5 Gy) might have been too low to elicit any functional detriment. Indeed, in terms of motor performance, none of the behavioral tests could identify a clear impairment following irradiation, as is discussed in more detail below.

Persistent Radiation-Induced Defects in the Adult Nervous System and the Preventive Role of FA

Because pre-natal exposure to ionizing radiation can induce gross congenital central nervous system defects (e.g., microphthalmos and anophthalmos) at moderate to high X-ray doses (0.5–1.0 Gy) (Craenen et al., 2017), we decided to explore whether this can elicit functional and morphological neurological defects that persist into adult age.

We used *in vivo* MRI to investigate whether X-ray exposure during neurulation has an effect on adult brain and eye morphology. Although we observed no global microcephaly, as was shown after irradiation during neurogenesis (Verreet et al., 2015, 2016a), volumetric analyses unveiled a decreased volume of some dedicated brain areas. More specifically, we observed that 0.5 Gy significantly reduces the size of the hippocampus, striatum, thalamus, midbrain and pons. These structures are involved in various mechanisms, ranging from cognition to visual acuity. For example, the thalamus is known for its importance in processing and relaying visual information (Tyll et al., 2011). The pons and midbrain are also involved in visual functioning, as anomalies within these brainstem regions can result in both horizontal and vertical gaze palsy (Strupp et al., 2014; Lin et al., 2018). Furthermore, we observed that irradiation with 0.5 Gy decreased the axial length of the adult eye, which can be relayed directly to an increased incidence of radiation-induced microphthalmia (Verma and Fitzpatrick, 2007; Craenen et al., 2017), and can be associated to an increased risk of refractive errors (Bhardwaj and Rajeshbhai, 2013). Supporting the MRI-based findings, SD-OCT revealed a decreased thickness of the NF+GCL layer in the adult eye, following 0.5 Gy at E7.5, which might lead to a decreased visual acuity (Moster et al., 2016).

Concomitant with these radiation-induced alterations in the brain and the observed eye anomalies, we indeed observed a decreased visual acuity following E7.5 irradiation. Interestingly, this was also observed in 0.1 Gy-irradiated animals, that did not show a decreased eye size and NF+GCL thickness nor a reduction in brain volumes, suggesting that other mechanisms might also be involved. The morphological defects underlying the radiation-induced loss of visual acuity may thus extend beyond changes in eye structure and warrants further investigation.

Even though pre-natal irradiation had no marked impact on the olfactory system in the adult brain, behavioral tests for olfaction were included in the test battery. This decision was based on a previous study that showed transient transcriptional disturbances in the embryonic head following 1.0 Gy irradiation at E7.5 that were related to the development of the olfactory epithelium (Craenen et al., 2020b). This is an important observation, as the olfactory system starts to develop during this neurulation period (Treloar et al., 2010). Besides, the overall process of olfaction extends well-beyond the olfactory lobe (Lehmkuhl et al., 2014), with congenital anomalies within the peripheral olfactory system (e.g., the olfactory epithelium) having been linked to hyposmia (Bergman et al., 2010). We are the first to demonstrate a decreased olfactory acuity in adult mice following 0.5 Gy at E7.5. In particular, we could demonstrate a

more pronounced anosmia for NS than S odors, which could be attributed to the functional importance of social odors and/or chemical differences between the respective odorants (Sinding et al., 2017). Since defects at this level might explain the observed hyposmia, it is of interest for future studies to more closely investigate this complex structure following prenatal X-irradiation.

In contrast to the morphological and sensory anomalies discussed above, we observed no effect of irradiation on cognition. Previous behavioral screenings demonstrated that irradiation of mice during neurogenesis had a marked effect on memory-based performance (Verreet et al., 2015, 2016a). However, when we irradiated animals during neurulation, no evidence for impaired memory formation or retention could be observed, both for spatial and fear-dependent learning. The observation that 0.5 Gy irradiation decreases hippocampal volume may appear paradoxical in that sense, but this volumetric decrease might not be directly related to a loss in function. Although irradiated animals had a notable loss of visual acuity, the defect may have been too small to influence MWM performance, which depends on large and distinct visual cues (Lindner et al., 1997; Brown and Wong, 2007; Phillips et al., 2013; Vorhees and Williams, 2014).

Many of the radiation-induced anomalies that were observed in adult mice could be (in part) prevented by FA fortification. FA fortification by itself had only a minimal impact on the adult tests. For instance, we observed a decreased social exploration in mice on the FA-fortified diet and also identified a lower volume of the basal ganglia (i.e., caudate putamen and adjacent structures). This might be related to the social impairments in these mice, since a decreased basal ganglia volume was already linked with autism-spectrum disorder (Barua et al., 2014), but whether FA fortification is a risk factor for abnormal social behavior remains controversial [reviewed in Wiens and DeSoto (2017)]. Furthermore, this volumetric loss might have played a role in the changes in cage activity as well, because the basal ganglia are involved in the regulation of the sleep-wake cycle (Qiu et al., 2010) and general activity (Portmann et al., 2014).

It is important to note that, depending on the causative factor, congenital defects may respond in a dose-dependent manner to FA fortification, with higher doses of FA yielding lower defect prevalences (Gray and Ross, 2009). Yet, in our study, the radioprotective role of FA did not appear dose-responsive and no added benefit was noted following 12 mg/kg FA fortification. Hence, we decided to limit the studies in adult animals to the 8 mg/kg FA diet. Radiation-induced volumetric decreases of the hippocampus, striatum, thalamus, midbrain and pons were prevented with FA fortification. In addition, FA rescued visual acuity loss following a dose of 0.1 Gy, but not 0.5 Gy. Yet, not all morphological eye anomalies were prevented by FA, for instance the radiation-induced reduction of NF+GCL thickness. Finally, radiation-induced hyposmia for NS odors was alleviated by AF. To the best of our knowledge, were are the first to highlight this radioprotective/antiteratogenic character of FA. In all, we can conclude that X-ray exposure during neurulation affects the adult nervous system at both a morphological and functional level, from a dose of 0.1 Gy onward, and that these defects can be in part prevented by FA food fortification. In the context of radiation protection, our study supports the use of FA fortification to increase the dose threshold required to elicit adult brain and eye anomalies.

Potential Mechanisms Underlying FA-Mediated Radioprotection

Although the exact mechanism through which FA elicits its radioprotective role is currently unknown, it is still of interest to highlight several likely modes of action. A first hallmark consequence of ionizing radiation exposure is the generation of reactive oxygen and nitrogen species, which can in turn damage various cellular structures. The detrimental impact of excessive oxidative stress in the developing embryo and pregnant mother has been repeatedly addressed in literature. Indeed, it appears that a disturbed redox status is a recurrent theme in the etiology of birth-defects caused by various chemicals, including thalidomide, phenytoin and ethanol (Dennery, 2007). FA is known to have antioxidative properties *in vitro*, which is suggestive of its potential radioprotective effect, but it remains unclear whether this antioxidative role persists at a systemic level *in vivo*. A second hallmark consequence of irradiation is DNA-damage (Reisz et al., 2014), which can theoretically be repaired more efficiently with an increased access to one-carbon donors such as FA. A third hallmark consequence of irradiation includes epigenetic alterations, in particular DNA methylation. Folates fulfill an important role in DNA methylation (Crider et al., 2012). As the key one-carbon donor behind the methylation process, it stands to reason that changes in the folate pool would affect this epigenetic process and potentially reverse radiation-induced DNA hypomethylation. The fourth potential mode-of-action lies in radiation-induced changes in the transcriptome and proteome. We previously demonstrated that X-irradiation (1.0 Gy) at E7.5 in mice reduced the expression of Lhx2, a key transcription factor for eye, brain and olfactory development (Craenen et al., 2020b). Furthermore, mutations in genes associated with Lhx2 are known to cause birth defects such as exencephaly (Barbera et al., 2002). As such, it is of interest to assess whether the radiation-induced suppression of Lhx2 transcription/translation can be alleviated by FA fortification. Although this study does not address any of the aforementioned modes-of-action directly, an exploration of the mechanisms that might be involved in the antiteratogenic and radioprotective effect of folic acid is warranted. Such novel insights might contribute to developing even more efficient means to protect the unborn child from genotoxic hazards such as radiation.

CONCLUSION

FA food fortification is effective at partially preventing the embryotoxic effects of X-ray exposure. Specifically severe defects such as anophthalmos, exencephaly and agnathia were responsive to FA. In addition, late fetal deaths, the incidence of resorptions, fetal weight and skeletal defects within the cervical and thoracal vertebrae were all negatively affected by 1.0 Gy X-irradiation at E7.5, which was in turn partially countered by FA. Behavioral

studies demonstrated that X-ray exposure to sub-lethal doses ($\leq$0.5 Gy) at E7.5 resulted in a decrease of visual acuity and olfactory performance in the habituation/dishabituation test. The impaired visual performance was supported by radiation-induced loss of NF+GCL thickness and a decreased eye diameter, at least for the highest dose of 0.5 Gy. We can conclude from our MRI data that irradiation during neurulation has more site-specific consequences than irradiation during neurogenesis (Verreet et al., 2015, 2016a). As such, it would be of interest to follow up this study with more sensitive behavioral tests, tailored more specifically to those brain regions that are decreased in volume following X-irradiation.

The increasing exposure of humans to ionizing radiation is a contemporary topic that deserves proper investigation. The heightened exposure to ionizing radiation finds its roots in the clinical environment, nuclear disasters, war or terrorist activities and natural sources such as Radon gas. With this research paper, the authors wish to address and promote novel radioprotection strategies such as FA fortification and the future implementation thereof in high-risk groups that currently do not have access to FA-fortified staple foods (or FA supplements). Included in these risk-groups are e.g., pregnant patients who require radiodiagnostics or radiotherapy and pregnant women living in radioisotope-contaminated regions. The fetal doses that can be expected during clinical exposure events (including conventional radiotherapy, computed tomography and nuclear medicine) range from 0.01 to 43.9 mGy (Lazarus et al., 2009). These doses are lower than those used in this study, as we opted to reduce the number of animals required to observe significant radiation effects. As such, it is difficult to make a direct extrapolation from the animal research presented here to the human exposure scenarios listed above. Nonetheless, as a proof of concept this study demonstrates the potential for using FA fortification to protect the unborn child against ionizing radiation. Protecting the unborn child from the detrimental effects of ionizing radiation will improve their quality of life, by preventing radiation-induced birth defects and sensory deprivation. Although our study in mice indicates that *ad libitum* FA food fortification at 8 mg/kg is sufficient to provide a radioprotective effect, the optimal concentration for humans remains to be studied in the context of radiation protection. Considering both the promising results and the limitations of this study, the authors support larger (epidemiological) studies (with lower fetal radiation doses) to explore the use of FA as a radioprotectant in humans.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Ethical Committees of SCK-CEN/VITO (ref. 02–012) and the Animal Welfare Committee of the KU Leuven.

AUTHOR CONTRIBUTIONS

MB and MV mentored KC, LM supervised KC as University promotor. KC, MV, LM, and MB planned the experiments and offered guidance regarding experimental design. KC performed the majority of the lab work and wrote the manuscript. LC, JB, and MN contributed significantly to lab work. KG processed the MRI images and WG assisted with the MRI image capture procedures. ZC-V and RD'H offered invaluable guidance and assistance regarding the behavioral tests. MB, MV, LM, KG, SB, ZC-V, RD'H, and UH reviewed the initial manuscript and offered crucial feedback that contributed to the submitted paper. KC was a joint PhD student of SCK CEN and KU Leuven funded through a scholarship of SCK CEN. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2020.609660/full#supplementary-material

REFERENCES

Andersen, G. S., Friis, H., Michaelsen, K. F., Rodrigues, A., Benn, C. S., Aaby, P., et al. (2010). Effects of maternal micronutrient supplementation on fetal loss and under-2-years child mortality: long-term follow-up of a randomised controlled trial from Guinea-Bissau. *Afr. J. Reprod. Health* 14, 17–26. Available online at: http://www.ncbi.nlm.nih.gov/pubmed/21243915 (accessed January 5, 2018).

Arbuckle, E. P., Smith, G. D., Gomez, M. C., and Lugo, J. N. (2015). Testing for odor discrimination and habituation in mice. *J. Vis. Exp.* 99:e52615. doi: 10.3791/52615

Armstrong, R. C., and Monie, I. W. (1966). Congenital eye defects on rats following maternal folic-acid deficiency during pregnancy. *J. Embryol. Exp. Morphol.* 16, 531–42. Available online at: http://www.ncbi.nlm.nih.gov/pubmed/4960285 (accessed August 22, 2014).

Balarajan, Y., Subramanian, S. V., and Fawzi, W. W. (2013). Maternal iron and folic acid supplementation is associated with lower risk of low birth weight in India. *J. Nutr.* 143, 1309–1315. doi: 10.3945/jn.112.172015

Barbera, J. P. M., Rodriguez, T. A., Greene, N. D. E., Weninger, W. J., Simeone, A., Copp, A. J., et al. (2002). Folic acid prevents exencephaly in Cited2 deficient mice. *Hum. Mol. Genet.* 11, 283–93. doi: 10.1093/hmg/11.3.283

Barua, S., Chadman, K. K., Kuizon, S., Buenaventura, D., Stapley, N. W., Ruocco, F., et al. (2014). Increasing maternal or post-weaning folic acid alters gene expression and moderately changes behavior in the offspring. *PLoS ONE* 9:e101674. doi: 10.1371/journal.pone.0101674

Bergman, J. E. H., Bosman, E. A., van Ravenswaaij-Arts, C. M. A., and Steel, K. P. (2010). Study of smell and reproductive organs in a mouse model for CHARGE syndrome. *Eur. J. Hum. Genet.* 18, 171–177. doi: 10.1038/ejhg.2009.158

Bhardwaj, V., and Rajeshbhai, G. P. (2013). Axial length, anterior chamber depth-a study in different age groups and refractive errors.

J. Clin. Diagn. Res. 7, 2211–2212. doi: 10.7860/JCDR/2013/7015.3473

Billington, C. J., Schmidt, B., Zhang, L., Hodges, J. S., Georgieff, M. K., Schotta, G., et al. (2013). Maternal diet supplementation with methyl donors and increased parity affect the incidence of craniofacial defects in the offspring of twisted gastrulation mutant mice. *J. Nutr.* 143, 332–339. doi: 10.3945/jn.112.168906

Blom, H. J., Shaw, G. M., den Heijer, M., and Finnell, R. H. (2006). Neural tube defects and folate: case far from closed. *Nat. Rev. Neurosci* 7, 724–731. doi: 10.1038/nrn1986

Bollen, B., Ramanantsoa, N., Naert, A., Matrot, B., Van den Bergh, O., D'Hooge, R., et al. (2015). Emotional disorders in adult mice heterozygous for the transcription factor Phox2b. *Physiol. Behav.* 141, 120–126. doi: 10.1016/j.physbeh.2015.01.012

Brown, R. E., and Wong, A. A. (2007). The influence of visual ability on learning and memory performance in 13 strains of mice. *Learn. Mem.* 14, 134–144. doi: 10.1101/lm.473907

Burrow, G. N., Hamilton, H. B., Hrubec, Z., Amamoto, K., Matsunaga, F., and Brill, A. B. (1964). Study of adolescents exposed in utero to the atomic bomb, Nagasaki, Japan. I. General aspects: clinical and laboratory data. *Yale J. Biol. Med.* 36, 430–44. Available online at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2604646andtool=pmcentrezandrendertype=abstract (accessed August 9, 2014).

Byrne, J. (2011). Periconceptional folic acid prevents miscarriage in Irish families with neural tube defects. *Ir J Med Sci.* 180, 59–62. doi: 10.1007/s11845-010-0629-5

Callaerts-Vegh, Z., Ahmed, T., Vermaercke, B., Marynen, P., Balschun, D., Froyen, G., et al. (2015). Nxf7 deficiency impairs social exploration and spatio-cognitive abilities as well as hippocampal synaptic plasticity in mice. *Front. Behav. Neurosci.* 9:179. doi: 10.3389/fnbeh.2015.00179

Chatterson, L. C., Leswick, D. A., Fladeland, D. A., Hunt, M. M., Webster, S., and Lim, H. (2014). Fetal shielding combined with state of the art CT dose reduction strategies during maternal chest CT. *Eur. J. Radiol.* 83, 1199–1204. doi: 10.1016/j.ejrad.2014.04.020

Cipollone, D., Carsetti, R., Tagliani, A., Rosado, M. M., Borgiani, P., Novelli, G., et al. (2009). Folic acid and methionine in the prevention of teratogen-induced congenital defects in mice. *Cardiovasc. Pathol.* 18, 100–109. doi: 10.1016/j.carpath.2008.02.007

Craenen, K., Verslegers, M., Baatout, S., and Abderrafi Benotmane, M. (2020a). An appraisal of folates as key factors in cognition and ageing-related diseases. *Crit. Rev. Food Sci. Nutr.* 60, 722–739. doi: 10.1080/10408398.2018.1549017

Craenen, K., Verslegers, M., Buset, J., Baatout, S., Moons, L., and Benotmane, M. A. (2017). A detailed characterization of congenital defects and mortality following moderate X-ray doses during neurulation. *Birth Defects Res.* 110, 467–482. doi: 10.1002/bdr2.1161

Craenen, K., Verslegers, M., Craeghs, L., Quintens, R., Janssen, A., Coolkens, A., et al. (2020b). Abnormal retinal pigment epithelium melanogenesis as a major determinant for radiation-induced congenital eye defects. *Reprod. Toxicol.* 91, 59–73. doi: 10.1016/j.reprotox.2019.10.002

Crider, K. S., Yang, T. P., Berry, R. J., and Bailey, L. B. (2012). Folate and DNA methylation: a review of molecular mechanisms and the evidence for folate's role. *Adv. Nutr.* 3, 21–38. doi: 10.3945/an.111.000992

Curhan, G. C., Willett, W. C., Rimm, E. B., Spiegelman, D., Ascherio, A. L., and Stampfer, M. J. (1996). Birth weight and adult hypertension, diabetes mellitus, and obesity in US men. *Circulation* 94, 3246–50. doi: 10.1161/01.CIR.94.12.3246

Czeizel, A. E., Bártfai, Z., and Bánhidy, F. (2011). Primary prevention of neural-tube defects and some other congenital abnormalities by folic acid and multivitamins: history, missed opportunity and tasks. *Ther. Adv. drug Saf.* 2, 173–188. doi: 10.1177/2042098611411358

De Groef, L., Andries, L., Siwakoti, A., Geeraerts, E., Bollaerts, I., Noterdaeme, L., et al. (2016). Aberrant collagen composition of the trabecular meshwork results in reduced aqueous humor drainage and elevated IOP in MMP-9 null mice. *Investig. Opthalmol. Vis. Sci.* 57:5984. doi: 10.1167/iovs.16-19734

Dennery, P. A. (2007). Effects of oxidative stress on embryonic development. *Birth Defects Res. C Embryo Today Rev.* 81, 155–162. doi: 10.1002/bdrc.20098

Di Majo, V., Ballardin, E., and Metalli, P. (1981). Comparative effects of fission neutron and X irradiation on 7.5-day mouse embryos. *Radiat. Res.* 87, 145–58. doi: 10.2307/3575548

Ferm, V. H., and Hanlon, D. P. (1986). Arsenate-induced neural tube defects not influenced by constant rate administration of folic acid. *Pediatr. Res.* 20, 761–762. doi: 10.1203/00006450-198608000-00012

Fujimori, K., Kyozuka, H., Yasuda, S., Goto, A., Yasumura, S., Ota, M., et al. (2014). Pregnancy and birth survey after the Great East Japan earthquake and Fukushima Daiichi nuclear power plant accident in Fukushima prefecture. *Fukushima J. Med. Sci.* 60, 75–81. doi: 10.5387/fms.2014-9

Gekas, J., Li, B., and Kamnasaran, D. (2010). Current perspectives on the etiology of agnathia-otocephaly. *Eur. J. Med. Genet.* 53, 358–366. doi: 10.1016/j.ejmg.2010.09.002

Godwin, K. A., Sibbald, B., Bedard, T., Kuzeljevic, B., Lowry, R. B., and Arbour, L. (2008). Changes in frequencies of select congenital anomalies since the onset of folic acid fortification in a Canadian birth defect registry. *Can. J. Public Health* 99, 271–5. doi: 10.1007/BF03403753

Gray, J. D., and Ross, M. E. (2009). Mechanistic insights into folate supplementation from Crooked tail and other NTD-prone mutant mice. *Birth Defects Res. A Clin. Mol. Teratol.* 85, 314–321. doi: 10.1002/bdra.20542

Harris, M. J. (2009). Insights into prevention of human neural tube defects by folic acid arising from consideration of mouse mutants. *Birth Defects Res. A Clin. Mol. Teratol.* 85, 331–339. doi: 10.1002/bdra.20552

Herman, S., Delio, M., Morrow, B., and Samanich, J. (2012). Agnathia–otocephaly complex: a case report and examination of the OTX2 and PRRX1 genes. *Gene* 494, 124–129. doi: 10.1016/j.gene.2011.11.033

Heyer, B. S., MacAuley, A., Behrendtsen, O., and Werb, Z. (2000). Hypersensitivity to DNA damage leads to increased apoptosis during early mouse development. *Genes Dev.* 14, 2072–2084. doi: 10.1101/gad.14.16.2072

Hindin, R., Brugge, D., and Panikkar, B. (2005). Teratogenicity of depleted uranium aerosols: a review from an epidemiological perspective. *Environ. Heal.* 4:17. doi: 10.1186/1476-069X-4-17

Imbard, A., Benoist, J.-F., and Blom, H. J. (2013). Neural tube defects, folic acid and methylation. *Int. J. Environ. Res. Public Health* 10, 4352–4389. doi: 10.3390/ijerph10094352

Jagtap, S. V., Saini, N., Jagtap, S., and Saini, S. (2015). Otocephaly: Agnathia-Microstomia-Synotia Syndrome- A Rare Congenital Anomaly. *J. Clin. Diagn. Res.* 9, ED03-4. doi: 10.7860/JCDR/2015/13636.6444

Jarmonenko, S. P. (1988). *Radiobiology of Humans and Animals*. Mir Publishers. Available online at: https://books.google.be/books/about/Radiobiology_of_Humans_and_Animals.html?id=qo8tAAAACAAJandredir_esc=y (accessed October 10, 2018).

Johnsson, I. W., Haglund, B., Ahlsson, F., and Gustafsson, J. (2015). A high birth weight is associated with increased risk of type 2 diabetes and obesity. *Pediatr. Obes.* 10, 77–83. doi: 10.1111/ijpo.230

Juriloff, D. M. (1985). Prevention of the eye closure defect inlgMl/lgMl fetal mice by thyroxine. *Teratology* 32, 73–86. doi: 10.1002/tera.1420320111

Kappen, C. (2013). Modeling anterior development in mice: diet as modulator of risk for neural tube defects. *Am. J. Med. Genet. C Semin. Med. Genet.* 163, 333–356. doi: 10.1002/ajmg.c.31380

Kim, S. H. R., Lee, J. H., Oh, H., Kim, S. H. R., Lee, C. S., Jo, S. K., et al. (2001). Dependence of malformation upon gestational age and exposed dose of gamma radiation. *J. Radiat. Res.* 42, 255–64. doi: 10.1269/jrr.42.255

Latif-Hernandez, A., Shah, D., Ahmed, T., Lo, A. C., Callaerts-Vegh, Z., Van der Linden, A., et al. (2016). Quinolinic acid injection in mouse medial prefrontal cortex affects reversal learning abilities, cortical connectivity and hippocampal synaptic plasticity. *Sci. Rep.* 6:36489. doi: 10.1038/srep36489

Lazarus, E., Debenedectis, C., North, D., Spencer, P. K., and Mayo-Smith, W. W. (2009). Utilization of imaging in pregnant patients: 10-year review of 5270 examinations in 3285 patients—1997-2006. *Radiology* 251, 517–524. doi: 10.1148/radiol.2512080736

Lehmkuhl, A. M., Dirr, E. R., and Fleming, S. M. (2014). Olfactory assays for mouse models of neurodegenerative disease. *J. Vis. Exp.* 90:e51804. doi: 10.3791/51804

Levin, M. (2005). Left-right asymmetry in embryonic development: a comprehensive review. *Mech. Dev.* 122, 3–25. doi: 10.1016/j.mod.2004.08.006

Li, J. M., Qu, P. F., Dang, S. N., Li, S. S., Bai, R. H., Qin, B. W., et al. (2016). Effect of folic acid supplementation in childbearing aged women during pregnancy on neonate birth weight in Shaanxi province. *Zhonghua Liu Xing Bing Xue Za Zhi* 37, 1017–20. doi: 10.3760/cma.j.issn.0254-6450.2016.07.022

Lin, C.-W., Lo, C.-P., and Tu, M.-C. (2018). Horizontal gaze palsy with progressive scoliosis: a case report with magnetic resonance

tractography and electrophysiological study. *BMC Neurol.* 18:75. doi: 10.1186/s12883-018-1081-9

Lindner, M. D., Plone, M. A., Schallert, T., and Emerich, D. F. (1997). Blind rats are not profoundly impaired in the reference memory Morris water maze and cannot be clearly discriminated from rats with cognitive deficits in the cued platform task. *Brain Res. Cogn. Brain Res.* 5, 329–33. doi: 10.1016/S0926-6410(97)00006-2

Lo, A. C., Callaerts-Vegh, Z., Nunes, A. F., Rodrigues, C. M. P., and D'Hooge, R. (2013). Tauroursodeoxycholic acid (TUDCA) supplementation prevents cognitive impairment and amyloid deposition in APP/PS1 mice. *Neurobiol. Dis.* 50, 21–29. doi: 10.1016/j.nbd.2012.09.003

Maestro-de-las-Casas, C., Pérez-Miguelsanz, J., López-Gordillo, Y., Maldonado, E., Partearroyo, T., Varela-Moreiras, G., et al. (2013). Maternal folic acid-deficient diet causes congenital malformations in the mouse eye. *Birth Defects Res. A Clin. Mol. Teratol.* 97, 587–596. doi: 10.1002/bdra.23176

Mangano, J., and Sherman, J. D. (2015). Changes in congenital anomaly incidence in West Coast and Pacific States (USA) after arrival of Fukushima fallout. *Open J. Pediatr.* 05, 76–89. doi: 10.4236/ojped.2015.51013

Martin, L. M., Marples, B., Lynch, T. H., Hollywood, D., and Marignol, L. (2014). Exposure to low dose ionising radiation: Molecular and clinical consequences. *Cancer Lett.* 349, 98–106. doi: 10.1016/j.canlet.2013.12.015

Mettler, F. A., Bhargavan, M., Faulkner, K., Gilley, D. B., Gray, J. E., Ibbott, G. S., et al. (2009). Radiologic and nuclear medicine studies in the United States and worldwide: frequency, radiation dose, and comparison with other radiation sources—1950-2007. *Radiology* 253, 520–531. doi: 10.1148/radiol.2532082010

Modat, M., Ridgway, G. R., Taylor, Z. A., Lehmann, M., Barnes, J., Hawkes, D. J., et al. (2010). Fast free-form deformation using graphics processing units. *Comput. Methods Programs Biomed.* 98, 278–284. doi: 10.1016/j.cmpb.2009.09.002

Moore, W., Bonvento, M. J., Lee, D., Dunkin, J., and Bhattacharji, P. (2015). Reduction of fetal dose in computed tomography using anterior shields. *J. Comput. Assist. Tomogr.* 39, 298–300. doi: 10.1097/RCT.0000000000000190

Moster, S. J., Moster, M. L., Scannell Bryan, M., and Sergott, R. C. (2016). Retinal Ganglion Cell and inner plexiform layer loss correlate with visual acuity loss in LHON: a longitudinal, segmentation OCT analysis. *Investig. Opthalmol. Vis. Sci.* 57:3872. doi: 10.1167/iovs.15-17328

Muralidharan, P., Sarmah, S., and Marrs, J. A. (2015). Zebrafish retinal defects induced by ethanol exposure are rescued by retinoic acid and folic acid supplement. *Alcohol* 49, 149–163. doi: 10.1016/j.alcohol.2014.11.001

Neel, J., and Schull, W. J. (1956). *Effect of Exposure to the Atomic Bombs on Pregnancy Termination in Hiroshima and Nagasaki*. Washington, DC: National Academies Press.

Owrangi, A. M., Roberts, D. A., Covington, E. L., Hayman, J. A., Masi, K. M., Lee, C., et al. (2016). Revisiting fetal dose during radiation therapy: evaluating treatment techniques and a custom shield. *J. Appl. Clin. Med. Phys.* 17, 34–46. doi: 10.1120/jacmp.v17i5.6135

Oyama, K., Sugimura, Y., Murase, T., Uchida, A., Hayasaka, S., Oiso, Y., et al. (2009). Folic acid prevents congenital malformations in the offspring of diabetic mice. *Endocr. J.* 56, 29–37. doi: 10.1507/endocrj.K08E-180

Pampfer, S., and Streffer, C. (1988). Prenatal death and malformations after irradiation of mouse zygotes with neutrons or X-rays. *Teratology* 37, 599–607. doi: 10.1002/tera.1420370609

Paranjothy, S., Broughton, H., Evans, A., Huddart, S., Drayton, M., Jefferson, R., et al. (2012). The role of maternal nutrition in the aetiology of gastroschisis: an incident case-control study. *Int. J. Epidemiol.* 41, 1141–1152. doi: 10.1093/ije/dys092

Phillips, J. B., Youmans, P. W., Muheim, R., Sloan, K. A., Landler, L., Painter, M. S., et al. (2013). Rapid learning of magnetic compass direction by C57BL/6 mice in a 4-armed "plus" water maze. *PLoS ONE* 8:e73112. doi: 10.1371/journal.pone.0073112

Plummer, G. (1952). Anomalies occurring in children exposed in utero to the atomic bomb in Hiroshima. *Pediatrics* 10, 687–693. Available online at: http://pediatrics.aappublications.org/content/10/6/687 (accessed August 27, 2017).

Portmann, T., Yang, M., Mao, R., Panagiotakos, G., Ellegood, J., Dolen, G., et al. (2014). Behavioral abnormalities and circuit defects in the basal ganglia of a mouse model of 16p11.2 deletion syndrome. *Cell Rep.* 7, 1077–1092. doi: 10.1016/j.celrep.2014.03.036

Qiu, M.-H., Vetrivelan, R., Fuller, P. M., and Lu, J. (2010). Basal ganglia control of sleep-wake behavior and cortical activation. *Eur. J. Neurosci.* 31, 499–507. doi: 10.1111/j.1460-9568.2009.07062.x

Ramakrishnan, U., Nguyen, P. H., Gonzalez-Casanova, I., Pham, H., Hao, W., Nguyen, H., et al. (2016). Neither preconceptional weekly multiple micronutrient nor iron-folic acid supplements affect birth size and gestational age compared with a folic acid supplement alone in rural vietnamese women: a randomized controlled trial. *J. Nutr.* 146, 1445S—1452S. doi: 10.3945/jn.115.223420

Regnier, C. H., Masson, R., Kedinger, V., Textoris, J., Stoll, I., Chenard, M.-P., et al. (2002). Impaired neural tube closure, axial skeleton malformations, and tracheal ring disruption in TRAF4-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* 99, 5585–5590. doi: 10.1073/pnas.052124799

Reisz, J. A., Bansal, N., Qian, J., Zhao, W., and Furdui, C. M. (2014). Effects of ionizing radiation on biological molecules–mechanisms of damage and emerging methods of detection. *Antioxid. Redox Signal.* 21, 260–292. doi: 10.1089/ars.2013.5489

Russell, L. B. (1950). X-ray induced developmental abnormalities in the mouse and their use in the analysis of embryological patterns. I. External and gross visceral changes. *J. Exp. Zool.* 114, 545–601. doi: 10.1002/jez.1401140307

Russell, L. B. (1956). X-ray-induced developmental abnormalities in the mouse and their use in the analysis of embryological patterns. II. Abnormalities of the vertebral column and thorax. *J. Exp. Zool.* 131, 329–395. doi: 10.1002/jez.1401310308

Scherb, H. H., Mori, K., and Hayashi, K. (2016). Increases in perinatal mortality in prefectures contaminated by the Fukushima nuclear power plant accident in Japan. *Medicine* 95:e4958. doi: 10.1097/MD.0000000000004958

Sergouniotis, P. I., Urquhart, J. E., Williams, S. G., Bhaskar, S. S., Black, G. C., Lovell, S. C., et al. (2015). Agnathia-otocephaly complex and asymmetric velopharyngeal insufficiency due to an in-frame duplication in OTX2. *J. Hum. Genet.* 60, 199–202. doi: 10.1038/jhg.2014.122

Shaw, G. M., Carmichael, S. L., Laurent, C., Louik, C., Finnell, R. H., Lammer, E. J., et al. (2007). Nutrient intakes in women and risks of anophthalmia and microphthalmia in their offspring. *Birth Defects Res. A Clin. Mol. Teratol.* 79, 708–713. doi: 10.1002/bdra.20398

Sinding, C., Valadier, F., Al-Hassani, V., Feron, G., Tromelin, A., Kontaris, I., et al. (2017). New determinants of olfactory habituation. *Sci. Rep.* 7:41047. doi: 10.1038/srep41047

Smith, R. S., Roderick, T. H., and Sundberg, J. P. (1994). Microphthalmia and associated abnormalities in inbred black mice. *Lab. Anim. Sci.* 44, 551–60. Available online at: http://www.ncbi.nlm.nih.gov/pubmed/7898027 (accessed August 25, 2016).

Stroobants, S., Leroy, T., Eckhardt, M., Aerts, J.-M., Berckmans, D., and D'Hooge, R. (2008). Early signs of neurolipidosis-related behavioural alterations in a murine model of metachromatic leukodystrophy. *Behav. Brain Res.* 189, 306–316. doi: 10.1016/j.bbr.2008.01.008

Strupp, M., Kremmyda, O., Adamczyk, C., Böttcher, N., Muth, C., Yip, C. W., et al. (2014). Central ocular motor disorders, including gaze palsy and nystagmus. *J. Neurol.* 261(Suppl. 2), S542–S558. doi: 10.1007/s00415-014-7385-9

Tamura, T., and Picciano, M. F. (2006). Folate and human reproduction. *Am. J. Clin. Nutr.* 83, 993–1016. doi: 10.1093/ajcn/83.5.993

Treloar, H. B., Miller, A. M., Ray, A., and Greer, C. A. (2010). *Development of the Olfactory System*. CRC Press/Taylor and Francis. Available online at: http://www.ncbi.nlm.nih.gov/pubmed/21882426 (accessed September 16, 2018).

Tustison, N. J., Avants, B. B., Cook, P. A., Yuanjie, Z.heng, Egan, A., Yushkevich, P. A., et al. (2010). N4ITK: improved N3 bias correction. *IEEE Trans. Med. Imaging* 29, 1310–1320. doi: 10.1109/TMI.2010.2046908

Tyll, S., Budinger, E., and Noesselt, T. (2011). Thalamic influences on multisensory integration. *Commun. Integr. Biol.* 4, 378–381. doi: 10.4161/cib.15222

Van Hove, I., Lefevere, E., De Groef, L., Sergeys, J., Salinas-Navarro, M., Libert, C., et al. (2016). MMP-3 deficiency alleviates endotoxin-induced acute inflammation in the posterior eye segment. *Int. J. Mol. Sci.* 17:1825. doi: 10.3390/ijms17111825

Verma, A. S., and Fitzpatrick, D. R. (2007). Anophthalmia and microphthalmia. *Orphanet J. Rare Dis.* 2:47. doi: 10.1186/1750-1172-2-47

Verreet, T., Quintens, R., Van Dam, D., Verslegers, M., Tanori, M., Casciati, A., et al. (2015). A multidisciplinary approach unravels early and persistent effects

of X-ray exposure at the onset of prenatal neurogenesis. *J. Neurodev. Disord.* 7:3. doi: 10.1186/1866-1955-7-3

Verreet, T., Rangarajan, J. R., Quintens, R., Verslegers, M., Lo, A. C., Govaerts, K., et al. (2016a). persistent impact of *in utero* irradiation on mouse brain structure and function characterized by MR imaging and behavioral analysis. *Front. Behav. Neurosci.* 10:83. doi: 10.3389/fnbeh.2016.00083

Verreet, T., Verslegers, M., Quintens, R., Baatout, S., and Benotmane, M. A. (2016b). Current evidence for developmental, structural, and functional brain defects following prenatal radiation exposure. *Neural Plast.* 2016:1243527. doi: 10.1155/2016/1243527

Vorhees, C. V., and Williams, M. T. (2014). Assessing spatial learning and memory in rodents. *ILAR J.* 55, 310–332. doi: 10.1093/ilar/ilu013

Wentzel, P., and Eriksson, U. J. (2005). A diabetes-like environment increases malformation rate and diminishes prostaglandin E2 in rat embryos: reversal by administration of vitamin E and folic acid. *Birth Defects Res. A Clin. Mol. Teratol.* 73, 506–511. doi: 10.1002/bdra.20145

Wertelecki, W., Koerblein, A., Ievtushok, B., Zymak-Zakutnia, N., Komov, O., Kuznietsov, I., et al. (2016). Elevated congenital anomaly rates and incorporated cesium-137 in the Polissia region of Ukraine. *Birth Defects Res. A. Clin. Mol. Teratol.* 106, 194–200. doi: 10.1002/bdra.23476

Wiens, D., and DeSoto, M. (2017). Is high folic acid intake a risk factor for autism?—a review. *Brain Sci.* 7:149. doi: 10.3390/brainsci7110149

Xu, Y., Li, Y., Tang, Y., Wang, J., Shen, X., Long, Z., et al. (2006). The maternal combined supplementation of folic acid and Vitamin B12 suppresses ethanol-induced developmental toxicity in mouse fetuses. *Reprod. Toxicol.* 22, 56–61. doi: 10.1016/j.reprotox.2005.12.004

Yanaguita, M. Y., Gutierrez, C. M., Ribeiro, C. N. M., Lima, G. A., Machado, H. R., and Peres, L. C. (2007). Pregnancy outcome in ethanol-treated mice with folic acid supplementation in saccharose. *Childs Nerv. Syst.* 24, 99–104. doi: 10.1007/s00381-007-0427-1

Yang, M., Abrams, D. N., Zhang, J. Y., Weber, M. D., Katz, A. M., Clarke, A. M., et al. (2012). Low sociability in BTBR T+tf/J mice is independent of partner strain. *Physiol. Behav.* 107, 649–662. doi: 10.1016/j.physbeh.2011.12.025

Yang, M., and Crawley, J. N. (2009). "Simple behavioral assessment of mouse olfaction," in *Current Protocols in Neuroscience* (Hoboken, NJ: John Wiley and Sons, Inc.) 8.24.1–8.24.12. doi: 10.1002/0471142301.ns0824s48

Yang, W., Carmichael, S. L., and Shaw, G. M. (2016). Folic acid fortification and prevalences of neural tube defects, orofacial clefts, and gastroschisis in California, 1989 to 2010. *Birth Defects Res. A Clin. Mol. Teratol.* 106, 1032–1041. doi: 10.1002/bdra.23514

Young, A. D., Phipps, D. E., and Astroff, A. B. (2000). Large-scale double-staining of rat fetal skeletons using Alizarin Red S and Alcian Blue. *Teratology* 61, 273–276. doi: 10.1002/(SICI)1096-9926(200004)61:4<273::AID-TERA5<3.0.CO;2-2

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Ontogenetic Oxycodone Exposure Affects Early Life Communicative Behaviors, Sensorimotor Reflexes, and Weight Trajectory in Mice

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Nationwide, opioid misuse among pregnant women has risen four-fold from 1999 to 2014, with commensurate increase in neonates hospitalized for neonatal abstinence syndrome (NAS). NAS occurs when a fetus exposed to opioids *in utero* goes into rapid withdrawal after birth. NAS treatment via continued post-natal opioid exposure has been suggested to worsen neurodevelopmental outcomes. We developed a novel model to characterize the impact of *in utero* and prolonged post-natal oxycodone (Oxy) exposure on early behavior and development. Via subcutaneous pump implanted before breeding, C57BL/6J dams were infused with Oxy at 10 mg/kg/day from conception through pup-weaning. At birth, *in utero* oxy-exposed pups were either cross-fostered (paired with non-Oxy exposed dams) to model opioid abstinence (*in utero* Oxy) or reared by their biological dams still receiving Oxy to model continued post-natal opioid exposure (prolonged Oxy). Offspring from vehicle-exposed dams served as cross-fostered (*in utero* Veh) or biologically reared (prolonged Veh) controls. *In utero* Oxy exposure resulted in sex-dependent weight reductions and altered spectrotemporal features of isolation-induced ultrasonic vocalization (USV). Meanwhile, prolonged Oxy pups exhibited reduced weight and sex-differential delays in righting reflex. Specifically, prolonged Oxy female offspring exhibited increased latency to righting. Prolonged Oxy pups also showed decreases in number of USV calls and changes to spectrotemporal USV features. Overall, ontogenetic Oxy exposure was associated with impaired attainment of gross and sensorimotor milestones, as well as alterations in communication and affective behaviors, indicating a need for therapeutic interventions. The model developed here will enable studies of withdrawal physiology and opioid-mediated mechanisms underlying these neurodevelopmental deficits.

Keywords: opioid, behavior, *in utero*, post-natal, oxycodone, neonatal abstinence syndrome

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INTRODUCTION

In the past two decades, illicit drug use and prescription opioid use in the United States have risen to epidemic proportions, with the United States Department of Health declaring a public health emergency in 2017. The United States Department of Health states that 46,802 people died from opioid overdose in 2018 and an estimated 2 million people have an opioid use disorder (ASPA, 2017). The public health crisis is largely driven by increased misuse of the prescription opioids hydrocodone, oxycodone (Oxy), and methadone (Kenan et al., 2012; Haight, 2018).

As a result of the opioid epidemic, the national prevalence of opioid use disorder among pregnant women has more than quadrupled, from 1.5 to 6.5 per 1,000 deliveries, from 1999 through 2014 (Haight, 2018). Consequently, there has been a significant increase in the number of neonates hospitalized for neonatal abstinence syndrome (NAS), a constellation of withdrawal symptoms affecting the nervous system, gastrointestinal tract, and respiratory system following *in utero* exposure to opioids (Wiles et al., 2014). Currently, medical management of NAS involves keeping the infant swaddled in a low-stimulation environment with promotion of maternal-infant bonding (Wiles et al., 2014). In cases of moderate-severe NAS, neonatal withdrawal is managed by opioid replacement therapy to alleviate withdrawal symptomatology (Wiles et al., 2014). Overall, clinical studies have not addressed whether long-term neurobehavioral outcomes are improved by managing withdrawal or whether continued post-natal exposure to opioids and adjunct agents used for withdrawal management worsen long-term outcomes (Hudak et al., 2012).

Epidemiological evidence suggests that *in utero* opioid exposure is associated with lower birth weight and adverse neurodevelopmental outcomes in childhood, including cognitive deficits, attention deficit hyperactivity disorder (ADHD), aggression, impaired language development, and decreased social maturity (Hunt et al., 2008; Azuine et al., 2019; Conradt et al., 2019). However, large epidemiological studies evaluating long-term behavioral outcomes of children exposed to *in utero* opioids have been difficult to perform due to confounding environmental variables including genetic and epigenetic factors, quality of caregiving, continued parental substance abuse with its impact on the maternal-infant dyad, and other socioeconomic variables which can significantly affect neurodevelopmental outcomes (Lutz and Kieffer, 2013). Consequently, the development of ontogenetic rodent models of opioid exposure is necessary to enable investigation of the biological mechanisms mediating deficits as well as testing alternative treatment avenues for post-natal withdrawal.

