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The role of maternal exercise on placental, behavioral and genetic alterations induced by prenatal stress

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ABSTRACT

The present study aimed to evaluate the effects of treadmill maternal exercise on alterations induced by prenatal stress in neonatal mice. Female and male Balb/c mice were divided into five groups: control (CON), prenatal restraint stress (PNS), prenatal restraint stress and physical exercise before pregnancy (PNS + EX1), prenatal restraint stress and physical exercise during pregnancy (PNS + EX2), and prenatal restraint stress and physical exercise before and during pregnancy (PNS + EX3). Exercise was performed using a treadmill, at a speed of 10 m/min, for 60 min, 5 days a week. Maternal behavior was assessed on days 3, 4 and 5 postpartum (PPD). Placental gene expression of glucocorticoid receptor (GR), 11-β-hydroxysteroid dehydrogenase 2 (11β-HSD2), 5-hydroxytryptamine receptor 1A ($5HT_{1A}R$), and corticotropin releasing hormone receptor 1 (CRHR1) were analyzed. In neonatal mice, the gene expression of GR, mineralocorticoid receptor (MR), CRHR1, 5HTr1, oxytocin Receptor 1 (OXTr1), tropomyosin related kinase B (TRκB), brain-derived neurotrophic factor exon I (BDNF I), and BDNF IV was analyzed in the brain (PND0) and hippocampus (PND10). Maternal exercise improved ($p < 0.05$) maternal care. In the placenta, maternal exercise prevented ($p < 0.01$) the increase in GR expression caused by PNS. In the brain from PND0, exercise before pregnancy prevented ($p = 0.002$) the decreased CRHR1 expression promoted by PNS. In the hippocampus of PND10 males, PNS decreased ($p = 0.0005$) GR expression, and exercise before pregnancy prevented ($p = 0.003$) this effect. In PND10 females, maternal exercise prevented ($p < 0.05$) the PNS-induced increase in MR expression. PNS + EX2 males showed increased ($p < 0.01$) BDNF I gene expression and PNS + EX1 females demonstrated increased ($p = 0.03$) BDNF IV expression. In conclusion, maternal physical exercise may play a role in modulating maternal-fetal health and may contribute to preventing neurodevelopmental changes induced by prenatal stress.

1. Introduction

Physical or emotional stress during pregnancy has been considered as an important factor in the development of metabolic, neuroendocrine, and behavioral diseases in adult life. The secretion of maternal glucocorticoids during stressful situations and the mechanisms related to the hypothalamic-pituitary-adrenal axis (HPA axis) regulation seem to be critical for the development of specific brain regions in the offspring. Thus, programming effects commonly observed in prenatally stressed individuals may result from the altered hippocampal expression of glucocorticoid (GR) and mineralocorticoid (MR) receptors, associated with decreased negative feedback control by glucocorticoids, leading to increased HPA axis activity and cortisol/corticosterone secretion (de Kloet et al., 2005). The glucocorticoid passage across the placental barrier is also proposed to explain the heightened fetal corticosteroid levels during maternal exposure to stress. The placental enzyme 11β-hydroxysteroid dehydrogenase (HSD) isoform 2 (11β-HSD2) exerts a protective role converting maternal cortisol (corticosterone in rodents) to inactive cortisone (Chapman et al., 2013). However, experimental studies have shown that prenatal stress reduces 11β-HSD2 expression and activity, increasing fetal exposure to maternal hormones (Challis et al., 2000; Zhou et al., 2020). Additionally, exposure to adverse events

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during pregnancy may also influence maternal behavior promoting important impacts on mother-infant interaction (Murgatroyd and Nephew, 2013; Pardon et al., 2000; Schmidt et al., 2018), including a reduced time spent with the pups and the capacity to differentiate offspring by odor (Bosch et al., 2007; de Souza et al., 2012).