To date, there have been a limited number of rodent studies evaluating early life developmental milestones following *in utero* opioid exposure. Current literature on early developmental effects of *in utero* opioid exposure in pre-clinical models demonstrates decreased birth weight following methadone and buprenorphine exposure (Kunko et al., 1996; Hung et al., 2013; Chiang et al., 2015). Increased latency to right has been observed following *in utero* morphine exposure and is suggestive of different classes of opioids having variable effects on developmental outcomes (Slamberová et al., 2005; Niu et al., 2009). Opioids exert their pharmacologic effects by activating the endogenous opioid system. While opioids are prescribed for their analgesic effects, acute activation of the μ-opioid receptor (MOR) by these medications has also been associated with feelings of euphoria, award reinforcement, and increased socio-emotional processing, which are linked to the drugs' potential for misuse (Vanderschuren et al., 1995). Despite the rising incidence of Oxy misuse, there is a paucity of literature evaluating the effects of Oxy, a μ- and κ-agonist, on early developmental behaviors. κ-agonists are of particular interest because over-activation of κ-opioid receptors (KOR) by dynorphin upregulation has been implicated in withdrawal physiology and depressed mood in humans, along with decreased social play in juvenile rodents (Vanderschuren et al., 1995; Li et al., 2016). In addition, most rodent opioid exposure models begin exposure mid-pregnancy, which may explain inconsistently documented or absent developmental changes (Richardson et al., 2006). To address the above concerns, we are adopting an ontogenetic model in which opioid exposure spans preconception through early offspring development. This new model also enables us to better understand the ontogenetic impact of short- and long-term opioid exposure on early development in the absence of confounding factors present in clinical observational studies. Specifically, we evaluated the effects of *in utero* Oxy exposure on early developmental and behavioral outcomes in male and female offspring of C57BL/6J mouse dams. We implemented a cross-fostering approach that allows us to compare the neurodevelopmental impact of continued post-natal opioid exposure (prolonged Oxy) to the impact of exposure only until birth (*in utero* Oxy) by pairing opioid exposed pups with non-oxy exposed dams.

Overall, we observed differences in the spectrotemporal features of affective vocalizations and sex-based differences in weight gain trajectories in offspring exposed to *in utero* Oxy. Continued post-natal Oxy exposure (prolonged Oxy) further impacted weight, communicative behavior, and sensorimotor reflexes. Our findings suggest that pups with continued post-natal opioid exposure showed worse overall developmental outcomes compared to pups following opioid cessation at birth, which may have implications regarding the safety of continued opioid treatment as mitigation for clinical NAS symptomology.

MATERIALS AND METHODS

Animals

Animal Ethics, Selection, and Welfare

All procedures using mice were approved by the Washington University Institutional Care and Use Committee and conducted in accordance with the approved Animal Studies Protocol. C57BL/6J mice (Jackson Laboratory, stock #: 000664) were housed in individually ventilated translucent plastic cages (IVC) measuring 36.2 × 17.1 × 13 cm (Allentown) with corncob bedding and *ad libitum* access to standard lab diet and

TABLE 1 | Litter and group size, including number of pups at the level of litter, group, and experiment.

Litter ID	Group	No. of pups
1	Prolonged Oxy	5
2	Prolonged Oxy	4
3	Prolonged Oxy	3
4	Prolonged Oxy	6
5	Prolonged Oxy	6
Total:		24
6	*In utero* Oxy	5
7	*In utero* Oxy	6
8	*In utero* Oxy	6
9	*In utero* Oxy	6
10	*In utero* Oxy	6
Total:		29
11	Prolonged Veh	6
12	Prolonged Veh	6
13	Prolonged Veh	6
14	Prolonged Veh	6
15	Prolonged Veh	6
Total:		30
16	*In utero* Veh	5
17	*In utero* Veh	6
18	*In utero* Veh	3
19	*In utero* Veh	6
20	*In utero* Veh	5
21	*In utero* Veh	3
Total:		28
	Total pups:	111

water. Animals were kept at 12/12 h light/dark cycle, and room temperature (20–23°C) and relative humidity (50%) were controlled automatically.

Adult male and female mice were used for breeding cohorts as described below. Sample sizes were determined by power analyses (f = 0.40, α = 0.05, 1-β = 0.80). A total of 24 dams were housed in pairs and randomly selected to receive either the Oxy or Vehicle (Veh) treatment infusion. In addition, another set of pair-housed, drug-naïve dams served as foster dams. The total sample size was 111 pups (**Table 1**). Since an inexperienced dam can exhibit poor maternal behavior with her first litter, all females were first bred to an age-matched male at post-natal day (P) 60. Following weaning of the first litter, treatment dams underwent surgical subcutaneous pump placement at P95 followed by a 1-week recovery period (**Figure 1A**). Afterward, each female dam was placed into an individual cage containing a male for breeding. Foster dams were bred at the same time and remained untreated throughout pregnancy. Following 20 days of co-habitation, cages were checked daily for pups. Upon detection, dam and litter were moved to a new cage, without the male, and culled to 6–8 pups per litter with equal males and females when possible. To evaluate the behavioral impact of early opioid cessation in the developing offspring, half of the litters (*in utero* Oxy and *in utero* Veh) were cross-fostered at this time to drug-naïve dams by removing the pups from their biological dam and transferring them to the nest of a lactating foster dam with two of her own pups of the same approximate age (**Figure 1B**; Lohmiller and Swing, 2006). The remaining litters were reared by the biological dam and exposed to post-natal vehicle (prolonged Veh) or Oxy (prolonged Oxy) through lactation (**Figures 1A,B**). To control for litter effects, each group included multiple, independent litters (**Table 1**). All mice were weaned at P21 and group-housed by sex with random assignment for drug/dam. A subset of the *in utero* Oxy mice required saline injections at P23-P25 due to skin tenting, hunched posture, and significant weight loss concerning for dehydration. Following saline injections, recovery was noted in two of the three affected mice with one associated mortality. Experimenters were all female and blinded to group designations during testing.

Drug Dosage

The dosage of Oxy (Sigma-Aldrich, Saint Louis, MO, United States; Lot#: SLBX4974) administration was guided by previous literature with concentrations ranging from 0.5 to 33 mg/kg/day (Enga et al., 2016; Sithisarn et al., 2017; Zanni et al., 2020). Based on this dosage range, we generated our own dosage curve through continuous Oxy administration to pregnant dams at 5, 10, or 15 mg/kg/day using the subcutaneous Alzet 2006 model pump (Durect Corporation, Cupertino, CA, United States; Lot #: 10376-17). We chose the dose of 10 mg/kg/day administered at 0.15 ul/h to pregnant dams as increased concentrations at 15 mg/kg/day resulted in lower litter success rate (vehicle, 5 and 10 mg/kg/d: 100% success rate; 15 mg/kg/d: 80% success rate). We chose to administer a consistent dose of opioids to the dam throughout pregnancy and lactation to model the current management of pregnant mothers with opioid use disorder. Women with opioid use disorder are now encouraged to enroll in opioid medication-assisted treatment programs with the goal of attaining a steady-state drug level. Maintaining a consistent level of opioid administration during pregnancy in mothers with opioid use disorder limits adverse maternal and fetal consequences associated with fluctuations related to higher opioid concentrations. Increases in opioid dosage can result in maternal respiratory depression and lethal overdose and may decrease fetal heart rate and variability (Krans et al., 2015; Rosenthal and Baxter, 2019).

Surgery and Drug Delivery System

Female dams were anesthetized at P95 with isoflurane (5% induction, 2% maintenance, 0.5 l/min) and placed in the mouse adapter (Stoelting, Wood Dale, IL, United States). Body temperature was maintained at 37°C using a heating pad. The dorsum of the back was shaved and a ∼1 cm horizontal incision was made below the scapulae with subsequent formation of a subcutaneous pocket. The Alzet pump was implanted and continuously infused with either Oxy or sterile 0.9% NaCl (Veh) over a period of 60 days. The pump duration allowed for adequate post-surgical recovery time, breeding, and administration of treatment through weaning of offspring at P21. In addition, the use of a subcutaneous pump limited unwanted maternal stress that can occur with daily injections.

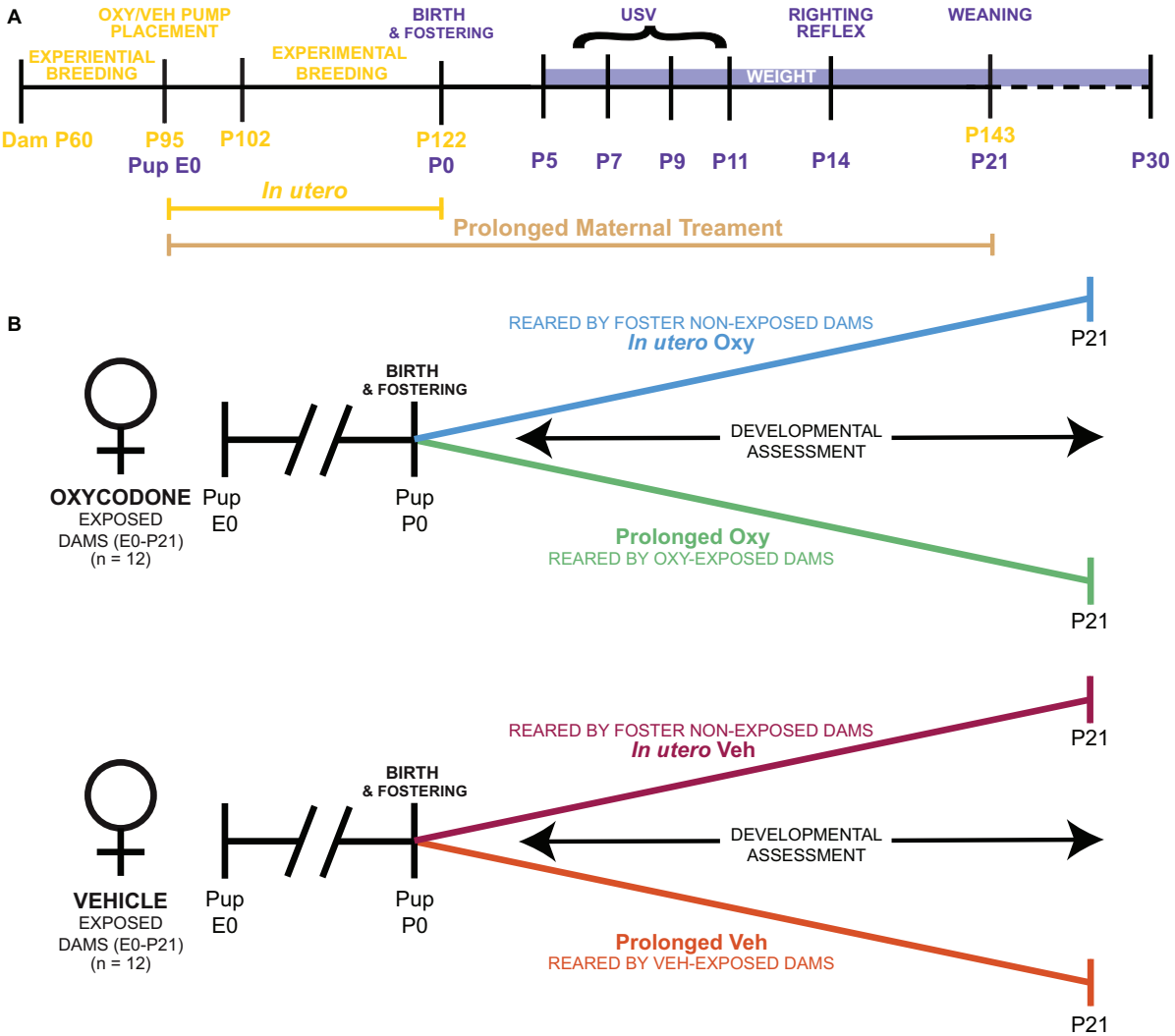

FIGURE 1 | Ontogenetic model of prolonged and limited maternal Oxy exposure. **(A)** Schematic of the paradigm for maternal Oxy exposure, including duration of *in utero* and prolonged maternal treatment, dam ages at experimental manipulations in yellow, and pup age for behavioral tests in purple. **(B)** Outline of the four different experimental groups, including Oxy or Veh exposure, and rearing dam exposure status.

Behavioral Testing

Maternal Isolation-Induced Ultrasonic Vocalization Recording

Neonates with NAS often exhibit excessively high-pitched crying, irritability, and prolonged periods of inconsolability (Anbalagan and Mendez, 2020). Affective characteristics of ultrasonic vocalizations (USVs) in rodents are generally thought to communicate different emotional states, such as aggression or pain. USV quantity, duration, pitch, frequency, and loudness (dB) of the calls allow for the assessment of call characteristics following *in utero* Oxy exposure (Vivian and Miczek, 1993a,b). USV recordings were performed on P5, P7, P9, and P11 (**Figure 1A**). Dams were removed from the home cage and placed into a clean IVC for the duration of testing. The home cage with the pups in nest was placed into a warming box (Harvard Apparatus) set to 34°C for 10 min prior to the start of testing. We maintained an average pup surface body temperature of 34°C prior to placement into the USV recording chamber, as low pup body temperature increases USV production (Branchi et al., 2001). The surface body temperature of all pups was assessed via a non-contact HDE Infrared Thermometer prior to placement into the recording chamber, and no differences in body surface temperature were observed between groups. The recording chamber was maintained at room temperature (22–23°C). For recording, pups were individually removed from the home cage and placed into an empty standard mouse cage (28.5 × 17.5 × 12 cm) inside a sound-attenuating chamber (Med Associates). USVs were recorded via an Avisoft UltraSoundGate CM16 microphone placed 5 cm away from the top of the cage, Avisoft UltraSoundGate 116H amplifier, and Avisoft Recorder software (gain = 3 dB, 16 bits, sampling rate = 250 kHz). Pups were recorded for 3 min,

after which they were weighed and returned to home cages. Frequency sonograms were prepared from USV recordings in MATLAB [frequency range = 25–120 kHz, Fast Fourier Transform (FFT) size = 512, overlap = 50%, time resolution 1.024 s, frequency resolution = 488.2 Hz]. Individual calls and other spectrotemporal features were identified from the sonograms adapted from validated procedures (Holy and Guo, 2005; Maloney et al., 2018a,b).

Developmental Reflexes and Milestones Assessment

Mice were evaluated for achievement of physical and behavioral milestones from early development through early juvenile stage. Weight was measured at 10 time points: P5, P7, P9, P11, P14, P23, P25, P27, and P30. A visual inspection of normal physical milestone attainment was performed with evaluation for detached pinnae at P5 and eye opening at P14. Righting reflex was assessed at P14 as follows: each mouse was placed prone onto its abdomen and quickly pronated 180° to its back in a smooth motion. The time for the mouse to right itself with all four paws positioned underneath the abdomen was recorded (Zanni et al., 2020). Each mouse underwent three timed trials, which were averaged for analysis.

Statistical Analyses

SPSS (IBM, v.25) was used for all statistical analyses. Data were screened for missing values, influential outliers, fit between distributions and the assumptions of normality and homogeneity of variance. Variables that violated assumptions of normality (including number of USV calls, mean pitch, pitch range, and peak power) were square root-transformed. Data were analyzed using hierarchical linear models with sex clustered within litters and age clustered within individual pups. Fixed factors were dam, drug, sex and, where appropriate, age. Age was also treated as a random repeated effect and was grand mean-centered for analysis. Interactions between the fixed factors are reported when significant. If an interaction effect was significant, *p*-values were obtained from the hierarchical linear model for simple main effects and reported for differences between different levels within the interaction. If sex had a significant main effect, findings are shown segregated by sex. As litter size can influence behavior and litter cannot be separated from drug treatment in this study, all models included litter size as a covariate. Probability value for all analyses was $p < 0.05$. Test statistics and analysis details are provided in **Table 2**. The datasets generated for this study are available upon reasonable request to the corresponding author.

TABLE 2 | Test statistics from hierarchical linear models.

Variable	Factor	Output	*p*-value
Weight (g)	Sex	F (1, 107) = 10.219	p = 0.002
	Drug	F (1, 109) = 5.448	p = 0.021
	Age	F (9, 901) = 785.001	p = 0.000
	Litter Size	F (1,102) = 9.922	p = 0.002
	Sex × Dam × Drug × Age	F (67, 685) = 4.232	p = 1.2916E^{-22}
Righting reflex	Dam	F (1,24) = 4.630	p = 0.042
	Sex × Dam × Drug	F (4,74) = 2.518	p = 0.048
	Litter Size	F (1,26) = 9.335	p = 0.005
Number of USV calls	Dam	F (1,156) = 7.015	p = 0.009
	Drug	F (1,151) = 12.000	p = 0.001
	Dam × Drug	F (1,159) = 5.223	p = 0.024
	Litter Size	F (1, 159) = 3.064	p = 0.082
Pitch range (Hz)	Drug	F (1,169) = 8.456	p = 0.004
	Dam × Drug	F (1,169) = 13.528	p = 0.000315
	Age × Dam × Drug × Sex	F (24, 319) = 1.385	p = 0.097
Mean pitch (Hz)	Drug	F (1,160) = 29.552	p = 1.9953E^{-7}
	Dam × Sex	F (1,160) = 5.373	p = 0.022
	Drug × Sex	F (1,160) = 5.354	p = 0.022
	Sex × Dam × Drug	F (1,160) = 8.365	p = 0.004
	Sex × Dam × Drug × Age	F (24, 301) = 1.617	p = 0.036
	Sex	F (1, 161) = 3.190	p = 0.076
Peak power (dB)	Dam	F (1,163) = 5.632	p = 0.019
	Drug	F (1,171) = 10.327	p = 0.002
	Dam × Drug	F (1,172) = 12.399	p = 0.001
	Litter Size	F (1,161) = 6.313	p = 0.013

Showing significant main and interaction effects. Age was grand mean-centered for analysis, where included. Litter size was included as a covariate.

RESULTS

Oxycodone Impacted Developmental Weight Trajectories Differentially by Sex and Exposure Duration

We examined the effects of Oxy administration on gross and sensorimotor development in mice from birth throughout the early juvenile stage (**Figures 2A,B**). To evaluate general health and gross development, we assessed the appearance of physical milestones and weight. No differences were observed between groups for pinnae detachment at P5 or eye opening by P14. In our analysis of weight, we found male mice weighed significantly more than females in all groups at all ages, and therefore, weight data are segregated by sex (**Figures 2C,D**) from the full factorial linear mixed model including sex, drug, and duration as factors.

Prolonged Oxy exposure led to an overall decrease in mean weight compared to prolonged Veh exposure, which was more pronounced in male offspring. Prolonged Oxy-exposed male offspring exhibited significantly reduced weights compared to prolonged Veh offspring post-weaning at P25, P27, and P30, with non-significant reductions at P23 (**Figure 2E**). Female prolonged Oxy offspring showed significantly reduced weight compared to prolonged Veh controls at P21, P23, and P25, with non-significant reductions at P27 and P30 (**Figure 2F**). These data indicate that overall prolonged Oxy exposure reduces weight across development in male and female offspring, with the effect on weight gain compounding once potentially compensatory maternal care is lost after weaning.

We then evaluated the potential effects of early opioid cessation (*in utero* Oxy) on weight gain in male and female offspring and once more found male offspring susceptible to Oxy effects. In contrast to prolonged exposure, an overall reduction in weight was not observed with *in utero* Oxy exposure compared to *in utero* Veh exposure. However, *in utero* Oxy males showed a precipitous decrease in weight gain trajectory after weaning

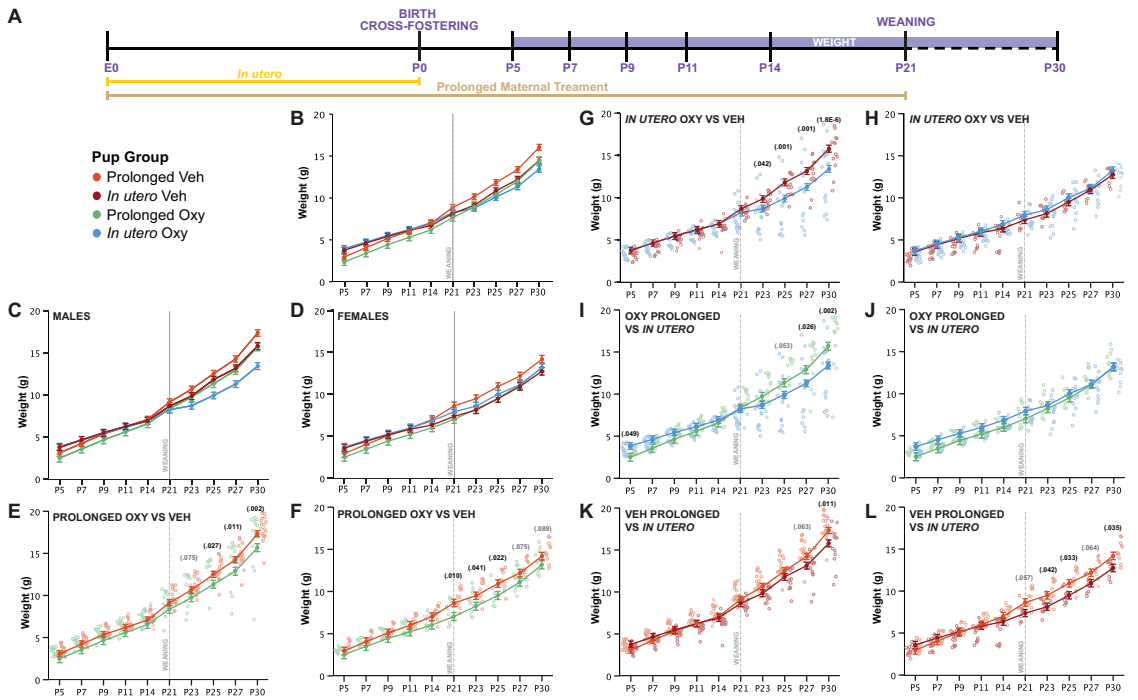

FIGURE 2 | Prolonged and *in utero* Oxy exposure, as well as cross-fostering, decreases weight gain after weaning. **(A)** Schematic of the treatment paradigm for maternal Oxy exposure and weight measurements throughout development. **(B)** Line graph of weight in all offspring (sex, $p = 0.002$; drug, $p = 0.021$; age, $p < 0.000$; sex × dam × drug × age, $p = 1.2916E^{-22}$). **(C,D)** Line graph of weight in **(C)** male and **(D)** female offspring. **(E,F)** Prolonged Oxy exposure, relative to prolonged Veh exposure, led to significantly lower weights post-weaning in **(E)** male (P23, $p = 0.075$; P25, $p = 0.027$; P27, $p = 0.011$; P30, $p = 0.002$) and **(F)** female offspring (P21, $p = 0.010$; P23, $p = 0.041$; P25, $p = 0.022$; P27, $p = 0.075$; P30, $p = 0.089$). **(G,H)** *In utero* Oxy exposure, relative to *in utero* Veh exposure, led to significantly lower weights post-weaning in **(G)** male offspring only (P23, $p = 0.042$; P25, $p = 0.001$; P27, $p = 0.001$; P30, $p = 0.000018$), with no effects in **(H)** female offspring. **(I,J)** *In utero* Oxy exposure with cross-fostering, relative to prolonged Oxy exposure, led to decreased weight in adolescence in **(I)** male pups only (P5, $p = 0.049$; P25, $p = 0.053$; P27, $p = 0.026$; P30, $p = 0.002$), with no effects in **(J)** female offspring. **(K,L)** *In utero* Veh exposure with cross-fostering, relative to prolonged Veh exposure, led to decreased weight post-weaning in **(K)** male (P27, $p = 0.063$; P30, $p = 0.011$) and **(L)** female offspring (P21, $p = 0.057$; P23, $p = 0.042$; P25, $p = 0.033$; P27, $p = 0.064$; P30, $p = 0.035$). Closed circles depict mean weight, with litter size as a covariate ($p = 0.002$), while open circles depict individual weights. Gray vertical line indicates date of weaning.

from the foster dam at P23, P25, P27, and P30 as compared to *in utero* Veh controls (**Figure 2G**). Female *in utero* Oxy offspring showed no difference in weight across development compared to *in utero* Veh controls (**Figure 2H**). Clinically, male infants are more susceptible to NAS (Charles et al., 2017), so the precipitous decrease in weight gain trajectory in the *in utero* Oxy male offspring may be associated with withdrawal symptomatology unmasked by cessation of care under a foster dam.

We also examined weight trajectories between *in utero* and prolonged vehicle-exposed groups. In vehicle-exposed males, cross-fostering was associated with decreased weights in the *in utero* Veh group at P30, with a trend toward decreased weight at P27, relative to the prolonged Veh group (**Figure 2K**). Of interest, cross-fostered female pups (*in utero* Veh) weighed less compared to prolonged Veh female pups post-weaning at P23, P25, and P30 (**Figure 2L**). These findings suggest that cross-fostering alone can influence post-weaning weight trajectories in a sex-dependent manner. However, it is noteworthy that the decrease of weight gain trajectories in the male *in utero* Oxy group persisted above and beyond the observed decreased weights in male *in utero* Veh controls, indicating *in utero* Oxy exposure affects weight gain when controlling for cross-fostering (**Figure 2G**).

We have shown so far that Oxy exposure, compared to Veh, decreased post-weaning weight following both long and short exposures. We next sought to determine how the duration of Oxy exposure influences weight by assessing the weight gain trajectory differences between prolonged and *in utero* Oxy pups. Comparisons between the male mice showed *in utero* Oxy pups initially weighed more than prolonged Oxy pups at P5. However, after the *in utero* Oxy male pups were weaned at P21, their weights decreased relative to the prolonged Oxy group at P27 and P30 (**Figure 2I**). No differences in weight gain between *in utero* and prolonged groups were detected in female Oxy-exposed pups (**Figure 2J**). Overall, early Oxy cessation was associated with increased weights at very early post-natal ages, followed by weight loss in males at weaning.

Prolonged Oxycodone Exposure Altered Sensorimotor Reflex in Female Offspring Only

Righting reflex at P14 was examined as an assessment of sensorimotor milestones, early gross locomotor abilities, and general strength (**Figures 3A,B**). In males, no difference in

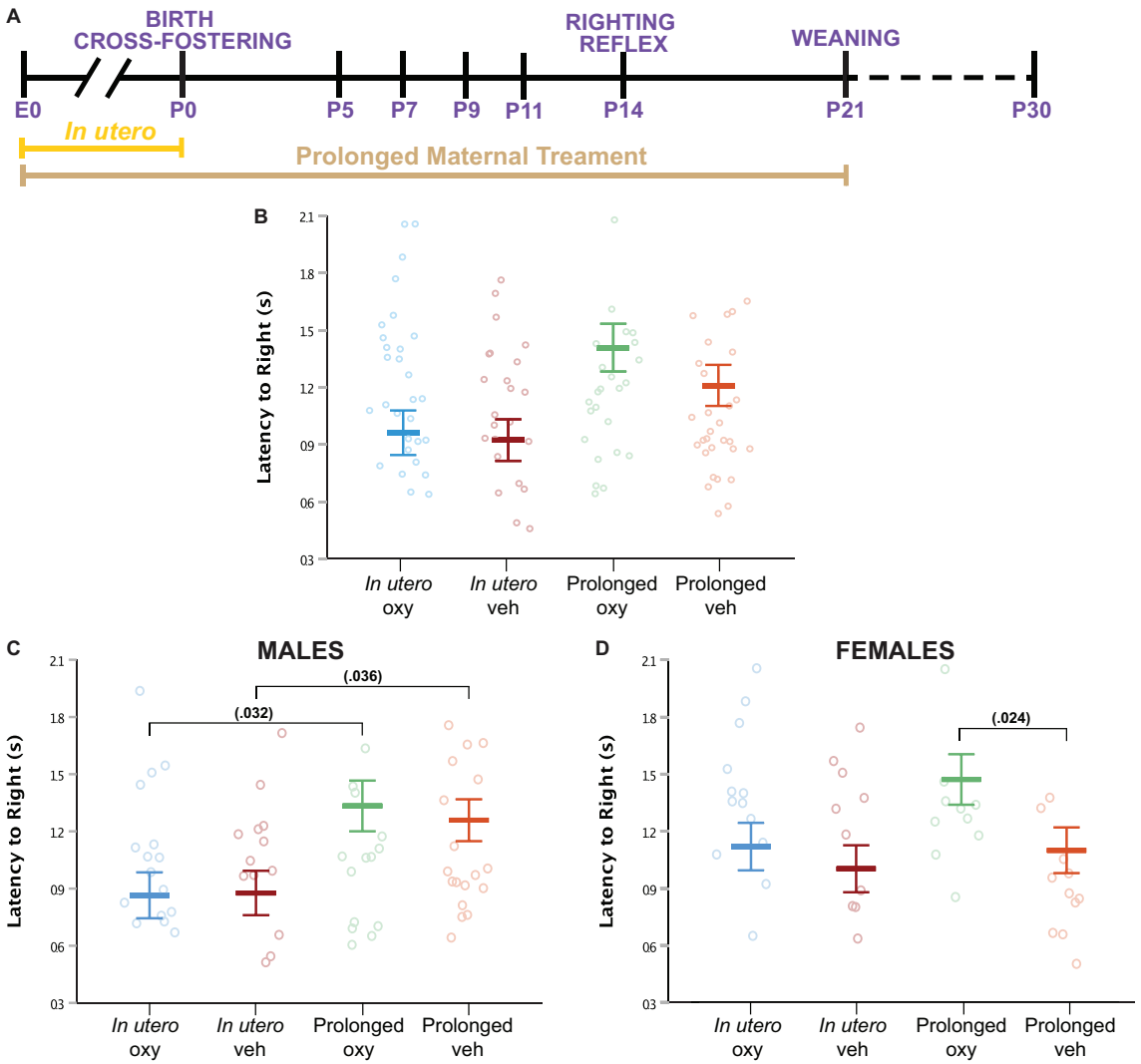

FIGURE 3 | Oxy exposure delays maturing of sensorimotor reflexes. **(A)** Schematic of the treatment paradigm for maternal Oxy exposure and righting reflex measurement throughout development. **(B)** Mean latency to right in all offspring (sex × dam × drug × age, $p = 0.048$; dam, $p = 0.042$). **(C,D)** Mean latency to right in **(C)** male and **(D)** female offspring. Prolonged Oxy exposure led to significantly longer latency to right relative to *in utero* Oxy ($p = 0.032$) in **(C)** male pups and relative to prolonged Veh ($p = 0.024$) in **(D)** female pups. Male pups exposed to prolonged Veh also show longer latency to right relative to *in utero* Veh ($p = 0.036$). Mean latencies, with litter size as a covariate ($p = 0.005$), while open circles depict individual latencies. Error bars represent standard error.

latency to right was noted between the prolonged Oxy or prolonged Veh groups (**Figure 3C**). An increased latency to right was demonstrated in prolonged Oxy male pups compared to *in utero* Oxy. However, cross-fostering may have a confounding effect on the righting reflex in male pups, since prolonged Veh males also exhibited an increased latency to right relative to *in utero* Veh males. In females, prolonged Oxy pups exhibited significantly increased latency to right relative to prolonged Veh controls (**Figure 3D**). No significant differences in the righting reflex were observed in the *in utero* Oxy or *in utero* Veh female offspring. Together, these data indicate that females, but not males, are susceptible to the effects of prolonged developmental Oxy exposure on attainment of the sensorimotor reflex.

Oxycodone Exposure Disrupts Early Communicative Behaviors

Language delays have been demonstrated in toddlers with prenatal opioid exposure (Conradt et al., 2019). Therefore, we assessed early affective and communicative behaviors by evaluating maternal isolation-induced USVs. USVs are an affective and communicative response that elicits maternal search and retrieval, lactation, and caretaking behaviors (Haack et al., 2009; Maloney et al., 2018a). As a result, characterization of quantity and quality of USV calls has been used in the rodent literature as a model for investigating early communicative deficits (Enga et al., 2016). Here, we quantified USV production and spectrotemporal features to examine the influence of Oxy on early communicative behaviors during the first 2 weeks of

life (**Figure 4A**). Overall, we detected a highly significant effect of continued Oxy exposure on USV production. Specifically, prolonged Oxy pups produced significantly fewer USVs relative to prolonged Veh pups and *in utero* Oxy pups (**Figure 4B**), which persisted from P5 through P11 (**Figure 4C**), an age at which the C57Bl/6J strain used here still has a detectable call rate (Rieger and Dougherty, 2016). While we did not see as substantial of a peak at P7/P9 as we normally see in these experiments, the effect of prolonged Oxy was consistent regardless.

Beyond call numbers, spectrotemporal USV features such as duration, pitch frequency, and power (loudness) inform of an affective component to USV characteristics (Wöhr and Schwarting, 2013). In previous analyses of USV spectrotemporal features in mouse models of intellectual and developmental disorder risk factors and early drug exposure models, we and others have demonstrated the vulnerability of these features to genetic and early environmental insults (Dougherty et al., 2013; Maloney et al., 2018a,b; Kopp et al., 2019). We examined call features including call duration, pitch range and mean, peak power, and fraction of calls with a pitch jump. Prolonged Oxy administration narrowed the USV pitch range compared to USVs produced by *in utero* Oxy pups and prolonged Veh controls (**Figure 5A**). Prolonged Oxy exposure also led to a highly significant reduction in mean pitch of USVs in prolonged Oxy male pups compared to *in utero* Oxy and prolonged Veh males (**Figure 5B**). Interestingly, USVs produced by prolonged Oxy female pups did not show a significant difference in pitch compared to prolonged Veh females (**Figure 5C**). However, female *in utero* Oxy offspring did exhibit USVs with significantly lower mean pitch relative to *in utero* Veh. A similar non-significant reduction in mean pitch was observed in USVs produced by *in utero* Oxy males relative to *in utero* Veh males (**Figure 5B**). Thus, *in utero* Oxy exposure was associated with changes in affective components of communication, the significance of which warrants further investigation. Since opioid withdrawal has been associated with high-pitched crying and increased agitation, we also assessed for alterations in USV peak power. *In utero* Oxy exposure resulted in louder USV calls compared to those produced by prolonged Oxy pups and *in utero* Veh controls (**Figure 5D**). The increased peak power, or loudness, in *in utero* Oxy pup calls only may be temporally related to onset of withdrawal after drug cessation at P0, relative to the prolonged Oxy pups which are weaned off the drug at P21.

Overall, prolonged Oxy pups demonstrated significant decreases in number of USVs along with a narrower pitch frequency range and mean pitch in male pups. *In utero* Oxy pups produced a similar number of USVs compared to controls, yet those calls were louder than controls and prolonged Oxy calls, and lower in mean pitch when produced by females. Together, our ontogenetic model of *in utero* Oxy exposure demonstrates some alterations in loudness of affective calls, while the prolonged Oxy exposure further shows alterations in number and spectrotemporal features of early communicative and affective behaviors.

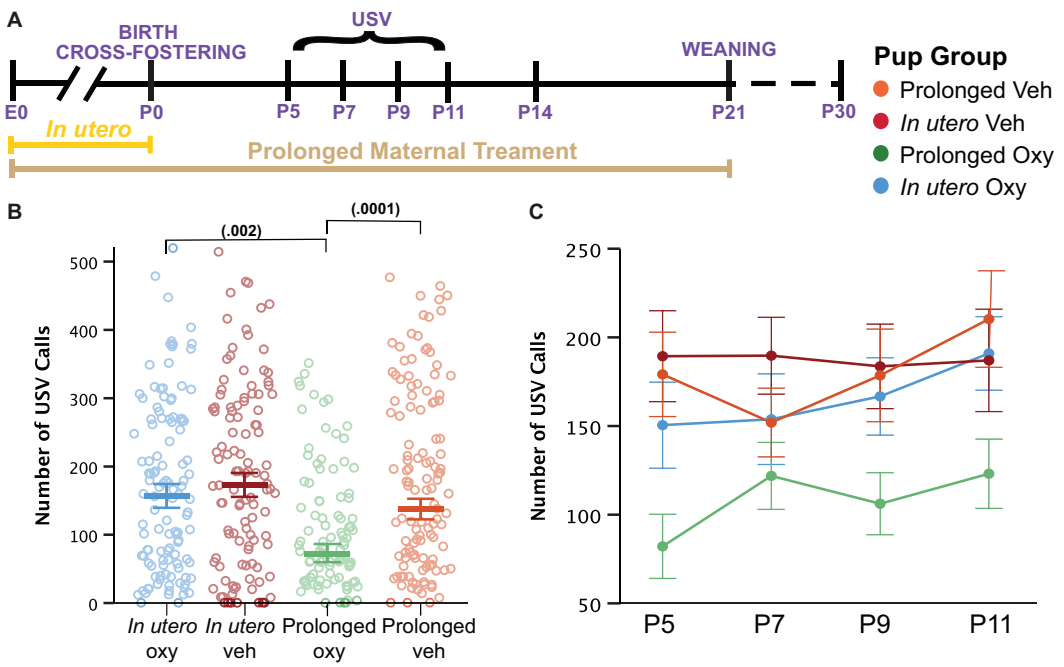

FIGURE 4 | Prolonged Oxy exposure decreases pup USV call production. **(A)** Schematic of the treatment paradigm for maternal Oxy exposure and USV measurements throughout development. **(B)** Cumulative means number of USV calls (dam, $p = 0.009$; drug, $p = 0.001$; dam × drug, $p = 0.024$). Prolonged Oxy exposure led to decreased number of calls relative to prolonged Veh ($p = 0.000126$) and relative to *in utero* Oxy exposure ($p = 0.002$). **(C)** Line graph of mean call number at all time points. Thick bars and closed circles depict mean call number, with litter size as a covariate, and open circles depict individual call numbers. Error bars represent standard error.

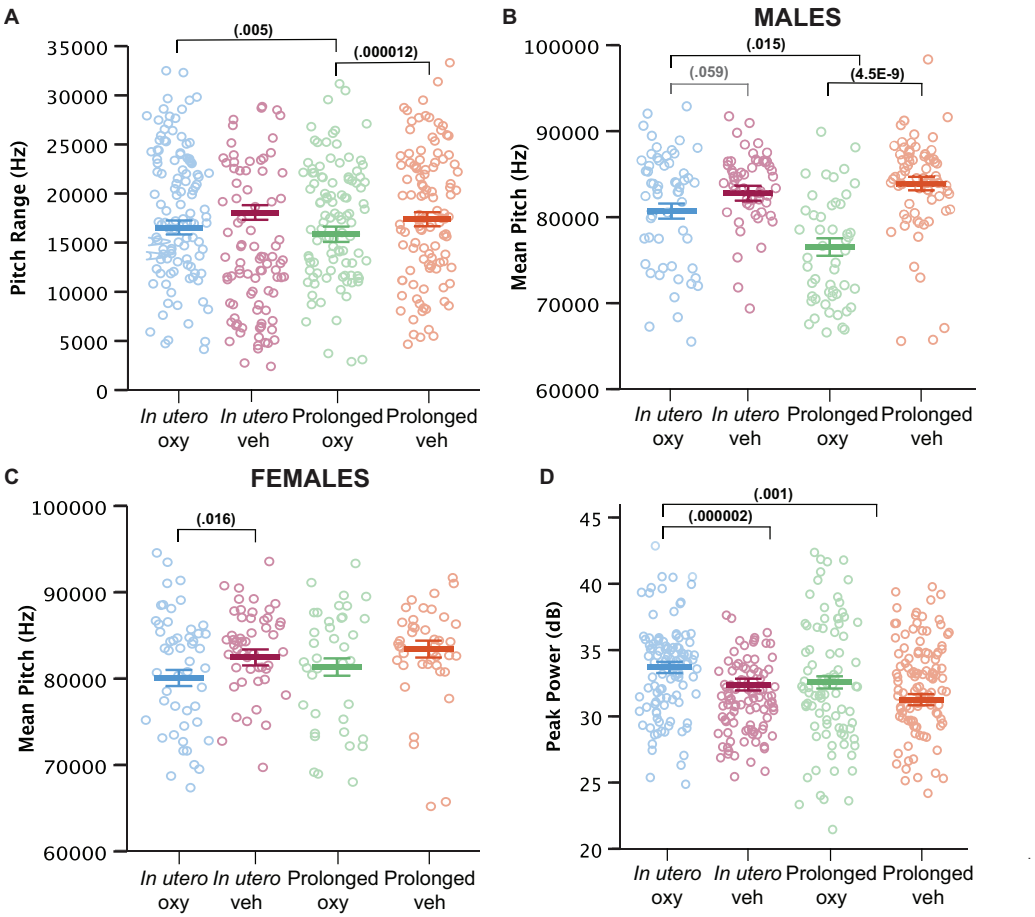

FIGURE 5 | Prolonged and *in utero* Oxy exposure affect spectrotemporal features of pup USV calls. **(A)** Cumulative means of pitch range (Hz) of USV calls (drug, $p = 0.004$; dam × drug, $p = 0.000315$; sex × dam × drug × age, $p = 0.097$). Prolonged Oxy exposure led to lower pitch range relative to prolonged Veh ($p = 0.000012$) and relative to *in utero* Oxy exposure ($p = 0.005$). **(B,C)** Cumulative mean pitch (Hz) in **(B)** male and **(C)** female offspring (drug, $p = 1.9953E^{-7}$; sex × dam, $p = 0.022$; sex × drug, $p = 0.022$; sex × dam × drug, $p = 0.004$; sex × dam × drug × age, $p = 0.036$; sex, $p = 0.076$). Prolonged Oxy exposure in **(B)** male pups led to decreased pitch relative to prolonged Veh ($p = 4.4816E^{-9}$) and relative to *in utero* Oxy ($p = 0.015$). *In utero* Oxy exposure led to a marginal decrease in pitch relative to *in utero* Veh ($p = 0.059$) in **(B)** male pups and a significant decrease in **(C)** female pups ($p = 0.016$). **(D)** Cumulative means of peak power (dB) (dam, $p = 0.019$; drug, $p = 0.002$; dam × drug, $p = 0.001$). *In utero* Oxy exposure leads to significantly higher peak power relative to prolonged Oxy exposure ($p = 0.001$) and relative to *in utero* Veh ($p = 0.000002$). Thick bars depict means, with litter size as a covariate, while open circles depict individual measurements. Error bars represent standard error.

DISCUSSION

Here we present a novel model to investigate the ontogenetic impact of *in utero* only versus prolonged mitigating opioid exposure on early neurodevelopmental outcomes, while controlling for confounding factors present in clinical observational studies. The utilization of a biological dam and cross-foster dam in our novel model of ontogenetic rodent exposure was based on an attempt to parallel opioid exposure through a method most consistent with clinical management and observations. Active maternal bonding and breast-feeding has been shown to decrease hospitalization length, decrease rates of pharmacotherapy administration, and decrease NAS severity in the NICU (Rosenthal and Baxter, 2019). The prolonged Oxy group remained with the biological dam and was weaned off Oxy through lactation, allowing for the assessment of continued post-natal exposure on early development. However, many infants with NAS experience decreased bonding time and skin–skin contact with the biological mother secondary to socioeconomic barriers and are cared for by healthcare staff for NAS. This type of setting may result in an environmental stressor to the neonate due to inconsistent maternal contact, frequent alteration of caregivers, along with a higher incidence of foster care placement following hospital discharge in neonates with maternal history of drug use (Brundage and Levine, 2019). As a result, we rationalized a cross-foster approach would be a feasible model for evaluating the effects of *in utero* opioid exposure on developmental impact in the *in utero* Oxy group.

In utero Oxy exposure decreased weight gain trajectory following weaning from foster dams, in male offspring. Further, *in utero* Oxy-exposed male and female pups

showed alterations in the spectrotemporal features of USVs. Meanwhile, offspring with prolonged Oxy exposure until weaning at P21 demonstrated poorer neurodevelopmental outcomes compared to mice exposed only until birth. Notably, continued post-natal Oxy exposure was associated with decreased weight gain trajectory, impaired motor reflexes, and abnormal early communication behaviors. Both male and female offspring in prolonged Oxy exposure groups had decreased weight gain following weaning at P21, with delayed latency to right observed in females. In addition, prolonged Oxy-exposed offspring had significantly reduced USV production and alterations in spectrotemporal features reflecting affective and early communicative impairment.

Oxycodone Exposure Impairs Attainment of Physical and Motor Development

Decreased fetal growth can be used as a general indicator of harmful *in utero* drug exposures (Haight, 2018). The association between birth weight and decreased infant survival is highly robust, though the underlying biological mechanisms are not always clearly understood (Basso et al., 2006; Yazdy et al., 2015). We did not obtain birth weights at P0 in order to minimize animal handling which can reduce behavioral and hormonal reactivity to stress and confound behavioral testing results (Luchetti et al., 2015). Initial weight assessment occurred at P5 with no observed effect of *in utero* or prolonged Oxy exposure relative to Veh controls. Overall, human literature shows low birth weight in the setting of maternal methadone use during pregnancy, but no evidence of low birth weight following *in utero* exposure to other opioids including codeine, tramadol, hydrocodone, or Oxy (Yazdy et al., 2015). Thus, our findings are consistent with existing Oxy human literature that shows no reported association between Oxy and low birth weight in neonates (Kelly et al., 2011; Yazdy et al., 2015).