Disturbances in early pregnancy are more likely to alter fetal neurodevelopment, increasing the vulnerability for long-lasting effects on offspring nervous system development (Atladottir et al., 2010; Khashan et al., 2008). Alterations in the expression of key genes also seem to contribute to the development of diseases in individuals stressed *in utero*. Serotonin (5HT) participates in essential processes during fetal development, such as neurogenesis, cell differentiation, and migration (Bonnin and Levitt, 2011). In addition, neurotrophins also directly interact with corticosteroids, participating in neuronal growth and differentiation. The brain-derived neurotrophic factor (BDNF) plays an important role in central nervous system development, neuronal differentiation, and cognition (Castren and Monteggia, 2021). Therefore, alterations in the expression of these genes can be associated with morphological and functional changes, contributing to abnormal fetal neurodevelopment.

On the other hand, physical exercise has been listed as an important tool in the prevention and treatment of several diseases, including cardiovascular, metabolic, and neurological (Balducci et al., 2014; Hotting and Roder, 2013; Schuttler et al., 2019). Regular physical exercise practice has been reported to have positive effects on several biological systems, including a decrease in the stress response by the HPA axis (Seo et al., 2019). Moreover, the protective effects, after stressful situations, have focused on responses related to the hippocampus, a region in which exercise plays an important role inducing neurogenesis and the expression of growth factors. We have recently demonstrated that exercise before pregnancy attenuates the effects of prenatal stress on gene expression in the hippocampus (Luft et al., 2021b).

Although the effects of prenatal stress on the offspring are already known and there is evidence for a possible protective role of physical exercise, very little is known on how maternal exercise may impact these effects in the early neurodevelopmental period. Hence, the current study evaluated the effects of maternal exercise on gene expression of stress response markers in the placenta and fetal brain of prenatally stressed mice. Moreover, we also investigated the effects of maternal exercise on the maternal behavior. We hypothesized here that maternal physical exercise would act as a protective intervention to prevent alterations induced by a stressful environment, contributing to improve maternal-fetal health.

2. Material and methods

2.1. Animals

Male and female Balb/c mice were acquired from the Center for Experimental Biological Models (CEMBE) of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS). All animals were housed in standard plastic mouse cages (22 cm × 16 cm × 14 cm), kept under controlled temperature (24 ± 2 °C) and ventilation, light/dark cycle of 12h, with food and water *ad libitum*. All procedures were approved by the Ethics Research Committee for Animal Use (CEUA-PUCRS, protocol number 8465).

2.2. Experimental design

This study was carried out using two experimental cohorts of animals (experiment 1 and experiment 2). In both cases, females were divided into five experimental groups: 1) CON - control; 2) PNS - prenatal restraint stress; 3) PNS + EX1 - prenatal restraint stress and physical exercise before pregnancy; 4) PNS + EX2 - prenatal restraint stress and physical exercise during pregnancy; 5) PNS + EX3 - prenatal restraint stress and physical exercise before and during pregnancy. Animals from the CON group remained in their cages and were handled only during the cleaning routine.

The same mating protocol was used for all experimental groups. The estrous cycle was verified under light microscopy prior to mating by collecting vaginal smears. During the fertile period, females were paired overnight with males from the in-house colony. The confirmation of the mating (presence of a semen plug) was considered gestational day 0 (G0). Pregnant females were caged individually from gestational day 8 (G8).

2.2.1. Experiment 1: effects of maternal exercise on placental and fetal brain (PND 0)

In the first cohort, dams were euthanized on G21, and both placenta and fetuses (PND0) were removed via cesarean section. The gene expression of GR, HSD11β2, 5HTr1 and CRHR1 were evaluated in the placenta. The gene expression of GR, MR, CRHR1, $5HT_{1A}R$, OXTr1, TRκB, BDNF I and BDNF IV were evaluated in the whole brain of fetuses. The experimental design is shown in Fig. 1A.

2.2.2. Experiment 2: effects of maternal exercise on the behavior of dams and hippocampus gene expression of neonatal mice (PND 10)

In the second cohort, maternal behavior was assessed at PND3, PND4 and PND5. Thus, during this period, animal handling was kept to a minimal. On PND10, male and female offspring were euthanized and brain was collected. The gene expression of GR, MR, CRHR1, $5HT_{1A}R$, OXTr1, TRκB, BDNF I and BDNF IV were evaluated in the hippocampus. The experimental design is shown in Fig. 1B.

2.3. Procedures

2.3.1. Prenatal stress

Prenatal stress was applied using a restraint stress protocol previously described by Luft et al., 2019, 2021a. Briefly, females from PNS, PNS + EX1, PNS + EX2 and PNS + EX3 groups were submitted to the protocol from the 8th day of gestation until birth. A closed cylinder was used for restraint, in sessions of 30 min, every other day. Females from the CON group remained in their cages without any intervention, other than cleaning, during the prenatal period.

2.3.2. Maternal exercise

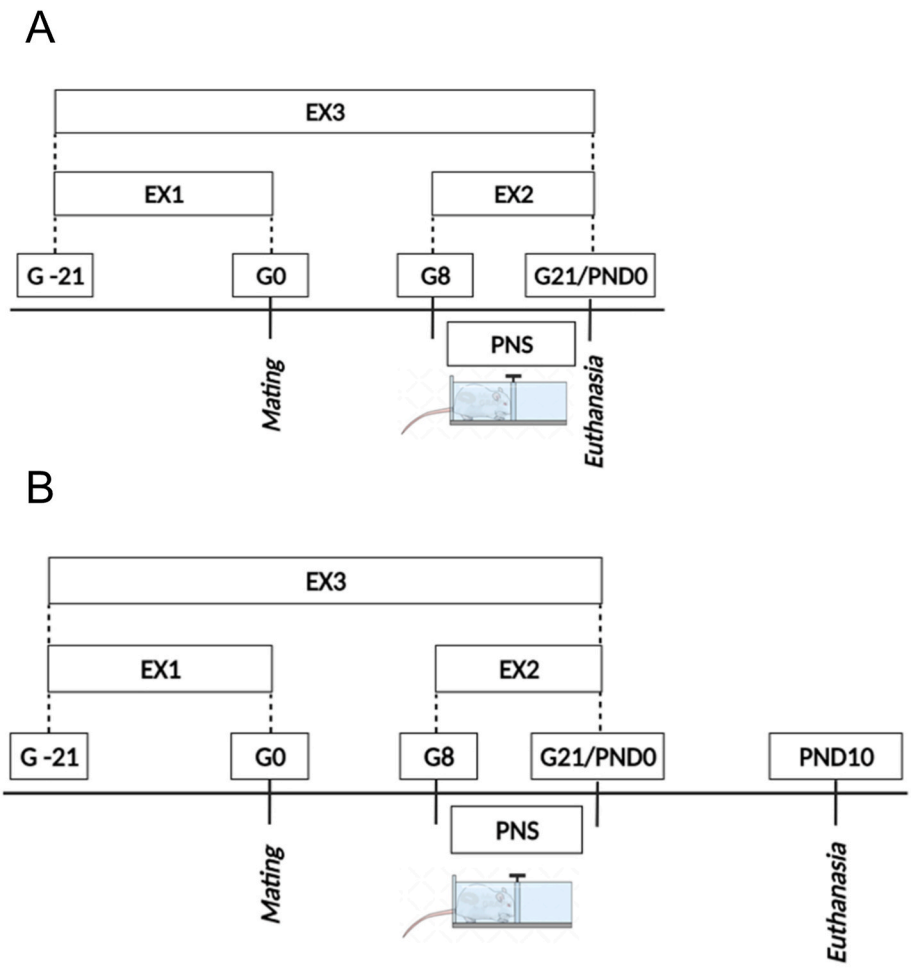
Females were submitted to physical exercise on a motorized treadmill. The physical exercise was performed at a speed of 10 m/min, for 60 min, 5 days a week (Luft et al., 2020). During the last week of gestation, females from the PNS + EX2 and PNS + EX3 groups had the speed reduced to 6 m/min, although duration was maintained at 60 min. Animals that refused to spontaneously exercise were excluded from the study.