Interestingly, female offspring exposed to continued Oxy, relative to Veh controls, showed a significant decrease in weight after weaning at P21, which persisted through P25, early juvenile development in mice. Neurodevelopmental processes occurring at these ages in the rodent occur in the human at approximately 2–3 years and pre-pubertal juvenile ages, respectively (Semple et al., 2013). Male weights, after prolonged exposure, showed a significant decline on P25–P30 as compared to Veh controls. Since these offspring were separated from the dam partially through a period during which they are naturally weaning from nursing (König and Markl, 1987), it is unclear whether the acute onset of decreased weight gain after separation from the dam at P21 is related to Oxy withdrawal symptomatology or not. It is certainly plausible. However, human studies of prenatal opioid exposure have described decreased adaptive behaviors during infancy through toddlerhood (Conradt et al., 2019). Cessation of care from the biological dam may potentially have uncovered deficiencies in self-care of offspring. The decreased weight gain post-weaning could also stem from maladaptive feeding behaviors secondary to Oxy exposure, since the opioid system has a strong role driving food intake homeostasis (Valbrun and Zvonarev, 2020).

Early Oxy cessation significantly decreased weight gain trajectory in a sex-specific manner not observed in the prolonged Oxy exposure cohort. Interestingly, *in utero* Oxy exposure led to significantly higher weight at P5 in males, though both groups show comparable averages at P21. Female weights following *in utero* Oxy exposure do not show any significant weight differences from controls. Males exposed to Oxy *in utero* showed a rapid, significant and persistent decrease in weight gain following weaning. Since the *in utero* exposure group was cross-fostered at birth to a non-drug exposed dam, normal weight gain trajectory was potentially maintained through adequate maternal care from the foster dam. A further explanation could be the "two-hit" hypothesis in which early life susceptibility, such as abstinence and withdrawal following *in utero* Oxy exposure, compounded with the post-natal stress of weaning precipitated a weight loss phenotype (Nederhof and Schmidt, 2012; Peña et al., 2019). Perhaps, males are more sensitive to early life stressors and may have long-term consequences from *in utero* opioid exposure compared to females. Male human neonates are more at risk for developing NAS compared to females, so long-term changes in opioid circuitry governing feeding behaviors could explain the abnormal weight trajectory in male mouse offspring post-weaning (Charles et al., 2017). Prospective human studies evaluating the long-term effects of *in utero* opioid and effects on weight trajectory during childhood through adulthood have not been performed to our knowledge. The altered weight trajectory findings in both the *in utero* Oxy and prolonged Oxy suggest a potential role of *in utero* opioid exposure on long-term impact on growth that requires further evaluation in the human literature.

In the vehicle-treated groups, cross-fostered pups showed decreased weight gain trajectory after weaning relative to pups reared by a biological dam. Our observation of decreased weight gain following weaning in Veh-exposed cross-fostered pups may be related to potential alterations in emotionality and stress responses secondary to confounders involved with cross-fostering, such as early handling (Luchetti et al., 2015). Regardless, the effect of cross-fostering on weight did not mask our ability to identify effects of *in utero* Oxy exposure on weight in males. Indeed, the effect of *in utero* Oxy exposure on weight occurred at additional younger ages and with a larger magnitude than *in utero* Veh exposure and persisted when controlling for effects of litter and cross-fostered dam status. Similarly, in females, weight reduction was observed following prolonged Oxy exposure compared to prolonged Veh controls, and *in utero* Veh exposure compared to the prolonged Veh exposure control group. Despite the independent effect on weight by cross-fostering, this method was valuable in allowing us to cease Oxy exposure at birth and thus observe effect of Oxy limited to *in utero* development. Furthermore, these findings highlight the importance of including proper cross-foster control groups in study designs for accurate interpretation of results.

Prenatal opioid exposure has also been associated with delays in attainment of motor milestones in children. A meta-analysis by Yeoh et al. (2019) detected significant delays in motor outcomes in children aged 0–6 years that experienced prenatal opioid exposure. We assessed the righting reflex at P14, the beginning of the visual critical period, and an age at which mice should be fully ambulatory (Williams and Scott, 1954; Hooks and Chen, 2007; Feather-Schussler and Ferguson, 2016). The righting reflex corrects the orientation of the body from an off axis position (Troiani et al., 2005). Proper execution of the reflex requires a combination of visual, vestibular, and somatosensory system inputs to make appropriate postural adjustments through neural pathways within the brain and cerebellum. Females in the prolonged Oxy exposure group had significantly increased latency to right compared to Veh controls. There was no significant difference in righting reflex latency between females exposed to Oxy and Veh *in utero*. Hence, only continued postnatal Oxy exposure seems to result in delayed sensorimotor development. Previously, increased latency to right has been demonstrated with *in utero* morphine exposure in both male and female rat pups, but rodent studies evaluating effects of opioids on the righting reflex have been limited (Slamberová et al., 2005). Though the exact mechanism of action is unclear, significant evidence in the literature demonstrates selective vulnerability of cerebellar granule neuroblasts to opioids, along with opioids' negative effects on neuronal somatosensory cortex development (Seatriz and Hammer, 1993; Hauser et al., 2003). Multiple studies have further linked opioid exposure to increased apoptosis and decreased differentiation of Purkinje cells in the cerebellum (Hauser et al., 2003). Additionally, perinatal morphine treatment in rats decreased total number of neurons in the somatosensory cortex (Seatriz and Hammer, 1993). Thus, it is possible multiple circuits are mediating the effect. Overall, the sex-specificity of the effect is also interesting. This observed sex bias could be related to sex-specific dimorphic alterations in catecholamine levels in the cerebellum as shown in a previous study of prenatal morphine exposure (Vathy et al., 1995). Interestingly, cross-fostering resulted in a shorter latency to exhibit the righting reflex in males compared to male pups reared by biological dams. This reflex is dependent on vestibular inputs that sense head movement but lacks cortical involvement. The righting reflex is frequently used in studies evaluating anesthesia reversal, sepsis survival or traumatic brain injuries (Gao and Calderon, 2020). In general, an increased latency to right is associated with decreased arousal or underlying neurological impairment but a decreased latency has not been shown to correlate with any particular pathophysiological condition or stressed anxiety state (Gao and Calderon, 2020). Decreased latency to right is unlikely to be linked to a behavioral or neurological impairment but could be secondary to a non-pathological increased arousal state due to cross-fostering in these litters. Overall, the shorter latency seen to right in the cross-fostered group is likely not indicative of altered development of sensorimotor reflexes. However, future studies will be necessary to delineate the mechanisms underlying this behavioral phenotype, and its sex-specific expression.

Ontogenetic Oxycodone Exposure May Delay Early Communicative Behaviors and Alter Spectrotemporal Features of USVs

Currently, studies exploring the effects of prenatal opioid exposure on language development in children demonstrate equivocal results (Conradt et al., 2019). Previous work has identified language development impairments following *in utero* exposure to methadone or heroin (Conradt et al., 2019). However, several of these analyses did not control for important confounders such as socioeconomic status or maternal use of other substances. In general, large epidemiological studies evaluating impact of prenatal opioid exposure on language development have been difficult to perform due to various confounding environmental variables including quality of caregiving, parental education level, and socioeconomic factors. Despite the advantage of limiting confounds through the use of rodent models, and indications that communicative circuits are conserved between rodents and humans (Arriaga et al., 2012), there have been minimal rodent studies evaluating the effects of prenatal opioid exposure on early communicative behaviors to date. Isolation-induced USVs are a strongly conserved adaptive response of young rodent pups to elicit maternal caregiving responses (Haack et al., 2009). Our observed collective decrease in mean USV production following prolonged Oxy exposure, compared to *in utero* Oxy exposure and Veh controls, is suggestive of impaired early communication. Previous studies have shown evidence for neuropharmacological modulation of USVs through alteration of mood or arousal state (Vivian and Miczek, 1991, 1993b; Maloney et al., 2018a). In addition to serving as an analgesic, Oxy is also a sedative and an anxiolytic agent, which may decrease USV calling in the prolonged Oxy exposure pups by actively suppressing USV circuitry secondary to reduced reactivity to surrounding environmental stressors and decreased respiratory rate (Rao and Desai, 2002). Furthermore, the interplay between pup communication and maternal care is complicated. In vocally impaired pups, decreased USV production has been shown to result in maternal neglect, because without calls the dams cannot locate the pups outside of the nest (Hernandez-Miranda et al., 2017). Thus, maternal care may be reduced in response to decreased USVs calling by prolonged Oxy-exposed pups. Maternal care has also been reported to be attenuated following morphine exposure during pregnancy, with a study reporting increased time to pup retrieval, decreased nursing and cleaning of pups, and increased maternal self-care time (Slamberová et al., 2001). This reduced maternal care could further disrupt neurodevelopment of the pup, and thus be a possible indirect influence on later adult behaviors. However, currently the literature on maternal care following Oxy exposure is conflicting. Two recent rodent studies found no changes in maternal behavior or maternal motivation following dam Oxy exposure (Watters et al., 2020; Zanni et al., 2020). Notably, in our study, *in utero* Oxy exposed offspring demonstrated comparable USV means to Veh controls. Normal USV call production in the *in utero* Oxy offspring at P5 suggests that,

in the prolonged exposure group, Oxy reduces USV production by acute suppression of USV circuits. Finally, the adequate weight trajectories before weaning in the Oxy group further indicate appropriate dam care. Hence, we would hypothesize that maternal care likely did not result in the behavioral deficits observed. Still, to our knowledge, the direct impact of Oxy exposure on maternal behaviors has not been examined. Furthermore, there is currently scant literature considering the effect of ongoing Oxy exposure on maternal behavior in rodents, with even more limited studies utilizing a mouse model. This warrants an individual study to assess reciprocity of interactions between pup and dam. Overall, deficits in observed USV production could be the result of a combination of factors, including acute drug effects on circuits, influence of dam care, and opioid-mediated effects on neural development and communication.

Affective characteristics of USVs in rodents are generally thought to communicate different emotional states, such as aggression or pain. Rodent USVs are particularly interesting as they occur only in salient situations such as exposure to painful stimuli, maternal behavior, sexual behavior, or aggression. As has been well-characterized in rats, affective features of rodent USVs may be reflected by alterations in duration, pitch, frequency, and loudness (dB) of the calls (Vivian and Miczek, 1993a,b). In our developmental cohort, pups exposed to prolonged Oxy demonstrated decreased mean pitch (in males only), and narrower pitch range. Interestingly, a previous study administered morphine to adult rats and observed decreased USV pitch, duration, and rate (Vivian and Miczek, 1993a). The decreased pitch and pitch range could be a result of Oxy's depressive effects on respiration, or of Oxy's anxiolytic drug properties which may dampen USV circuity. For the *in utero* Oxy exposure group, we predicted increased USV production along with increased duration, pitch, frequency, and amplitude of USVs secondary to discomfort associated with withdrawal. The assumption is based on the current human description of NAS characterized by human neonates exhibiting prolonged periods of high-pitched crying and inconsolability secondary to withdrawal (Anbalagan and Mendez, 2020). However, human studies do not formally characterize the spectrotemporal features of crying in infants with NAS, so the description of a high-pitched cry may be subjective in nature. We found that that continued opioid exposure during the post-natal period in the prolonged Oxy group resulted in less USV calls compared to both the *in utero* Oxy and vehicle controls. We did not expect the prolonged Oxy group to be undergoing withdrawal at this time, thus we did not hypothesize an increase in call rate in this group. A reduction in call rate is likely more consistent with an acute dampening of the circuits mediating USV production by the continued presence of Oxy in these pups during recordings. Indeed, reduced call rates were observed in rat pups following injection of μ- or δ-opioid receptor agonists (Carden et al., 1991). Future studies are needed to evaluate the impact of early suppression of these social communicative circuits by opioid agonists on social behavior consequences at older ages.

Our novel Oxy administration paradigm enables future exploration of withdrawal periods, to determine if NAS following Oxy cessation can be appropriately modeled in rodents. If so, testing of novel agents for treatment of withdrawal symptoms will be possible, with the goal of limiting continued post-natal opioid exposure given potential long-term side effects of early post-natal opioid administration on neurodevelopment (Attarian et al., 2014). Finally, few mouse models of *in utero* opioid exposure currently exist, with the majority of the literature utilizing rat perinatal opioid models. Thus, our model will facilitate genetic manipulations using established cutting-edge genetic tools available in the mouse to broaden understanding of the mechanisms mediating consequences of early opioid exposure on neurodevelopment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Washington University in St. Louis.

AUTHOR CONTRIBUTIONS

EM contributed to the conceptualization, methodology, investigation, data curation, writing (original draft preparation and editing), and funding acquisition for this project. SS contributed to the methodology, validation, formal analysis, writing (original draft preparation and editing), and visualization for this project. MM, KB, and KM contributed to the methodology for this project. JD contributed to the conceptualization, methodology, validation, resources, writing (draft editing), supervision, project administration, and funding acquisition for this project. RA-H contributed to the conceptualization, methodology, validation, writing (draft editing), supervision, project administration, and funding acquisition for this project. SM contributed to the conceptualization, methodology, data curation, resources, writing (draft editing), validation, supervision, project administration, and funding acquisition for this project. All authors contributed to the article and approved the submitted version.

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REFERENCES

Anbalagan, S., and Mendez, M. D. (2020). "Neonatal abstinence syndrome," in *StatPearls* (Treasure Island, FL: StatPearls Publishing).

Arriaga, G., Zhou, E. P., and Jarvis, E. D. (2012). Of mice, birds, and men: the mouse ultrasonic song system has some features similar to humans and song-learning birds. *PLoS One* 7:e46610. doi: 10.1371/journal.pone.0046610

ASPA (2017). *What is the U.S. Opioid Epidemic? HHS.gov*. Available online at: https://www.hhs.gov/opioids/about-the-epidemic/index.html (accessed December 4, 2017)

Attarian, S., Tran, L. C., Moore, A., Stanton, G., Meyer, E., and Moore, R. P. (2014). The neurodevelopmental impact of neonatal morphine administration. *Brain Sci.* 4, 321–334. doi: 10.3390/brainsci4020321

Azuine, R. E., Ji, Y., Chang, H.-Y., Kim, Y., Ji, H., DiBari, J., et al. (2019). Prenatal risk factors and perinatal and postnatal outcomes associated with maternal opioid exposure in an Urban, low-income, multiethnic US population. *JAMA Netw. Open* 2:e196405. doi: 10.1001/jamanetworkopen.2019.6405

Basso, O., Wilcox, A. J., and Weinberg, C. R. (2006). Birth weight and mortality: causality or confounding? *Am. J. Epidemiol.* 164, 303–311. doi: 10.1093/aje/kwj237

Branchi, I., Santucci, D., and Alleva, E. (2001). Ultrasonic vocalisation emitted by infant rodents: a tool for assessment of neurobehavioural development. *Behav. Brain Res.* 125, 49–56. doi: 10.1016/S0166-4328(01)00277-7

Brundage, S. C., and Levine, C. (2019). *The Ripple Effect: The Impact of the Opioid Epidemic on Children and Families*. New York, NY: United Hospital Fund. 46.

Carden, S. E., Barr, G. A., and Hofer, M. A. (1991). Differential effects of specific opioid receptor agonists on rat pup isolation calls. *Dev. Brain Res.* 62, 17–22. doi: 10.1016/0165-3806(91)90185-L

Charles, M. K., Cooper, W. O., Jansson, L. M., Dudley, J., Slaughter, J. C., and Patrick, S. W. (2017). Male sex associated with increased risk of neonatal abstinence syndrome. *Hosp. Pediatr.* 7, 328–334. doi: 10.1542/hpeds.2016-0218

Chiang, Y.-C., Ye, L.-C., Hsu, K.-Y., Liao, C. W., Hung, T. W., Lo, W. J., et al. (2015). Beneficial effects of co-treatment with dextromethorphan on prenatally methadone-exposed offspring. *J. Biomed. Sci.* 22:19. doi: 10.1186/s12929-015-0126-2

Conradt, E., Flannery, T., Aschner, J. L., Annett, R. D., Croen, L. A., Duarte, C. S., et al. (2019). Prenatal opioid exposure: neurodevelopmental consequences and future research priorities. *Pediatrics* 144:e20190128. doi: 10.1542/peds.2019-0128

Dougherty, J. D., Maloney, S. E., Wozniak, D. F., Rieger, M. A., Sonnenblick, L., Coppola, G., et al. (2013). The disruption of Celf6, a gene identified by translational profiling of serotonergic neurons, results in autism-related behaviors. *J. Neurosci.* 33, 2732–2753. doi: 10.1523/JNEUROSCI.4762-12.2013

Enga, R. M., Jackson, A., Damaj, M. I., and Beardsley, P. M. (2016). Oxycodone physical dependence and its oral self-administration in C57BL/6J mice. *Eur. J. Pharmacol.* 789, 75–80. doi: 10.1016/j.ejphar.2016.07.006

Feather-Schussler, D. N., and Ferguson, T. S. (2016). A battery of motor tests in a neonatal mouse model of cerebral palsy. *J. Vis. Exp.* 117:53569. doi: 10.3791/53569

Gao, S., and Calderon, D. P. (2020). Robust alternative to the righting reflex to assess arousal in rodents. *Sci. Rep.* 10:20280. doi: 10.1038/s41598-020-77162-3

Haack, B., Markl, H., and Ehret, G. (2009). *Sound Communication Between Parents and Offspring*. New York, NY: Psychology Press. doi: 10.18725/OPARU-1174

Haight, S. C. (2018). Opioid use disorder documented at delivery hospitalization — United States, 1999–2014. *MMWR Morb. Mortal Wkly. Rep.* 67, 845–849. doi: 10.15585/mmwr.mm6731a1

Hauser, K. F., Khurdayan, V. K., Goody, R. J., Nath, A., Saria, A., and Pauly, J. R. (2003). Selective vulnerability of cerebellar granule neuroblasts and their progeny to drugs with abuse liability. *Cerebellum Lond. Engl.* 2, 184–195. doi: 10.1080/14734220310016132

Hernandez-Miranda, L. R., Ruffault, P.-L., Bouvier, J. C., Murray, A. J., Morin-Surun, M. P., Zampieri, N., et al. (2017). Genetic identification of a hindbrain nucleus essential for innate vocalization. *Proc. Natl. Acad. Sci. U.S.A.* 114, 8095–8100. doi: 10.1073/pnas.1702893114

Holy, T. E., and Guo, Z. (2005). Ultrasonic songs of male mice. *PLoS Biol.* 3:e386. doi: 10.1371/journal.pbio.0030386

Hooks, B. M., and Chen, C. (2007). Critical periods in the visual system: changing views for a model of experience-dependent plasticity. *Neuron* 56, 312–326. doi: 10.1016/j.neuron.2007.10.003

Hudak, M. L., Tan, R. C., Committee On Drugs, Committee On Fetus and Newborn, and American Academy of Pediatrics (2012). Neonatal drug withdrawal. *Pediatrics* 129, e540–e560. doi: 10.1542/peds.2011-3212

Hung, C.-J., Wu, C.-C., Chen, W.-Y., Chang, C. Y., Kuan, Y. H., Pan, H. C., et al. (2013). Depression-like effect of prenatal buprenorphine exposure in rats. *PLoS One* 8:e82262. doi: 10.1371/journal.pone.0082262

Hunt, R. W., Tzioumi, D., Collins, E., and Jeffery, H. E. (2008). Adverse neurodevelopmental outcome of infants exposed to opiate in-utero. *Early Hum. Dev.* 84, 29–35. doi: 10.1016/j.earlhumdev.2007.01.013

Kelly, L., Dooley, J., Cromarty, H., Minty, B., Morgan, A., Madden, S., et al. (2011). Narcotic-exposed neonates in a First Nations population in northwestern Ontario: incidence and implications. *Can. Fam. Phys.* 57, e441–e447.

Kenan, K., Mack, K., and Paulozzi, L. (2012). Trends in prescriptions for oxycodone and other commonly used opioids in the United States, 2000–2010. *Open Med.* 6, e41–e47.

König, B., and Markl, H. (1987). Maternal care in house mice. *Behav Ecol Sociobiol.* 20, 1–9. doi: 10.1007/BF00292161

Kopp, N., McCullough, K., Maloney, S. E., and Dougherty, J. D. (2019). Gtf2i and Gtf2ird1 mutation do not account for the full phenotypic effect of the Williams syndrome critical region in mouse models. *Hum. Mol. Genet.* 28, 3443–3465. doi: 10.1093/hmg/ddz176

Krans, E. E., Cochran, G., and Bogen, D. L. (2015). Caring for opioid dependent pregnant women: prenatal and postpartum care considerations. *Clin. Obstet. Gynecol.* 58, 370–379. doi: 10.1097/GRF.0000000000000098

Kunko, P. M., Smith, J. A., Wallace, M. J., Maher, J. R., Saady, J. J., and Robinson, S. E. (1996). Perinatal methadone exposure produces physical dependence and altered behavioral development in the rat. *J. Pharmacol. Exp. Ther.* 277, 1344–1351.

Li, W., Sun, H., Chen, H., Yang, X., Xiao, L., Liu, R., et al. (2016). Major depressive disorder and kappa opioid receptor antagonists. *Transl. Perioper Pain Med.* 1, 4–16.

Lohmiller, J., and Swing, S. (2006). "Reproduction and Breeding," in *The Laboratory Rat*, eds M. A. Suckow, S. H. Weisbroth, and C. L. Franklin (Amsterdam: Elsevier), 147–164. doi: 10.1016/B978-012074903-4/50009-1

Luchetti, A., Oddi, D., Lampis, V., Centofante, E., Felsani, A., Battaglia, M., et al. (2015). Early handling and repeated cross-fostering have opposite effect on mouse emotionality. *Front. Behav. Neurosci.* 9:93. doi: 10.3389/fnbeh.2015.00093

Lutz, P.-E., and Kieffer, B. L. (2013). Opioid receptors: distinct roles in mood disorders. *Trends Neurosci.* 36, 195–206. doi: 10.1016/j.tins.2012.11.002

Maloney, S. E., Akula, S., Rieger, M. A., McCullough, K. B., Chandler, K., Corbett, A. M., et al. (2018a). Examining the reversibility of long-term behavioral disruptions in progeny of maternal SSRI exposure. *eNeuro* 5, 1–27. doi: 10.1523/ENEURO.0120-18.2018

Maloney, S. E., Chandler, K. C., Anastasaki, C., Rieger, M. A., Gutmann, D. H., and Dougherty, J. D. (2018b). Characterization of early communicative behavior in mouse models of neurofibromatosis type 1. *Autism Res.* 11, 44–58. doi: 10.1002/aur.1853

Minakova, E., Sarafinovska, S., Mikati, M. O., Barclay, K., McCullough, K. B., Dougherty, J. D., et al. (2020). Ontogenetic oxycodone exposure affects early-life communicative behaviors, sensorimotor reflexes, and weight trajectory in mice. *bioRxiv* [Preprint] doi: 10.1101/2020.09.30.321372

Nederhof, E., and Schmidt, M. V. (2012). Mismatch or cumulative stress: toward an integrated hypothesis of programming effects. *Physiol. Behav.* 106, 691–700. doi: 10.1016/j.physbeh.2011.12.008

Niu, L., Cao, B., Zhu, H., Mei, B., Wang, M., Yang, Y., et al. (2009). Impaired in vivo synaptic plasticity in dentate gyrus and spatial memory in juvenile rats induced by prenatal morphine exposure. *Hippocampus* 19, 649–657. doi: 10.1002/hipo.20540

Peña, C. J., Nestler, E. J., and Bagot, R. C. (2019). Environmental programming of susceptibility and resilience to stress in adulthood in male mice. *Front. Behav. Neurosci.* 13:40. doi: 10.3389/fnbeh.2019.00040

Rao, R., and Desai, N. S. (2002). OxyContin and neonatal abstinence syndrome. *J. Perinatol.* 22, 324–325. doi: 10.1038/sj.jp.7210744

Richardson, K. A., Yohay, A.-L. J., Gauda, E. B., and McLemore, G. L. (2006). Neonatal animal models of opiate withdrawal. *ILAR J.* 47, 39–48. doi: 10.1093/ilar.47.1.39

Rieger, M. A., and Dougherty, J. D. (2016). Analysis of within subjects variability in mouse ultrasonic vocalization: pups exhibit inconsistent, state-like patterns of call production. *Front. Behav. Neurosci.* 10:182. doi: 10.3389/fnbeh.2016.00182

Rosenthal, E. W., and Baxter, J. K. (2019). Obstetric management of women with opioid use disorder. *Semin. Perinatol.* 43, 168–172. doi: 10.1053/j.semperi.2019.01.006

Seatriz, J. V., and Hammer, R. P. (1993). Effects of opiates on neuronal development in the rat cerebral cortex. *Brain Res. Bull.* 30, 523–527. doi: 10.1016/0361-9230(93)90078-P

Semple, B. D., Blomgren, K., Gimlin, K., Ferriero, D. M., and Noble-Haeusslein, L. J. (2013). Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Prog. Neurobiol.* 106-107, 1–16. doi: 10.1016/j.pneurobio.2013.04.001

Sithisarn, T., Legan, S. J., Westgate, P. M., Wilson, M., Wellmann, K., Bada, H. S., et al. (2017). The effects of perinatal oxycodone exposure on behavioral outcome in a rodent model. *Front. Pediatr.* 5:180. doi: 10.3389/fped.2017.00180

Slamberová, R., Riley, M. A., and Vathy, I. (2005). Cross-generational effect of prenatal morphine exposure on neurobehavioral development of rat pups. *Physiol. Res.* 54, 655–660.

Slamberová, R., Szilágyi, B., and Vathy, I. (2001). Repeated morphine administration during pregnancy attenuates maternal behavior. *Psychoneuroendocrinology* 26, 565–576. doi: 10.1016/s0306-4530(01)00012-9

Troiani, D., Ferraresi, A., and Manni, E. (2005). Head-body righting reflex from the supine position and preparatory eye movements. *Acta Otolaryngol.* 125, 499–502. doi: 10.1080/00016480510036448

Valbrun, L. P., and Zvonarev, V. (2020). The opioid system and food intake: use of opiate antagonists in treatment of binge eating disorder and abnormal eating behavior. *J. Clin. Med. Res.* 12, 41–63. doi: 10.14740/jocmr4066

Vanderschuren, L. J., Niesink, R. J., Spruijt, B. M., and Van Ree, J. M. (1995). Mu- and kappa-opioid receptor-mediated opioid effects on social play in juvenile rats. *Eur. J. Pharmacol.* 276, 257–266. doi: 10.1016/0014-2999(95)00040-r

Vathy, I., Rimanoczy, A., Eaton, R. C., and Katay, L. (1995). Sex dimorphic alterations in postnatal brain catecholamines after gestational morphine. *Brain Res. Bull.* 36, 185–193. doi: 10.1016/0361-9230(94)00192-4

Vivian, J. A., and Miczek, K. A. (1991). Ultrasounds during morphine withdrawal in rats. *Psychopharmacology (Berl.)* 104, 187–193. doi: 10.1007/BF02244177

Vivian, J. A., and Miczek, K. A. (1993a). Diazepam and gepirone selectively attenuate either 20-32 or 32-64 kHz ultrasonic vocalizations during aggressive encounters. *Psychopharmacology (Berl.)* 112, 66–73. doi: 10.1007/BF02247364

Vivian, J. A., and Miczek, K. A. (1993b). Morphine attenuates ultrasonic vocalization during agonistic encounters in adult male rats. *Psychopharmacology (Berl.)* 111, 367–375. doi: 10.1007/BF02244954

Watters, A. B., Sands, L. P., and Cammack, K. M. (2020). Pregestational oxycodone exposure does not impact future maternal care or drug preference in the postpartum mouse. *Exp. Clin. Psychopharmacol.* doi: 10.1037/pha0000427 [Epub ahead of print].

Wiles, J. R., Isemann, B., Ward, L. P., Vinks, A. A., and Akinbi, H. (2014). Current management of neonatal abstinence syndrome secondary to intrauterine opioid exposure. *J. Pediatr.* 165, 440. doi: 10.1016/j.jpeds.2014.05.010

Williams, E., and Scott, J. (1954). The development of social behavior patterns in the mouse, in relation to natural periods 1. *Behavier* 6, 35–64. doi: 10.1163/156853954X00031

Wöhr, M., and Schwarting, R. K. W. (2013). Affective communication in rodents: ultrasonic vocalizations as a tool for research on emotion and motivation. *Cell Tissue Res.* 354, 81–97. doi: 10.1007/s00441-013-1607-9

Yazdy, M. M., Desai, R. J., and Brogly, S. B. (2015). Prescription opioids in pregnancy and birth outcomes: a review of the literature. *J. Pediatr. Genet.* 4, 56–70. doi: 10.1055/s-0035-1556740

Yeoh, S. L., Eastwood, J., Wright, I. M., Morton, R., Melhuish, E., Ward, M., et al. (2019). Cognitive and motor outcomes of children with prenatal opioid exposure: a systematic review and meta-analysis. *JAMA Netw. Open* 2:e197025. doi: 10.1001/jamanetworkopen.2019.7025

Zanni, G., Robinson-Drummer, P. A., Dougher, A. A., Deutsch, H. M., DeSalle, M. J., Teplitsky, D., et al. (2020). Maternal continuous oral oxycodone self-administration alters pup affective/social communication but not spatial learning or sensory-motor function. *bioRxiv* [Preprint] doi: 10.1101/2020.04.04.022533

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The Long-Term Effects of Neonatal Inflammatory Pain on Cognitive Function and Stress Hormones Depend on the Heterogeneity of the Adolescent Period of Development in Male and Female Rats

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Exposure to stress at an early age programs the HPA axis which can lead to cognitive deficits in adults. However, it is not known whether these deficits emerge in adulthood or are expressed earlier in life. The aims of the study were to investigate (1) the immediate effects of early injury-induced stress in one-day-old (P1) and repeated stress on at P1 and P2 rat pups on plasma corticosterone levels; and (2) examine the subsequent long-term effects of this early stress on spatial learning and memory, and stress reactivity in early P26-34 and late P45-53 adolescent male and female rats. Intra-plantar injection of formalin induced prolonged and elevated levels of corticosterone in pups and impaired spatial learning and short- and long-term memory in late adolescent males and long-term memory in early adolescent females. There were sex differences in late adolescence in both learning and short-term memory. Performance on the long-term memory task was better than that on the short-term memory task for all early adolescent male and female control and stressed animals. Short-term memory was better in the late age control rats of both sexes and for formalin treated females as compared with the early age rats. These results are consistent with an impaired function of structures involved in memory (the hippocampus, amygdala, prefrontal cortex) after newborn pain. However, activation of the HPA axis by neonatal pain did not directly correlate with spatial learning and memory outcomes and the consequences of neonatal pain remain are likely multi-determined.

Keywords: neonatal pain, corticosterone, adolescence, spatial memory, sex differences, spatial learning

Abbreviations: The HPA axis, the hypothalamo-pituitary-adrenocortical system; the HPG axis, the hypothalamus-pituitary-gonadal system; MWM, the Morris water maze; CFA, complete Freund's adjuvant; GD, gestational day; P1, P2, postnatal day1, postnatal day 2; PVN, paraventricular nucleus; CA1 hippocampus; GR, glucocorticoid receptor; CRH, corticotrophin-releasing hormone; ACTH, adrenocorticotropic hormone; NMDA receptor, the N-methyl-D-aspartate receptor; PFC, prefrontal cortex.

INTRODUCTION

The longitudinal analysis of outcomes of pain for premature infants in a neonatal clinic found that pain in these infants resulted in impaired cognitive function in the child and adolescent (Haley et al., 2006; Grunau et al., 2009; Doesburg et al., 2013; Vinall et al., 2014; Chau et al., 2017). Whereas the long-term influence of non-pain stress (e.g., maternal separation, mother and offspring isolation, early handling or the limited nesting model) on cognitive behavior and memory in animal models is abundant in the literature (Krugers and Joëls, 2014; Schroeder et al., 2018; Bonapersona et al., 2019; Cordier et al., 2021), the long-term consequences of pain stress have been understudied (Khawla et al., 2017), especially considering it prevalence in the clinical setting (Ranger and Grunau, 2014; Mooney-Leber and Brummelte, 2017) and the strong connection between neonatal pain and disturbances of central nervous system maturation (Schwaller and Fitzgerald, 2014; Brewer and Baccei, 2020; Williams and Lascelles, 2020). The neonatal period is a critical period of development due to the rapidly changing neurobiological processes, which result in altered sensitivity to external stimuli and a high level of plasticity of the nervous system (Lupien et al., 2009). The nervous system during the neonatal period is highly sensitive to painful stimuli (Goksan et al., 2015; Williams and Lascelles, 2020) with the critical period for later effects on adult pain ending around the end of the first week of life in the rat (Ren et al., 2004). It is known that repeated pain in the neonatal period disrupts the balance between the processes of excitation and inhibition in the central nervous system (Brewer and Baccei, 2020), modifies brain development (Schwaller and Fitzgerald, 2014), programming the HPA axis (Mooney-Leber and Brummelte, 2017), therefore modifying multiple types of behavior (Williams and Lascelles, 2020). Moreover, there is a close neuroanatomical and physiological interaction between pain and the HPA axis, which is regulated in part by the hypothalamus, amygdala, hippocampus, prefrontal cortex (PFC), and thalamus (Ulrich-Lai and Herman, 2009; Victoria et al., 2013; Timmers et al., 2019). The features of this interaction in response to damaging stimuli at an early age are poorly understood. Because there are multiple physiological systems affecting pain and the HPA axis in the neonatal period (Mooney-Leber and Brummelte, 2017, 2020; Van Bodegom et al., 2017) the data on the effect of pain on the HPA axis, both in the clinic and in animals, are incomplete (Walker et al., 2003; Victoria et al., 2013; Victoria and Murphy, 2016). Moreover, the effects of stress and pain on the HPA are age dependent, even in infancy (Van Bodegom et al., 2017). Further research is needed to clarify the relationship between neonatal pain and the HPA axis, since there is a multifaceted relationship between the type of pain, severity, gender, and age of pain exposure and the response of the HPA axis.

In adolescence, problems of neurodevelopment and behavior caused by infant stressful influences first appear. To correct the behavior during the adolescent period, it is important to know the features of the age-related intervals of an adolescent development period. Adolescence is characterized by intensive processes of synaptogenesis and myelinization, especially in the prefrontal cortex, hippocampus and amygdala (Brenhouse and Andersen, 2011), reorganization in the hormonal, neurotransmitter, and reproductive systems (McCormick et al., 2004, 2016; Romeo and McEwen, 2006; Romeo, 2010; McCormick, 2011), and behavioral and cognitive maturation (McCormick and Mathews, 2010; McCormick et al., 2012; Siddiqui and Romeo, 2019). The trajectory of these processes in adolescence can be altered by neonatal stress, including clinically necessary painful procedures (Amaral et al., 2015; Chen et al., 2016; Mooney-Leber and Brummelte, 2017; Xia et al., 2020), which can modify behavior and cognitive abilities in adolescents (Mooney-Leber and Brummelte, 2020; see Williams and Lascelles, 2020 for a review). Thus, early-life pain can give rise to numerous clinical, social, educational problems in adolescents which may differ from those of adults.

Based on behavioral and the nervous system maturation, adolescence in rats ranges from 28-48 days (P28-48) with adults defined as P60 and older (Spear, 2000). A more granular view of this critical period of development divides adolescence into three sub-periods: early P21-P34, middle P34-P46 and late P46-P59 (Tirelli et al., 2003). These epochs within adolescence vary somewhat depending on which criteria are used (morphogenetic, behavioral, neurohormonal, and neural). Much attention has been paid to the different neurobiological systems within the age-related intervals of adolescence (McCormick et al., 2016, 2020; Bailey et al., 2020; Marcolin et al., 2020; Gore-Langton et al., 2021). One of the main factors determining the timing of adolescence is the maturation of the hypothalamic-pituitary-adrenal system (the HPA axis), and its feedback mechanisms, which continue to mature during adolescence. Glucocorticoids, the secretion of which is controlled by the HPA axis, affect brain development, including neurogenesis, synaptogenesis, and cell death (McEwen, 2000). The development of the HPA axis during adolescence may be modified by stress experienced early in life (Van Bodegom et al., 2017). The timing of adolescence can also be determined by sexual maturation and its relationship with the HPA axis (McCormick and Mathews, 2007). The pubertal onset of sexual maturation, determined by vaginal opening in female rats and preputial separation in male rats, occurs earlier in females (P35 $\pm$ 2) than in males (P42 $\pm$ 2) (McCormick and Mathews, 2007, 2010), making it important to include males and females at different stages of adolescence in studies. The peripheral steroid hormone of the HPA axis, cortisol in humans, corticosterone in rodents, plays an important role in learning and memory (Akirav et al., 2004). The effects of inflammatory pain on the secretion of corticosterone in newborns, and the consequences of these effects on the development of brain structures involved in cognitive function and in the formation of HPA axis before puberty are still largely unknown. Repeated prick needles of the pad of hind paws have been used as a pain stressor in newborn rodents (Anand et al., 1999; Walker et al., 2003; Nuseir et al., 2015, 2017; Ranger et al., 2019); however, the results are often mixed. For example, repeated needle pricks to the pad of the hind paws of newborn rodents did not change the level of corticosterone in adult rats (Anand et al., 1999; Walker et al., 2003), but did decrease stress reactivity of the HPA axis in

adolescence, and impaired the ability to retain spatial memory in prepubertal male rats (Chen et al., 2016). In prepubertal mice, similar injury impaired spatial learning and short-term (Nuseir et al., 2015) and long-term (Nuseir et al., 2017) memory, whereas in adult mice, it impaired short-term memory, but did not alter spatial learning ability and long-term memory (Ranger et al., 2019).

We are aware of only a few rodent studies that investigated the effect of neonatal inflammatory pain on memory. For instance, inflammatory pain caused by the intraplantar injection of carrageenan (1%) on the day of birth (P0), resulted in spatial memory deficits in adult rats (Henderson et al., 2015), and also changed the regulation of the HPA axis (Victoria et al., 2013); complete Freund's adjuvant on P1 did not affect short-or long-term memory in male or female rats on P60, but resulted in spatial learning deficits in males (Amaral et al., 2015). Formalin-induced pain in newborn rats, which produces less prolonged pain than does carrageenan or CFA, impaired visual-spatial learning and memory in the radial 8-arm maze, which uses food reinforcement, in adult rats (Anand et al., 2007).

There are many models of neonatal pain, each with their advantages and disadvantages. All are meant to model the experience of the infant in the NICU who experiences many skin breaking experiences each day (Grunau et al., 2006). The four most common are repeated needle stabs, or carrageenan, CFA or formalin injection, although others exist (e.g., paw incision; local capsaicin treatment). Formalin has the advantage of producing a reliable short-lived behavioral response (<1 h) and accompanying edema (Anand et al., 1999), without long-term immune activation or substantial disruption of mother-pup interactions, therefore limiting the duration of pain and allowing precise control over the age of injury.

We previously showed that inflammatory pain on P1and P2 did not alter spatial learning in 33-day-old adolescent female rats (Butkevich et al., 2020). A more granular study of the consequences of inflammatory painful effects on cognitive abilities and the stress-hormonal system in adolescence is especially important, since neurobiological and behavioral changes vary greatly at different stages of adolescence and these changes, caused by stressful effects at an early age, may be manifested specifically at one or more of these stages. Identifying this unique developmental pattern would help direct efforts to ameliorate any untoward effects of early stress pain more precisely.

Most of the basic research has been and is being done on males (Chen et al., 2016; Xia et al., 2020). When both sexes are tested, females have been found to be more vulnerability to the noxious influences at an early age by some (Gildawie et al., 2021), whereas others report increased vulnerability in males (Van Dammen et al., 2020). Androgens in adults inhibit the activity of the HPA, whereas estrogens, on the contrary, increase it (Handa et al., 1994; McCormick et al., 2002). Pain-related sex differences are present from birth (Verriotis et al., 2018; Gursul et al., 2019). Pain stress is often presented in the neonatal clinic, and it is important to know how consequences of pain stress are manifested in different term intervals of adolescence in order to correct the behavior in this period of postnatal development.

The aim here was to investigate the immediate effect of formalin-induced pain in one-day-old and two-day-old (P1, P2) rat pups on corticosterone level in blood plasma and the long-term effects of early-life pain stress on spatial learning and memory in the Morris water maze, and stress reactivity of the HPA axis in male and female rats of early P26-34 and late P45-53 age groups of adolescence.

MATERIALS AND METHODS

Animals

Subjects were the offspring of Wistar rats (parents: males, $n = 35$ and females $n = 62$) from the biocollection of Pavlov Institute of Physiology of the Russian Academy of Sciences. After two days of adapting to new quarters, the rats were mated, and a vaginal smear was examined next morning to verify insemination. The days of insemination and birth were considered as gestational day (G) 0 and postnatal day (P) 0, respectively. Pregnant dams were housed four per cage, then individually after the 17th day of pregnancy. All animals were maintained under standard conditions (12 h light, 12 h dark, lights on at 08:00, 20–22°C) in standard plastic rat cages with food and water available *ad libitum*. The birth of offspring was checked at 8, 13, 17 and 20 h. A day after the birth of the offspring, litters were reduced to 8 rat pups (4 males and 4 females if possible). From each mother, one male and one female were included in the experiment; in two cases, two rats of each sex from one dam were used. In the latter case, data from these two animals were averaged to produce a "litter" mean (see the statistics section below). The remaining rats in a litter were used in other experiments. All procedures were approved by the Local Ethics Committee for Animal Experiments of the I. P. Pavlov Institute of Physiology, Russian Academy of Sciences (Saint Petersburg, Russia) and followed the guidelines published by the Committee for Research and Ethical Issues of the IASP on ethical standards for investigations of experimental pain in animals.

Neonatal Inflammatory Pain

On the first and second day of life (P1 and P2) offspring of both sexes were injected with the inflammatory agent formalin (2.5%, 0.5 μl) into the pad of the left hind paw; as a control, a single prick needle or saline injection was used. In preliminary experiments, in which P1 and P2 rat pups were subjected to a single needle prick ($n = 5$ in each adolescent age and sex group) or a single injection of saline solution into the pad of the left hind paw ($n = 5$ in each adolescent age and sex group), the animals were tested at P26-34 and P45-53. No differences were evident in spatial learning and memory in the Morris water maze (MWM) or in the stress reactivity of corticosterone between the pricked and saline animals in both age and sex groups (see **Supplementary Figures 1–4**), so needle prick rats were used as a control for formalin injection in the MWM experiments and saline injection as control for the corticosterone assays in newborn rat pups. In addition, the rats designed to determine basal corticosterone levels were handled at the same time as Control or Formalin rats but otherwise untreated. P1 and P2 in rats roughly correspond

to extreme prematurity in human (gestation weeks 24), based on development of the brain and pain system in rats and humans (Dobbing, 1981; Fitzgerald et al., 1988). All animals in each litter were randomized to the inflammatory pain (Formalin) and Control. Formalin rats were labeled with a weak solution of picric acid along the back, control rats remained unmarked; remaining rats designed for other experiments, had their heads painted with picric acid.

Morris Water Maze (MWM)

A modified version of the MWM was used to assess spatial learning, spatial short-term and long-term memory (Morris, 1981; Vorhees and Williams, 2014). The MWM consisted of a round tank (120 cm diameter, 72 cm deep) filled to 40 cm with opaque with chalk-clouded water (24 ± 2°C) to eliminate the platform's visibility. The tank, located in a room with several strongly contrasting extra maze cues, was visually divided into four equal quadrants West (W), South (S), East (E) and North (N). A steel platform (39 cm height, 12 cm diameter) was placed in the SW quadrant approximately 40 cm from the side wall, and its location fixed for all the animals during all training days. The investigator was visible to the rats, and her location was constant throughout all experiments. The installation was illuminated by two lamps (250 W), the light from which was directed to the ceiling to obtain soft diffused lighting.

Spatial Learning Assessment

Spatial learning tests were conducted at two age groups during the adolescent period: early (P26-34) (formalin rats $n = 15$ males and $n = 12$ females, and control rats $n = 16$ males and $n = 14$ females) and late (P45-53) (Formalin rats $n = 15$ males and $n = 15$ females, and Control rats $n = 16$ males and $n = 16$ females). Rats tested at the early age were with their mother until the end of the experiments (on the 34th day of life). The rats of the late age group were weaned also at 34 days, and males and females placed in different cages, 3–4 per cage. Both cohorts were tested identically.