2.3.3. Maternal behavior

Maternal behavioral evaluation was performed according to Pardo et al. (2016), with modifications. The maternal behavior was observed, for 72 min, in 3 periods of the light cycle (10 a.m., 1 p.m., 4 p.m.) and one period of the dark cycle (7 p.m.), at PND3, PND4 and PND5. Behavioral observations were recorded every 3 min. The following behaviors were measured: time spent in breastfeeding, maternal self-maintenance (lactating female drinking water or eating food) and no interaction with pups (lactating female out of the nest with no interaction with pups). The percentage of time spent in each behavior was calculated.

2.3.4. Euthanasia

The animals were euthanized by decapitation at G21/PND0 or PND10. The placenta and brains were rapidly removed and stored in RNA-later (Applied Biosystems, USA) for 24h at 4 °C and then transferred to −80 °C until final processing.



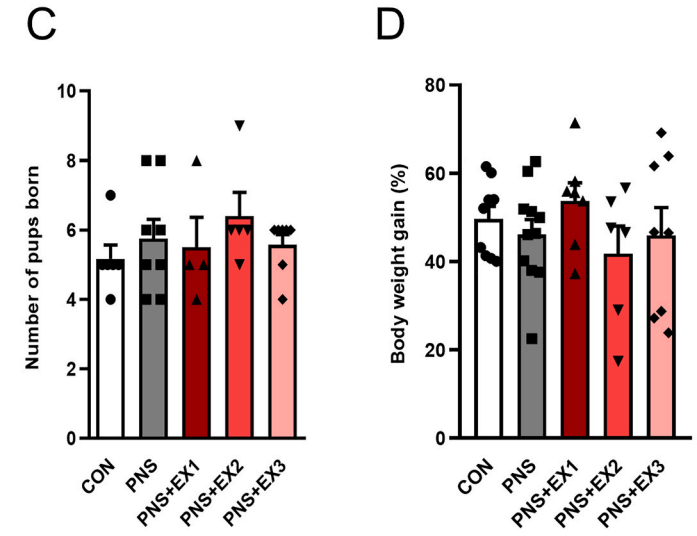


Fig. 1. Experimental design of the study (A and B), number of pups born (C), and maternal body weight gain (D). The timeline is presented in days. Data are shown as mean and standard error of the mean (SEM), and groups were compared by one-way ANOVA followed by the LSD post-hoc test. G-21: 21 days before G0; G0: gestational day 0/mating day; G8: gestational day 8; G21: gestational day 21; PND 0: post-natal day 0/offspring birth; PND 10: post-natal day 10; CON: control; PNS: prenatal restraint stress; PNS + EX1: prenatal restraint stress and physical exercise before pregnancy; PNS + EX2: prenatal restraint stress and physical exercise during pregnancy; PNS + EX3: prenatal restraint stress and physical exercise before and during pregnancy.

2.3.5. Gene expression

The total cellular mRNA from placenta, fetal brain and hippocampus (PND10) were extracted using the Trizol method (ThermoFisher - Scientific) according to the manufacturer's instructions. The mRNA was resuspended in 20 μL of nuclease-free water (Ambion - ThermoFisher - Scientific) and converted into complementary DNA (cDNA) (GoScript™ Reverse Transcription System Protocol - Promega), according to the protocol indicated by the manufacturer. Gene expression was performed in real-time quantitative PCR (Step One Plus - Applied Biosystems - ThermoFisher – Scientific). The relative mRNA expression was calculated by the ΔΔCt method using beta-2-microglobulin (B2M) as the endogenous reference gene. A negative control for each primer was used on each plate to check for possible contamination. The measurements of the reagents for amplification were calculated based on the incorporation of the fluorescent marker SYBR® Green (Applied Biosystems - ThermoFisher - Scientific) in the double strand of cDNA for each

amplification reaction. The set of specific primers for each gene is shown in Table 1.