In the MWM, each rat was trained for 5 consecutive days to locate a platform with eight training trials per day, divided into four trials with an interval between them of 4 minutes. For each training trial, the rat was placed in the water facing the tank wall, in the first training trial into the NW quadrant, then consistently in the SW, SE and NE quadrants. The rat was allowed to search for the platform for 60 s. Failing that, the experimenter placed the rat on a platform, where it remained for 20 s to learn its location relative to the extra-maze visual cues. Then the rat was transferred to a dry cage with paper towels as bedding for 15 s, after which the training trials were continued. The latency (from when the rat was submerged in the water tank to when it located the platform) was recorded in each trial. If the rat did not locate the platform during a trial, it was assigned the maximum trial duration 60 s as the score for that trial. The average latency in the first four training trials and the average latency in second four training trials were used as measures of learning.

In addition to latency, we used the index of acquisition and the savings index as additional measures of spatial learning during training tests (Whiting and Kokiko-Cochran, 2016; Tucker et al., 2018). The index of acquisition describes the learning that occurs within one day of testing, and is calculated by taking the difference between the latency in the first and last tests and averaging this difference for all days of spatial learning. The savings index is the measure of how well, on the first test of each day, the rats remember what was learned on the previous day. This value is calculated as the difference between the latency in the last test of a given day and the latency in the first test of the next day and averaged over all days of spatial learning. Thus, the savings index reflects consolidating and storing memory and/or its retrieval process.

Spatial Memory Assessment

On the fifth and last training day, the rats were exposed only to the first four training trials, and then dried and returned to their cages in a different room. After one hour, each rat was placed back into the pool and the spatial short-term memory was examined without the platform. This short-term assessment reflects a combination of reference and working memory (Vorhees and Williams, 2014). The NW quadrant start location was used for short- and long-term memory assessment. Long-term spatial memory, memory retention, was examined 96 h after the short-term memory study (34th and 53d days of life in the early and late age groups), by placing each animal sequentially in water without a platform. Animals were allowed to swim freely for 60 s in the water tank without the platform. The latency to locate the spot where the hidden platform had been previously and the amount of time the animal spent in the target quadrant (SW quadrant) were the scores for the short- and long-term memory probe trials. Behaviors in the short- and long-term memory tests were recorded using a webcam with automatic focusing (Microsoft 5WH-00002) and also visually in real time.

Blood Collection and Corticosterone Determination

There were 56 newborn rat pups (basal, $n = 19$; saline (control) $n = 18$; formalin, $n = 19$) used for determination of corticosterone in the blood. Blood samples were collected by rapid decapitation without anesthesia. Basal samples were taken at 9 AM. To determine the effect of inflammatory pain in newborn rats on the activity of the HPA axis, blood samples were collected following decapitation of one female and one male into a single test tube (the volume of blood from one rat neonate is very small, so we combined samples from one male and one female in one test tube) 30 minutes after subcutaneous injection of formalin (2.5%, 0.5 μl) or saline into the pad of the left hind paw. The time course of the effects of the formalin treatment on corticosterone levels were evaluated one day and seven days after injection of formalin or saline. In early adolescent rats (basal, $n = 8$ males and $n = 8$ females; control (needle prick), $n = 10$ males and $n = 9$ females; formalin, $n = 10$ males and $n = 8$ females) and late adolescent rats (basal, $n = 7$ males and $n = 11$ females; control (needle prick), $n = 6$ males and $n = 6$ females; formalin, $n = 6$ males and $n = 6$ females), corticosterone reactivity to the forcing swimming was determined 30 min after the long-term spatial memory test in MWM. Here, unlike the corticosterone assay in newborn rat pups, blood of males and females was collected in separate test tubes by rapid decapitation without anesthesia. Following

collection, blood was centrifuged, and blood plasma was stored in a freezer (−20°C). Corticosterone was determined in duplicate by immune-enzyme analysis, using standard kits ("Xema-Medica Co" Cat No: K210R; Russia); the intra-assay coefficient was 3.8.

Statistical Analysis

Mixed ANOVA was used for spatial learning Analysis I for the first four training trials and Analysis II for the second four training trials, the within-subjects factor was day of testing (days 1,2,3,4,5), and between-subjects factors were age (26–30 days/45–49 days), sex (males/females), exposure (formalin P1 & P2 or needle prick at P1 & P2); when Mauchly's Test of Sphericity was significant, we used Greenhouse-Geisser method. A mixed ANOVA was used for comparison of the first four and second four training trials, Analysis III, the within-subjects factors for day number (1,2,3,4 on each of four days), between-subjects factors were the same as Analyses I and II. For the index of acquisition, three-way analysis of variance ANOVA was used; the factors were age, sex, exposure. For the savings index, three-way (factors: age, sex, exposure) and two-way (factors: age, exposure) analyses of variance ANOVA were used. For memory, mixed ANOVA was used, within-subjects factors: memory (short-time memory, STM/long-term memory LTM) for latency (time to find platform location) and for time the animal spent in the target quadrant, and between-subjects factors age (30 and 34 days for STM and 49 and 53 days for LTM), sex (males/females) and exposure (formalin P1 & P2/needle prick P1 & P2). For corticosterone in newborns, a one-dimensional two-factor analysis of variance ANOVA was used. The dependent variables were corticosterone, day of test (30 minutes, first day, seventh day) and exposure (basal level, saline, formalin); for corticosterone of adolescent rats, three-way univariate analysis of variance ANOVA was used, and the between factors were day of test (34 days/53days), sex (males/females), exposure (basal/needle prick/formalin). Comparison of corticosterone separately for males and females did not reveal sex differences; therefore, a variant of paired comparisons was made for males and females in total. *Post hoc* comparisons were made with Bonferroni multiple comparisons test. For two cases, when two rats of each sex from one dam were used, we averaged the data from littermates (where they were 2 from a litter) to create a single data point per litter. We ran the analysis of variance for litters per group, subjected to the same exposure. The analysis showed that in the majority of the groups of the rats there is no principal differences between the results of dimension in different litters, this indicates that the litters are uniform for the most part and we re-ran the statistical analysis this way.

RESULTS

Details of the analyses of main experimental data are in **Table 1** and summarized below.

Spatial Learning, Latency to Find the Platform

Both Control and Formalin rats in both age groups and of both sexes showed spatial learning, since the latency to find the platform decreased on the fifth training day in all the animals (**Figure 1**). However, there were differences in spatial learning between P26-P34 and P45-P53 rats. For early adolescent period (P26-P34) (**Figures 1A,B**) *post hoc* analysis found no differences in the latency to find the platform between the Control rats (males $n = 16$, females $n = 14$) and Formalin rats (males $n = 15$, females $n = 12$) in either training trial on each of the 5 training days. Neonatal formalin pain did not alter the latency to find the platform. For late adolescent period (P45-P53) (**Figures 1C,D**) *post hoc* analysis found that neonatal pain significantly increased the latency to find the platform in Formalin males ($n = 15$) compared to the latency in Control males ($n = 16$) (**Figure 1C**).

Age and sex differences in the latency to find the platform are presented in **Figure 2**. The latency was longer in P26-34 rats of both sexes. Age differences were found in Control males (**Figures 2A,C**) and Control females (**Figures 2B,D**) in the first four (**Figures 2A,B**) and second four (**Figures 2C,D**) training trials. It was found, that in the first training day, that characterizes the greatest response to the stimulus in MWM (Vorhees and Williams, 2014), neonatal pain significantly increased the latency to find the platform in P45-53 males in both four training trials to the levels of the younger males, whereas in females, similar effects of formalin were not evident. The greater latency to find the platform in the late adolescent period in Formalin P45-53 males compared to the latency in Formalin P45-53 females indicates their greater vulnerability to the effects of neonatal inflammatory pain on spatial learning in males than in females (**Figure 2C**).

The Index of Acquisition (Figure 3A) and the Savings Index (Figure 3B) for the Latency

We used the index of acquisition and the savings index as supplemental ways to assess the effectiveness of spatial learning. The index of acquisition (**Figure 3A**) and the savings index (**Figure 3B**) support the data presented in **Figures 1**, **2**, indicating that the spatial learning within a day was more successful (latency was shorter) in Control P45-53 rats than in Control P25-34 rats ($p < 0.05$, males and females). The savings index (**Figure 3B**) demonstrated that Control P45-53 females, compared to Control P25-34 females, ($p < 0.05$), recalled better on the first trial of each day what was learned on the last trial of the previous day. Neonatal formalin pain neutralized these age-related differences. In the early age group of rats of both sexes, neither index showed effects in the formalin treated animals. Indeed, the index of acquisition and the savings index indicated that formalin treated P45-53 females showed deficits in the both learning and memory (the latency was greater) compared to Control females of the same age. These data are not consistent with those data in **Figure 1**. It is likely that the scoring mechanism underlying this alternative method determines these differences, as reflected in the sex differences in the first four and second four training trials.

Short- and Long-Term Spatial Memory, Latency to Find the Platform (Figure 4)

In latency to the target quadrant, there were no age or sex differences in the treated groups for either the short- and

TABLE 1 | Details of the statistical analyses.

Spatial learning, latency to find the platform	
Main effects for early and late adolescent periods of development for the first four training trials (Analysis I)	
Within subjects effects	$F(3,1, 342,4) = 224$, $^{***}p < 0,001$, $\eta^2 = 0,670$,
day	$F(3,1, 342,4) = 5,724$, $^{***}p = 0,001$, $\eta^2 = 0,049$,
day X age:	*Note. Mauchly's Test of Sphericity $p < 0,001$,*
Between subjects effects	*Greenhouse-Geisser method Df1 = 3,084, Df2 = 342,359.*
age	$F(1,111) = 20,208$, $^{***}p < 0,001$, $\eta^2 = 0,154$.
Main effects for early and late adolescent periods of development for the second four training trials (Analysis II)	
Within subjects effects	$F(2,5, 272,4) = 64,1$, $^{***}p < 0,001$, $\eta^2 = 0,366$,
day	$F(2,5, 272,4) = 8,9$, $^{***}p < 0,001$, $\eta^2 = 0,074$,
day X age:	*Note. Mauchly's Test of Sphericity $p < 0,001$,*
Between subjects effects	*Greenhouse-Geisser Df1 = 2,454, Df2 = 272,378.*
age	$F(1,111) = 20,5$, $^{***}p < 0,001$, $\eta^2 = 0,156$,
exposure	$F(1,111) = 4,3$, $^{*}p = 0,041$, $\eta^2 = 0,037$,
age X sex	$F(1,111) = 4,7$, $^{*}p < 0,032$, $\eta^2 = 0,041$.
Analysis III (try1-2)	
Age and sex differences in the latency to find the platform	
Effect of training day	$F(4, 108) = 71$, $^{***}p < 0,001$, $\eta^2 = 0,725$
Effect of training day in X age X sex	$F(4,108) = 2,6$, $p = 0,04$, $\eta^2 = 0,088$,
Tests of between-subjects effects	$F(1, 111) = 12,2$, $^{**}p = 0,001$, $\eta^2 = 0,099$, $F(1, 111) = 25,9$,
Age	$^{***}p < 0,001$, $\eta^2 = 0,189$, $F(1, 111) = 15,9$, $^{***}p < 0,001$,
Posthoc analysis	$\eta^2 = 0,126$ (the first, second and third day respectively)
The index of acquisition for the latency	$p = 0.042$, $p = 0.041$, $p = 0.022$, the first, third and fourth
age	training days
age X exposure	$F(1,111) = 4,304$, $p = 0,040$, $\eta^2 = 0,037$
The savings index for the latency	$F(1,111) = 4,416$, $p = 0,038$, $\eta^2 = 0,038$;
age X exposure	$F(1,111) = 4,046$, $p = 0,046$, $\eta^2 = 0,035$
Short- and long-term spatial memory, latency to find the platform and time spent in target quadrant	
Within subjects effects	
memory	
memory X age	$F(2, 107) = 20,5$, $^{***}p < 0,001$, $\eta^2 = 0,277$,
memory X exposure	$F(2, 107) = 19,48$, $^{***}p < 0,001$, $\eta^2 = 0,267$
Between subjects effects	$F(2, 107) = 7,32$, $^{***}p = 0,001$, $\eta^2 = 0,120$;
age	$F(2, 107) = 5,87$, $^{***}p = 0,004$, $\eta^2 = 0,099$
exposure.	$F(2, 107) = 3,76$, $^{*}p = 0,026$, $\eta^2 = 0,06$
Comparison between short- and long-term memory	
Within subjects effects	$F(1, 108) = 8,9$, $^{**}p = 0,003$, $\eta^2 = 0,076$,
latency	$F(1, 108) = 13$, $^{**}p < 0,001$, $\eta^2 = 0,107$,
memory X exposure	
Within subjects effects	
time spent in target quadrant	$F(1, 108) = 37$, $^{***}p < 0,001$, $\eta^2 = 0,255$
memory X age	$F(1, 108) = 38,4$ $^{***}p < 0,001$, $\eta^2 = 0,263$;
memory X exposure	$F(1, 108) = 3,4$ $p = 0,068$, $\eta^2 = 0,031$,
memory X age X sex	$F(1, 108) = 3,1$ $p = 0,079$, $\eta^2 = 0,028$;
Between subjects effects, latency	
age	$F(1, 108) = 4,51$, $^{*}p = 0,036$, $\eta^2 = 0,040$
Between subjects effects,	
time spent in target quadrant	$F(1, 108) = 9,17$, $^{**}p = 0,003$, $\eta^2 = 0,078$,
age	$F(1, 108) = 7,4$, $^{**}p = 0,008$, $\eta^2 = 0,064$.
exposure	
In newborn rats, main effects for corticosterone	
day of test,	
exposure,	$F(2,47) = 75,6$, $p < 0,001$, $\eta^2 = 0,763$;
day of test X exposure,	$F(2,47) = 26,3$, $p < 0,001$, $\eta^2 = 0,528$;
In adolescent rats, main effects for corticosterone	$F(4,47) = 9,66$, $p < 0,001$, $\eta^2 = 0,451$.
day of test,	
exposure	$F(1,83) = 27,45$, $^{***}p < 0,001$, $\eta^2 = 0,249$
day of test X exposure	$F(2,83) = 66,28$ $^{***}p < 0,001$, $\eta^2 = 0,615$,
	$F(2,83) = 10,7$ $^{***}p < 0,001$, $\eta^2 = 0,205$.

long-term memory measures. However, the formalin treated males, but not females, showed long-term memory deficits at both ages (**Figures 4B,B1**) with no differences in the short-term memory task (**Figure 4A**). Control P26-34 rats of both sexes found the quadrant where the platform had been previously more quickly in the long-term compared to the short-term memory task ($p = 0.018$ and $p = 0.006$, males and females respectively) as did P45-53 females ($p = 0.003$) (**Figures 4A,B**). Thus, neonatal pain impaired long-, but not short-term memory, in the males, but not females, in both ages. Formalin pain

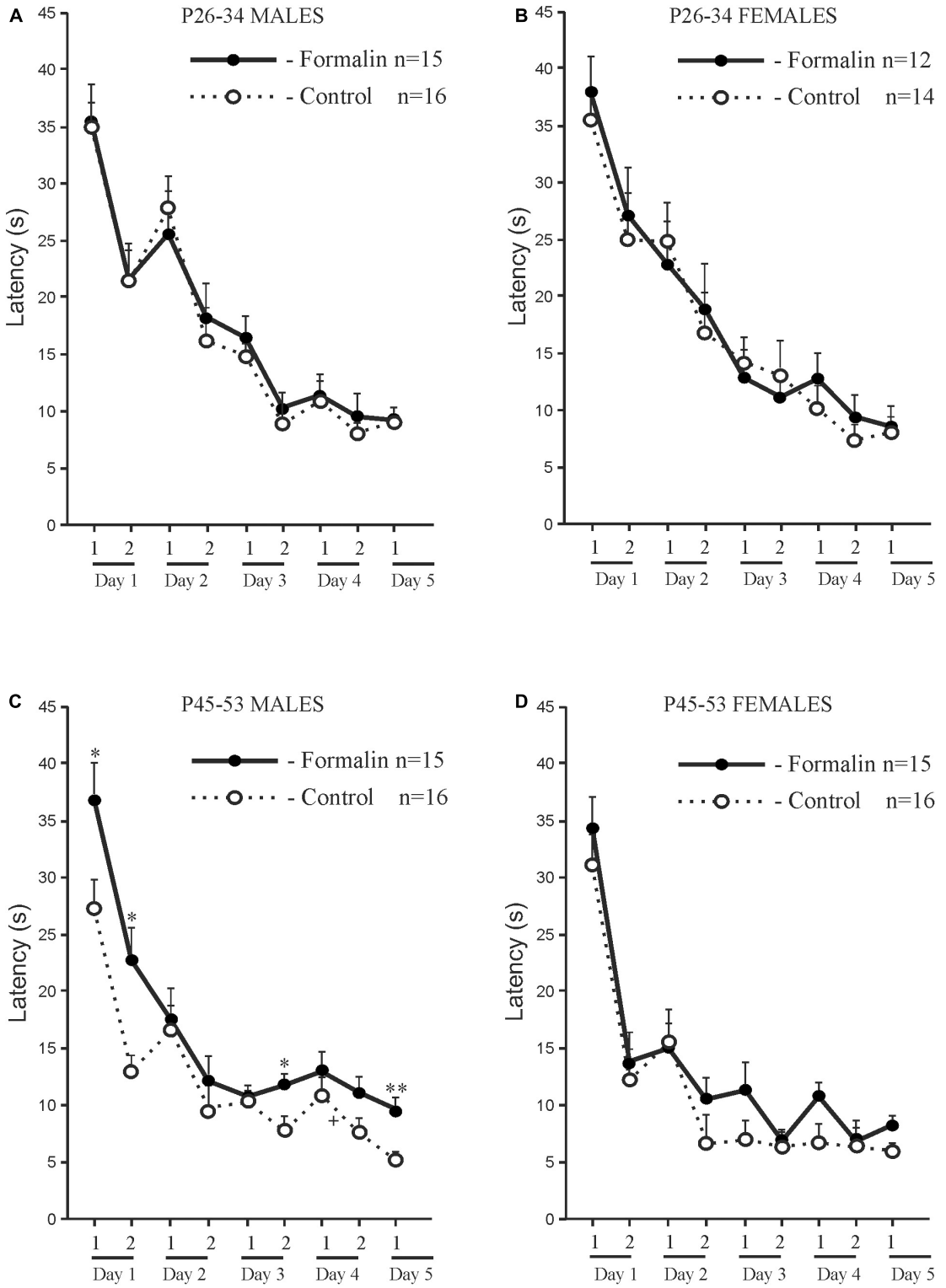

FIGURE 1 | Mean (±SEM) latency to find the platform in the first four training trials for 5 days and second four training trials for four training days of spatial learning in Control and Formalin male and female rats of early (P26-34) and late (P45-53) age groups. Panels **(A,B)** show data for male and female rats at the early age group. Panels **(C,D)** show data for male and female rats at the late age group. The abscissa shows the first and second training four trials (1 and 2) in each of the five training days. $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ significant differences in Control rats between the first four training trials and the second four training trials each day. $*p < 0.05$, $**p < 0.01$ Formalin rats *vs* Control rats.

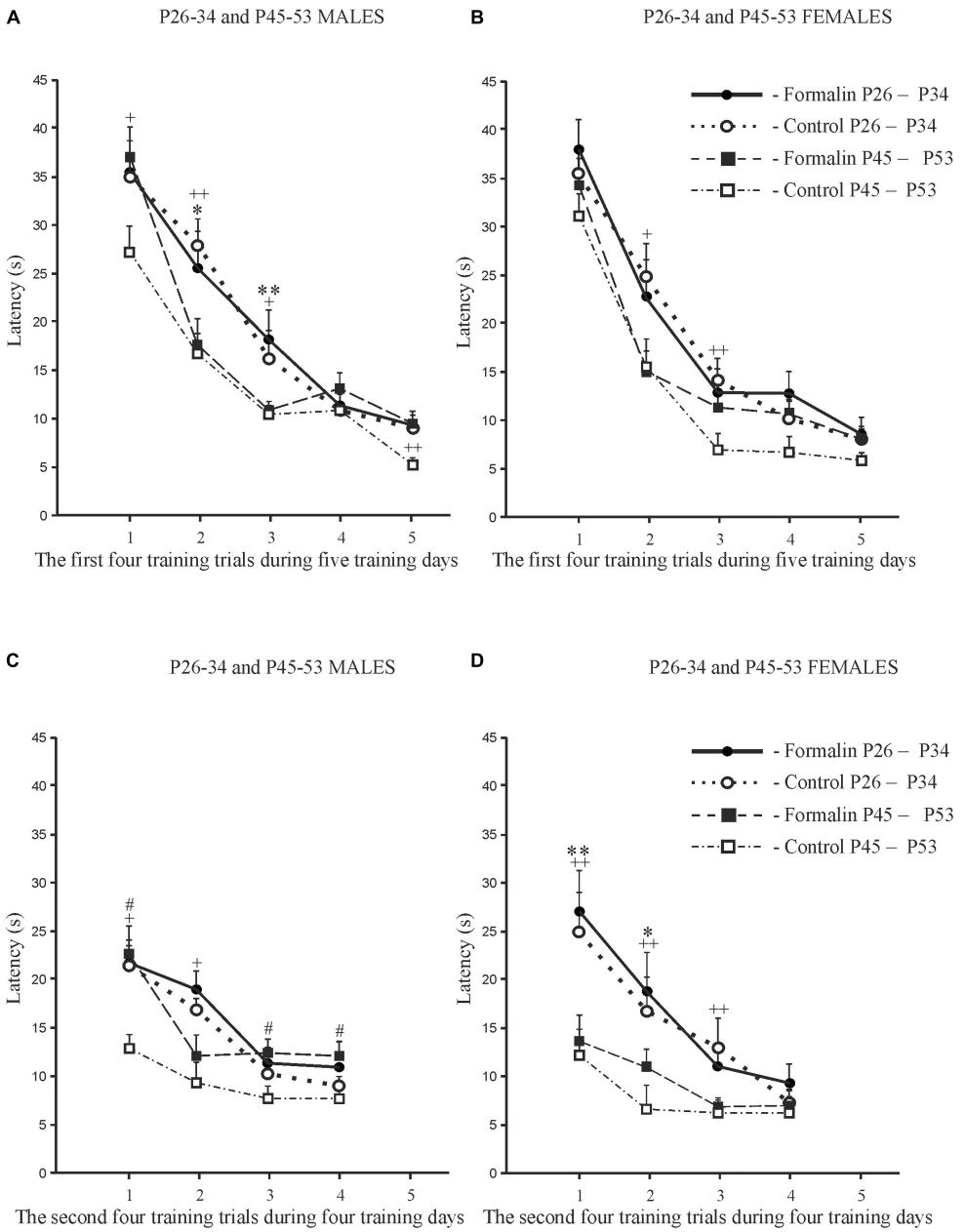

FIGURE 2 | Age and Sex effects in the mean (±SEM) latency to find the platform in Control and Formalin male and female rats in the first four training trials during five training days [early P26-34 and late P45-53 age groups **(A,B)**] and in the second four training trials during four training days [early P26-34 and late P45-53 age groups **(C,D)**]. $^{*}p < 0.05$, $^{**}p < 0.01$ age differences in Formalin rats, $^{+}p < 0.05$, $^{++}p < 0.01$ age differences in Control rats. $^{\#}p < 0.05$ sex differences in P45-P48 Formalin rats. Abscissa, training days. The number of the rats in the groups corresponds to the number of rats in **Figure 1**.

neutralized the differences in latency between short-term and long-term memory, which were found in Control animals.

Short- and Long-Term Spatial Memory, the Time Spent in Target Quadrant (Figure 5)

For the time in the target quadrant, Formalin P45-53 male rats spent less time in target quadrant in the short-term memory task than did the same-age Control males (**Figures 5A,A1**). There were age differences in the short-term memory task in Control males and females and Formalin females (**Figure 5A**). In the long-term memory task, less time was spent in target quadrant in Formalin P45-53 males and Formalin P26-34 females as compared to the time in Control rats of the same ages (**Figures 5B,B1**). There were differences between short-and term-long memory performance in P26-34 animals, in both Control and Formalin rats of both sexes (**Figures 5A,B**). The time spent in

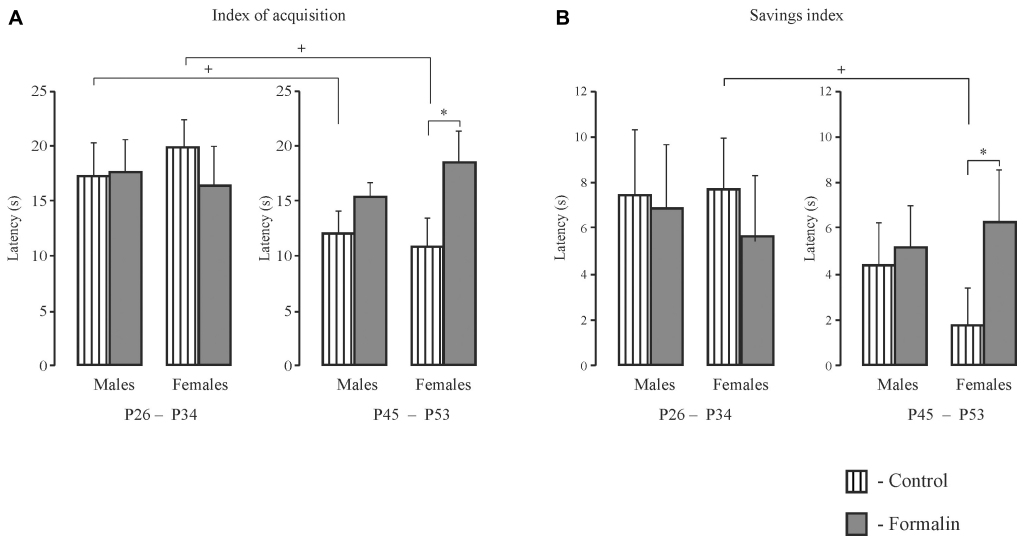

FIGURE 3 | The index of acquisition **(A)** and the savings index **(B)** for latency to find the platform during spatial learning in male and female rats of early (P26-34) and late (P45-53) age groups. The data are: Mean (±SEM) latency differences between the first and last training trials of each of the five training days of spatial learning **(A)**. Mean (±SEM) latency differences between the last training trial of a given day and the first training trial of the next day during the five-day spatial learning **(B)**. Both indices illustrate a decrease of latency to find the platform with age in Control rats; neonatal formalin-induced pain leveled the age differences. $^{*}p < 0.05$ Formalin *vs* Control **(A,B)**; $^{+}p < 0.05$ Control P45-49 rats *vs* Control P26-34 rats **(A,B)**. The number of the rats in the groups corresponds to the number of rats in **Figures 1**, **2**.

the target quadrant was less in the short-term memory than in the long-term memory task. Sex differences were found in the short-term memory test since females spent more the time in the target quadrant in Formalin P45-53 rats than did males (**Figure 5A**).

Corticosterone Determination in Newborn Rats (Figure 6) and Adolescent Rats (Figure 7)

In newborn rats, 30 min after formalin injection corticosterone levels were higher in the Control and Formalin pups compared to basal levels, and higher in the Formalin pups than in Control pups (**Figure 6**). Likewise, twenty-four hours after formalin injection, the Formalin pups had a higher corticosterone level than the Control and the basal pups. Seven days after formalin injection, there were no significant differences in the corticosterone levels among the three groups of pups.

In adolescent rats, there were no sex differences in corticosterone levels and therefore male and female data were combined. Thirty min after forced swimming, which rats were subjected to after long-term memory testing, corticosterone levels were higher compared to basal levels in both the Control and Formalin groups in early and late ages (**Figure 7**). Age differences were found in both sexes in the Control and Formalin rats with greater corticosterone levels in early age group.

DISCUSSION

The goal of this work was to examine the acute and subacute effects of an injury to the pad of the hind paw on plasma corticosterone, a marker of stress reactivity, in newborn rat pups. Corticosterone was elevated quickly and that elevation was maintained for at least 24 h compared to basal levels and saline injection controls. This suggests that neonatal inflammatory pain could modify the development of the HPA. We hypothesized that this could lead to changes in stress reactivity and cognitive abilities in adolescent rats. Indeed, there were differences in adolescent rats' plasma corticosterone in response to a swim stress and in aspects of spatial learning and memory depending on whether early and late age rats were tested. In the early P26-34 and late P45-53 age groups, the effects of repetitive neonatal peripheral inflammatory pain on spatial learning, short-term and long-term memory, strongly indicate a pronounced heterogeneity of the effects of early pain during the adolescent period of rat development. However, the long-term effects of early formalin injury and subsequent HPA activation cannot explain the adolescent effects in any simple way.

In neonates, the HPA axis rapidly develops and responds to strong stressors (Wood and Walker, 2015). We had previously shown that even during the stress hyporesponsive period of the HPA axis development, formalin-induced pain caused a gradual over an hour an increase in plasma corticosterone levels in 7-day-old male rats; twenty-four hours after injection of formalin corticosterone still exceeded the basal value (Butkevich et al., 2013). We also showed that neonatal formalin-induced pain caused an increase in an inflammatory pain response, depression-like behavior, and impairment of learning in adolescent male rats (Butkevich et al., 2016). But the question remains open as to whether the altered endogenous cortisol following early pain stress may play a role in learning and memory performance, as has been suggested in children (Mooney-Leber and Brummelte, 2017), especially in the prepubertal period in both sexes.

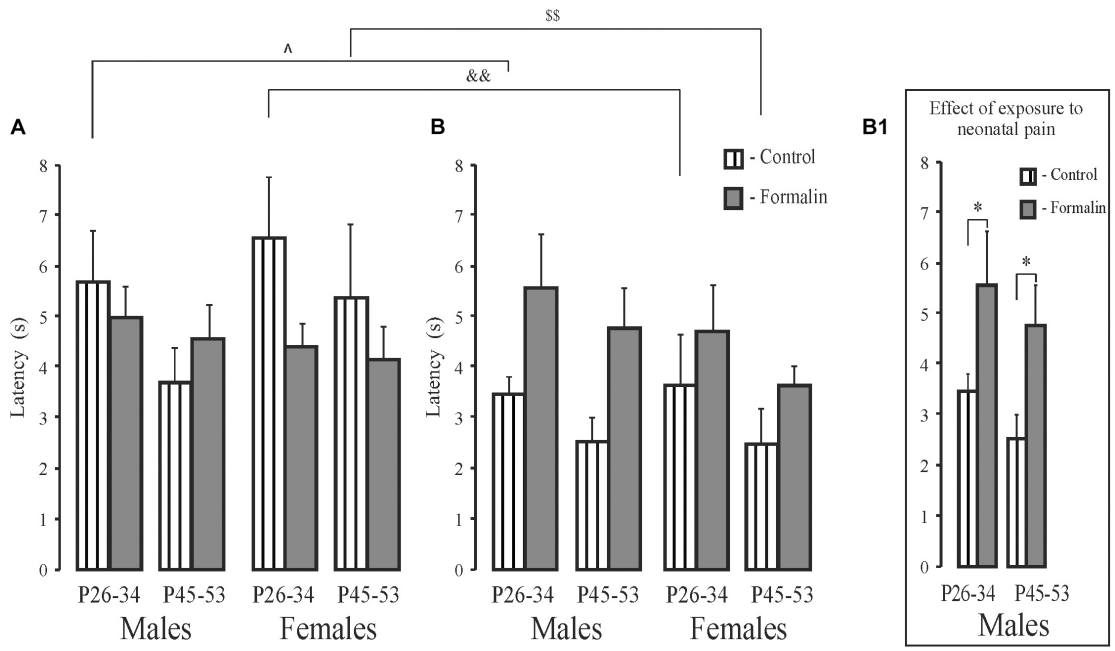

FIGURE 4 | Mean (±SEM) latency to find the platform **(A,B)** for short-term **(A)** and long-term **(B)** spatial memory in the Formalin or Control male and female rats for the early (P26-34) and late (P45-53) age groups. Differences in latency between short-term and long-term memory were found in the Control male and female rats of the early age group and in females of the late age group. Formalin *vs* Control rats; differences between short- and long-term memory: in latency, $^{\wedge}p < 0.05$, $^{\&\&}p < 0.01$, in Control P26-34 males and females, and $^{\$\$}p < 0.001$, in Control P45-53 females. The number of the rats in the groups corresponds to the number of rats in the groups in **Figures 1**, **2**. The graphs on the right illustrate significant results of statistical analysis. $^{*}p < 0.05$ significant effect of exposure.

Weaning is stressful for the offspring. In the present work, weaning occurred on P34 in both age groups. Experiments with rats of the early group were conducted before weaning, the pups after the experiment were in the home nest with their mother, while the pups of the late group were without the mother, but with their sisters or brothers. If weaning took place in P25, as is usual in our laboratory (Butkevich et al., 2017), then testing with P26 would be more stressful for the early group of rats than testing with P45 for the late group of rats. So, the rats had weaning at a later age. The rats of the late age group had enough time (11 days) for adaptation of life without the mother. In the MWM, the early age Control rats were capable of spatial learning, which is consistent with the literature (Vorhees and Williams, 2014), but which is in contrast to earlier work that found that effective strategies for spatial learning in the Morris water maze appear relatively late in adolescence (P42) (Schenk, 1985). Probably, the differences in the line of rats (Wistar and Hooded rats) and the testing methodology (in Schenk, 1985, Hooded rats were allowed to swim only for 30 s) underlie these differences. Formalin treated males and females of both age groups also demonstrated spatial learning, as evidenced by the gradual decrease in the latency of finding the platform during five training days. However, compared to controls, older male but not younger male formalin-induced neonatal pain rats took longer to find the platform for both four training trials. This difference (35%) was particularly pronounced on the first training day and it is first training day that is an important criterion for the learning process (Vorhees and Williams, 2014). In contrast to males, females of neither age group showed differences between Formalin and Controls on the first training day. Differences in spatial learning between age groups in Control and Formalin females only appeared in the second four trials. These sex differences may be due to different rates of adolescent sexual maturation which occurs later in males (∼P42 ± 2), than in females (∼P35 ± 2) (McCormick and Mathews, 2007, 2010). This suggests that sex hormones can be one of the reasons for these differences between males and females. Another reason for these differences may be the different reactivity of the HPA axis in males and females. However, no differences in corticosterone reactivity were found between sexes after assessing long-term memory. Interestingly, when using other metrics for spatial learning, the index of acquisition and the savings index (Whiting and Kokiko-Cochran, 2016), we found an increase in the latency to find the platform, which is impairment of cognition, in Formalin P45-53 females, as compared to Control P45-53 females, but not in males of the same age group. The two different metrics measuring learning and memory can explain this difference. The index of acquisition – measure of the learning within one day of testing, and is calculated by taking the difference between the latency in the first and last tests and averaging this difference for all days of learning. The savings index is the measure of how well, on the first test of each day, the rats remember what was learned on the previous day. This value is calculated as the difference between the latency in the last test of a given day and the latency in the first test of the next day and averaged over all days of learning. However, the absence of differences in the latency to find the platform

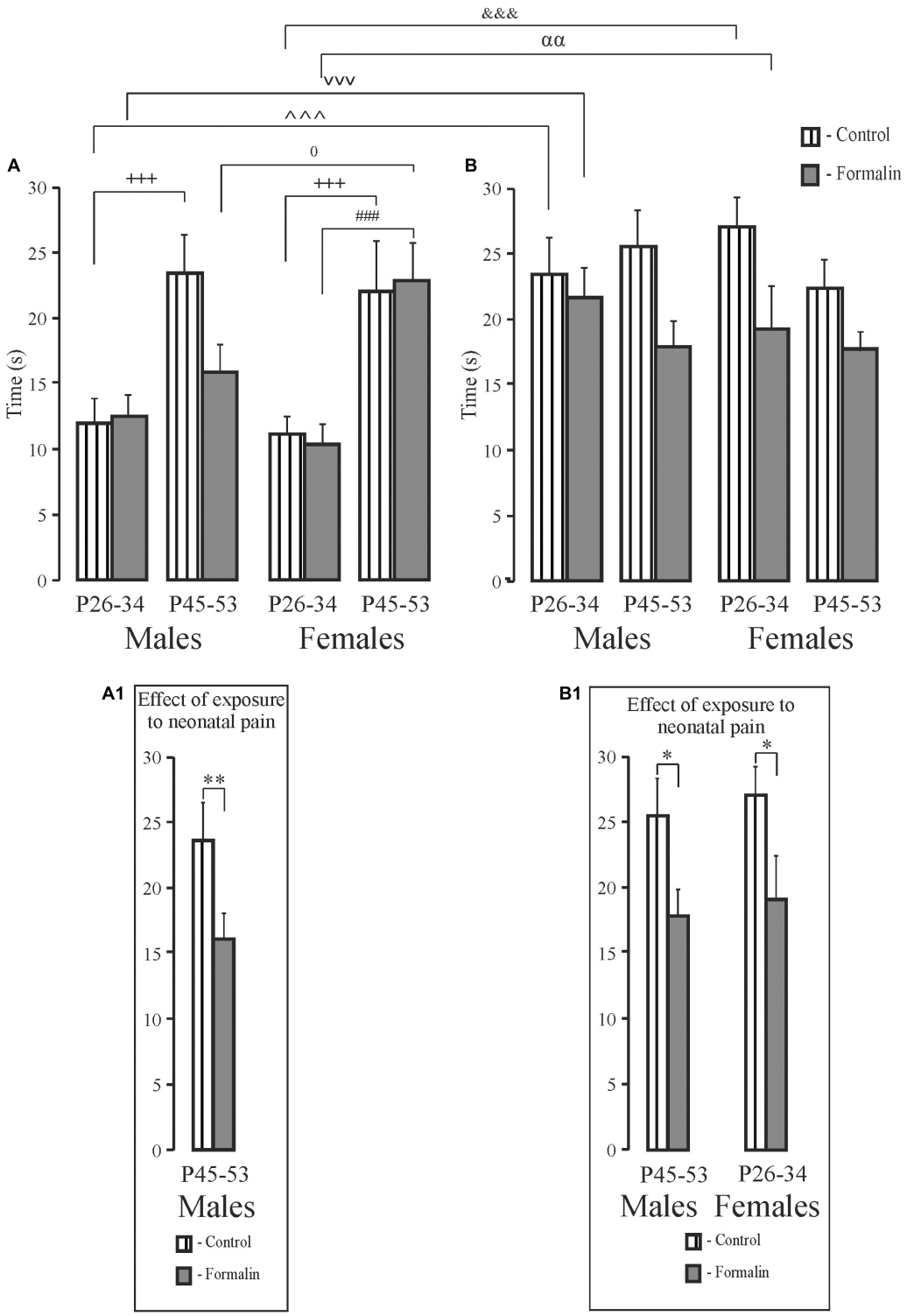

FIGURE 5 | Mean (±SEM) time in target quadrant **(A,B)** for short-term **(A)** and long-term **(B)** spatial memory in the Formalin or Control male and female rats for the early (P26-34) and late (P45-53) age groups. Differences in time in target quadrant between short-term and long-term memory were found in Control and Formalin males and females of early age groups. In all cases, the time in target quadrant was shorter in the short-term memory **(A,B)**. $^{+++}p < 0.001$, age differences in Control rats; $^{###}p < 0.001$, age differences in Formalin rats. Differences between short- and long-term memory: in time in target quadrant, $^{\wedge\wedge\wedge}p < 0.001$, $^{\&\&\&}p < 0.001$ in Control P26-34 males and females, $^{vv}p < 0.001$, $^{\alpha\alpha}p < 0.01$, in Formalin P26-34 males and females; $^{0}p < 0.05$, sex differences in P45-53 Formalin rats. The number of the rats in the groups corresponds to the number of rats in **Figures 1**, **2**. The graphs below illustrate significant results of statistical analysis. $^{*}p < 0.05$, $^{**}p < 0.01$ significant effect of exposure. Graphs **(A1)** and **(B1)** illustrate the significant outcomes of the statistical analyses.

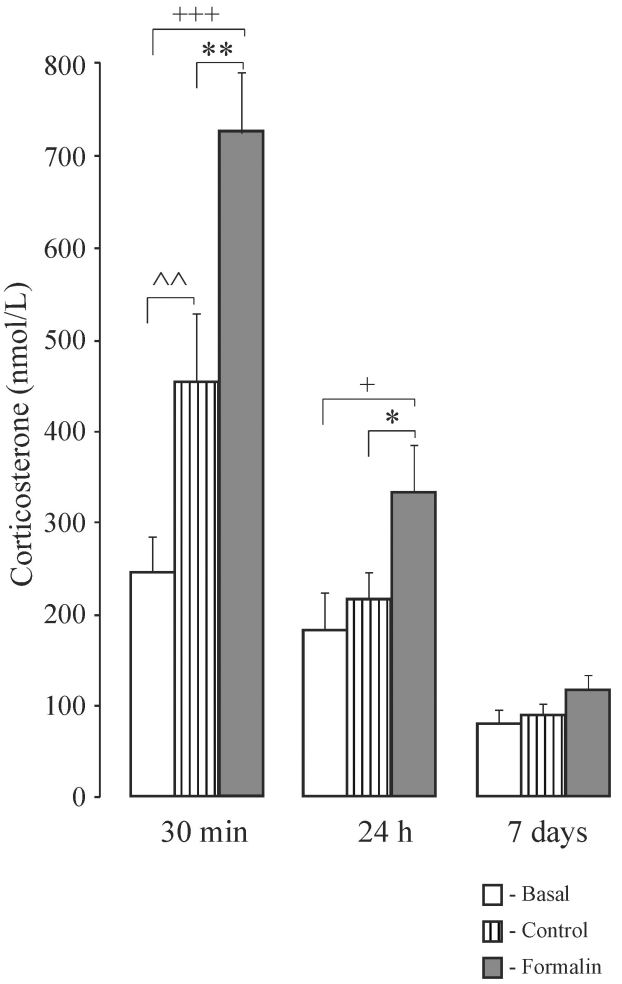

FIGURE 6 | Mean (±SEM) corticosterone levels in blood plasma in neonatal pups under basal conditions, or 30 min, 24 h and 7 days after injection of Formalin (2.5%, 0.5 μl) or Control into the pad of the left hind paw. $^{*}p < 0.05$, $^{**}p < 0.01$, Formalin *vs* saline; $^{+}p < 0.05$, $^{+++}p < 0.001$, Formalin *vs* basal; $^{\wedge\wedge}p < 0.01$, saline *vs* basal.

between Formalin and Control rats in the early P26-34 age group was the same as the results obtained using these indices and analysis I and II.

When assessing memory by the latency to find the platform, neonatal pain caused deficits only in long-term memory in males in both age groups, whereas when assessing memory by the time spent in the target quadrant, neonatal pain decreased it in males of the late age group in both short- and long-term memory and also in females of early age group. Only control rats of both sexes of the early age group showed differences between short- and long-term memory in both latency and time spent in the target quadrant, with shorter latency and longer time spent in the target quadrant in long-term memory. In the time spent in the target quadrant, Formalin rats of the early age group, showed the similar behavior. Note, age differences were found only in short-term memory in Control rats of both sexes and Formalin females, and only in the target quadrant, with a longer parameter in the late age group. Differences identified in memory processes using latency to find the platform and the duration to stay in the target quadrant indicate participation of different brain structures in these behavioral characteristics of memory.

We are aware of only a few rodent studies that investigated the effect of neonatal inflammatory pain on memory. For instance, formalin-induced pain in newborn rats impaired visual-spatial learning and memory in the radial 8-arm maze, which uses food reinforcement, in adult rats (Anand et al., 2007). Inflammatory pain caused by the intra-plantar injection of carrageenan (1%) on the day of birth, P0, resulted in spatial memory deficits also in adult rats (Henderson et al., 2015), and dysregulated the HPA axis (Victoria et al., 2013). Complete Freund's adjuvant on P1 did not affect short-or long-term memory in male or female rats on P60, but resulted in spatial learning deficits in males (Amaral et al., 2015). Therefore, although there are some inconsistencies, in general early experiences of painful injury can disrupt adult spatial learning/memory processes. When assessed, the single injection of carrageenan on the day of birth activated the infant HPA axis in rat pups (Victoria et al., 2014). Daily needle pricks in each paw at 6-h intervals until P7 (Chen et al., 2016), decreased serum corticosterone in P24, had no effect at P45 and increased corticosterone in adult rats. Thus, these early insults can have long-term effects on subsequent HPA axis function. However, we know of no comparable data for testing the effects of early inflammatory injury in adolescence.

We measured HPA reactivity in response to forced swimming in the rats after testing in the MWM, and found no differences in corticosterone levels in adolescent rats between Formalin and Control rats of either sex. Importantly, both Control and Formalin rats at the early age showed greater corticosterone levels compared to those of the late age group. The forced swim test is known to stimulate the HPA activity in rats (Mathews et al., 2008), and HPA axis reactivity is modified by previous stress history, especially during critical periods of rapid brain development (reviewed in Meaney and Szyf, 2005). Stress at an early age changes adaptive behavior. For example, we have previously shown that the formalin test preceding the forced swim test sharply reduced the immobility time only in 7-day-old rat pups that had been prenatally stressed but not control pups (Mikhailenko et al., 2010). Our long-term experience with the forced swim test indicates that the severe physical and emotional stress experienced by the rat in this test can obviate the effects of other varied types of stress. It is important to note, that the absence of differences in the reactivity of the HPA axis between Formalin and Control rats in our current study could be a consequence of the cumulative effects of testing in MWM and forced swimming on the activity of the HPA axis. The interaction of different types of stress, especially during critical periods of development, can lead to unexpected results (Sokołowski et al., 2020). Especially interesting and of practical importance is the consequence of suppressing the adverse effects of one stress by another adverse stress (Van Bodegom et al., 2017). Our data using the formalin pain stress in newborns showed suppression of the expected pronociceptive effect of prenatal stress in the formalin test in adolescent rats, but did not reduce depressive-like behavior (Butkevich et al., 2020).

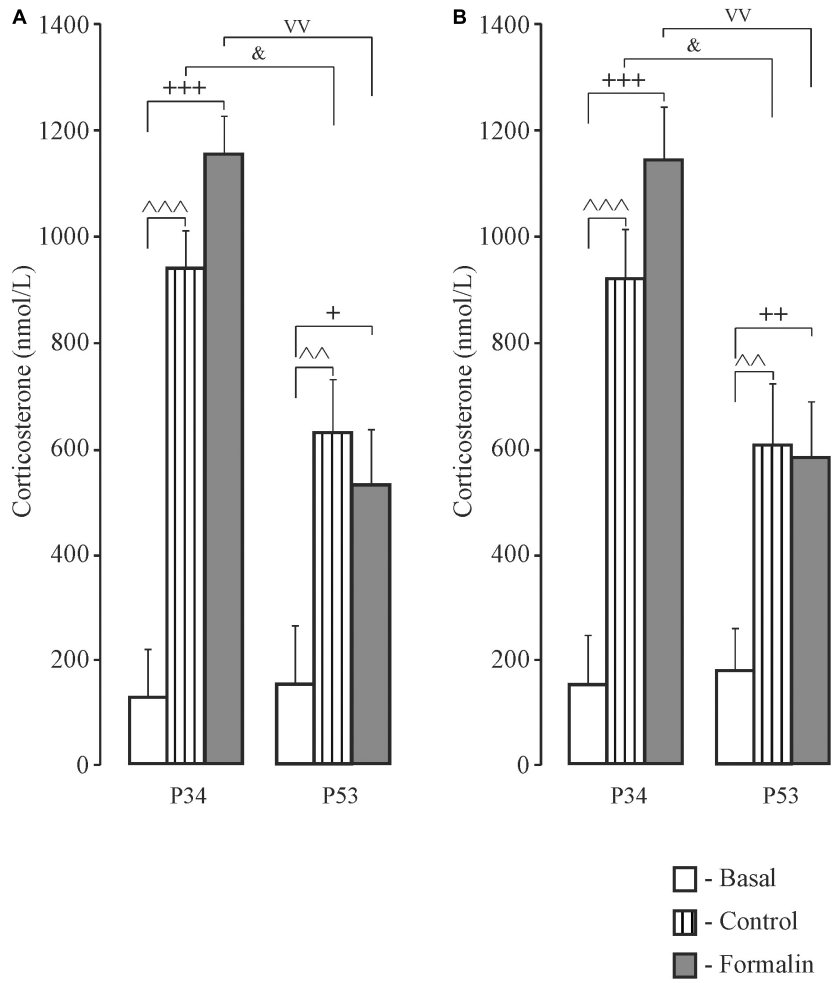

FIGURE 7 | Mean (±SEM) corticosterone levels in blood plasma in response to forced swimming in Control and Formalin rats at the age of P34 and P53 after testing in the Morris Water Maze. $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, Formalin *vs* basal; $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ Control *vs* basal; $^{\&}p < 0.05$, age differences between Control rats; $^{W}p < 0.01$, age differences between Formalin rats. The number of the rats in the groups corresponds to the number of rats in **Figures 1**, **2**.

Our present experiments have shown that the activation of the HPA axis by neonatal pain has no direct relationship with spatial learning and memory in rats in adolescence. Other physiological systems besides the HPA axis may be involved in the effects of inflammatory pain in newborns, such as the immune system, which responds to inflammation and stress and can affect brain neurons and cognitive function. The immune system closely interacts with the HPA axis (Gaillard, 2003). Sex differences in microglia, neuroimmune cells, begin to emerge during the prenatal organizational period for sexual differentiation of the brain (Schwarz et al., 2012). Immunocompetent cells of the brain express steroid hormone receptors and are regulated by hormones and activation of the immune system is determined by sex hormones (Lombardo et al., 2021). Moreover, the immune system acts as a regulator of sex differences in brain development and behavior (Nelson and Lenz, 2017; VanRyzin et al., 2018). The immune and sexual systems interact with the HPA axis (Bereshchenko et al., 2018). One can suggest that the sex differences reported here following neonatal pain depend on the balance in maturation of the HPA and the hypothalamus-pituitary-gonadal (the HPG) axis. Neonatal pain, by disrupting the processes of inhibition or excitation in the central nervous system, could modify the synchronization of development of the HPA and HPG systems, which closely interact and affect the neuroplasticity of learning and memory. The hippocampus, medial prefrontal cortex, and amygdala, brain structures implicated in the control of the HPA axis (Herman et al., 2003) and cognition (Euston et al., 2012; Méndez-Couz et al., 2014), mature rapidly during adolescence (Spear, 2000) and can influence sensitivity of the HPA axis to sex hormones and alter cognitive abilities.

The relatively long-lasting high level of corticosterone evoked by formalin-induced pain in newborn rat could impair the development of the PVN. In the newborn rat, the PVN and CA1 of the hippocampus contain GR mRNA expression (Pryce, 2008). The CRH hippocampal system regulates neurogenesis in the hippocampus which is involved in spatial learning and memory (Koutmani et al., 2019). Elevated levels of glucocorticoids have

also been shown to impair working and reference memory (Stylianakis et al., 2018). CRH neurosecretory systems release glutamate, in addition to neuropeptides, into the pericapillary space of hypophysial portal vessels, and there is expression of the mRNA for vesicular glutamate transporter-2 in the rat CRH neurons in the PVH (Hrabovszky et al., 2005). Glutamatergic neurons are one of the main links in the processes of learning and memorization (review, Mooney-Leber and Brummelte, 2017). Excessive levels of glucocorticoids enhance the release of glutamate, causing neurotoxicity, which enhances apoptosis, as shown in the hippocampus and other brain regions during the first postnatal week in the rat (Lu et al., 2003; Dührsen et al., 2013). The role of glutamate during development has been primarily associated with the NMDA receptor, which is present at P0 in the rats (Behuet et al., 2019). During normal early development when the NMDA receptor containing the NR2B subunit in the hippocampus of the newborn rat is activated, the corresponding channel remains in the open position much longer than in the mature receptor. In addition, neurons with such receptors develop long-term potentiation, a form of activity-dependent synaptic strengthening, more quickly, which contributes to memory strengthening. The selective loss of NR2B protein and subsequent synaptic dysfunction weakens prelimbic PFC function during development and may underlie early cognitive impairments (Gulchina et al., 2017). We hypothesize that the impairment of the NR2B subunit caused by increased corticosterone in rats with neonatal pain may be associated with the abnormalities in spatial memory that we found.

Short and long exposures to corticosterone differentially tune NMDAR signaling in hippocampus by altering the expression and synaptic presence of NMDAR subunits, allowing adaptations of glutamate synapses (Mikasova et al., 2017). Taking into account the different roles of metabotropic and inotropic glutamatergic and GABAergic receptors in the effect of stress on learning and memory, as well as the mechanism of co-transmission of glutamate with GABAergic neurons (Trudeau and Mestikawy, 2018), it is possible that these complex relationships are involved both in the differences we found in the effect of early pain stress on cognitive abilities in adolescent rats, and in the absence of differences in the reactivity of the HPA axis to stress in the adolescent Formalin and Control rats. It is known that the serotonergic, the HPA axis, glutamatergic, and GABAergic systems are all involved in nociception and are affected by stress (Goudet et al., 2009; Quintero et al., 2011; Bannister et al., 2017; Hernández-Vázquez et al., 2019). Formalin-induced neonatal pain effects various neurotransmitter systems, disrupts the balance between excitation and inhibition in the central nervous system, modifies the development of functional activity of the HPA axis, and thus affects the neurophysiological mechanisms underlying cognitive processes.

In conclusion, we found that activation of the HPA axis by neonatal pain did not directly correlate with spatial learning and memory in adolescence, and therefore the consequences of newborn pain remain are likely multi-determined. Neonatal pain impaired spatial learning and long- and short-term memory in late adolescent males and long-term memory in early adolescent females. The comparative analysis of the memory scores revealed that long-term memory performance was more robust than short-term memory. The differences found in spatial memory performance in MWM in P25-34 and P45-53 rats provide strong evidence of the heterogeneity in the development of cognitive processes in the two age groups of the adolescence. These behavioral changes suggest that neonatal pain causes changes in various structures and neurotransmitters involved in spatial short-term and long-term memory only in P45-53 rats. The effect of stress at an early age on memory and the HPA axis, as well as brain structures involved in memory processes in adulthood are well studied (Krugers and Joëls, 2014; Schroeder et al., 2018; Bonapersona et al., 2019; Cordier et al., 2021), but information on the effects of neonatal pain stress on memory and the participation of the HPA axis in this process is very meager. Our work is the first, as far as we know, aimed at studying the effects of early-life inflammatory pain on spatial learning and memory, and the HPA reactivity at different age intervals within the adolescent period. It was also important in our study to include male and female rats, as very few studies have included rats of both sexes in adolescence, and our results show clear differences in the effects in males and females that might be accounted for by different developmental trajectories during adolescence. The limitation of the work was that we analyzed corticosterone not after MWM, but after the further stress of the forced swim, to determine the reactivity of the HPA axis in Formalin and Control rats. We also conducted that assay only once, and thus did not evaluate the dynamics of corticosterone change. It will be interesting to investigate changes in these behavioral and endocrine systems in adult rats exposed to inflammatory neonatal pain to determine if the age and sex differences that we identified here continue into adulthood or are unique features of the adolescent period.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

All procedures were approved by the Local Ethics Committee for Animal Experiments of the I. P. Pavlov Institute of Physiology, Russian Academy of Sciences (Saint Petersburg, Russia) and followed the guidelines published by the Committee for Research and Ethical Issues of the IASP on ethical standards for investigations of experimental pain in animals.

AUTHOR CONTRIBUTIONS

IB and VM: experimental design. IB, VM, and EV: collection of data and conduction of statistical analyses. IB, VM, EV, and GB: interpretation and analysis of data, participation in the drafting and revising of the manuscript, and reviewing and approving the final submitted manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2021.691578/full#supplementary-material

REFERENCES

Akirav, I., Kozenicky, M., Tal, D., Sandi, C., Venero, C., and Richter-Levin, G. (2004). A facilitative role for corticosterone in the acquisition of a spatial task under moderate stress. *Learn. Mem.* 11, 188–195. doi: 10.1101/lm.61704

Amaral, C., Antonio, B., Oliveira, M. G., Hamani, C., Guinsburg, R., and Covolan, L. (2015). Early postnatal nociceptive stimulation results in deficits of spatial memory in male rats. *Neurobiol. Learn. Mem.* 125, 120–125. doi: 10.1016/j.nlm.2015.08.012

Anand, K. J. S., Coskun, V., Thrivikraman, K. V., Nemeroff, C. B., and Plotsky, P. M. (1999). Long-term behavioral effects of repetitive pain in neonatal rat pups. *Physiol. Behav.* 66, 627–637. doi: 10.1016/s0031-9384(98)00338-2

Anand, K. J., Garg, S., Rovnaghi, C. R., Narsinghani, U., Bhutta, A. T., and Hall, R. W. (2007). Ketamine reduces the cell death following inflammatory pain in newborn rat brain. *Pediatr. Res.* 62, 283–290. doi: 10.1203/pdr.0b013e3180986d2f

Bailey, C. D. C., Gerlai, R., Cameron, N. M., Marcolin, M. L., and McCormick, C. M. (2020). Preclinical methodological approaches investigating of the effects of alcohol on perinatal and adolescent neurodevelopment. *Neurosci. Biobehav. Rev.* 116, 436–451. doi: 10.1016/j.neubiorev.2020.07.011

Bannister, K., Lockwood, S., Goncalves, L., Patel, R., and Dickenson, A. H. (2017). An investigation into the inhibitory function of serotonin in diffuse noxious inhibitory controls in the neuropathic rat. *Eur. J. Pain* 21, 750–760. doi: 10.1002/ejp.979

Behuet, S., Cremer, J. N., Cremer, M., Palomero-Gallagher, N., Zilles, K., and Amunts, K. (2019). Developmental changes of glutamate and GABA receptor densities in wistar rats. *Front. Neuroanat.* 13:100. doi: 10.3389/fnana.2019.00100

Bereshchenko, O., Bruscoli, S., and Riccardi, C. (2018). Glucocorticoids, sex hormones, and immunity. *Front. Immunol.* 9:1332. doi: 10.3389/fimmu.2018.01332

Bonapersona, V., Kentrop, J., Van Lissa, C. J., van der Veen, R., Joëls, M., and Sarabdjitsingh, R. A. (2019). The behavioral phenotype of early life adversity: a 3-level meta-analysis of rodent studies. *Neurosci. Biobehav. Rev.* 102, 299–307. doi: 10.1016/j.neubiorev.2019.04.021

Brenhouse, H. C., and Andersen, S. L. (2011). Developmental trajectories during adolescence in males and females: a cross-species understanding of underlying brain changes. *Neurosci. Biobehav. Rev.* 35, 1687–1703. doi: 10.1016/j.neubiorev.2011.04.013

Brewer, C. L., and Baccei, M. L. (2020). The development of pain circuits and unique effects of neonatal injury. *J. Neural. Transm.* 127, 467–479. doi: 10.1007/s00702-019-02059-z

Butkevich, I. P., Mikhailenko, V. A., and Vershinina, E. A. (2020). The influence of perinatal stress and antidepressants on different types of adaptive behavior and cognitive abilities of prepubertal female rats. *J. Evol. Biochem. Physiol.* 56, 133–144. doi: 10.1139/cjpp-2020-0307

Butkevich, I. P., Mikhailenko, V. A., Bagaeva, T. R., Vershinina, E. A., Aloisi, A. M., and Otellin, V. A. (2013). Inflammatory pain and corticosterone response in infant rats: effect of 5-HT1A agonist buspirone prior to gestational stress. *Mediat. Inflamm.* 2013:915189. doi: 10.1155/2013/915189

Butkevich, I. P., Mikhailenko, V. A., Lavrova, lu. A, and Ulanova, N. A. (2016). Repeated inflammation-related pain syndrome in neonatal male rats alters adaptive behavior during the adolescent period of development. *Neurosci. Behav. Physiol.* 46, 461–466. doi: 10.1007/s11055-016-0258-1

Butkevich, I. P., Mikhailenko, V. A., Vershinina, E. A., Aloisi, A. M., and Barr, G. A. (2017). Long-term effects of chronic buspirone during adolescence reduce the adverse influences of neonatal inflammatory pain and stress on adaptive behavior in adult male rats. *Front. Behav. Neurosci.* 11:11. doi: 10.3389/fnbeh.2017.00011

Chau, C. M., Cepeda, I. L., Devlin, A. M., Weinberg, J., and Grunau, R. E. (2017). The Val66Met brain-derived neurotrophic factor gene variant interacts with early pain exposure to predict cortisol dysregulation in 7-year-old children born very preterm: implications for cognition. *Neuroscience* 7, 188–199. doi: 10.1016/j.neuroscience.2015.08.044

Chen, M., Xia, D., Min, C., Zhao, X., Chen, Y., Liu, L., et al. (2016). Neonatal repetitive pain in rats leads to impaired spatial learning and dysregulated hypothalamic-pituitary-adrenal axis function in later life. *Sci. Rep.* 14:39159. doi: 10.1038/srep39159

Cordier, J. M., Aguggia, J. P., Danelon, V., Mir, F. R., Rivarola, M. A., and Mascó, D. (2021). Postweaning enriched environment enhances cognitive function and brain-derived neurotrophic factor signaling in the hippocampus in maternally separated rats. *Neuroscience* 453, 138–147. doi: 10.1016/j.neuroscience.2020.09.058

Dobbing, J. (1981). "Nutritional growth restriction and the nervous system," in *The Molecular Basis of Neuropathology*, eds A. N. Davidson and R. H. S. Thompson (London: Edward Arnold Co), 221–233.

Doesburg, S. M., Chau, C. M., Cheung, T. P. L., Moiseev, A., Ribary, U., Herdman, A. T., et al. (2013). Neonatal pain-related stress, functional cortical activity and visual-perceptual abilities in school-age children born at extremely low gestational age. *Pain* 154, 1946–1952. doi: 10.1016/j.pain.2013.04.009

Dührsen, L., Simons, S. H., Dzietko, M., Genz, K., Boos, V., Sifringer, M., et al. (2013). Effects of repetitive exposure to pain and morphine treatment on the neonatal rat brain. *Neonatology* 103, 35–43. doi: 10.1159/000341769

Euston, D. R., Gruber, A. J., and McNaughton, B. L. (2012). The role of medial prefrontal cortex in memory and decision making. *Neuron* 76, 1057–1070. doi: 10.1016/j.neuron.2012.12.002

Fitzgerald, M., Shaw, A., and MacIntosh, N. (1988). Postnatal development of the cutaneous flexor reflex: comparative study of preterm infants and newborn rat pups. *Dev. Med. Child Neurol.* 30, 520–526. doi: 10.1111/j.1469-8749.1988.tb04779.x

Gaillard, R. C. (2003). Interactions between the immune and neuroendocrine systems: clinical implications. *J. Soc. Biol.* 197, 89–95.

Gildawie, K. R., Ryll, L. M., Hexter, J. C., Peterzell, S., Valentine, A. A., and Brenhouse, H. C. (2021). A two-hit adversity model in developing rats reveals sex-specific impacts on prefrontal cortex structure and behavior. *Dev. Cogn. Neurosci.* 48:100924. doi: 10.1016/j.dcn.2021.100924

Goksan, S., Hartley, C., Emery, F., Cockrill, N., Poorun, R., Moultrie, F., et al. (2015). fMRI reveals neural activity overlap between adult and infant pain. *Elife* 4:e06356. doi: 10.7554/elife.06356

Gore-Langton, J. K., Werner, D. F., and Spear, L. P. (2021). The effects of age, sex, and handling on behavioral parameters in the multivariate concentric square field^{TM} test. *Physiol. Behav.* 229:113243. doi: 10.1016/j.physbeh.2020.113243

Goudet, C., Magnaghi, V., Landry, M., Nagy, F., Gereau, R. W., and Pin, J.-P. (2009). Metabotropic receptors for glutamate and GABA in pain. *Brain Res. Rev.* 60, 43–56. doi: 10.1016/j.brainresrev.2008.12.007

Grunau, R. E., Holsti, L., and Peters, J. W. (2006). Long-term consequences of pain in human neonates. *Semin. Fetal Neonatal Med.* 11, 268–275. doi: 10.1016/j.siny.2006.02.007

Grunau, R. E., Whitfield, M. F., Petrie-Thomas, J., Synnes, A. R., Cepeda, I. L., Keidar, A., et al. (2009). Neonatal pain, parenting stress and interaction, in relation to cognitive and motor development at 8 and 18 months in preterm infants. *Pain* 143, 138–146. doi: 10.1016/j.pain.2009.02.014

Gulchina, Y., Xu, S.-J., Snyder, M. A., Elefant, F., and Gao, W.-J. (2017). Epigenetic mechanisms underlying NMDA receptor hypofunction in the prefrontal cortex of juvenile animals in the MAM model for schizophrenia. *J. Neurochem.* 143, 320–333. doi: 10.1111/jnc.14101

Gursul, D., Hartley, C., and Slater, R. (2019). Nociception and the neonatal brain. *Semin. Fetal. Neonatal. Med.* 24:101016. doi: 10.1016/j.siny.2019.05.008

Haley, D. W., Weinberg, J., and Grunau, R. E. (2006). Cortisol, contingency learning, and memory in preterm and full-term infants. *Psychoneuroendocrinology* 31, 108–117. doi: 10.1016/j.psyneuen.2005.06.007

Handa, R. J., Nunley, K. M., Lorens, S. A., Louie, J. P., McGivern, R. F., and Bollnow, M. R. (1994). Androgen regulation of adrenocorticotropin and corticosterone secretion in the male rat following novelty and foot shock stressors. *Physiol. Behav.* 55, 117–124. doi: 10.1016/0031-9384(94)90018-3

Henderson, Y. O., Victoria, N. C., Inoue, K., Murphy, A. Z., and Parent, M. B. (2015). Early life inflammatory pain induces long-lasting deficits in hippocampal-dependent spatial memory in male and female rats. *Neurobiol. Learn. Mem.* 118, 30–41. doi: 10.1016/j.nlm.2014.10.010

Herman, J. P., Figueiredo, H., Mueller, N. K., Ulrich-Lai, Y., Ostrander, M. M., Choi, D. C., et al. (2003). Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. *Front. Neuroendocrinol.* 24:151–180. doi: 10.1016/j.yfrne.2003.07.001

Hernández-Vázquez, F., Garduño, J., and Hernández-López, S. (2019). GABAergic modulation of serotonergic neurons in the dorsal raphe nucleus. *Rev. Neurosci.* 30, 289–303. doi: 10.1515/revneuro-2018-0014

Hrabovszky, E., Wittmann, G., Turi, G. F., Liposits, Z., and Fekete, C. (2005). Hypophysiotropic thyrotropin-releasing hormone and corticotropin-releasing hormone neurons of the rat contain vesicular glutamate transporter-2. *Endocrinology* 146, 341–347. doi: 10.1210/en.2004-0856

Khawla, Q., Alzoubi, N. K. H., Alhusban, A., Bawaane, A., Al-Azzani, M., and Khabour, O. F. (2017). Sucrose and naltrexone prevent increased pain sensitivity and impaired long-term memory induced by repetitive neonatal noxious stimulation: role of BDNF and β-endorphin. *Physiol. Behav.* 179, 213–219.

Koutmani, Y., Gampierakis, I. A., Polissidis, A., Ximerakis, M., Koutsoudaki, P. N., Polyzos, A., et al. (2019). CRH promotes the neurogenic activity of neural stem cells in the adult hippocampus. *Cell. Rep.* 29, 932–945.e7. doi: 10.1016/j.celrep.2019.09.037

Krugers, H. J., and Joëls, M. (2014). "Long-lasting consequences of early life stress on brain structure, emotion and cognition," in *Current Topics in Behavioral Neurosciences*, Vol. 18, eds C. M. Pariante and M. D. Lapiz-Bluhm (Berlin: Springer-Verlag), 81–92.

Lombardo, G., Mondelli, V., Dazzan, P., and Pariante, C. M. (2021). Sex hormones and immune system: a possible interplay in affective disorders? A systematic review. *J. Affect. Disord.* 290, 1–14. doi: 10.1016/j.jad.2021.04.035

Lu, J., Goula, D., Sousa, N., and Almeida, O. F. X. (2003). Ionotropic and metabotropic glutamate receptor mediation of glucocorticoid-induced apoptosis in hippocampal cells and the neuroprotective role of synaptic N-methyl-D-aspartate receptors. *Neuroscience* 121, 123–131. doi: 10.1016/s0306-4522(03)00421-4

Lupien, S. J., McEwen, B. S., Gunnar, M. R., and Heim, C. (2009). Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nat. Rev. Neurosci.* 10, 434–445. doi: 10.1038/nrn2639

Marcolin, M. L., Baumbach, J. L., Hodges, T. E., and McCormick, C. M. (2020). The effects of social instability stress and subsequent ethanol consumption in adolescence on brain and behavioral development in male rats. *Alcohol* 82, 29–45. doi: 10.1016/j.alcohol.2019.08.003

Mathews, I. Z., Wilton, A., Styles, A., and McCormick, C. M. (2008). Increased depressive behaviour in females and heightened corticosterone release in males to swim stress after adolescent social stress in rats. *Behav. Brain Res.* 190, 33–40. doi: 10.1016/j.bbr.2008.02.004

McCormick, C. M. (2011). Effect of neonatal ovariectomy and estradiol treatment on corticosterone release in response to stress in the adult female rat. *Stress* 14, 82–87. doi: 10.3109/10253890.2010.490309

McCormick, C. M., and Mathews, I. Z. (2007). HPA function in adolescence: role of sex hormones in its regulation and the enduring consequences of exposure to stressors. *Pharmacol. Biochem. Behav.* 86, 220–233. doi: 10.1016/j.pbb.2006.07.012

McCormick, C. M., and Mathews, I. Z. (2010). Adolescent development, hypothalamic-pituitary-adrenal function, and programming of adult learning and memory. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 34, 756–765. doi: 10.1016/j.pnpbp.2009.09.019

McCormick, C. M., Green, M. R., and Simone, J. J. (2016). Translational relevance of rodent models of hypothalamic-pituitary-adrenal function and stressors in adolescence. *Neurobiol. Stress* 29, 31–43. doi: 10.1016/j.ynstr.2016.08.003

McCormick, C. M., Linkroum, W., Sallinen, B. J., and Miller, N. W. (2002). Peripheral and central sex steroids have differential effects on the HPA axis of male and female rats. *Stress* 5, 235–247. doi: 10.1080/1025389021000061165

McCormick, C. M., Robarts, D., Gleason, E., and Kelsey, J. E. (2004). Stress during adolescence enhances locomotor sensitization to nicotine in adulthood in female, but not male, rats. *Horm. Behav.* 46, 458–466. doi: 10.1016/j.yhbeh.2004.05.004

McCormick, C. M., Smith, K., Baumbach, J. L., de Lima, A. P. N., Shaver, M., Hodges, T. E., et al. (2020). Adolescent social instability stress leads to immediate and lasting sex-specific changes in the neuroendocrine-immune-gut axis in rats. *Horm. Behav.* 126:104845. doi: 10.1016/j.yhbeh.2020.104845

McCormick, C. M., Thomas, C. M., Sheridan, C. S., Nixon, F., and Flynn, J. A. (2012). Mathews IZ. Social instability stress in adolescent male rats alters hippocampal neurogenesis and produces deficits in spatial location memory in adulthood. *Hippocampus* 22, 1300–1312. doi: 10.1002/hipo.20966

McEwen, B. S. (2000). Protective and damaging effects of stress mediators: central role of the brain. *Prog. Brain Res.* 122, 25–34. doi: 10.1016/s0079-6123(08)62128-7

Meaney, M. J., and Szyf, M. (2005). Environmental programming of stress responses through DNA methylation: life at the interface between a dynamic environment and a fixed genome. *Dialogues Clin. Neurosci.* 7, 103–123. doi: 10.31887/dcns.2005.7.2/mmeaney

Méndez-Couz, M., Conejo, N. M., Vallejo, G., and Arias, J. L. (2014). Spatial memory extinction: a c-Fos protein mapping study. *Behav. Brain Res.* 260, 101–110. doi: 10.1016/j.bbr.2013.11.032

Mikasova, L., Xiong, H., Kerkhofs, A., Bouchet, D., Krugers, H. J., and Groc, L. (2017). Stress hormone rapidly tunes synaptic NMDA receptor through membrane dynamics and mineralocorticoid signalling. *Sci. Rep.* 7:8053. doi: 10.1038/s41598-017-08695-3

Mikhailenko, V. A., Butkevich, I. P., Vershinina, E. A., and Semenov, P. O. (2010). Interrelationship between measures of pain reactions in inflammation and levels of depression in prenatally stressed rat pups. *Neurosci. Behav. Physiol.* 40, 179–184. doi: 10.1007/s11055-009-9241-4

Mooney-Leber, S. M., and Brummelte, S. (2017). Neonatal pain and reduced maternal care: early-life stressors interacting to impact brain and behavioral development. *Neuroscience* 7, 21–36. doi: 10.1016/j.neuroscience.2016.05.001

Mooney-Leber, S. M., and Brummelte, S. (2020). Neonatal pain and reduced maternal care alter adult behavior and hypothalamic-pituitary-adrenal axis reactivity in a sex-specific manner. *Dev. Psychobiol.* 62, 631–643. doi: 10.1002/dev.21941

Morris, R. G. M. (1981). Spatial localization does not require the presence of local cues. *Learn. Motiv.* 12, 239–260.

Nelson, L. H., and Lenz, K. H. (2017). The immune system as a novel regulator of sex differences in brain and behavioral development. *J. Neurosci. Res.* 95, 447–461. doi: 10.1002/jnr.23821

Nuseir, K. Q., Alzoubi, K. H., Alabwaini, J., Khabour, O. F., and Kassab, M. I. (2015). Sucrose-induced analgesia during early life modulates adulthood learning and memory formation. *Physiol. Behav.* 145, 84–90. doi: 10.1016/j.physbeh.2015.04.002

Nuseir, K. Q., Alzoubi, K. H., Alhusban, A., Bawaane, A., Al-Azzani, M., and Khabour, O. F. (2017). Sucrose and naltrexone prevent increased pain sensitivity and impaired long-term memory induced by repetitive neonatal noxious stimulation: role of BDNF and β-endorphin. *Physiol. Behav.* 179, 213–219. doi: 10.1016/j.physbeh.2017.06.015

Pryce, C. R. (2008). Postnatal ontogeny of expression of the corticosteroid receptor genes in mammalian brains: inter-species and intra-species differences. *Brain Res. Rev.* 57, 596–605. doi: 10.1016/j.brainresrev.2007.08.005

Quintero, L., Cardenas, R., and Suarez-Roca, H. (2011). Stress-induced hyperalgesia is associated with a reduced and delayed GABA inhibitory control that enhances post-synaptic NMDA receptor activation in the spinal cord. *Pain* 152, 1909–1922. doi: 10.1016/j.pain.2011.04.017

Ranger, M., and Grunau, R. E. (2014). Early repetitive pain in preterm infants in relation to the developing brain. *Pain Manag.* 4, 57–67. doi: 10.2217/pmt.13.61

Ranger, M., Tremblay, S., Chau, C. M. Y., Holsti, L., Grunau, R. E., and Goldowitz, D. (2019). Adverse behavioral changes in adult mice following neonatal repeated exposure to pain and sucrose. *Front. Psychol.* 9:2394. doi: 10.3389/fpsyg.2018.02394

Ren, K., Anseloni, V., Zou, S.-P., Wade, E. B., Novikova, S. I., Ennis, M., et al. (2004). Characterization of basal and re-inflammation-associated long-term alteration in pain responsivity following short-lasting neonatal local inflammatory insult. *Pain* 110, 588–596. doi: 10.1016/j.pain.2004.04.006

Romeo, R. D. (2010). Pubertal maturation and programming of hypothalamic-pituitary-adrenal reactivity. *Front. Neuroendocrinol.* 31:232–240. doi: 10.1016/j.yfrne.2010.02.004

Romeo, R. D., and McEwen, B. S. (2006). Stress and the adolescent brain. *Ann. N.Y. Acad. Sci.* 1094, 202–214. doi: 10.1196/annals.1376.022

Schenk, F. (1985). Development of place navigation in rats from weaning to puberty. *Behav. Neural Biol.* 43, 69–85. doi: 10.1016/s0163-1047(85)91510-9

Schroeder, A., Notaras, M., Du, X., and Hill, R. A. (2018). On the developmental timing of stress: delineating sex-specific effects of stress across development on adult behavior. *Brain Sci.* 8:121. doi: 10.3390/brainsci8070121

Schwaller, F., and Fitzgerald, M. (2014). The consequences of pain in early life: injury-induced plasticity in developing pain pathways. *Eur. J. Neurosci.* 39, 344–352. doi: 10.1111/ejn.12414

Schwarz, J. M., Sholar, P. W., and Bilbo, S. D. (2012). Sex differences in microglial colonization of the developing rat brain. *J. Neurochem.* 120, 948–963. doi: 10.1111/j.1471-4159.2011.07630.x

Siddiqui, A., and Romeo, R. D. (2019). Sex differences and similarities in hippocampal cellular proliferation and the number of immature neurons during adolescence in rats. *Dev. Neurosci.* 41, 132–138. doi: 10.1159/000502056

Sokołowski, A., Folkierska-Żukowska, M., Jednoróg, K., Moodie, C. A., and Dragan, W. Ł (2020). The relationship between early and recent life stress and emotional expression processing: a functional connectivity study. *Cogn. Affect. Behav. Neurosci.* 20, 588–603. doi: 10.3758/s13415-020-00789-2

Spear, L. P. (2000). The adolescent brain and age-related behavioral manifestations. *Neurosci. Biobehav. Rev.* 24, 417–463. doi: 10.1016/s0149-7634(00)00014-2

Stylianakis, A. A., Harmon-Jones, S. K., Richardson, R., and Baker, K. D. (2018). Differences in the persistence of spatial memory deficits induced by a chronic stressor in adolescents compared to juveniles. *Dev. Psychobiol.* 60, 805–813. doi: 10.1002/dev.21750

Timmers, I., Quaedflieg, C. W. E. M., Hsu, C., Heathcote, L. C., Rovnaghi, C. R., and Simons, L. E. (2019). The interaction between stress and chronic pain through the lens of threat learning. *Neurosci. Biobehav. Rev.* 107, 641–655. doi: 10.1016/j.neubiorev.2019.10.007

Tirelli, E., Laviola, G., and Adriani, W. (2003). Ontogenesis of behavioral sensitization and conditioned place preference induced by psychostimulants in laboratory rodents. *Neurosci. Biobehav. Rev.* 27, 163–178. doi: 10.1016/s0149-7634(03)00018-6

Trudeau, L. E., and Mestikawy, S. (2018). Glutamate cotransmission in cholinergic, GABAergic and monoamine systems: contrasts and commonalities. *Front. Neural. Circuits* 12:113. doi: 10.3389/fncir.2018.00113

Tucker, L. B., Velosky, A. G., and McCabe, J. T. (2018). Applications of the morris water maze in translational traumatic brain injury research. *Neurosci. Biobehav. Rev.* 88, 187–200. doi: 10.1016/j.neubiorev.2018.03.010

Ulrich-Lai, Y. M., and Herman, J. P. (2009). Neural regulation of endocrine and autonomic stress responses. *Nat. Rev. Neurosci.* 10, 397–409. doi: 10.1038/nrn2647

Van Bodegom, M., Homberg, J. R., and Henckens, M. J. A. G. (2017). Modulation of the hypothalamic-pituitary-adrenal axis by early life stress exposure. *Front. Cell Neurosci.* 11:87. doi: 10.3389/fncel.2017.00087

Van Dammen, L., de Rooij, S. R., Behnsen, P. M., and Huizink, A. C. (2020). Sex-specific associations between person and environment-related childhood adverse events and levels of cortisol and DHEA in adolescence. *PLoS One* 15:e0233718. doi: 10.1371/journal.pone.0233718

VanRyzin, J. W., Pickett, L. A., and McCarthy, M. M. (2018). Microglia: driving critical periods and sexual differentiation of the brain. *Dev. Neurobiol.* 78, 580–592. doi: 10.1002/dneu.22569

Verriotis, M., Jones, L., Whitehead, K., Laudiano-Dray, M., Panayotidis, I., Patel, H., et al. (2018). The distribution of pain activity across the human neonatal brain is sex dependent. *Neuroimage* 178, 69–77. doi: 10.1016/j.neuroimage.2018.05.030

Victoria, N. C., and Murphy, A. Z. (2016). Long-term impact of early life pain on adult responses to anxiety and stress: historical perspectives and empirical evidence. *Exp. Neurol.* 275, 261–273. doi: 10.1016/j.expneurol.2015.07.017

Victoria, N. C., Inoue, K., Young, L. J., and Murphy, A. Z. (2013). Long-term dysregulation of brain corticotrophin and glucocorticoid receptors and stress reactivity by single early-life pain experience in male and female rats. *Psychoneuroendocrinology* 38, 3015–3028. doi: 10.1016/j.psyneuen.2013.08.013

Victoria, N. C., Karom, M. C., Eichenbaum, H., and Murphy, A. Z. (2014). Neonatal injury rapidly alters markers of pain and stress in rat pups. *Dev. Neurobiol.* 74, 42–51. doi: 10.1002/dneu.22129

Vinall, J., Miller, S. P., Bjornson, B. H., Fitzpatrick, K. P., Poskitt, K. J., Brant, R., et al. (2014). Invasive procedures in preterm children: brain and cognitive development at school age. *Pediatrics* 133, 412–421. doi: 10.1542/peds.2013-1863

Vorhees, C. V., and Williams, M. T. (2014). Assessing spatial learning and memory in rodents. *ILAR J.* 55, 310–332. doi: 10.1093/ilar/ilu013

Walker, S. M., Meredith-Middleton, J., Cooke-Yarborough, C., and Fitzgerald, M. (2003). Neonatal inflammation and primary afferent terminal plasticity in the rat dorsal horn. *Pain* 105, 185–195. doi: 10.1016/s0304-3959(03)00201-x

Whiting, M. D., and Kokiko-Cochran, O. N. (2016). Assessment of cognitive function in the water maze task: maximizing data collection and analysis in animal models of brain injury. *Methods Mol. Biol.* 1462, 553–571. doi: 10.1007/978-1-4939-3816-2_30

Williams, M. D., and Lascelles, B. D. X. (2020). early neonatal pain-a review of clinical and experimental implications on painful conditions later in life. *Front. Pediatr.* 8:30. doi: 10.3389/fped.2020.00030

Wood, C. E., and Walker, C. D. (2015). Fetal and neonatal HPA axis. *Compar. Physiol.* 6, 33–62. doi: 10.1002/cphy.c150005

Xia, D., Min, C., Chen, Y., Ling, R., Chen, M., and Li, X. (2020). Repetitive pain in neonatal male rats impairs hippocampus-dependent fear memory later in life. *Front. Neurosci.* 14:722. doi: 10.3389/fnins.2020.00722

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The Impact of Adolescent Alcohol Exposure on Nicotine Behavioral Sensitization in the Adult Male Neonatal Ventral Hippocampal Lesion Rat

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Nicotine and alcohol use is highly prevalent among patients with serious mental illness, including those with schizophrenia (SCZ), and this co-occurrence can lead to a worsening of medical and psychiatric morbidity. While the mechanistic drivers of co-occurring SCZ, nicotine use and alcohol use are unknown, emerging evidence suggests that the use of drugs during adolescence may increase the probability of developing psychiatric disorders. The current study used the neonatal ventral hippocampal lesion (NVHL) rat model of SCZ, which has previously been shown to have enhanced nicotine behavioral sensitization and, following adolescent alcohol, increased alcohol consumption. Given how commonly alcohol is used by adolescents that develop SCZ, we used the NVHL rat to determine how exposure to adolescent alcohol impacts the development of nicotine behavioral sensitization in adulthood. Male Sprague-Dawley rats underwent the NVHL surgery or a sham (control) surgery and subsequently, half of each group was allowed to drink alcohol during adolescence. Nicotine behavioral sensitization was assessed in adulthood with rats receiving subcutaneous injections of nicotine (0.5 mg/kg) each day for 3 weeks followed by a nicotine challenge session 2 weeks later. We demonstrate that all groups of rats became sensitized to nicotine and there were no NVHL-specific increases in nicotine behavioral sensitization. We also found that NVHL rats appeared to develop sensitization to the nicotine paired context and that adolescent alcohol exposure blocked this context sensitization. The current findings suggest that exposure to alcohol during adolescence can influence behaviors that manifest in the adult NVHL rat (i.e., context sensitization). Interestingly, nicotine behavioral sensitization levels were not altered in the NVHL groups regardless of adolescent alcohol exposure in contrast to prior reports.

Keywords: adolescent alcohol, NVHL, co-occurring disorders, mental illness, smoking, nicotine behavioral sensitization

INTRODUCTION

Smoking is highly prevalent among patients with serious mental illness and this co-occurrence leads to medical and psychiatric morbidity (Green et al., 1999, 2008; Mallet et al., 2019) as well as an increased mortality risk (Tran et al., 2009; McGinty et al., 2012; Dickerson et al., 2018). Specifically, patients with schizophrenia (SCZ) have higher smoking rates than the general population (de Leon and Diaz, 2005) with lifetime prevalence reported at 60–90% (Volkow, 2009). In one investigation studying patients with SCZ or bipolar disorder, current smokers showed worse cognitive functioning and had poorer functional outcomes than past or never smokers. These effects were observed regardless of diagnosis, however, the patients with SCZ were twice as likely to be smokers compared to those with bipolar disorder (Depp et al., 2015). Moreover, in a recent study, 31% of current smokers were readmitted to a psychiatric hospital within 1 year of discharge compared to 26% of never smokers (Kagabo et al., 2019). Collectively, these studies indicate a correlation between smoking in patients with a serious mental illness and increased psychiatric morbidity and mortality.

The underlying causes of co-occurring mental illness and substance use disorders are largely unknown. However, there is evidence indicating that genetic factors combined with prenatal and/or postnatal developmental insults (including the use of drugs during adolescence; Khokhar et al., 2017), contribute to the development of these disorders. A number of studies suggest cannabis use (Fergusson et al., 2003), and tobacco smoking (Gage and Munafo, 2015; Kendler et al., 2015) may be associated with increased psychotic symptoms. Additionally, for many patients substance use precedes psychosis, with reports finding that substance use rates among patients with first episode psychosis are 30–70% (Abdel-Baki et al., 2017). Thus it is important to study substance use during adolescence and its potential role in contributing to an individual's risk of developing a psychiatric diagnosis.

One developmental insult used in rats that results in several dysregulated behavioral endophenotypes is the neonatal ventral hippocampal lesion (NVHL). NVHL rats display symptoms resembling those occurring across psychiatric disorders, though they are often used as a model of SCZ (Lipska et al., 1993, 1995; Sams-Dodd et al., 1997; Brady et al., 2010; Gruber et al., 2010; Placek et al., 2013). Moreover NVHL rats self-administer drugs, including nicotine, at a higher rate than normal rats (Chambers and Self, 2002; Berg et al., 2011; Sentir et al., 2020), as well as demonstrate enhanced nicotine behavioral sensitization (Berg and Chambers, 2008). Behavioral sensitization is the progressive increase of drug-induced locomotion with repeated exposure to a drug (Robinson and Berridge, 1993) and is a phenomenon documented in both humans and animals (Kalivas and Stewart, 1991; Robinson and Berridge, 2008). This behavior is indicative of neuroadaptations occurring in motivation related brain regions underlying drug-wanting and craving (Robinson and Berridge, 2008) and can be affected by perturbations occurring in adolescence (McCormick et al., 2004; Mathews et al., 2008; McCormick, 2010; Garcia et al., 2017). Furthermore, cross-sensitization has also been shown, where the repeated exposure of one drug yields sensitization to another drug (Kalivas and Stewart, 1991; Steketee and Kalivas, 2011).

Neonatal ventral hippocampal lesion rats have also been shown to increase alcohol consumption in adulthood after voluntary adolescent alcohol intake (Jeanblanc et al., 2015). Alcohol remains one of the most commonly used drugs by adolescents [Johnston et al., 2020; Substance Abuse and Mental Health Services Administration (SAMHSA), 2020], and as such, combining the NVHL developmental insult with adolescent alcohol exposure can be used to study the complex dynamics between adolescent drug use, SCZ, and increased smoking. In the present study, we used the NVHL rat to determine how exposure to adolescent alcohol affects nicotine behavioral sensitization in adulthood. In humans, alcohol use during adolescence has been linked to increased substance use in adulthood (Ellickson et al., 2003; Grant et al., 2006; Ryan et al., 2019), therefore, we hypothesized NVHL animals with alcohol exposure would demonstrate increased nicotine behavioral sensitization.

MATERIALS AND METHODS

Subjects and Housing

Lactating Sprague-Dawley female rats (n = 4) with 10 male pups each were ordered from Charles River (Wilmington, MA, United States) and arrived on the pups' postnatal day (PD) 2. We specifically chose to use the outbred rat strain Sprague-Dawley in order to maximize the genetic and epigenetic variability, as any behavioral signals would likely be more generalizable to other rats. Additionally, as reports indicate that the prevalence rates of any current tobacco product use is higher in men than women in the general population (Higgins et al., 2015; Cornelius et al., 2020) and in patients with SCZ (Kelly and McCreadie, 1999; Ohi et al., 2018), we used male rats. All rats were housed on a reverse 12-h light cycle with *ad libitum* access to food and water. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) and were approved by the Institutional Animal Care and Use Committee of Dartmouth College.

Neonatal Ventral Hippocampal Lesion Surgery

Neonatal ventral hippocampal lesion or sham (control) surgeries were carried out following previously published guidelines (Chambers and Lipska, 2011). On PD 7 when pups weighed between 15 and 20 *g*, they were anesthetized via hypothermia and then placed on a Styrofoam platform attached to a stereotaxic frame (Kopf Instruments, Tujunga, CA, United States). Half of the pups (NVHL; n = 20) were bilaterally injected with 0.3 μl excitotoxic ibotenic acid [10 μg/μl ibotenic acid (Tocris, Minneapolis, MN, United States) in artificial cerebrospinal fluid (aCSF)] into the ventral hippocampi (from bregma: AP −3.0 mm, ML ± 3.5 mm, DV −5.0 mm). The remaining pups (Sham; n = 20) were injected with 0.3 μl of aCSF at the same coordinates. After surgery, wounds were closed with surgical glue (VetOne Surgical Adhesive, Boise, ID, United States) and the pups were

warmed on a heating pad until their activity level was restored, at which time they were returned to their home cages. In order to control for litter/dam effects, half of each litter underwent the NVHL surgery and the other half was sham-operated. Rats were weaned on PD 21 and housed individually. One sham rat did not recover after surgery.

Alcohol Drinking in Adolescence

We followed the methods from previously published studies (Jeanblanc et al., 2015; Khokhar and Todd, 2017), but briefly, half of each group [NVHL with alcohol exposure (NVHL AE); sham with alcohol exposure (Sham AE)] was given free access to 10% v/v ethanol (EtOH) in water solution in their home cage for 24 h per day from PD 28–42. Alcohol intake, water intake, and body weights were measured daily and the position of the alcohol and water bottles was alternated each day to prevent development of a side preference. At the end of PD 42, the alcohol bottle was removed, and the rats had access to water only for the duration of the study.

Nicotine Sensitization

Nicotine behavioral sensitization began on PD 60 and was performed during the active cycle (the time when the animal rooms are dark between 0700 and 1,900 h). Nicotine bitartrate dihydrate (MilliporeSigma, Burlington, MA, United States; 0.5 mg/mL) was dissolved in 0.9% sterile saline, adjusted to 7.4 pH, and administered with a volume of 1 mL/kg bodyweight. Locomotor activity was assessed in four open field arenas (60 cm × 60 cm × 33 cm) located in an animal behavior room, separate from the rats' housing room. The lights were turned on in the behavior room during nicotine behavioral sensitization (average light intensity was 297.7 lux), and the paradigm was conducted so each round of four animals was comprised of both NVHL and sham rats. The arena used for each individual rat remained consistent throughout the entire experiment and in between each round of four animals, the arenas were thoroughly cleaned.

The injection series occurred Monday through Friday for three consecutive weeks (15 sessions). During each session, rats were first placed in the arena for 30 min (i.e., preinjection). After 30 min, each rat was given a subcutaneous (s.c.) injection of nicotine (0.5 mg/kg in 1 mL/kg) and returned to the same arena for 60 min (i.e., postinjection). After the 15th session, rats were given a 2 week washout period where they remained in their home cage. Following the washout period, rats underwent a challenge session where, again, they had a 30 min preinjection period, followed by an injection of nicotine (0.5 mg/kg), and remained in the arena for a 60 min postinjection period (**Figure 1**). Every pre- and post-injection session was videotaped using a Defeway Security camera system (Shenzhen, China) and analyzed using Noldus EthoVision XT tracking software (Wageningen, Netherlands) for distance traveled (cm), velocity (cm/s), and location within the chamber (i.e., center zone). One NVHL AE rat had to be euthanized after completing the 15 sessions but prior to the challenge session due to seizures. One NVHL no AE rat died before completing the nicotine behavioral sensitization paradigm. Final numbers for the four groups were: NVHL AE = 10; Sham AE = 10; NVHL no AE = 8; and Sham no AE = 9.

Anxiety-Like Behavior

Anxiety-like behavior was assessed using latency to center, frequency in center, and total duration in center zone for preinjection and postinjection on days 1, 5, 10, 15, and challenge. The center zone (20 cm × 20 cm) was created using EthoVision XT arena settings by dividing the arena floor into nine equal-sized zones. The rat was considered in the center zone if the center tracking point (while using three-point tracking) was within 2 cm of the defined center zone. In sessions where the rat never entered the center zone, the variable latency to center was recorded as the maximum number of seconds for that session (i.e., 1,800 or 3,600 s for preinjection and postinjection, respectively).

Lesion Verification

At the end of the experiment, rats were euthanized by CO_2 overdose, brains were extracted and flash frozen using 2-methylbutane on dry ice. Tissue was stored at −20°C prior to being sectioned at 40 μm using a Leica Biosystems CM1850 cryostat (Buffalo Grove, IL, United States) and stained with thionin. Lesion size was verified using an AmScope light microscope (Irvine, CA, United States). Lesions include cell loss, cellular disorganization, and ventricle enlargement (**Figure 2**). NVHL rats with extra-hippocampal damage or unilateral damage were excluded from analysis. One NVHL rat was removed due to an exceedingly large lesion.

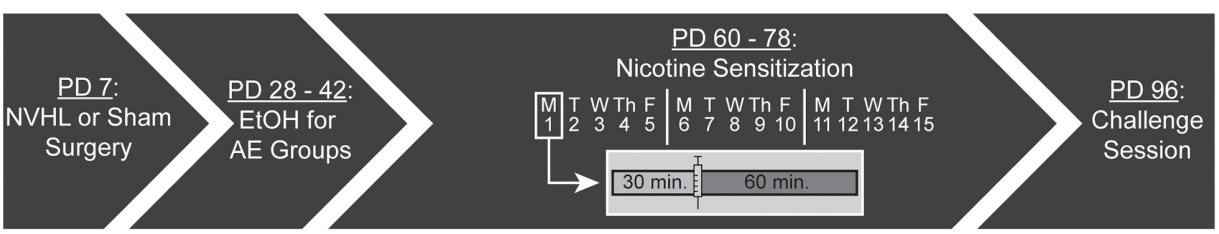

FIGURE 1 | Experimental timeline. NVHL or sham surgery was performed on postnatal day (PD) 7. Half of each group received access to 10% ethanol (EtOH) in their home cage from PD 28–42. Nicotine sensitization began on PD 60 and occurred Monday through Friday for three consecutive weeks (15 sessions). Each session had a 30 min preinjection phase before the rat received a subcutaneous injection of 0.5 mg/kg nicotine followed by a 60 min postinjection phase. After a 2 week washout, all rats had a challenge session on PD 96.

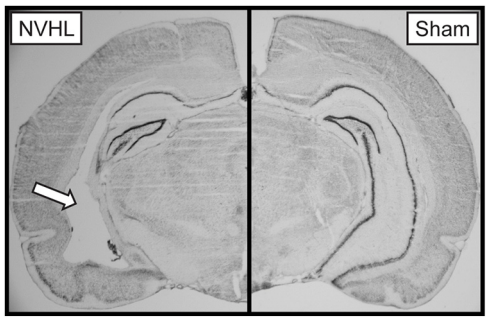

FIGURE 2 | Representative image of a NVHL **(left)** and sham **(right)** brain. The arrow points to the NVHL lesion in the ventral hippocampus.

Data Analyses

Alcohol Intake

The alcohol intake (g EtOH/kg bodyweight) for each group (NVHL AE or Sham AE) was averaged for each day. A repeated measures analyses of variance (RMANOVA) was used to compare the average alcohol intake between the groups across alcohol exposure time.

Nicotine Sensitization

Total distance traveled during preinjection and postinjection was calculated for each day and averaged across groups. To account for individual differences in locomotor activity and to determine the level of nicotine behavioral sensitization, the distance traveled during the first 30 min of postinjection was compared to that days' preinjection for each rat and expressed as a percentage change. A three-way RMANOVA was run with day (day 1–15) and treatment (preinjection or postinjection) as within-subject factors and group (NVHL AE, Sham AE, NVHL no AE, Sham no AE) as the between group factor. Two-way RMANOVAs were subsequently used to determine group differences in preinjection, postinjection, and percentage change. ANOVAs were used to compare distance traveled between the groups on challenge day. If the assumption of sphericity was violated, the Greenhouse-Geisser correction was used. Any significant effects were further analyzed using Bonferroni *post hoc* tests.

Velocity

A three-way RMANOVA was run with day (day 1, 5, 10, 15) and treatment (preinjection or postinjection) as within-subject factors and group as the between group factor. Two-way RMANOVAs were used to compare preinjection and postinjection average velocity between the groups. ANOVAs were used to compare preinjection and postinjection velocity between the groups on challenge day.

Anxiety-Like Behavior

Repeated measures analyses of variances were used to compare preinjection and postinjection latency to center, center frequency, and total center duration over days 1, 5, 10, and 15. ANOVAs were used to compare groups during preinjection and postinjection on challenge day. To determine the effect of nicotine on anxiety and to account for an increase in total distance traveled after nicotine, the ratio of center crosses to total distance traveled was calculated for preinjection and postinjection on challenge day. A RMANOVA was used to assess the preinjection and postinjection ratio between the four groups.

RESULTS

Adolescent Alcohol Intake

As shown in **Figure 3**, RMANOVA revealed that alcohol intake during adolescence did not differ between NVHL AE and Sham AE groups [F (1,16) = 0.068, p = 0.798].

Nicotine Sensitization

Three-way RMANOVA revealed a significant treatment*day interaction [F (3.869,127.671) = 73.656, $p < 0.001$] indicating that distance traveled during postinjection was greater than preinjection, demonstrating nicotine behavioral sensitization. There was also a significant treatment*day*group interaction [F (11.606,127.671) = 2.763, p = 0.003]. Two-way RMANOVA revealed a significant group effect in distance traveled during the preinjection phase across the 15 nicotine sessions [F (3,33) = 4.639, p = 0.008; **Figure 4A**]. Bonferroni *post hoc* analyses showed that the NVHL no AE group traveled significantly further than every other group: Sham no AE (p = 0.027), NVHL AE (p = 0.021), and Sham AE (p = 0.027). A similar pattern emerged when focusing on the postinjection phase. Two-way RMANOVA showed a significant group effect across the 15 nicotine sessions [F (3,33) = 10.206, $p < 0.001$; **Figure 4B**]. Bonferroni *post hoc* analyses showed that the NVHL no AE rats traveled significantly further following nicotine injection than Sham no AE ($p < 0.001$), NVHL AE (p = 0.009), and Sham AE ($p < 0.001$) rats.

Looking at the challenge day, an ANOVA indicated a significant difference in distance traveled between groups during the preinjection phase [F (3,32) = 7.864, $p < 0.001$; **Figure 4C**]. Bonferroni *post hoc* showed that the NVHL no AE group traveled significantly further than Sham no AE (p = 0.002), NVHL AE (p = 0.012), and Sham AE (p = 0.001) groups. Additionally, an ANOVA showed a significant difference in distance traveled between groups during the postinjection phase on the challenge

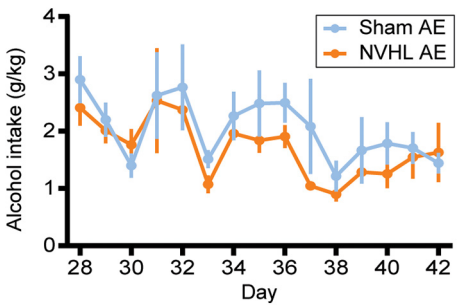

FIGURE 3 | Adolescent alcohol intake. Alcohol Intake (g EtOH/kg bodyweight) from PD 28–42 did not differ between NVHL AE and Sham AE rats. Data is shown as group mean ± SEM.

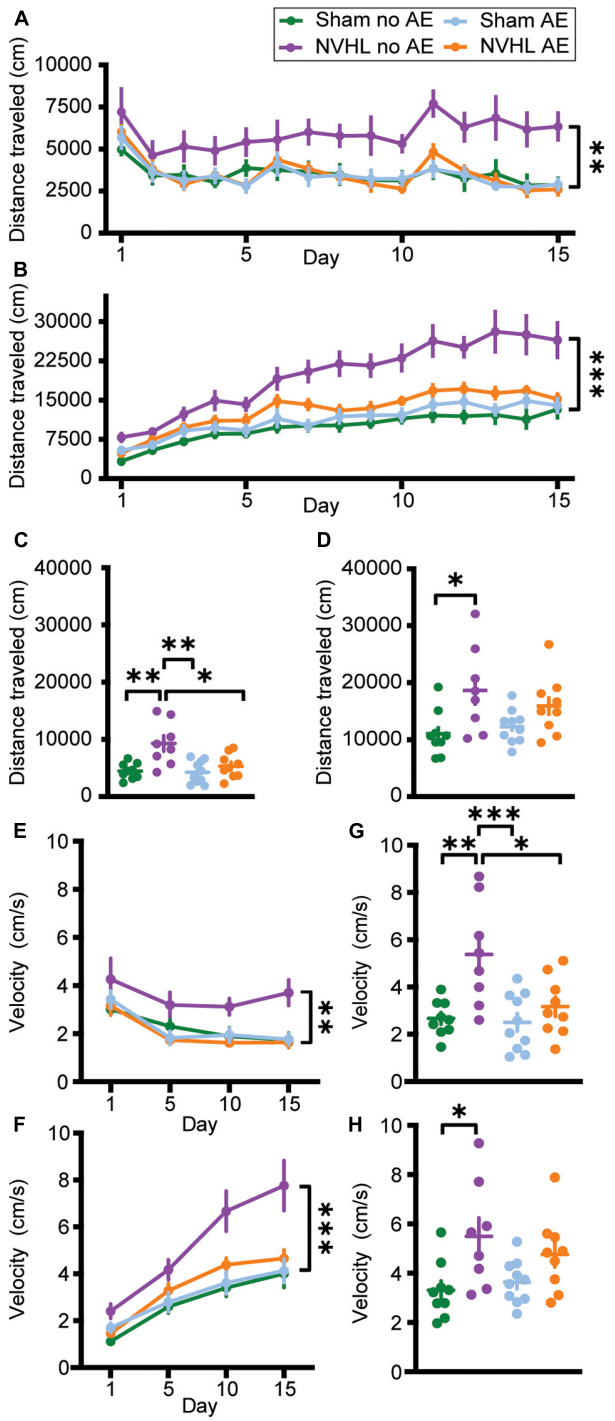

FIGURE 4 | Total distance traveled and average velocity. **(A)** Total distance traveled before an injection of 0.5 mg/kg nicotine across the 15 sensitization sessions. **(B)** Total distance traveled after an injection of 0.5 mg/kg nicotine across the 15 sensitization sessions. The NVHL no AE group showed significantly greater distance traveled compared to the Sham no AE, NVHL AE, and Sham AE groups during both the preinjection and postinjection phases. **(C)** On the preinjection phase of the challenge day, the NVHL no AE group showed significantly more distance traveled than the other three groups. **(D)** During the postinjection phase on the challenge day, the NVHL no AE group only traveled significantly further than the Sham no AE group. **(E)** NVHL no AE rats had significantly elevated average velocity during the preinjection phase on days 1, 5, 10, and 15. **(F)** NVHL no AE rats had significantly elevated average velocity during the postinjection phase on days 1, 5, 10, and 15. **(G)** Average velocity of the NVHL no AE rats remained elevated during the preinjection phase on the challenge day. **(H)** During the postinjection phase, NVHL no AE rats only had significantly increased velocity compared to Sham no AE rats. Data is shown as group mean ± SEM. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; and $^{***}p \leq 0.001$.

day [F (3,32) = 4.051, p = 0.015; **Figure 4D**]. Bonferroni *post hoc* analyses indicated that the NVHL no AE group traveled significantly further than the Sham no AE group (p = 0.012). However, the NVHL no AE group was no longer significantly different from the NVHL AE or the Sham AE group following the injection of nicotine.

Since the NVHL no AE group showed significantly greater distance traveled in both the preinjection and postinjection phase, the level of nicotine behavioral sensitization when controlling for any nicotine induced context sensitization was determined by calculating the percentage change in distance traveled from the preinjection phase to the first 30 min of the postinjection phase on each day for each rat. RMANOVA revealed no group differences in the level of nicotine behavioral sensitization across the 15 sessions [F (3,33) = 0.380, p = 0.768; **Figure 5**], however, a significant effect of day using the Greenhouse-Geisser correction again indicates that all groups did become sensitized to nicotine [F (3.879,128.006) = 16.872, p < 0.001]. An ANOVA on percentage change in distance traveled on the challenge day showed no differences between groups [F (3,32) = 2.259, p = 0.1; **Figure 5**].

Velocity

Three-way RMANOVAs revealed significant treatment*day, day*group, and group*treatment interactions. Two-way RMANOVAs showed a group effect in average velocity during the preinjection [F (3,33) = 5.165, p = 0.005; **Figure 4E**] and postinjection [F (3,33) = 7.949, p < 0.001; **Figure 4F**] phase across days 1, 5, 10, and 15. Bonferroni *post hoc* analyses showed that NVHL no AE rats moved with greater average velocity during both preinjection and postinjection sessions compared to Sham no AE (p = 0.027 and p = 0.002 for pre- and post-injection, respectively), NVHL AE (p = 0.006 and p = 0.013), and Sham AE (p = 0.023 and p = 0.001) groups.

During the challenge day, ANOVA showed a significant difference in average velocity between groups during the preinjection phase [F (3,32) = 7.397, p = 0.01; **Figure 4G**] and the postinjection phase [F (3,32) = 4.154, p = 0.014; **Figure 4H**]. Bonferroni *post hoc* analyses indicated that during the preinjection phase NVHL no AE rats had greater average velocity than all other groups (Sham no AE [p = 0.001], NVHL AE [p = 0.018], NVHL no AE [p = 0.003]). However, during the postinjection phase, NVHL no AE rats had significantly increased velocity compared to only Sham no AE rats (p = 0.025).

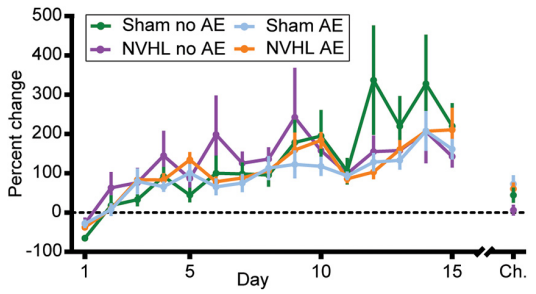

FIGURE 5 | Percentage change in distance traveled. The level of nicotine sensitization was determined by calculating the percentage change in distance traveled from the preinjection phase to the first 30 min of the postinjection phase across each of the initial 15 sessions and on the challenge day for each rat. No group differences were observed but a significant effect of day across the 15 sessions indicates that all groups became sensitized to nicotine. Data is shown as group mean ± SEM.

Anxiety-Like Behavior

Repeated measures analyses of variances found no significant differences between groups in latency to center zone (**Supplementary Figures 1A,B**), frequency in center (**Figures 6A,B**), and total duration in center (**Supplementary Figures 1E,F**) in both preinjection and postinjection phases across days 1, 5, 10, and 15.

Similarly, ANOVAs found no significant differences between groups in latency to center zone (**Supplementary Figures 1C,D**) and total duration in center (**Supplementary Figures 1G,H**) during preinjection and postinjection on the challenge day. However, there was a significant difference between groups in frequency in center during the preinjection phase on challenge day [F (3,32) = 5.518, p = 0.004; **Figure 6C**]. Bonferroni *post hoc* analysis showed that NVHL no AE rats entered the center zone more frequently than Sham no AE (p = 0.027), NVHL AE (p = 0.028), and Sham AE (p = 0.004). Following the nicotine injection on challenge day, the significant differences between groups in center frequency no longer remained (**Figure 6D**). To assess the effect of nicotine on anxiety-like behaviors and to account for an increase in locomotive behavior after nicotine, the ratio of number of center crosses to total distance traveled was calculated for preinjection and postinjection on the challenge day. RMANOVA revealed a significant increase in the center crosses-to-distance ratio during the postinjection phase [F (1,32) = 37.161, p < 0.001] with no significant group differences (**Figure 6E**).

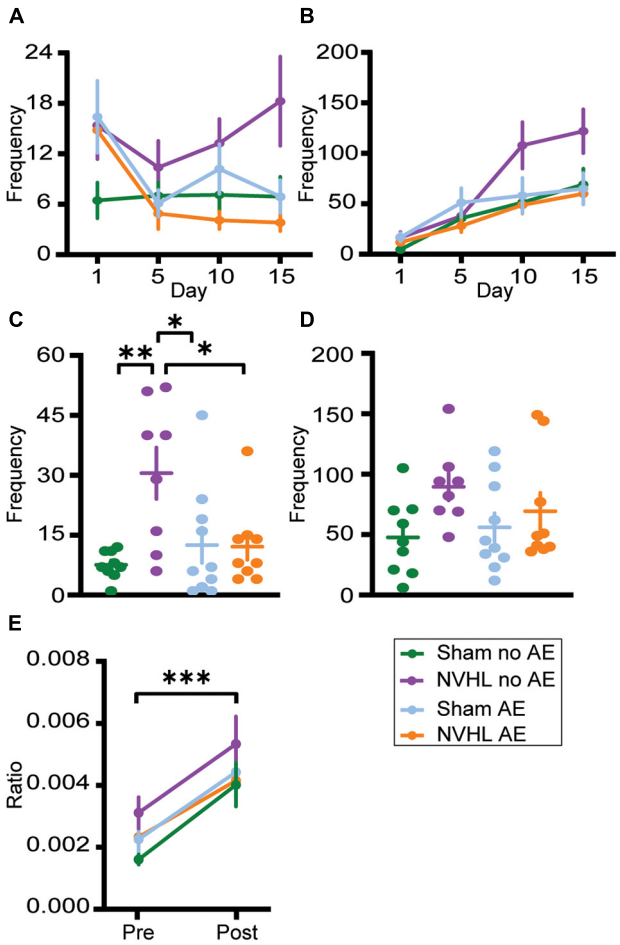

FIGURE 6 | Center zone frequency. **(A)** Frequency of center zone crosses before the injection of nicotine on days 1, 5, 10, and 15. **(B)** Frequency of center zone crosses after the injection of nicotine on days 1, 5, 10, and 15. No significant group differences were observed. **(C)** NVHL no AE rats had significantly increased frequency of center crosses during preinjection on the challenge day. **(D)** Following the nicotine injection on challenge day, no group differences were observed. **(E)** There was a significant increase in the ratio of frequency of center crosses to distance traveled following nicotine on the challenge day with no group differences. Data is shown as group mean ± SEM. *$p \leq 0.05$; **$p \leq 0.01$; and ***$p \leq 0.001$.

DISCUSSION

Here, we sought to determine the effects that alcohol exposure during adolescence would have on nicotine behavioral sensitization in the NVHL model of SCZ. The results suggest that adolescent alcohol exposure from PD 28–42 did not alter the amount of nicotine behavioral sensitization. When controlling for baseline differences in distance traveled, there were no differences in the amount of nicotine behavioral sensitization between NVHL and sham rats, regardless of adolescent alcohol exposure. Importantly though, all groups did sensitize to nicotine, as demonstrated by a significant treatment*day interaction in distance traveled and the significant increase in percentage change across the 15 sessions (**Figure 5**).

The NVHL no AE group showed a significant increase in distance traveled during the preinjection phase of the 15 sessions (**Figure 4A**), in addition to a significant increase in velocity (**Figure 4E**). While previous studies have found that postpubertal NVHL rats show spontaneous hyperlocomotion (Lipska et al., 1993; Sams-Dodd et al., 1997), as well as increased locomotor response to a novel environment (Berg and Chambers, 2008), we saw no group differences in distance traveled during the preinjection phase on Day 1. However, we did observe

that the NVHL no AE group showed significant increases in distance traveled during the preinjection phase across days once nicotine injections began, suggesting the development of context sensitization, a phenomenon that has previously been reported in the literature in normal rats. Rats treated with nicotine (0.6 mg/kg) for 9 days showed an increase in locomotor activity compared to saline treated animals in the 30 min prior to drug administration (Kosowski and Liljequist, 2005). Similarly, an environment repeatedly paired with nicotine (0.6 mg/kg) acquired the ability to elicit increases in activity even in the absence of nicotine (Walter and Kuschinsky, 1989; Bevins et al., 2001). These data are the first to report that the NVHL rat has enhanced context sensitization, possibly pointing to an increase in the salience of nicotine, and a shift of that salience from nicotine to the context, in this group. Furthermore, it appears that adolescent alcohol exposure impairs the formation of context sensitization, possibly by dampening the salience of nicotine, in the NVHL AE rat. An increase in the salience of nicotine is supported by previous work demonstrating that NVHL rats have increased nicotine seeking behavior during extinction than their sham counterparts (Berg et al., 2013; Rao et al., 2016; Sentir et al., 2020). Thus the current results lend further support to the NVHL rat as a model to better understand SCZ and the increased prevalence of nicotine use.

While additional research is needed to elucidate the exact mechanism, one potential reason for the reduction in context sensitization seen in the NVHL AE group when compared to the NVHL no AE group, may be the impact that alcohol has on developing brain regions. Clinical studies show that alcohol use during adolescence impacts the volume of several brain regions such as the prefrontal cortex (PFC; De Bellis et al., 2005), nucleus accumbens (Thayer et al., 2012), hippocampus (De Bellis et al., 2000; Nagel et al., 2005; Medina et al., 2007), and amygdala (Wilson et al., 2015). Preclinical studies corroborate these findings with alcohol causing numerous anatomical and functional alterations, including decreases in neurogenesis and region-specific brain damage and cell death (Crews et al., 2000; Spear, 2015, 2016). As many of these brain regions play a role in incentive salience, it is possible that disrupted cortical development stemming from alcohol exposure during adolescence dampened the salience of nicotine in the NVHL AE group which prevented the development of context sensitization.

Another neurobiological mechanism that may underlie behavioral changes within the NVHL rat are disruptions in nicotinic acetylcholine receptor (nAChR) function. Extensive literature exists demonstrating that patients with SCZ have disrupted nAChR function and decreased receptor density (Freedman et al., 1995; Breese et al., 2000; Durany et al., 2000; D'Souza and Markou, 2011; Esterlis et al., 2014). These results are supported by a preclinical study showing that NVHL rats have a 12% reduction in nAChR binding in the PFC compared to their sham counterparts (Berg et al., 2015). Furthermore, additional cholinergic alterations exist in this model. *In vivo* acetylcholine release was hyper reactive to both peripheral and local administration of a dopamine $(D)_1$ agonist in NVHL rats, and receptor autoradiography showed an increase in muscarinic $(M)_1$-like receptor binding sites in the PFC (Laplante et al., 2004a). Another study indicated that tail-pinch stress resulted in a significantly greater increase in PFC acetylcholine release in the NVHL rats, which was subsequently blocked by D_1 and D_2 antagonists (Laplante et al., 2004b). Interestingly, nAChRs have been shown to be involved in alcohol-related behaviors where blocking nAChRs partially prevented alcohol-induced locomotor activity (Blomqvist et al., 1992). While some preclinical studies suggest that moderate lengths of alcohol exposure (15–17 days) do not alter nicotinic receptor binding (de Fiebre and Collins, 1993; Ribeiro-Carvalho et al., 2009), chronic alcohol treatment (28 weeks) in rats produced long-lasting reductions in acetylcholine levels, acetylcholinesterase activity, choline uptake, and acetylcholinesterase-positive neurons (Arendt et al., 1988, 1989). Similarly, non-human primates chronically treated with alcohol for 4 weeks had decreased nAChR availability in cortical and thalamic regions (Cosgrove et al., 2010). Though the NVHL AE and Sham AE rats in the current study were only exposed to 14 days of alcohol, there is potential that alcohol exposure during adolescence could alter nAChR function.

Using percentage change as a measure of the amount of nicotine behavioral sensitization, we found that there were no differences between NVHL and sham groups, regardless of whether they received alcohol during adolescence. Our results contrast previously published results showing that NVHL rats (without adolescent alcohol exposure) have enhanced nicotine behavioral sensitization (Berg and Chambers, 2008). A possible explanation for the discrepancies between these studies is the post-weaning housing conditions of the animals. The rats in the current study were singly housed so that alcohol intake during adolescence could be determined for each individual. The rats used in the previously published study (Berg and Chambers, 2008) were pair housed after weaning. Several studies have shown that housing conditions can influence not only the locomotor response to a novel environment, but also the behavioral response to drugs, including nicotine. Rats housed in isolation show increased locomotor response in a novel environment compared to those housed in pairs (Garcia et al., 2017) and those housed in groups (Smith et al., 1997; Cheeta et al., 2001). Furthermore, rats housed in isolation have enhanced sensitization to the locomotor effects of repeated administration of amphetamine (Smith et al., 1997). Additionally, female rats that underwent chronic social stress during adolescence (isolation for 1 h each day and then housed with a new partner) show increased locomotor sensitization in response to amphetamine (Mathews et al., 2008) and nicotine (McCormick et al., 2004). Therefore it is reasonable that isolated housing led to an increase in the nicotine behavioral sensitization of the sham groups, and combined with a potential ceiling effect in the nicotine behavioral sensitization of the NVHL groups, any group differences were masked.

Using measures related to the center zone of the open field arena as a proxy for anxiety-like behaviors (latency to enter the center zone and the duration of time spent in the center zone), we found no differences between NVHL and sham groups, regardless of alcohol exposure (**Supplementary Figure 1**). The NVHL no AE group did have a significantly

increased number of center entrances compared to the other groups, but only during the preinjection phase during the challenge day (**Figure 6C**). With no other increases in anxiety-like behaviors and the significant increase in both total distance traveled and average velocity, it is likely that the significantly elevated center frequency in the NVHL no AE rats was due to their increased context sensitization. Previous studies assessing anxiety-related behaviors in the NVHL rat have found mixed results based on the method used to measure anxiety. In one study, male NVHL rats demonstrated persistent anxiety as adolescents and adults compared to control rats, spending less time in the central zone of an open field task (Sams-Dodd et al., 1997). However, several other studies found that male and female NVHL rats spend more time in the open arm of an elevated plus maze, suggesting less anxiety (Wood et al., 2003; Beninger et al., 2009). Although the current results found that there were no group differences in anxiety-like behavior using an open field task, future studies assessing anxiety in the NVHL rat should take into account locomotor differences that may confound the results.

In order to assess the effect of nicotine treatment on anxiety-like behaviors and to control for an increase in movement after an injection of nicotine, the ratio of number of center crosses to total distance traveled was calculated for preinjection and postinjection on the challenge day. The significant increase in this ratio during postinjection demonstrates that nicotine had an anxiolytic effect on all groups (**Figure 6E**). This is consistent with some previous literature showing that 7 days of nicotine treatment (Irvine et al., 2001) or chronic nicotine administered via drinking water (Onaivi et al., 1994) both resulted in anxiolytic effects on elevated plus maze behaviors.

One limitation of the current study was not using both sexes, though there is variability in the literature as to whether a sex difference in nicotine behavioral sensitization exists. Nevertheless, some studies have found that female rats show more locomotor activity in response to nicotine behavioral sensitization (Booze et al., 1999; Harrod et al., 2004), while others find that sex does not have marked influences on this behavior (Kanyt et al., 1999; Ericson et al., 2010). A limited number of studies have used both male and female NVHL rats to assess cognitive abilities (Chambers et al., 1996; Beninger et al., 2009), neurotransmitter release (Beninger et al., 2009), and expression of G-protein coupled receptor kinases (Bychkov et al., 2011). However, to our knowledge, no work has been done exploring sex differences in nicotine behavioral sensitization specifically in the NVHL rat. Another limitation of the current study was the absence of a saline injected group which would serve to control for any handling and injection stress. While this is an important control group, many previous studies have demonstrated that repeated subcutaneous or intraperitoneal injections of saline do not increase locomotor activity (McCormick et al., 2004; Kosowski and Liljequist, 2005; Varvel et al., 2007; Marin et al., 2009; Gomez et al., 2012; Hamilton et al., 2014; Carrara-Nascimento et al., 2020; Trujillo and Heller, 2020). Additional studies corroborate these findings specifically in NVHL rats (Conroy et al., 2007; Berg and Chambers, 2008; Chambers et al., 2013). Therefore, it is highly unlikely that repeated saline injections in our hands would cause behavioral sensitization.

In this study, we found that NVHL rats demonstrated an apparent sensitization to the nicotine paired context, and adolescent alcohol exposure prevented the formation of this context sensitization in the NVHL AE rats. We found that exposure to alcohol during adolescence did not impact the amount of nicotine behavioral sensitization in adulthood. Surprisingly, NVHL rats (regardless of alcohol exposure) did not show increased nicotine behavioral sensitization as had been previously reported, potentially due to post-weaning housing conditions. Nicotine treatment had an anxiolytic effect during the postinjection phase of the challenge day, however, there were no group differences. Future studies could more specifically test the impact of social isolation on nicotine behavioral sensitization and the development of context sensitization in the NVHL rat, as well as expand this work to females.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Dartmouth College.

AUTHOR CONTRIBUTIONS

ES contributed to planning the experimental design and wrote the manuscript. ES, LL, and EB collected the behavioral data. ES and DW performed the NVHL surgeries and DW contributed to editing the manuscript. AH contributed to data analysis and editing the manuscript. EB contributed to the histological assessment. JK and WD contributed to planning the experimental design and editing the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2021.760791/full#supplementary-material

Supplementary Figure 1 | Latency to and duration in center zone. **(A)** There were no significant group differences in latency to center zone during the preinjection phase on days 1, 5, 10, and 15. **(B)** There were no significant group differences in latency to center zone during the postinjection phase on days 1, 5, 10, and 15. **(C)** There were no significant group differences in latency to center zone during preinjection phase on the challenge day. **(D)** There were no significant group differences in latency to center zone during postinjection phase on the challenge day. **(E)** There were no significant group differences in the total duration of time spent in the center zone during the preinjection phase on days 1, 5, 10, and 15. **(F)** There were no significant group differences in the total duration of time spent in the center zone during the postinjection phase on days 1, 5, 10, and 15. **(G)** There were no significant group differences in the total duration of time spent in the center zone during the preinjection phase of the challenge day. **(H)** There were no significant group differences in the total duration of time spent in the center zone during the postinjection phase of the challenge day. Data is shown as group mean ± SEM.

REFERENCES

Abdel-Baki, A., Ouellet-Plamondon, C., Salvat, E., Grar, K., and Potvin, S. (2017). Symptomatic and functional outcomes of substance use disorder persistence 2 years after admission to a first-episode psychosis program. *Psychiatry Res.* 247, 113–119. doi: 10.1016/j.psychres.2016.11.007

Arendt, T., Allen, Y., Marchbanks, R. M., Schugens, M. M., Sinden, J., Lantos, P. L., et al. (1989). Cholinergic system and memory in the rat: effects of chronic ethanol, embryonic basal forebrain brain transplants and excitotoxic lesions of cholinergic basal forebrain projection system. *Neuroscience* 33, 435–462. doi: 10.1016/0306-4522(89)90397-7

Arendt, T., Hennig, D., Gray, J. A., and Marchbanks, R. M. (1988). Loss of neurons in the rat basal forebrain cholinergic projection system after prolonged intake of Ethanol. *Brain Res. Bull.* 21, 563–570. doi: 10.1016/0361-9230(88)90193-1

Beninger, R. J., Tuerke, K. J., Forsyth, J. K., Giles, A., Xue, L., Boegman, R. J., et al. (2009). Neonatal ventral hippocampal lesions in male and female rats: effects on water maze, locomotor activity, plus-Maze and Prefrontal Cortical GABA and Glutamate Release in Adulthood. *Behav. Brain Res.* 202, 198–209. doi: 10.1016/j.bbr.2009.03.044

Berg, S. A., and Chambers, R. A. (2008). Accentuated behavioral sensitization to nicotine in the neonatal ventral hippocampal lesion model of schizophrenia. *Neuropharmacology* 54, 1201–1207. doi: 10.1016/j.neuropharm.2008.03.011

Berg, S. A., Czachowski, C. L., and Chambers, R. A. (2011). Alcohol seeking and consumption in the NVHL Neurodevelopmental Rat Model of Schizophrenia. *Behav. Brain Res.* 218, 346–349. doi: 10.1016/j.bbr.2010.12.017

Berg, S. A., Sentir, A. M., Bell, R. L., Engleman, E. A., and Chambers, R. A. (2015). Nicotine effects in adolescence and adulthood on cognition and α4β2-nicotinic receptors in the neonatal ventral hippocampal lesion rat model of schizophrenia. *Psychopharmacology* 232, 1681–1692. doi: 10.1007/s00213-014-3800-2

Berg, S. A., Sentir, A. M., Cooley, B. S., Engleman, E. A., and Chambers, R. A. (2013). Nicotine is more addictive, not more cognitively therapeutic in a neurodevelopmental model of schizophrenia produced by neonatal ventral hippocampal lesions. *Addict. Biol.* 19, 1020–1031. doi: 10.1111/adb.12082

Bevins, R. A., Besheer, J., and Pickett, K. S. (2001). Nicotine-conditioned locomotor activity in rats: dopaminergic and GABAergic influences on conditioned expression. *Pharmacol. Biochem. Behav.* 68, 135–145. doi: 10.1016/s0091-3057(00)00451-2

Blomqvist, O., Soderpalm, B., and Engel, J. A. (1992). Ethanol-induced locomotor activity: involvement of central nicotinic acetylcholine receptors? *Brain Res. Bull.* 29, 173–178. doi: 10.1016/0361-9230(92)90023-q

Booze, R. M., Welch, M. A., Wood, M. L., Billings, K. A., Apple, S. R., and Mactutus, C. F. (1999). Behavioral sensitization following repeated intravenous nicotine administration: gender differences and gonadal hormones. *Pharmacol. Biochem. Behav.* 64, 827–839. doi: 10.1016/s0091-3057(99)00169-0

Brady, A. M., Saul, R. D., and Wiest, M. K. (2010). Selective deficits in spatial working memory in the neonatal ventral hippocampal lesion rat model of schizophrenia. *Neuropharmacology* 59, 605–611. doi: 10.1016/j.neuropharm.2010.08.012

Breese, C. R., Lee, M. J., Adams, C. E., Sullivan, B., Logel, J., Gillen, K. M., et al. (2000). Abnormal regulation of high affinity nicotinic receptors in subjects with schizophrenia. *Neuropsychopharmacology* 23, 351–364. doi: 10.1016/S0893-133X(00)00121-4

Bychkov, E., Ahmed, M. R., and Gurevich, E. V. (2011). Sex differences in the activity of signalling pathways and expression of G-protein-coupled receptor kinases in the neonatal ventral hippocampal lesion model of schizophrenia. *Int. J. Neuropsychopharmacol.* 14, 1–15. doi: 10.1017/S1461145710000118

Carrara-Nascimento, P. F., Hoffmann, L. B., Flório, J. C., Planeta, C. S., and Camarini, R. (2020). Effects of ethanol exposure during adolescence or adulthood on locomotor sensitization and dopamine levels in the reward system. *Front. Behav. Neurosci.* 14:31. doi: 10.3389/fnbeh.2020.00031

Chambers, R. A., and Lipska, B. K. (2011). "A method to the madness: producing the neonatal ventral hippocampal lesion rat model of schizophrenia," in *Animal Models of Schizophrenia and Related Disorders. Neuromethods*, ed. P. O'Donnell (Totowa: Humana Press), 1–24.

Chambers, R. A., McClintick, J. N., Sentir, A. M., Berg, S. A., Runyan, M., Choi, K. H., et al. (2013). Cortical-striatal gene expression in neonatal hippocampal lesion (nvhl)-amplified cocaine sensitization. *Genes Brain Behav.* 12, 564–575. doi: 10.1111/gbb.12051

Chambers, R. A., Moore, J., McEvoy, J. P., and Levin, E. D. (1996). Cognitive effects of neonatal hippocampal lesions in a rat model of schizophrenia. *Am. Coll. Neuropsychopharmacol.* 15, 587–594. doi: 10.1016/S0893-133X(96)00132-7

Chambers, R. A., and Self, D. W. (2002). Motivational responses to natural and drug rewards in rats with neonatal ventral hippocampal lesions: an animal model of dual diagnosis schizophrenia. *Neuropsychopharmacology* 27, 889–905. doi: 10.1016/S0893-133X(02)00365-2

Cheeta, S., Irvine, E., and File, S. E. (2001). Social isolation modifies nicotine's effects in animal tests of anxiety. *Br. J. Pharmacol.* 132, 1389–1395. doi: 10.1038/sj.bjp.0703991

Conroy, S. K., Rodd, Z., and Chambers, R. A. (2007). Ethanol sensitization in a neurodevelopmental lesion model of schizophrenia in rats. *Pharmacol. Biochem. Behav.* 86, 386–394. doi: 10.1016/j.pbb.2006.07.017

Cornelius, M. E., Wang, T. W., Jamal, A., Loretan, C. G., and Neff, L. J. (2020). Tobacco Product Use Among Adults — United States, 2019. *MMWR Morb. Mortal. Wkly Rep.* 69, 1736–1742. doi: 10.15585/mmwr.mm6946a4

Cosgrove, K. P., Kloczynski, T., Bois, F., Pittman, B., Tamagnan, G., Seibyl, J. P., et al. (2010). Decreased Beta2*-nicotinic acetylcholine receptor availability after chronic ethanol exposure in nonhuman primates. *Synapse* 64, 729–732. doi: 10.1002/syn.20795

Crews, F. T., Braun, C. J., Hoplight, B., Switzer, R. C., and Knapp, D. J. (2000). Binge Ethanol consumption causes differential brain damage in young adolescent rats compared with adult rats. *Alcohol. Clin. Exp. Res.* 24, 1712–1723.

De Bellis, M. D., Clark, D. B., Beers, S. R., Soloff, P. H., Boring, A. M., Hall, J., et al. (2000). Hippocampal volume in adolescent-onset alcohol use disorders. *Am. J. Psychiatry* 157, 737–744. doi: 10.1176/appi.ajp.157.5.737

De Bellis, M. D., Narasimhan, A., Thatcher, D. L., Keshavan, M. S., Soloff, P., and Clark, D. B. (2005). Prefrontal cortex, thalamus, and cerebellar volumes in adolescents and young adults with adolescent-onset alcohol use disorders and comorbid mental disorders. *Alcohol. Clin. Exp. Res.* 29, 1590–1600. doi: 10.1097/01.alc.0000179368.87886.76

de Fiebre, C. M., and Collins, A. C. (1993). A comparison of the development of tolerance to ethanol and cross-tolerance to nicotine after chronic Ethanol

treatment in long-and short-sleep mice. *J. Pharmacol. Exp. Ther.* 266, 1398–1406.

de Leon, J., and Diaz, F. J. (2005). A meta-analysis of worldwide studies demonstrates an association between schizophrenia and tobacco smoking behaviors. *Schizophr. Res.* 76, 135–157. doi: 10.1016/j.schres.2005.02.010

Depp, C. A., Bowie, C. R., Mausbach, B. T., Wolyniec, P., Thornquist, M. H., Luke, J. R., et al. (2015). Current smoking is associated with worse cognitive and adaptive functioning in serious mental illness. *Acta Pscyhiatr. Scand.* 131, 333–341. doi: 10.1111/acps.12380

Dickerson, F., Origoni, A., Schroeder, J., Adamos, M., Katsafanas, E., Khushalani, S., et al. (2018). Natural cause mortality in persons with serious mental illness. *Acta Pscyhiatr. Scand.* 137, 371–379. doi: 10.1111/acps.12880

D'Souza, M. S., and Markou, A. (2011). Neuronal mechanisms underlying development of nicotine dependence: implications for novel smoking-cessation treatments. *Addict. Sci. Clin. Pract.* 6, 4–16.

Durany, N., Zochling, R., Boissl, K. B., Paulus, W., Ransmayr, G., Tatschner, T., et al. (2000). Human post-mortem striatal A4b2 Nicotinic acetylcholine receptor density in schizophrenia and Parkinson's syndrome. *Neurosci. Lett.* 287, 109–112. doi: 10.1016/s0304-3940(00)01144-7

Ellickson, P. L., Tucker, J. S., and Klein, D. J. (2003). Ten-year prospective study of public health problems associated with early drinking. *Pediatrics* 111, 949–955. doi: 10.1542/peds.111.5.949

Ericson, M., Norrsjö, G., and Svensson, A. I. (2010). Behavioral Sensitization to Nicotine in Female and Male Rats. *J. Neural Transm.* 117, 1033–1039. doi: 10.1007/s00702-010-0449-9

Esterlis, I., Ranganathan, M., Bois, F., Pittman, B., Picciotto, M. R., Shearer, L., et al. (2014). In Vivo evidence for β_2 Nicotinic acetylcholine receptor subunit upregulation in smokers as compared with nonsmokers with schizophrenia. *Biol. Psychiatry* 76, 495–502. doi: 10.1016/j.biopsych.2013.11.001

Fergusson, D. M., Horwood, L. J., and Swain-Campbell, N. R. (2003). Cannabis dependence and psychotic symptoms in young people. *Psychol. Med.* 33, 15–21. doi: 10.1017/s0033291702006402

Freedman, R., Hall, M., Adler, L. E., and Leonard, S. (1995). Evidence in postmortem brain tissue for decreased numbers of hippocampal nicotinic receptors in schizophrenia. *Biol. Psychiatry* 38, 22–33. doi: 10.1016/0006-3223(94)00252-X

Gage, S. H., and Munafo, M. R. (2015). Rethinking the association between smoking and schizophrenia. *Lancet Psychiatry* 2, 118–119. doi: 10.1016/S2215-0366(14)00057-1

Garcia, E. J., Haddon, T. N., Saucier, D. A., and Cain, M. E. (2017). Differential housing and novelty response: protection and risk from locomotor sensitization. *Pharmacol. Biochem. Behav.* 154, 20–30. doi: 10.1016/j.pbb.2017.01.004

Gomez, A. M., Midde, N. M., Mactutus, C. F., Booze, R. M., and Zhu, J. (2012). Environmental enrichment alters nicotine-mediated locomotor sensitization and phosphorylation of DARPP-32 and CREB in rat prefrontal cortex. *PLoS One* 7:e44149. doi: 10.1371/journal.pone.0044149

Grant, J. D., Scherrer, J. F., Lynskey, M. T., Lyons, M. J., Eisen, S. A., Tsuang, M. T., et al. (2006). Adolescent alcohol use is a risk factor for adult alcohol and drug dependence: evidence from a twin design. *Psychol. Med.* 36, 109–118. doi: 10.1017/S0033291705006045

Green, A. I., Noordsy, D. L., Brunette, M. F., and O'Keefe, C. (2008). Substance abuse and schizophrenia: pharacotherapeutic intervention. *J. Subst. Abuse Treat.* 34, 61–71. doi: 10.1016/j.jsat.2007.01.008

Green, A. I., Zimmet, S. V., Strous, R. D., and Schildkraut, J. J. (1999). Clozapine for comorbid substance use disorder and schizophrenia: do patients with schizophrenia have a reward-deficiency syndrome that can be ameliorated by clozapine? *Harv. Rev. Psychiatry* 6, 287–296. doi: 10.3109/10673229909017206

Gruber, A. J., Calhoon, G. G., Shusterman, I., Schoenbaum, G., Roesch, M. R., and O'Donnell, P. (2010). More is less: a disinhibited prefrontal cortex impairs cognitive flexibility. *J. Neurosci.* 30, 17102–17110. doi: 10.1523/JNEUROSCI.4623-10.2010

Hamilton, K. R., Elliott, B. M., Berger, S. S., and Grunberg, N. E. (2014). Environmental enrichment attenuates nicotine behavioral sensitization in male and female rats. *Exp. Clin. Psychopharmacol.* 22, 356–363. doi: 10.1037/a0037205

Harrod, S. B., Mactutus, C. F., Bennett, K., Hasselrot, U., Wu, G., Welch, M., et al. (2004). Sex differences and repeated intravenous nicotine: behavioral sensitization and dopamine receptors. *Pharmacol. Biochem. Behav.* 78, 581–592. doi: 10.1016/j.pbb.2004.04.026

Higgins, S. T., Kurti, A. N., Redner, R., White, T. J., Gaalema, D. E., Roberts, M. E., et al. (2015). A literature review on prevalence of gender differences and intersections with other vulnerabilities to tobacco use in the United States, 2004-2014. *Prevent. Med.* 80, 89–100. doi: 10.1016/j.ypmed.2015.06.009

Irvine, E. E., Cheeta, S., and File, S. E. (2001). Tolerance to Nicotine's effects in the elevated plus-maze and increased anxiety during withdrawal. *Pharmacol. Biochem. Behav.* 68, 319–325. doi: 10.1016/s0091-3057(00)00449-4

Jeanblanc, J., Balguerie, K., Coune, F., Legastelois, R., Jeanblanc, V., and Naassila, M. (2015). Light alcohol intake during adolescence induces alcohol addiction in a neurodevelopmental model of schizophrenia. *Addict. Biol.* 20, 490–499. doi: 10.1111/adb.12146

Johnston, L. D., Miech, R. A., O'Malley, P. M., Bachman, J. G., Schulenberg, J. E., and Patrick, M. E. (2020). *Monitoring the Future National Survey Results on Drug Use 1975-2019: Overview, Key Findings on Adolescence Drug Use*. Ann Arbor: University of Michigan.

Kagabo, R., Kim, J., Zubieta, J., Kleinschmit, K., and Okuyemi, K. (2019). Association between smoking, and hospital readmission among inpatients with psychiatric illness at an academic inpatient psychiatric facility, 2000–2015. *Addict. Behav. Rep.* 9:100181. doi: 10.1016/j.abrep.2019.100181

Kalivas, P. W., and Stewart, J. (1991). Dopamine transmission in the initiation and expression of drug-and stress-induced sensitization of motor activity. *Brain Res. Rev.* 16, 223–244. doi: 10.1016/0165-0173(91)90007-u

Kanyt, L., Stolerman, I. P., Chandler, C. J., Saigusa, T., and Pögun, Ş (1999). Influence of sex and female hormones on nicotine-induced changes in locomotor activity in rats. *Pharmacol. Biochem. Behav.* 62, 179–187. doi: 10.1016/s0091-3057(98)00140-3

Kelly, C., and McCreadie, R. G. (1999). Smoking habits, current symptoms, and premorbid characteristics of schizophrenic patients in Nithsdale, Scotland. *Am. J. Psychiatry* 156, 1751–1757. doi: 10.1176/ajp.156.11.1751

Kendler, K. S., Lönn, S. L., Sundquist, J., and Sundquist, K. (2015). Smoking and schizophrenia in population cohorts of swedish women and men: a prospective co-relative control study. *Am. J. Psychiatry* 172, 1092–1100. doi: 10.1176/appi.ajp.2015.15010126

Khokhar, J. Y., Dwiel, L. L., Henricks, A. M., Doucette, W. T., and Green, A. I. (2017). The link between schizophrenia and substance use disorder: a unifying hypothesis. *Schizophr. Res.* 194, 78–85. doi: 10.1016/j.schres.2017.04.016

Khokhar, J. Y., and Todd, T. P. (2017). Behavioral Predictors of Alcohol Drinking in a Neurodevelopmental Rat Model of Schizophrenia and Co-Occurring Alcohol Use Disorder. *Schizophr. Res.* 194, 91–97. doi: 10.1016/j.schres.2017.02.029

Kosowski, A. R., and Liljequist, S. (2005). Behavioural sensitization to nicotine precedes the onset of nicotine-conditioned locomotor stimulation. *Behav. Brain Res.* 156, 11–17. doi: 10.1016/j.bbr.2004.05.004

Laplante, F., Srivastava, L. K., and Quirion, R. (2004a). Alterations in dopaminergic modulation of prefrontal cortical acetylcholine release in post-pubertal rats with neonatal ventral hippocampal lesions. *J. Neurochem.* 89, 314–323. doi: 10.1111/j.1471-4159.2004.02351.x

Laplante, F., Stevenson, C. W., Gratton, A., Srivastava, L. K., and Quirion, R. (2004b). Effects of neonatal ventral hippocampal lesion in rats on stress-induced acetylcholine release in the prefrontal cortex franc. *J. Neurochem.* 91, 1473–1482. doi: 10.1111/j.1471-4159.2004.02831.x

Lipska, B. K., Jaskiw, G. E., and Weinberger, D. R. (1993). Postpubertal emergence of hyperresponsiveness to stress and to amphetamine after neonatal excitotoxic hippocampal damage: a potential animal model of schizophrenia. *Neuropsychopharmacology* 9, 67–75. doi: 10.1038/npp.1993.44

Lipska, B. K., Swerdlow, N. R., Geyer, M. A., Jaskiw, G. E., Braff, D. L., and Weinberger, D. R. (1995). Neonatal excitotoxic hippocampal damage in rats causes post-pubertal changes in prepulse inhibition of startle and its disruption by apomorphine. *Psychopharmacology* 122, 35–43. doi: 10.1007/BF02246439

Mallet, J., le Strat, Y., Schürhoff, F., Mazer, N., Portalier, C., Andrianarisoa, M., et al. (2019). Tobacco smoking is associated with antipsychotic medication, physical aggressiveness, and alcohol use disorder in schizophrenia: results from the FACE-SZ national cohort. *Eur. Arch. Psychiatry Clin. Neurosci.* 269, 449–457. doi: 10.1007/s00406-018-0873-7

Marin, M. T., Berkow, A., Golden, S. A., Koya, E., Planeta, C. S., and Hope, B. T. (2009). Context-specific modulation of cocaine-induced locomotor sensitization and ERK and CREB phosphorylation in the rat nucleus

accumbens. *Eur. J. Neurosci.* 30, 1931–1940. doi: 10.1111/j.1460-9568.2009.06982.x

Mathews, I. Z., Mills, R. G., and McCormick, C. M. (2008). Chronic social stress in adolescence influenced both amphetamine conditioned place preference and locomotor sensitization. *Dev. Psychobiol.* 50, 451–459. doi: 10.1002/dev.20299

McCormick, C. M. (2010). An animal model of social instability stress in adolescence and risk for drugs of abuse. *Physiol. Behav.* 99, 194–203. doi: 10.1016/j.physbeh.2009.01.014

McCormick, C. M., Robarts, D., Gleason, E., and Kelsey, J. E. (2004). Stress during adolescence enhances locomotor sensitization to nicotine in adulthood in female, but not male, rats. *Horm. Behav.* 46, 458–466. doi: 10.1016/j.yhbeh.2004.05.004

McGinty, E. E., Zhang, Y., Guallar, E., Ford, D. E., Steinwachs, D., Dixon, L. B., et al. (2012). Cancer incidence in a sample of Maryland residents with serious mental illness. *Psychiatric Serv.* 63, 714–717. doi: 10.1176/appi.ps.201100169

Medina, K. L., Schweinsburg, A. D., Cohen-Zion, M., Nagel, B. J., and Tapert, S. F. (2007). Effects of alcohol and combined marijuana and alcohol use during adolescence on hippocampal volume and asymmetry. *Neurotoxicol. Teratol.* 29, 141–152. doi: 10.1016/j.ntt.2006.10.010

Nagel, B. J., Schweinsburg, A. D., Phan, V., and Tapert, S. F. (2005). Reduced hippocampal volume among adolescents with alcohol use disorders without psychiatric comorbidity. *Psychiatry Res.* 139, 181–190. doi: 10.1016/j.pscychresns.2005.05.008

Ohi, K., Shimada, T., Kuwata, A., Kataoka, Y., Okubo, H., Kimura, K., et al. (2018). Smoking rates and number of cigarettes smoked per day in schizophrenia: a large cohort meta-analysis in a Japanese population. *Int. J. Neuropsychopharmacol.* 22, 19–27. doi: 10.1093/ijnp/pyy061

Onaivi, E. S., Payne, S., Brock, J. W., Hamdi, A., Faroouqui, S., and Prasad, C. (1994). Chronic nicotine reverses age-associated increases in tail-flick latency and anxiety in rats. *Life Sci.* 54, 193–202. doi: 10.1016/0024-3205(94)00588-5

Placek, K., Dippel, W. C., Jones, S., and Brady, A. M. (2013). Impairments in set-shifting but not reversal learning in the neonatal ventral hippocampal lesion model of schizophrenia: further evidence for medial prefrontal deficits. *Behav. Brain Res.* 256, 405–413. doi: 10.1016/j.bbr.2013.08.034

Rao, K. N., Sentir, A. M., Engleman, E. A., Bell, R. L., Hulvershorn, L. A., Breier, A., et al. (2016). Toward early estimation and treatment of addiction vulnerability: radial arm maze and N-Acetyl cysteine before cocaine sensitization or nicotine self-administration in neonatal ventral hippocampal lesion rats. *Psychopharmacology* 233, 3933–3945. doi: 10.1007/s00213-016-4421-8

Ribeiro-Carvalho, A., Lima, C. S., Medeiros, A. H., Siqueira, N. R., Filgueiras, C. C., Manhães, A. C., et al. (2009). Combined exposure to nicotine and ethanol in adolescent mice: effects on the central cholinergic systems during short and long term withdrawal. *Neuroscience* 162, 1174–1186. doi: 10.1016/j.neuroscience.2009.05.032

Robinson, T. E., and Berridge, K. C. (1993). The Neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Res. Rev.* 18, 247–291. doi: 10.1016/0165-0173(93)90013-P

Robinson, T. E., and Berridge, K. C. (2008). The incentive sensitization theory of addiction: some current issues. *Philos. Trans. R. Soc. B.* 363, 3137–3146. doi: 10.1098/rstb.2008.0093

Ryan, S. A., Kokotailo, P., and Committee on Substance Use and Prevention (2019). Alcohol use by Youth. *Pediatrics* 144:e20191357. doi: 10.1542/peds.2019-1357

Sams-Dodd, F., Lipska, B. K., and Weinberger, D. R. (1997). Neonatal lesions in the rat ventral hippocampus result in hyperlocomotion and deficits in social behaviour in adulthood. *Psychopharmacology* 132, 303–310. doi: 10.1007/s002130050349

Sentir, A. M., Bell, R. L., Engleman, E. A., and Chambers, R. A. (2020). Polysubstance addiction vulnerability in mental illness: concurrent alcohol and nicotine self-administration in the neurodevelopmental hippocampal lesion rat model of schizophrenia. *Addict. Biol.* 25:e12704. doi: 10.1111/adb.12704

Smith, J. K., Neill, J. C., and Costall, B. (1997). Post-weaning housing conditions influence the behavioural effects of Cocaine and d-Amphetamine. *Psychopharmacology* 131, 23–33. doi: 10.1007/s002130050261

Spear, L. P. (2015). Adolescent alcohol exposure: are there separable vulnerable periods within adolescence? *Physiol. Behav.* 148, 122–130. doi: 10.1016/j.physbeh.2015.01.027

Spear, L. P. (2016). Consequences of adolescent use of alcohol and other drugs: studies using rodent models. *Neurosci. Biobehav. Rev.* 70, 228–243. doi: 10.1016/j.neubiorev.2016.07.026

Steketee, J. D., and Kalivas, P. W. (2011). Drug wanting: behavioral sensitization and relapse to drug-seeking behavior. *Pharmacol. Rev.* 63, 348–365. doi: 10.1124/pr.109.001933

Substance Abuse and Mental Health Services Administration [SAMHSA] (2020). *Key Substance Use and Mental Health Indicators in the United States: Results from the 2019 National Survey on Drug Use and Health*. Rockville: SAMHSA.

Thayer, R. E., Crotwell, S. M., Callahan, T. J., Hutchison, K. E., and Bryan, A. D. (2012). Nucleus accumbens volume is associated with frequency of alcohol use among juvenile justice-involved adolescents. *Brain Sci.* 2, 605–618. doi: 10.3390/brainsci2040605

Tran, E., Rouillon, F., Loze, J. Y., Casadebaig, F., Philippe, A., Vitry, F., et al. (2009). Cancer mortality in patients with schizophrenia: an 11-year prospective cohort study. *Cancer* 115, 3555–3562. doi: 10.1002/cncr.24383

Trujillo, K. A., and Heller, C. Y. (2020). Ketamine sensitization: influence of dose, environment, social isolation and treatment interval. *Behav. Brain Res.* 378:112271. doi: 10.1016/j.bbr.2019.112271

Varvel, S. A., Martin, B. R., and Lichtman, A. H. (2007). Lack of behavioral sensitization after repeated exposure to THC in Mice and comparison to methamphetamine. *Psychopharmacology* 193, 511–519. doi: 10.1007/s00213-007-0811-2

Volkow, N. D. (2009). Substance use disorders in schizophrenia–clinical implications of comorbidity. *Schizophr. Bull.* 35, 469–472. doi: 10.1093/schbul/sbp016

Walter, S., and Kuschinsky, K. (1989). Conditioning of nicotine effects on motility and behaviour in rats. *Naunyn Schmiedebergs Arch. Pharmacol.* 339, 208–213. doi: 10.1007/BF00165145

Wilson, S., Malone, S. M., Thomas, K. M., and Iacono, W. G. (2015). Adolescent drinking and brain morphometry: a co-twin control analysis. *Dev. Cogn. Neurosci.* 16, 130–138. doi: 10.1016/j.dcn.2015.07.005

Wood, G. K., Quirion, R., and Srivastava, L. K. (2003). Early environment contributes to developmental disruption of MPFC After neonatal ventral hippocampal lesions in rats. *Synapse* 50, 223–232. doi: 10.1002/syn.10265

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High-Salt Diet in the Pre- and Postweaning Periods Leads to Amygdala Oxidative Stress and Changes in Locomotion and Anxiety-Like Behaviors of Male Wistar Rats

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High-salt (HS) diets have recently been linked to oxidative stress in the brain, a fact that may be a precursor to behavioral changes, such as those involving anxiety-like behavior. However, to the best of our knowledge, no study has evaluated the amygdala redox status after consuming a HS diet in the pre- or postweaning periods. This study aimed to evaluate the amygdala redox status and anxiety-like behaviors in adulthood, after inclusion of HS diet in two periods: preconception, gestation, and lactation (preweaning); and only after weaning (postweaning). Initially, 18 females and 9 male Wistar rats received a standard (n = 9 females and 4 males) or a HS diet (n = 9 females and 5 males) for 120 days. After mating, females continued to receive the aforementioned diets during gestation and lactation. Weaning occurred at 21-day-old Wistar rats and the male offspring were subdivided: control-control (C-C)—offspring of standard diet fed dams who received a standard diet after weaning (n = 9–11), control-HS (C-HS)—offspring of standard diet fed dams who received a HS diet after weaning (n = 9–11), HS-C—offspring of HS diet fed dams who received a standard diet after weaning (n = 9–11), and HS-HS—offspring of HS diet fed dams who received a HS diet after weaning (n = 9–11). At adulthood, the male offspring performed the elevated plus maze and open field tests. At 152-day-old Wistar rats, the offspring were euthanized and the amygdala was removed for redox state analysis. The HS-HS group showed higher locomotion and rearing frequency in the open field test. These results indicate that this group developed hyperactivity. The C-HS group had a higher ratio of entries and time spent in the open arms of the elevated plus maze test in addition to a higher head-dipping frequency. These results suggest less anxiety-like behaviors. In the analysis of

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the redox state, less activity of antioxidant enzymes and higher levels of the thiobarbituric acid reactive substances (TBARS) in the amygdala were shown in the amygdala of animals that received a high-salt diet regardless of the period (pre- or postweaning). In conclusion, the high-salt diet promoted hyperactivity when administered in the pre- and postweaning periods. In animals that received only in the postweaning period, the addition of salt induced a reduction in anxiety-like behaviors. Also, regardless of the period, salt provided amygdala oxidative stress, which may be linked to the observed behaviors.

Keywords: high-sodium, open-field, elevated plus-maze, pre-natal, post-natal, redox state

INTRODUCTION

Sodium chloride (NaCl), also known worldwide as salt, is one of the most widely used condiments in food processing (Steffensen et al., 2018). It is estimated that current salt intake averages are 6 g/day in most countries (86% greater than the optimal amount), with varying usages ranging from food preservation to flavor enhancement (Afshin et al., 2019; Tan et al., 2021). Excessive use of salt in the diet is responsible for the development mainly of cardiovascular diseases (Huang et al., 2020; Neal et al., 2021), but also stomach cancer (Ge et al., 2012), kidney diseases (Garofalo et al., 2018), and osteoporosis (Fatahi et al., 2018). Moreover, recent data indicates that high-salt diets were directly related to approximately three million deaths in 1 year, being classified as one of the top 3 dietary risk factors for health (Bill and Foundation, 2019; He et al., 2020).

In addition to the known harmful health effects, the use of high-salt diets has recently been linked to cerebrovascular diseases and cognitive impairment in humans (Heye et al., 2016). Studies in rodents that used dietary or water salt supplementation (2–8%) confirm these findings, reporting impaired cognition, aggravation of cerebral ischemic injury, and high-stress responsivity (Ge et al., 2017; Faraco et al., 2018, 2019; Mitchell et al., 2018; Gilman et al., 2019a; Zhang et al., 2020). Importantly, preclinical studies suggest that the maternal high-salt diet can also induce changes in locomotion, inhibition, and anxiety in the offspring, when fed in the preconception, gestation, or lactation periods (Mcbride et al., 2008; Mecawi and Almeida, 2017; Dingess et al., 2018). During these periods, the offspring is highly susceptible to dietary salt, which may impact on development, potentially leading to lifelong changes in metabolism and behavior. These changes are related to the Developmental Origin of Health and Disease (DOHaD), which proposes that adversities in early life can result in persistent changes in physiology, leading to an increased risk of developing diseases in adulthood (O'Donnell and Meaney, 2016; Klein et al., 2018; de Souza et al., 2020a).

One of the main possible mechanisms for behavioral changes caused by salt consumption is related to the oxidative stress (Santisteban and Iadecola, 2018; He et al., 2020). Evidence indicates that a high-salt diet can reduce nitric oxide (NO) production (Dong et al., 2011; Kouyoumdzian et al., 2016; Zheng et al., 2019), suppress the activity of antioxidant enzymes (Kitiyakara et al., 2003; Huang et al., 2017), and increase the production of nitrogen and oxygen-free radicals (Kitiyakara et al., 2003; Huang et al., 2017; Zheng et al., 2019). Also, it is highlighted that a high-salt diet causes oxidative stress in the hippocampus, hypothalamus, and cerebellum, important brain regions for behavior and cognition (Bai et al., 2017; Ge et al., 2017; Stocher et al., 2018). However, to the best of our knowledge, there are no studies evaluating the amygdala redox status after administration of a high-salt diet, either before weaning (preweaning) or after weaning (postweaning). Noteworthy, the amygdala is a major brain region in the interpretation of environmental threats, possibly related to anxiety-like and fear behaviors in rodents (Calhoon and Tye, 2015; Wilson et al., 2015; dos Santos et al., 2017).

Therefore, this study aimed to evaluate the effects of the high-salt diet on amygdala redox status and anxiety-like behaviors at adulthood, considering: (1) the inclusion of the salt in the preconception, gestation, and lactation periods (preweaning) and (2) the addition of salt in the diet only after weaning until adulthood (postweaning). The main hypothesis was that the high-salt diet may result in amygdala oxidative stress regardless of the period, which, in turn, would promote changes in anxiety-like behaviors at adulthood.

MATERIALS AND METHODS

Ethics

This experimental protocol was approved by the Ethics Committee on the Use of Animals of Universidade Federal dos Vales do Jequitinhonha e Mucuri (CEUA-UFVJM) (protocol 025/2018). These are also in agreement to the ethical principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). All the rats (Wistar—*Rattus norvegicus*) were obtained from Laboratório de Pós-Graduação e Pesquisa (LPP-UFVJM) and housed in conditions of natural moisture, temperature of 22 ± 2°C (controlled by an air conditioner), and a 12-h cycle of light and darkness, with the light cycle beginning at 7:00 am. All the animals had free access to potable water and their respective diets.

Experimental Design

Initially, 18 female and 9 male Wistar rats aged 21 days were used. The animals were housed in 3 per box according to sex in order to randomly receive the diets for a duration of

120 days: **control (C)**: received standard diet (laboratory chow for rodents: Nuvilab® CR-1, Quimtia S/A, Paraná, Brazil) (n = 9 females and 4 males) or **high-salt (HS) diet**: received laboratory chow with added salt (4% NaCl non-iodized, Mossoró®—purity 96.04% bought at the local store) (n = 9 females and 5 males). Copulation was evaluated every morning and confirmed by the presence of sperm in the vaginal smear, which was considered the beginning of gestation. All the animals received food and water *ad libitum*. After this period, the nulliparous female rats (141 days old) were placed for mating with males (1 male to 3 females) during the dark cycle (7:00 pm to 7:00 am) every day. Parents during mating (males and females) and dams during gestation and lactation continued receiving the aforementioned diets (control or HS). At birth, the litters were culled to eight pups (6 males and 2 females).

In the postweaning period, only male offspring were used, housed 3 animals per box. Male offspring was randomly allocated to receive either control (laboratory chow Nuvilab® CR-1) or HS diets (laboratory chow with added salt 4% NaCl non-iodized). Therefore, the offspring were subdivided into the following groups: **control-control (C-C)**—offspring of standard diet fed dams who received a standard diet after weaning (n = 9–11), **control-high-salt (C-HS)**—offspring of standard diet fed dams who received a HS diet (laboratory chow with added salt at 4% NaCl non-iodized) after weaning (n = 9–11), **HS-C**—offspring of HS diet fed dams who received a standard diet after weaning (n = 9–11), and **HS-HS**—offspring of HS diet fed dams who received a HS diet (laboratory chow with added salt at 4% NaCl non-iodized) after weaning (n = 9–11).

The male offspring received the aforementioned diets until adulthood (141 day-old), when behavioral tests were carried out. Approximately, 1–2 animals from each litter were used for the behavioral and redox status analyses, in order to reduce litter effects. The experimental design is shown in **Figure 1**.

Offspring Behavior

All the tests were performed in an isolated room (130 lux) and in a double-blind manner. The offspring performed the elevated plus maze (EPM) (141 day-old) and open field (OF) (151 day-old) tests, both during the morning period (7:00–12:00 am). A camera (Sony Handycam®) was positioned above the arena and two independent, blinded, and experienced studies later evaluated the randomly arranged videos. Between the performances of the two behavioral tests (EPM and OF), the animals were kept with their respective diets in the conditions mentioned previously. All the equipment used was cleaned with 70% ethanol between each test to eliminate olfactory cues.

The EPM test is based on the aversion to open and high spaces of the rodents and is a classic test for assessing anxiety-like behaviors (Pellow et al., 1985; de Souza et al., 2020b). The EPM is made of wood, with two closed arms (50 cm × 10 cm × 40 cm) perpendicular to two open arms (50 cm × 10 cm), besides a central area (10 cm × 10 cm), raised 50 cm high from the floor. Each rat was placed individually in the central area of the EPM with its head facing toward one closed arm and its movements were filmed for 10 min (Teixeira et al., 2020). The ratio of entries (considered as the animal inserting all the four paws) in each arm (closed or open) and the time spent in them were evaluated (Teixeira et al., 2020). In addition, to analyze the risk assessment of animal, the frequency of head-dipping (the head flexes below the edge of the open arms), rearing (frequency with which the animal stands on its hind legs), and grooming (frequency of time which the animal spent licking or scratching itself while stationary) was recorded (Plescia et al., 2015; Guedine et al., 2018; Riul and Almeida, 2020).

The OF test is widely used to check locomotion of animal through distance covered, but is also used to evaluate anxiety-like behaviors over the conflict between exploring a new environment and exposed to an open arena (Montgomery, 1955). The OF is a square wooden arena, with total dimensions of 70 cm × 70 cm × 50 cm (dimensions of central zone of the arena: 35 cm × 35 cm), being subdivided into 16 quadrants (17.5 cm × 17.5 cm). Each animal was placed in the center of the OF and free exploration was allowed for 10 min (Teixeira et al., 2020). The parameters of center zone entries frequency (defined when the animal inserted the four paws in the central zone), time spent in the center zone, distance covered (quadrants), rearing, and grooming frequency were observed (Teixeira et al., 2020; Rocha-Gomes et al., 2021a).

Redox State

The animals were euthanized by decapitation when they were at 152 day-old. The whole brain was rapidly removed (<1 min) and submerged on cold (4°C) phosphate-buffered saline (PBS) (50 mM; pH 7.0), followed by the amygdala dissection (Paxinos and Watson, 2014). After, the tissues were homogenized in cold PBS (4°C; 50 mM; pH 7.0) and centrifuged at 750 × g for 10 min at 4°C (Melo et al., 2019). Both the sides of the amygdala were used for the analysis of the total antioxidant capacity, activity of antioxidant enzymes, and oxidative stress marker.

The total antioxidant capacity was evaluated using the ferric reducing antioxidant power (FRAP) method (Benzie and Strain, 1996). The assay is based on the ability of the antioxidant compounds of the sample to reduce the ferric-tripyridyltriazine complex to ferrous tripyridyltriazine, monitored at 550 nm. Ferrous sulfate ($FeSO_4$) was used as standard and the results were reported as nM of $FeSO_4$/mg protein (Freitas et al., 2019).

For the activity of the antioxidant enzyme superoxide dismutase (SOD), a solution containing 50 mM potassium dihydrogen phosphate (KH_2PO_4) and 1 mM diethylene-triamine-pentaacetic acid (DTPA) was added to the tissue homogenate. Following this, 0.2 mM of pyrogallol was added and its oxidation was measured at 420 nm for 250 s at interval of 10 s. The results were defined as one unit (U) of SOD per mg protein in the sample (U/mg protein) (Marklund and Marklund, 1974; Melo et al., 2019).

Catalase (CAT) activity was assessed by metabolizing hydrogen peroxide (Nelson and Kiesow, 1972). To perform this test, 5 μl of hydrogen peroxide (0.3 M) was added to a solution containing potassium phosphate buffer (50 mM; pH 7.0; 25°C) and 30 μl of sample. The readings were performed in a microplate reader every 15 s for 1 min (at 25°C). CAT activity was expressed in ΔE/min/mg of protein (Freitas et al., 2019).

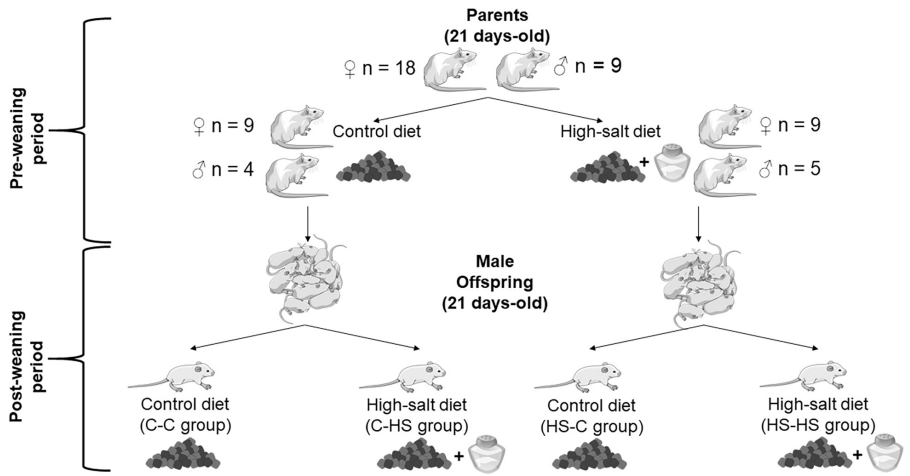

FIGURE 1 | Representation of the experimental design. Control (standard diet; laboratory chow Nuvilab® CR-1) or high-salt (HS) (laboratory chow with added salt 4% NaCl non-iodized) diets were provided for parents at 21 day-old. They received their respective diets for 120 days, until they reached adulthood. During the periods of mating, gestation, and lactation, the dams remained on the aforementioned diets. The preconception, gestation, and lactation periods were classified as "preweaning." At 21 day-old, the offspring were weaned and received until adulthood the control or HS diets. The period from weaning to adulthood was classified as "postweaning." Therefore, the offspring were subdivided into the groups: control-control (C-C), offspring of standard diet fed dams who received a standard diet after weaning; control-HS (C-HS), offspring of standard diet fed dams who received a HS diet after weaning; HS-C, offspring of HS diet fed dams who received a standard diet after weaning; and HS-HS, offspring of HS diet fed dams who received a HS diet after weaning.

Glutathione S-transferase (GST) activity was estimated spectrophotometrically as previously described (Habig et al., 1974). The assay occurred according to the formation of glutathione conjugated with 2,4-dinitrochlorobenzene (molar coefficient extinction: ε340 = 9.6 mmol × L^{-1} × cm^{-1}). One unit of GST activity was defined as the amount of the enzyme that catalyzed the formation of one μmol of product × min^{-1} × mL^{-1} (Rocha-Gomes et al., 2021a).

The lipid peroxidation evaluation was performed using the thiobarbituric acid reactive substances (TBARS) method and is classified as an oxidative stress marker (Ohkawa et al., 1979). A solution containing acetic acid (2.5 M; pH 3.4), thiobarbituric acid (0.8%), and sodium dodecyl sulfate (8.1%) was added to the tissue sample for 90 min at 95°C. The TBARS formation was evaluated at 532 nm using malondialdehyde (MDA) (1,1,3,3-tetramethoxypropane) as the standard. The results are expressed in nmol MDA/mg protein (Freitas et al., 2019).

All the redox analyses were performed in triplicate using a plate reader (UV/Visible U-200 L Spectrophotometer). Protein content was quantified using bovine serum albumin (BSA) (1 mg/ml) as the standard (Bradford, 1976). The results of the redox state were corrected for the amount of protein in the samples.

Statistical Analysis

Statistical analysis was performed with Statistica software (version 10.0, StatSoft®, Hamburg, Germany). Graphics were made using the GraphPad Prism® version 7.0 (GraphPad, La Jolla, CA, United States). Sample normality was evaluated using the Shapiro–Wilk test. Data with normal distribution were analyzed using the two-way ANOVA, with the factors: preweaning (received standard or HS diets until weaning) and postweaning (received standard or HS diets only after weaning). The Newman–Keuls was used as a *post hoc* test when appropriate ($p < 0.05$). Data with non-normal distributions were analyzed by the Kruskal–Wallis test with the Dunn's *post hoc* test. Results are expressed as a mean and SEM.

RESULTS

In the EPM test, the ratio of entries in the open arms showed a significant difference in the preweaning factor [$F_{PRE(1,36)} = 9.19$, $p < 0.01$]. The offspring who received a HS diet until weaning entered less in the open arms compared to the offspring of standard diet fed dams ($p < 0.01$). In addition, an interaction in the factors pre- and postweaning was observed [$F_{PRE \times POST(1,36)} = 4.93$, $p < 0.05$]. The C-HS group showed higher ratio of entries in the open arms compared to the C-C ($p < 0.05$), HS-C ($p < 0.01$), and HS-HS ($p < 0.01$) groups (**Figure 2A**). Similarly, the ratio of time spent in the open arms showed a difference in the preweaning factor [$F_{PRE(1,36)} = 4.36$, $p < 0.05$]. The offspring who received a HS diet until weaning spent less time in the open arms compared to the offspring of standard diet fed dams ($p < 0.05$). Also, an interaction in the factors pre- and postweaning was observed [$F_{PRE \times POST(1,36)} = 4.28$, $p < 0.05$]. The C-HS group spent more time in the open arms in relation to the C-C ($p < 0.05$) and HS-HS ($p < 0.05$) groups (**Figure 2B**). For the head-dipping frequency, a significant difference in the preweaning factor could be seen [$F_{PRE(1,36)} = 12.52$, $p < 0.01$]. The offspring who received a HS diet until weaning showed lower head-dipping frequency compared to the offspring of standard diet fed dams ($p < 0.01$). Moreover, a difference in the interaction of pre- and postweaning factors was observed

[$F_{PRE \times POST(1,36)} = 1.97$, $p < 0.05$]. The C-HS group performed head-dipping more frequently compared to the HS-C ($p < 0.01$) and HS-HS ($p < 0.01$) groups (**Figure 2C**). No differences were found in the evaluation of rearing ($p = 0.06$) and grooming frequency ($p = 0.73$) in the EPM test (**Figures 2D,E**).

In the evaluation of the time spent in the OF central zone, a difference was found with respect to the preweaning diet [$F_{PRE(1,32)} = 5.12$, $p < 0.05$]. The offspring who received a HS diet until weaning remained more time in the central zone of the OF test compared to the offspring of standard diet fed dams ($p < 0.05$) (**Figure 3B**). The total distance covered in the OF test showed a difference in the interaction of pre- and postweaning diets [$F_{PRE \times POST(1,32)} = 16.59$, $p < 0.01$]. The HS-HS group reported higher locomotion in relation to the C-C ($p < 0.05$), C-HS ($p < 0.01$), and HS-C ($p < 0.01$) groups (**Figure 3D**). The rearing frequency in the OF test showed a difference in the interaction of pre- and postweaning diets [$F_{PRE \times POST(1,32)} = 0.16$, $p < 0.05$]. The C-HS and HS-C groups accomplished lower numbers of rearing in relation to the C-C and HS-HS groups ($p < 0.05$) (**Figure 3E**). No differences were shown in the evaluation of latency to escape of center zone ($p = 0.27$) and grooming frequency ($p = 0.35$) in the OF test (**Figures 3A,C,F**).

In the amygdala redox state evaluation, a difference with respect to the postweaning diet factor was shown for SOD analysis [$F_{POST(1,20)} = 29.92$, $p < 0.001$]. The offspring who received a HS diet after weaning reported less SOD activity compared to the offspring of standard diet fed dams ($p < 0.001$). In addition, an interaction in the pre- and postweaning diets was observed [$F_{PRE \times POST(1,20)} = 0.13$, $p < 0.05$]. The C-HS and HS-HS groups showed less SOD activity compared to the C-C and HS-C groups ($p < 0.01$) (**Figure 4B**). For GST activity, a difference was shown in the postweaning diet [$F_{POST(1,20)} = 5.69$, $p < 0.05$]. The offspring who received a HS diet after weaning displayed less GST activity compared to the offspring of standard diet fed dams ($p < 0.05$). Also, an interaction in the pre- and postweaning diets was found [$F_{PRE \times POST(1,20)} = 0.42$, $p < 0.05$]. The HS-HS group reported less GST activity with respect to the C-C group (**Figure 4D**). In the TBARS evaluation, a difference in the postweaning diet was observed [$F_{POST(1,20)} = 5.21$, $p < 0.05$]. The offspring who received a HS diet after weaning reported higher TBARS compared to the offspring of standard diet fed dams ($p < 0.05$). Moreover, an interaction of pre- and postweaning diets was observed [$F_{PRE \times POST(1,20)} = 2.14$, $p < 0.05$]. The C-HS, HS-C, and HS-HS groups showed the higher TBARS levels compared to the C-C group ($p < 0.05$) (**Figure 4E**). No differences were reported in the FRAP ($p = 0.16$) and CAT ($p = 0.57$) evaluations (**Figures 4A,C**).

DISCUSSION

High-salt diets are consumed worldwide and are associated with cardiovascular morbidity and mortality. Noteworthy, HS intake has also been linked to behavioral changes in rodents. This study evaluated differential effects of HS and standard diet combinations given in the pre- or postweaning period. In this study, an increase in locomotion was showed in the group of animals that received a HS diet in, both, the pre- and postweaning period (HS-HS group). In addition, animals that received the HS diet only after weaning displayed a decrease in anxiety-like behaviors (C-HS group). Furthermore, both the groups showed amygdala oxidative stress, which may explain the behavioral changes observed.

The HS-HS group received the HS diet in both the periods (pre- and postweaning) resulting in adulthood hyperactivity measured by higher locomotion in the OF test. Also, this group presented an increase in the rearing frequency, which can be classified as a vertical exploration, confirming a high activity (Borta and Schwarting, 2005; Wardwell et al., 2020). Interestingly, with a similar protocol, Mcbride et al. (2008) observed that both the male Wistar rats treated with HS diet (4% NaCl) in the pre- (preconception and gestation periods) and postnatal periods (lactation) had increased locomotion in the OF test. In combination, these data indicate that a HS diet can induce hyperactivity in rodents. Moreover, these animals were more sensitive to the stimulating effect on locomotion produced by the administration of amphetamine compared to the group that received a standard diet (Mcbride et al., 2008). This result leads to the assumption that a HS diet of this study could also sensitize offspring to the effects of amphetamines. Interestingly, we have previously showed that cafeteria or calorie-restricted diets during lactation and postlactation can alter anxiety and locomotion of offspring after ephedrine (psychostimulant drug) application, reaffirming the role of diets in sensitization to some drugs by mechanisms that are not yet clearly established (Rocha-Gomes et al., 2021b).

Curiously, the spontaneously hypertensive rats (SHR) model consistently exhibits hyperactivity in the OF test (Botanas et al., 2016; Aparicio et al., 2019; Chen et al., 2019). This model was initially developed for the study of deleterious effects of cardiovascular diseases. However, due to its behavioral characteristics of hyperactivity, high impulsivity, and learning disabilities, SHR rats are also used as a model of attention-deficit/hyperactivity disorder (ADHD) (Leffa et al., 2019). It is important to note that the excessive salt consumption is recognized as a risk factor for the development of arterial hypertension (Valenzuela et al., 2021). In addition, rodents on HS diets during the pre- or postnatal periods can develop hypertension in adulthood (Contreras et al., 2000; Swenson et al., 2004). Although we did not use the SHR model in this study and did not check the blood pressure of animals, we speculated in relation to the similarities between the results presented by the HS-HS group and the SHR model. It is possible that a HS diet in the HS-HS group has programmed the mechanisms for controlling blood pressure and also induced hyperactive behavior in adulthood, similar to that observed in the studies with the SHR model. Therefore, a hyperactivity phenotype is suggested for the HS-HS group. However, further studies are needed to assess whether the phenotype presented by this group may have any relation to ADHD.

Furthermore, one of our main hypotheses was that a HS diet could promote changes in anxiety-like behavior at adulthood. In this study, the C-HS group reported less anxiety-like behavior in the EPM test, due to the higher ratio of

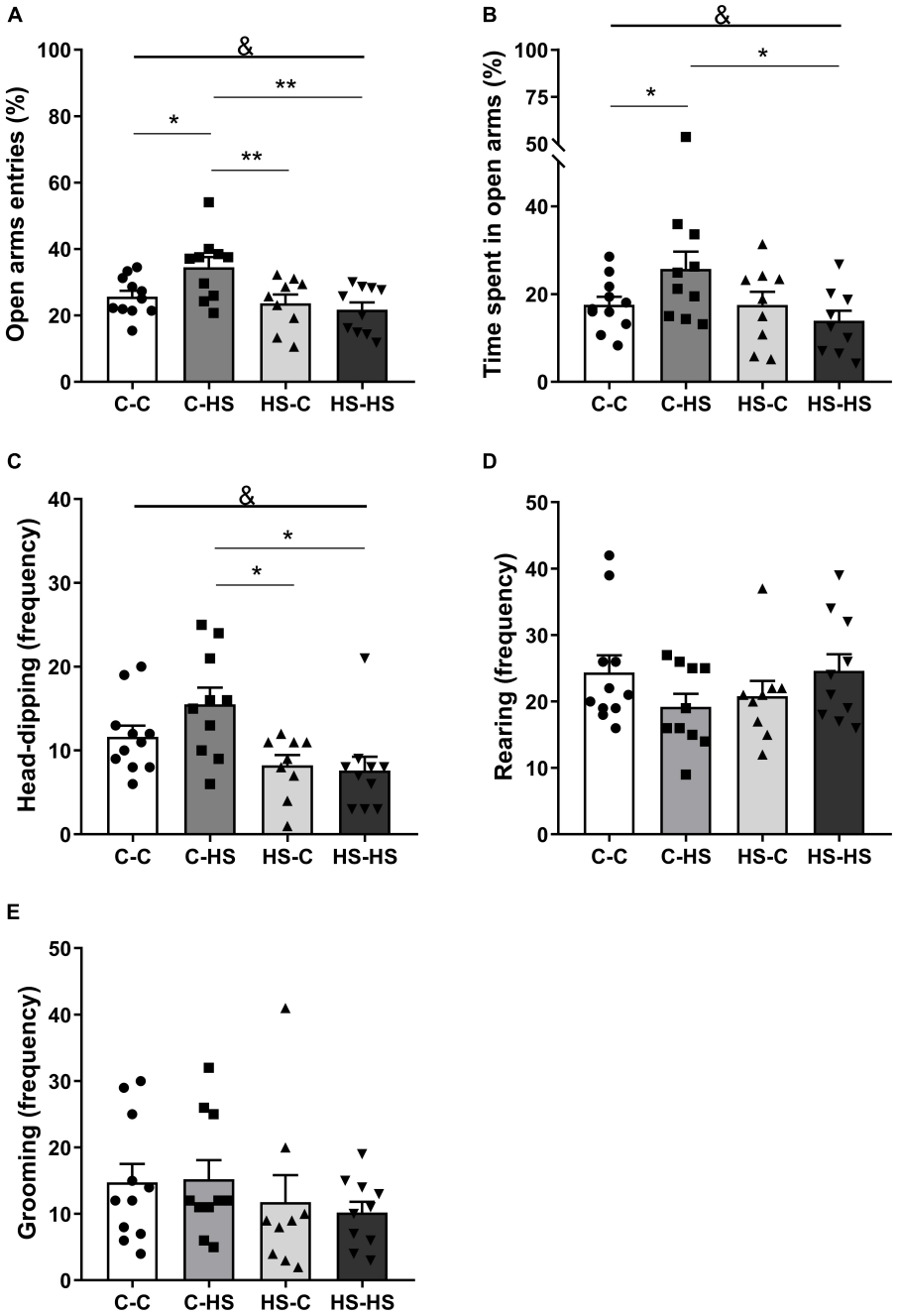

FIGURE 2 | Ratio of entries **(A)** and time spent in the open arms **(B)**; head-dipping **(C)**, rearing **(D)**, and grooming **(E)** frequency in the elevated plus maze test. C-C, offspring of standard diet fed dams who received a standard diet after weaning; C-HS, offspring of standard diet fed dams who received a HS diet after weaning; HS-C, offspring of HS diet fed dams who received a standard diet after weaning; and HS-HS, offspring of HS diet fed dams who received a HS diet after weaning. Data are shown as mean and SEM; n = 9–11; &$p < 0.05$ (preweaning factor); *$p < 0.05$, **$p < 0.01$ (interaction of the pre- and postweaning factors) using the ANOVA and the Newman–Keuls tests.

entries and time spent in the open arms, in addition to the higher head-dipping frequency (Souto et al., 2020). Gilman et al. (2019a) observed that after a short exposure to a HS diet (4% NaCl; during 7 days), rodents reduced behavioral inhibition under relatively low-threat conditions. In particular, this means that a HS diet can decrease anxiety-like behavior in situations that would be naturally aversive to rodents, as in the EPM test. This has important implications; as by exposing themselves to open or higher spaces, these animals may be more exposed to risky conditions or even increasing their visibility to predators (Gilman et al., 2019a). This result of a higher activity in potentially aversive situations was

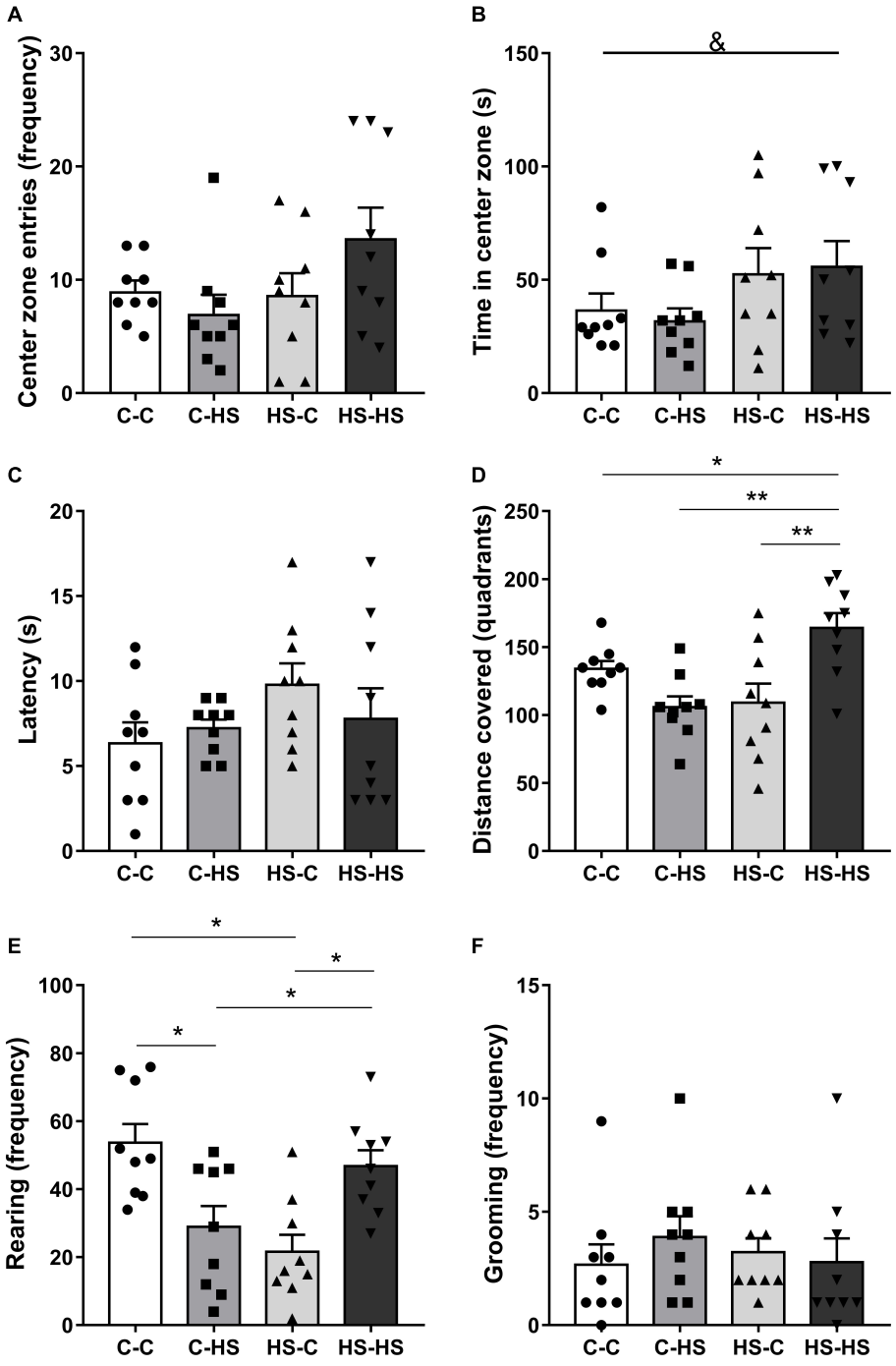

FIGURE 3 | Entries frequency **(A)**, time spent at the central zone **(B)**, latency to leave the center zone **(C)**, distance covered **(D)**, rearing **(E)**, and grooming frequency **(F)** in the open field test. C-C, offspring of standard diet fed dams who received a standard diet after weaning; C-HS, offspring of standard diet fed dams who received a HS diet after weaning; HS-C, offspring of HS diet fed dams who received a standard diet after weaning; and HS-HS, offspring of HS diet fed dams who received a HS diet after weaning. Data are shown as mean and SEM; $n = 9$. &$p < 0.05$ (preweaning factor); *$p < 0.05$, **$p < 0.01$ (interaction of the pre- and postweaning factors) using the ANOVA and the Newman–Keuls tests.

found in male mice (C57BL/6J) using other paradigms after consuming a HS diet (4% NaCl; during 7 days) such as the forced swim test (Mitchell et al., 2018; Gilman et al., 2019b). In addition, the abovementioned studies observed amygdala inflammation (Mitchell et al., 2018; Gilman et al., 2019b), possibly establishing a link to a HS intake, low anxiety-like behavior, and cellular damages in a specific brain region. Although we cannot distinguish anxiety-like from impulsive

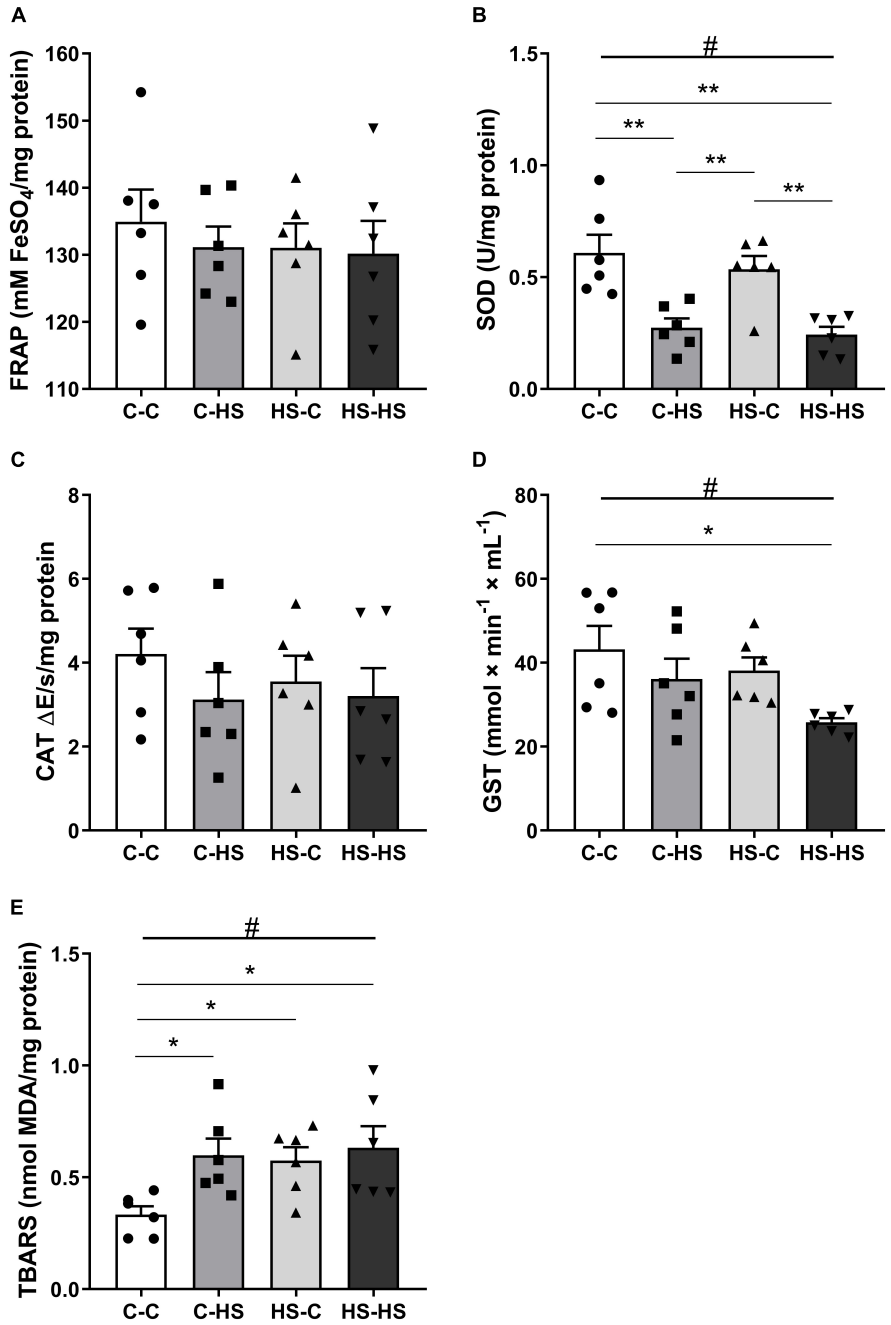

FIGURE 4 | The ferric reducing antioxidant power (FRAP) total antioxidant capacity **(A)**, superoxide dismutase (SOD) **(B)**, catalase (CAT) **(C)**, glutathione S-transferase (GST) activity **(D)**, and the thiobarbituric acid reactive substances (TBARS) **(E)** concentration in the amygdala. C-C, offspring of standard diet fed dams who received a standard diet after weaning; C-HS, offspring of standard diet fed dams who received a HS diet after weaning; HS-C, offspring of HS diet fed dams who received a standard diet after weaning; and HS-HS, offspring of HS diet fed dams who received a HS diet after weaning. Data are shown as mean and SEM; $n = 6$. #$p < 0.05$ (postweaning factor); *$p < 0.05$, **$p < 0.01$ (interaction of the pre- and postweaning factors) using the ANOVA and the Newman–Keuls tests.

behaviors, increased exploratory (horizontal and vertical) activity in new environments is a characteristic of impulsive behavior, which may also be caused by alterations in specific brain areas related to decision-making in adverse situations (Almeida et al., 1993). However, the reasons why the C-HS group had a lower frequency of rearing in the OF and a lower tendency in the EPM (with no statistical difference) tests remain to be clarified in future studies.

It is well established that experiences of mother during preweaning periods can modify the developmental health trajectory of her offspring. However, in some cases, no significant deleterious effects are observed, as demonstrated in the EPM test

by the groups that received a HS diet in the preweaning period (HS-C and HS-HS). These observations are combined with the Predictive Adaptive Response (PAR) hypothesis, which argues that some changes that occur in early life in response to aversive stimuli are important to provide an advantage later in life. The PAR hypothesis predicts that these changes occur through epigenetic programming, which may also bring specific costs in the adult environment, making the animal maladapted on certain occasions (Raubenheimer et al., 2012; St-Cyr and McGowan, 2018). However, further studies are suggested to assess epigenetic changes that may be related to the results obtained here.

Reactive oxygen and nitrogen species can be considered as essential for the full development of neuronal functions when occurring in low or moderate amounts. However, at excessive levels, they are harmful and can lead to oxidative/nitrosative stress, causing damage to proteins, lipids, and nucleic acids (da Silva et al., 2014; Salim, 2017). In turn, this can lead to the release of inflammatory signals, resulting in neuroinflammation, loss of function, and, consequently, in behavioral changes (Hatanaka et al., 2016; Cirulli et al., 2020; Dias et al., 2020; Maciel August et al., 2020). Previous studies in rodents have shown that HS diets caused an imbalance in the brain redox state, with decreased cognition (Liu et al., 2014; Ge et al., 2017; Faraco et al., 2019) and increased reactivity to stressful situations (Bai et al., 2017; Dingess et al., 2018). Moreover, a HS diet in the preconception, gestation, and lactation periods has been shown to negatively influence the redox state of the cerebellum, hypothalamus, and hippocampus of the offspring (Stocher et al., 2018). These findings indicate a role of salt-rich diets with respect to the brain redox status, being able to induce oxidative stress in regions of fundamental importance for behavior and cognition.

The brain is very vulnerable to the excessive reactive oxygen and nitrogen species production, due to its high O_2 consumption and modest antioxidant defenses (Bakunina et al., 2015; Salim, 2017). In addition, regions such as the hippocampus and the amygdala have been reported as the most susceptible to oxidative stress, consequently being more prone to functional decline (Bouayed et al., 2009; Salim, 2017). In this study, amygdala oxidative stress was observed, due to high levels of the TBARS (C-HS, HS-C, and HS-HS groups), in addition to the low activity of SOD (C-HS and HS-HS groups) and GST (HS-HS group) antioxidant enzymes. It is important to note that the amygdala plays a key role in the interpretation of environmental threats. Sensory stimuli are received in the amygdala that imbues them with emotional value and processing the outcomes as negative or positive valence, directly influencing anxiety-like behaviors mainly through the serotonergic system (Calhoon and Tye, 2015; dos Santos et al., 2017; de Lima et al., 2020). It is possible that diet-associated amygdala oxidative stress may be related to the behavioral alterations observed in the EPM and the OF tests; however, no clear patterns linking behavioral and redox readouts were noticeable in this study. Future studies are needed to better characterize this hypothetical relationship by also analyzing potential mediators that could serve as a link between changes in amygdala redox status and behavior.

In relation to the mechanism by which a HS diet can trigger oxidative stress of brain tissues, some suggestions based on previously published data are raised. The nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that regulates the expression of several proteins, among them some involved in antioxidant defense system of cells. For example, antioxidant enzymes such as CAT, SOD, and GST are produced after activating the Nrf2 pathway (Iranshahy et al., 2018; Liu et al., 2020). Previously, Liu et al. (2020) showed a downregulation of the Nrf2 expression in renal tissue of rats receiving a HS diet. Similarly, Wang et al. (2020) reported high levels of reactive oxygen species and low activity of SOD and CAT in the hippocampus of HS diet rats. This result indicates that the downregulation of the Nrf2 pathway can occur not only at the systemic level, but also in the brain after consuming a HS diet. In addition, a HS diet can provide a reduction in NO production (Kouyoumdzian et al., 2016; Zheng et al., 2019). In situations where there is oxidative stress of the tissue, reactive oxygen species can inactivate NO ($NO + O2^- \rightarrow ONOO^-$). The radical $ONOO^-$ is a very powerful oxidant and nitrosating agent. Thus, besides generates a toxic molecule ($ONOO^-$), this reaction decreases the NO availability. NO plays an important role as a vasodilator, thus reducing it also contributing to arterial hypertension (Modlinger et al., 2004; Vaziri and Rodríguez-Iturbe, 2006). Notably, hypertension is strongly linked to oxidative stress (González et al., 2014; Ahmad et al., 2017; Guzik and Touyz, 2017; Small et al., 2018).

This study has some limitations. First, to better understand the relationship between amygdala oxidative stress and observed behavioral changes, it is necessary in the future the use of drugs that alter the production of reactive oxygen and nitrogen species and the evaluation of the Nrf2 expression. Second, the assessment of inflammation in the amygdala would be important to understand the real impact of a HS diet at the cellular level and the extent of tissue damage. Also, it is also important to evaluate serotonin levels in this brain region, since its concentration in the amygdala is directly related to anxiety-like behaviors. Third, the use of females is necessary, since sexual dimorphism is common in behavioral assessment studies. Females could have different responses due to other developmental vulnerabilities, altered neuroendocrine regulation, or placental and epigenetic different effects. Fourth, the evaluation of other tests related to anxiety-like (light-dark box and hole-board tests) and hyperactivity (SHR model; use of drugs that affect locomotion) behaviors must be performed to better understand the outcomes of this model. Fifth, finally, the next studies should assess blood pressure and heart rate, in an attempt to establish a link between these physiological responses and the observed behaviors.

In summary, this study demonstrated negative effects of a HS diet on the amygdala redox state. In addition, a HS diet promoted hyperactivity when administered in the combination of pre- and postweaning periods and decreased anxiety-like behaviors when offered only in the postweaning period. To the best of our knowledge, this is the first study that indicates damage to the amygdala in addition to behavior changes, regardless of the period in which salt is added to the diet. This fact is highlighted, due to the large consumption of salt in the world (Steffensen et al., 2018), its relationship with the development of cardiovascular diseases (Bill and Foundation, 2019; He et al., 2020), and with

the hypotheses of behavioral changes and cognitive deficits after HS consumption also in humans (Heye et al., 2016; Abdoli, 2017; Afroz and Alviña, 2019).

CONCLUSION

A HS diet promoted hyperactivity when administered in the pre- and postweaning periods. In animals that received only in the postweaning period, the addition of salt induced a reduction in anxiety-like behaviors. Regardless of the administration period, salt provided amygdala oxidative stress, which may be linked to the observed behaviors.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee on the Use of Animals of Universidade Federal dos Vales do Jequitinhonha e Mucuri (CEUA-UFVJM; protocol 025/2018).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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REFERENCES

Abdoli, A. (2017). Hypothesis: high salt intake as an inflammation amplifier might be involved in the pathogenesis of neuropsychiatric disorders. *Clin. Exp. Neuroimmunol.* 8, 146–157. doi: 10.1111/cen3.12389

Afroz, K. F., and Alviña, K. (2019). Maternal elevated salt consumption and the development of autism spectrum disorder in the offspring. *J. Neuroinflammation* 16:265. doi: 10.1186/s12974-019-1666-2

Afshin, A., Sur, P. J., Fay, K. A., Cornaby, L., Ferrara, G., Salama, J. S., et al. (2019). Health effects of dietary risks in 195 countries, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet* 393, 1958–1972. doi: 10.1016/S0140-6736(19)30041-8

Ahmad, K. A., Yuan Yuan, D., Nawaz, W., Ze, H., Zhuo, C. X., Talal, B., et al. (2017). Antioxidant therapy for management of oxidative stress induced hypertension. *Free Radic. Res.* 51, 428–438. doi: 10.1080/10715762.2017.1322205

Almeida, S. S., Garcia, R. A., and de Oliveira, L. M. (1993). Effects of early protein malnutrition and repeated testing upon locomotor and exploratory behaviors in the elevated plus-maze. *Physiol. Behav.* 54, 749–752. doi: 10.1016/0031-9384(93)90086-U

Aparicio, C. F., Hennigan, P. J., Mulligan, L. J., and Alonso-Alvarez, B. (2019). Spontaneously hypertensive (SHR) rats choose more impulsively than Wistar-Kyoto (WKY) rats on a delay discounting task. *Behav. Brain Res.* 364, 480–493. doi: 10.1016/j.bbr.2017.09.040

Bai, J., Yu, X.-J., Liu, K.-L., Wang, F.-F., Li, H.-B., Shi, X.-L., et al. (2017). Tert-butylhydroquinone attenuates oxidative stress and inflammation in hypothalamic paraventricular nucleus in high salt-induced hypertension. *Toxicol. Lett.* 281, 1–9. doi: 10.1016/j.toxlet.2017.08.018

Bakunina, N., Pariante, C. M., and Zunszain, P. A. (2015). Immune mechanisms linked to depression via oxidative stress and neuroprogression. *Immunology* 144, 365–373. doi: 10.1111/imm.12443

Benzie, I. F. F., and Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": the FRAP assay. *Anal. Biochem.* 239, 70–76. doi: 10.1006/abio.1996.0292

Bill, F., and Foundation, M. G. (2019). Health effects of dietary risks in 195 countries, 1990 – 2017: a systematic analysis for the Global Burden of Disease Study. *Lancet* 393, 1958–1972.

Borta, A., and Schwarting, R. K. W. (2005). Inhibitory avoidance, pain reactivity, and plus-maze behavior in Wistar rats with high versus low rearing activity. *Physiol. Behav.* 84, 387–396. doi: 10.1016/j.physbeh.2005.01.009

Botanas, C. J., Lee, H., de la Peña, J. B., Dela Peña, I. J., Woo, T., Kim, H. J., et al. (2016). Rearing in an enriched environment attenuated hyperactivity and inattention in the spontaneously hypertensive rats, an animal model of attention-deficit hyperactivity disorder. *Physiol. Behav.* 155, 30–37. doi: 10.1016/j.physbeh.2015.11.035

Bouayed, J., Rammal, H., and Soulimani, R. (2009). Oxidative stress and anxiety: relationship and cellular pathways. *Oxid. Med. Cell. Longev.* 2:623654. doi: 10.4161/oxim.2.2.7944

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1006/abio.1976.9999

Calhoon, G. G., and Tye, K. M. (2015). Resolving the neural circuits of anxiety. *Nat. Neurosci.* 18, 1394–1404. doi: 10.1038/nn.4101

Chen, V. C.-H., Chiu, C.-C., Weng, J.-C., Chen, L.-J., Siow, J. Y., Hsu, T.-C., et al. (2019). Taurine reduces hyperactive behavior in SHR rats through upregulating the proportion of CD4+CD25+Foxp3+ regulatory T cells. *J. Funct. Foods* 56, 312–320. doi: 10.1016/j.jff.2019.03.032

Cirulli, F., Musillo, C., and Berry, A. (2020). Maternal obesity as a risk factor for brain development and mental health in the offspring. *Neuroscience* 447, 122–135. doi: 10.1016/j.neuroscience.2020.01.023

Contreras, R. J., Wong, D. L., Henderson, R., Curtis, K. S., and Smith, J. C. (2000). High dietary NaCl early in development enhances mean arterial pressure of adult rats. *Physiol. Behav.* 71, 173–181. doi: 10.1016/S0031-9384(00)00331-0

da Silva, A. I., Galindo, L. C. M., Nascimento, L., Freitas, C. M., Manhaes-de-Castro, R., Lagranha, C. J., et al. (2014). Fluoxetine treatment of rat neonates significantly reduces oxidative stress in the hippocampus and in behavioral indicators of anxiety later in postnatal life. *Can. J. Physiol. Pharmacol.* 92, 330–337. doi: 10.1139/cjpp-2013-0321

de Lima, R. M. S., dos Santos Bento, L. V., di Marcello Valladão Lugon, M., Barauna, V. G., Bittencourt, A. S., Dalmaz, C., et al. (2020). Early life stress and the programming of eating behavior and anxiety: sex-specific relationships with serotonergic activity and hypothalamic neuropeptides. *Behav. Brain Res.* 379:112399. doi: 10.1016/j.bbr.2019.112399

de Souza, J. A., da Silva, M. C., Costa, F. C. O., de Matos, R. J. B., de Farias Campina, R. C., do Amaral Almeida, L. C., et al. (2020a). Early life stress induced by maternal separation during lactation alters the eating behavior and serotonin system in middle-aged rat female offspring. *Pharmacol. Biochem. Behav.* 192:172908. doi: 10.1016/j.pbb.2020.172908

de Souza, J. A., do Amaral Almeida, L. C., Tavares, G. A., Falcão, L., de, A. L., Beltrão, L. C., et al. (2020b). Dual exposure to stress in different stages of development affects eating behavior of male Wistar rats. *Physiol. Behav.* 214:112769. doi: 10.1016/j.physbeh.2019.112769

Dias, C. T., Curi, H. T., Payolla, T. B., Lemes, S. F., Betim Pavan, I. C., Torsoni, M. A., et al. (2020). Maternal high-fat diet stimulates proinflammatory pathway and increases the expression of Tryptophan Hydroxylase 2 (TPH2) and brain-derived neurotrophic factor (BDNF) in adolescent mice hippocampus. *Neurochem. Int.* 139:104781. doi: 10.1016/j.neuint.2020.104781

Dingess, P. M., Thakar, A., Zhang, Z., Flynn, F. W., and Brown, T. E. (2018). High-salt exposure during perinatal development enhances stress sensitivity. *Dev. Neurobiol.* 78, 1131–1145. doi: 10.1002/dneu.22635

Dong, Y., Kataoka, K., Tokutomi, Y., Nako, H., Nakamura, T., Toyama, K., et al. (2011). beneficial effects of combination of valsartan and amlodipine on salt-induced brain injury in hypertensive rats. *J. Pharmacol. Exp. Ther.* 339, 358–366. doi: 10.1124/jpet.111.182576

dos Santos, T. M., Kolling, J., Siebert, C., Biasibetti, H., Bertó, C. G., Grun, L. K., et al. (2017). Effects of previous physical exercise to chronic stress on long-term aversive memory and oxidative stress in amygdala and hippocampus of rats. *Int. J. Dev. Neurosci.* 56, 58–67. doi: 10.1016/j.ijdevneu.2016.12.003

Faraco, G., Brea, D., Garcia-Bonilla, L., Wang, G., Racchumi, G., Chang, H., et al. (2018). Dietary salt promotes neurovascular and cognitive dysfunction through a gut-initiated TH17 response. *Nat. Neurosci.* 21, 240–249. doi: 10.1038/s41593-017-0059-z

Faraco, G., Hochrainer, K., Segarra, S. G., Schaeffer, S., Santisteban, M. M., Menon, A., et al. (2019). Dietary salt promotes cognitive impairment through tau phosphorylation. *Nature* 574, 686–690. doi: 10.1038/s41586-019-1688-z

Fatahi, S., Namazi, N., Larijani, B., and Azadbakht, L. (2018). The association of dietary and urinary sodium with bone mineral density and risk of osteoporosis: a systematic review and meta-analysis. *J. Am. Coll. Nutr.* 37, 522–532. doi: 10.1080/07315724.2018.1431161

Freitas, D. A., Rocha-Vieira, E., De Sousa, R. A. L., Soares, B. A., Rocha-Gomes, A., Chaves Garcia, B. C., et al. (2019). High-intensity interval training improves cerebellar antioxidant capacity without affecting cognitive functions in rats. *Behav. Brain Res.* 376:112181. doi: 10.1016/j.bbr.2019.112181

Garofalo, C., Borrelli, S., Provenzano, M., De Stefano, T., Vita, C., Chiodini, P., et al. (2018). Dietary salt restriction in chronic kidney disease: a meta-analysis of randomized clinical trials. *Nutrients* 10:732. doi: 10.3390/nu10060732

Ge, Q., Wang, Z., Wu, Y., Huo, Q., Qian, Z., Tian, Z., et al. (2017). High salt diet impairs memory-related synaptic plasticity via increased oxidative stress and suppressed synaptic protein expression. *Mol. Nutr. Food Res.* 61:1700134. doi: 10.1002/mnfr.201700134

Ge, S., Feng, X., Shen, L., Wei, Z., Zhu, Q., and Sun, J. (2012). Association between habitual dietary salt intake and risk of gastric cancer: a systematic review of observational studies. *Gastroenterol. Res. Pract.* 2012:808120. doi: 10.1155/2012/808120

Gilman, T. L., George, C. M., Andrade, M. A., Mitchell, N. C., Toney, G. M., and Daws, L. C. (2019a). High salt intake lowers behavioral inhibition. *Front. Behav. Neurosci.* 13:271. doi: 10.3389/fnbeh.2019.00271

Gilman, T. L., Mitchell, N. C., Daws, L. C., and Toney, G. M. (2019b). Neuroinflammation contributes to high salt intake- augmented neuronal activation and active coping responses to acute stress. *Int. J. Neuropsychopharmacol.* 22, 137–142. doi: 10.1093/ijnp/pyy099

González, J., Valls, N., Brito, R., and Rodrigo, R. (2014). Essential hypertension and oxidative stress: new insights. *World J. Cardiol.* 6, 353–366. doi: 10.4330/wjc.v6.i6.353

Guedine, C. R. C., Pordeus, L. C. M., Riul, T. R., Jordao, A. A. J., and Almeida, S. S. (2018). Cafeteria diet during lactation and/or post-lactation altered lipid profile/lipid peroxidation and increased anxiety-like behavior in male rat offspring. *Nutr. Neurosci.* 23, 526–536. doi: 10.1080/1028415X.2018.1529283

Guzik, T., and Touyz, R. (2017). Oxidative stress, inflammation, and vascular aging in hypertension. *Hypertension* 70, 660–667. doi: 10.1161/HYPERTENSIONAHA.117.07802

Habig, W. H., Pabst, M. J., and Jakoby, W. B. (1974). Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* 249, 7130–7139.

Hatanaka, Y., Wada, K., and Kabuta, T. (2016). Maternal high-fat diet leads to persistent synaptic instability in mouse offspring via oxidative stress during lactation. *Neurochem. Int.* 97, 99–108. doi: 10.1016/J.NEUINT.2016.03.008

He, F. J., Tan, M., Ma, Y., and MacGregor, G. A. (2020). Salt reduction to prevent hypertension and cardiovascular disease: JACC State-of-the-Art review. *J. Am. Coll. Cardiol.* 75, 632–647. doi: 10.1016/j.jacc.2019.11.055

Heye, A. K., Thrippleton, M. J., Chappell, F. M., Valdés Hernández, M., del, C., Armitage, P. A., et al. (2016). Blood pressure and sodium: association with MRI markers in cerebral small vessel disease. *J. Cereb. Blood Flow Metab.* 36, 264–274. doi: 10.1038/jcbfm.2015.64

Huang, L., Trieu, K., Yoshimura, S., Neal, B., Woodward, M., Campbell, N. R. C., et al. (2020). Effect of dose and duration of reduction in dietary sodium on blood pressure levels: systematic review and meta-analysis of randomised trials. *BMJ* 368:m315. doi: 10.1136/bmj.m315

Huang, P., Shen, Z., Yu, W., Huang, Y., Tang, C., Du, J., et al. (2017). Hydrogen sulfide inhibits high-salt diet-induced myocardial oxidative stress and myocardial hypertrophy in dahl rats. *Front. Pharmacol.* 8:128. doi: 10.3389/fphar.2017.00128

Iranshahy, M., Iranshahi, M., Abtahi, S. R., and Karimi, G. (2018). The role of nuclear factor erythroid 2-related factor 2 in hepatoprotective activity of natural products: a review. *Food Chem. Toxicol.* 120, 261–276. doi: 10.1016/j.fct.2018.07.024

Kitiyakara, C., Chabrashvili, T., Chen, Y., Blau, J., Karber, A., Aslam, S., et al. (2003). Salt intake, oxidative stress, and renal expression of NADPH oxidase and superoxide dismutase. *J. Am. Soc. Nephrol.* 14, 2775–2782. doi: 10.1097/01.asn.0000092145.90389.65

Klein, M. O., MacKay, H., Edwards, A., Park, S.-B., Kiss, A. C. I., Felicio, L. F., et al. (2018). POMC and NPY mRNA expression during development is increased in rat offspring brain from mothers fed with a high fat diet. *Int. J. Dev. Neurosci.* 64, 14–20. doi: 10.1016/j.ijdevneu.2017.03.004

Kouyoumdzian, N. M., Mikusic, N. R., Cao, G., Choi, M. R., Penna, S. D., Fernández, B. E., et al. (2016). Adverse effects of tempol on hidrosaline balance in rats with acute sodium overload. *Biotech. Histochem.* 91, 510–521. doi: 10.1080/10520295.2016.1249029

Leffa, D. T., Panzenhagen, A. C., Salvi, A. A., Bau, C. H. D., Pires, G. N., Torres, I. L. S., et al. (2019). Systematic review and meta-analysis of the behavioral effects of methylphenidate in the spontaneously hypertensive rat model of attention-deficit/hyperactivity disorder. *Neurosci. Biobehav. Rev.* 100, 166–179. doi: 10.1016/j.neubiorev.2019.02.019

Liu, M., Deng, M., Luo, Q., Dou, X., and Jia, Z. (2020). High-salt loading downregulates Nrf2 expression in a sodium-dependent manner in renal collecting duct cells. *Front. Physiol.* 10:1565. doi: 10.3389/fphys.2019.01565

Liu, Y. Z., Chen, J. K., Li, Z. P., Zhao, T., Ni, M., Li, D. J., et al. (2014). High-salt diet enhances hippocampal oxidative stress and cognitive impairment in mice. *Neurobiol. Learn. Mem.* 114, 10–15. doi: 10.1016/j.nlm.2014.04.010

Maciel August, P., Hözer, R., dos Santos Rodrigues, K., Gindri dos Santos, B., Moura Maurmann, R., Crestani Scortegagna, M., et al. (2020). Effect of maternal exercise on diet-induced redox imbalance in hippocampus of adult offspring. *Neuroscience* 437, 196–206. doi: 10.1016/j.neuroscience.2020.04.046

Marklund, S., and Marklund, G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem.* 47, 469–474. doi: 10.1111/j.1432-1033.1974.tb03714.x

Mcbride, S. M., Culver, B., Flynn, F. W., Regul, J. P., Comp, I., and Physiol, R. (2008). Dietary sodium manipulation during critical periods in development sensitize adult offspring to amphetamines. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 82071, 899–905. doi: 10.1152/ajpregu.00186.2008

Mecawi, A. S., and Almeida, L. F. (2017). Increased exposure to sodium during pregnancy and lactation changes basal and induced behavioral and neuroendocrine responses in adult male offspring. *Physiol. Rep.* 5:e13210. doi: 10.14814/phy2.13210

Melo, C. S., Rocha-Vieira, E., Freitas, D. A., Soares, B. A., Rocha-Gomes, A., Riul, T. R., et al. (2019). A single session of high-intensity interval exercise increases antioxidants defenses in the hippocampus of Wistar rats. *Physiol. Behav.* 211:112675. doi: 10.1016/j.physbeh.2019.112675

Mitchell, N. C., Gilman, T. L., Daws, L. C., and Toney, G. M. (2018). High salt intake enhances swim stress-induced PVN vasopressin cell activation and active stress coping. *Psychoneuroendocrinology* 93, 29–38. doi: 10.1016/j.psyneuen.2018.04.003

Modlinger, P. S., Wilcox, C. S., and Aslam, S. (2004). Nitric oxide, oxidative stress, and progression of chronic renal failure. *Semin. Nephrol.* 24, 354–365. doi: 10.1016/j.semnephrol.2004.04.007

Montgomery, K. C. (1955). The relation between fear induced by novel stimulation and exploratory behavior. *J. Comp. Physiol. Psychol.* 48, 254–260. doi: 10.1037/h0043788

Neal, B., Wu, Y., Feng, X., Zhang, R., Zhang, Y., Shi, J., et al. (2021). Effect of salt substitution on cardiovascular events and death. *N. Engl. J. Med.* 385, 1067–1077. doi: 10.1056/nejmoa2105675

Nelson, D. P., and Kiesow, L. A. (1972). Enthalpy of decomposition of hydrogen peroxide by catalase at 25 degrees C (with molar extinction coefficients of H 2 O 2 solutions in the UV). *Anal. Biochem.* 49, 474–478. doi: 10.1016/0003-2697(72)90451-4

O'Donnell, K. J., and Meaney, M. J. (2016). Fetal origins of mental health: the developmental origins of health and disease hypothesis. *Am. J. Psychiatry* 174, 319–328. doi: 10.1176/appi.ajp.2016.16020138

Ohkawa, H., Ohishi, N., and Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* 95, 351–358. doi: 10.1016/0003-2697(79)90738-3

Paxinos, G., and Watson, C. (2014). *The Rat Brain in Stereotaxic Coordinates*, 7th Edn. San Diego, CA: Elsevier.

Pellow, S., Chopin, P., File, S. E., and Briley, M. (1985). Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J. Neurosci. Methods* 14, 149–167. doi: 10.1016/0165-0270(85)90031-7

Plescia, F., Brancato, A., Venniro, M., Maniaci, G., Cannizzaro, E., Sutera, F. M., et al. (2015). Acetaldehyde self-administration by a two-bottle choice paradigm: consequences on emotional reactivity, spatial learning, and memory. *Alcohol* 49, 139–148. doi: 10.1016/j.alcohol.2015.01.002

Raubenheimer, D., Simpson, S. J., and Tait, A. H. (2012). Match and mismatch: conservation physiology, nutritional ecology and the timescales of biological adaptation. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 367, 1628–1646. doi: 10.1098/rstb.2012.0007

Riul, T. R., and Almeida, S. S. (2020). Feed restriction since lactation has reduced anxiety in adult Wistar rats. *Rev. Nutr.* 33:e190143. doi: 10.1590/1678-9865202033e190143

Rocha-Gomes, A., Teixeira, A. E., de Oliveira, D. G., Santiago, C. M. O., da Silva, A. A., Riul, T. R., et al. (2021a). LPS tolerance prevents anxiety-like behavior and amygdala inflammation of high-fat-fed dams' adolescent offspring. *Behav. Brain Res.* 411:113371. doi: 10.1016/j.bbr.2021.113371

Rocha-Gomes, A., Teixeira, A. E., Lima, D. S. S., Rocha, L., dos, S., da Silva, A. A., et al. (2021b). Caloric restriction or cafeteria diet from birth to adulthood increases the sensitivity to ephedrine in anxiety and locomotion in Wistar rats. *Physiol. Behav.* 236:113430. doi: 10.1016/j.physbeh.2021.113430

Salim, S. (2017). Oxidative stress and the central nervous system. *J. Pharmacol. Exp. Ther.* 360, 201–205. doi: 10.1124/jpet.116.237503

Santisteban, M. M., and Iadecola, C. (2018). Hypertension, dietary salt and cognitive impairment. *J. Cereb. Blood Flow Metab.* 38, 2112–2128. doi: 10.1177/0271678X18803374

Small, H. Y., Migliarino, S., Czesnikiewicz-Guzik, M., and Guzik, T. J. (2018). Hypertension: focus on autoimmunity and oxidative stress. *Free Radic. Biol. Med.* 125, 104–115. doi: 10.1016/j.freeradbiomed.2018.05.085

Souto, T. S., Nakao, F. S. N., Giriko, C. Á, Dias, C. T., Cheberle, A. I. P., Lambertucci, R. H., et al. (2020). Lard-rich and canola oil-rich high-fat diets during pregnancy promote rats' offspring neurodevelopmental delay and behavioral disorders. *Physiol. Behav.* 213:112722. doi: 10.1016/j.physbeh.2019.112722

St-Cyr, S., and McGowan, P. O. (2018). Adaptation or pathology? The role of prenatal stressor type and intensity in the developmental programing of adult phenotype. *Neurotoxicol. Teratol.* 66, 113–124. doi: 10.1016/j.ntt.2017.12.003

Steffensen, I.-L., Frølich, W., Dahl, K. H., Iversen, P. O., Lyche, J. L., Lillegaard, I. T. L., et al. (2018). Benefit and risk assessment of increasing potassium intake by replacement of sodium chloride with potassium chloride in industrial food products in Norway. *Food Chem. Toxicol.* 111, 329–340. doi: 10.1016/j.fct.2017.11.044

Stocher, D. P., Klein, C. P., Saccomori, A. B., August, P. M., Martins, N. C., Couto, P. R. G., et al. (2018). Maternal high-salt diet alters redox state and mitochondrial function in newborn rat offspring's brain. *Br. J. Nutr.* 119, 1003–1011. doi: 10.1017/S0007114518000235

Swenson, S. J., Speth, R. C., and Porter, J. P. (2004). Effect of a perinatal high-salt diet on blood pressure control mechanisms in young Sprague-Dawley rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 286, 764–770. doi: 10.1152/ajpregu.00492.2003

Tan, S.-Y., Sotirelis, E., Bojeh, R., Maan, I., Medalle, M., Chik, X. S. F., et al. (2021). Is dietary intake associated with salt taste function and perception in adults? A systematic review. *Food Qual. Prefer.* 92:104174. doi: 10.1016/j.foodqual.2021.104174

Teixeira, A. E., Rocha-Gomes, A., Pereira dos Santos, T., Amaral, B. L. S., da Silva, A. A., Malagutti, A. R., et al. (2020). Cafeteria diet administered from lactation to adulthood promotes a change in risperidone sensitivity on anxiety, locomotion, memory, and social interaction of Wistar rats. *Physiol. Behav.* 220:112874. doi: 10.1016/j.physbeh.2020.112874

Valenzuela, P. L., Carrera-Bastos, P., Gálvez, B. G., Ruiz-Hurtado, G., Ordovas, J. M., Ruilope, L. M., et al. (2021). Lifestyle interventions for the prevention and treatment of hypertension. *Nat. Rev. Cardiol.* 18, 251–275. doi: 10.1038/s41569-020-00437-9

Vaziri, N. D., and Rodríguez-Iturbe, B. (2006). Mechanisms of disease: oxidative stress and inflammation in the pathogenesis of hypertension. *Nat. Clin. Pract. Nephrol.* 2, 582–593. doi: 10.1038/ncpneph0283

Wang, Z., Ge, Q., Wu, Y., Zhang, J., Gu, Q., and Han, J. (2020). Impairment of long-term memory by a short-term high-fat diet via hippocampal oxidative stress and alterations in synaptic plasticity. *Neuroscience* 424, 24–33. doi: 10.1016/j.neuroscience.2019.10.050

Wardwell, J., Watanasriyakul, W. T., Normann, M. C., Akinbo, O. I., McNeal, N., Ciosek, S., et al. (2020). Physiological and behavioral responses to observing a sibling experience a direct stressor in prairie voles. *Stress* 23, 444–456. doi: 10.1080/10253890.2020.1724950

Wilson, M. A., Grillo, C. A., Fadel, J. R., and Reagan, L. P. (2015). Stress as a one-armed bandit: differential effects of stress paradigms on the morphology, neurochemistry and behavior in the rodent amygdala. *Neurobiol. Stress* 1, 195–208. doi: 10.1016/j.ynstr.2015.06.001

Zhang, T., Wang, D., Li, X., Jiang, Y., Wang, C., Zhang, Y., et al. (2020). Excess salt intake promotes M1 microglia polarization via a p38/MAPK/AR-dependent pathway after cerebral ischemia in mice. *Int. Immunopharmacol.* 81:106176. doi: 10.1016/j.intimp.2019.106176

Zheng, X., Li, X., Chen, M., Yang, P., Zhao, X., Zeng, L., et al. (2019). The protective role of hawthorn fruit extract against high salt-induced hypertension in Dahl salt-sensitive rats: impact on oxidative stress and metabolic patterns. *Food Funct.* 10, 849–858. doi: 10.1039/c8fo01818a

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