2.3.6. Statistical analyzes

The normality of data was verified using the Shapiro-Wilk test and possible outliers were excluded from analyses. The results were expressed as mean ± standard error of the mean (SEM). The comparisons between experimental groups were performed through One-way analysis of variance (ANOVA), followed by Fisher's LSD post-test. The level of significance adopted was set at 5% ($p \leq 0.05$). Data were analyzed using the software Prism GraphPad (version 8.0, GraphPad Software Inc, USA).

3. Results

3.1. Maternal exercise does not alter the number of pups and body weight during pregnancy

No significant differences between groups were found in the number of animals born (Fig. 1C). Similarly, when body weight was assessed, dams did not present significant differences in body weight gain during pregnancy ($F_{(4,36)} = 0.825$, $p = 0.51$) (Fig. 1D).

3.2. Gestational exercise decreases the effects of prenatal stress on placental GR gene expression

The possible mechanisms related to the effects of prenatal stress on placental barrier were assessed. Regarding the effects on GR ($F_{(4,19)} = 3.716$, $p = 0.02$), a significant increase ($p = 0.03$) was observed in the GR gene expression in the placenta from PNS animals when compared to the CON group (Fig. 2A). The PNS + EX1 ($p = 0.002$) and PNS + EX2 ($p = 0.008$) groups decreased significantly the gene expression of GR when compared to CON mice (Fig. 2A). When evaluating the expression of $5HT_{1A}R$ ($F_{(4,24)} = 4.070$, $p = 0.01$), mice from PNS + EX1 ($p = 0.01$), PNS + EX2 ($p = 0.001$) and PNS + EX3 ($p = 0.03$) showed reduced expression when compared to PNS animals (Fig. 2C). No significant differences for 11β-HSD2 (Fig. 2B) and CRHR1 (Fig. 2D) mRNA analyzes were found.

Table 1
Primer sequences for real-time PCR analysis.

Gene	Primer sequences
$5HT_{1A}R$	Forward: 5′ GCGTTGTTGGGTGCCATAAT 3′ Reverse: 5′ CCGGATTGAGCAGGGAGTT 3′
B2M	Forward: 5′ CCCCAGTGAGACTGATACATACG 3′ Reverse: 5′ CGATCCCAGTAGACGGTCTTG 3′
HSD11β2	Forward: 5′ ATCACCGGTTGTGACACT 3′ Reverse: 5′ TCTAGGGCACCAGGGCTATT 3′
CRHR1	Forward: 5′ TGAGTGTTAGCGATGCCTTG 3′ Reverse: 5′ TCCTACCACTGAGGACTGG 3′
BDNF I	Forward: 5′ GCGTTGAGAAAGCTGCTTCAG 3′ Reverse: 5′ GAATGAGCGAGGTTACCAATGA 3′
BDNF IV	Forward: 5′ GCAGCTGCCTTGATGTTTAC 3′ Reverse: 5′ CCGTGGACGTTTACTTCTTTC 3′
GR	Forward: 5′ GGAATAGGTGCCAAGGGTCT 3′ Reverse: 5′ GAGCACACCAGGCAGAGTTT 3′
MR	Forward: 5′ CCAGTTCTCCGTTCTCTGTA 3′ Reverse: 5′ CTTGAGCACCAATCCGGTAG 3′
OXTr1	Forward: 5′ GGAGCGTCTGGGACGTCAAT 3′ Reverse: 5′ AGGAAGCGCTGCACGAGTT 3′
TRκB	Forward: 5′ TGGTGCATTCCATTCACTGT 3′ Reverse: 5′ CGTGGTACTCCGTGTGATTG 3′

Abbreviations: $5HT_{1A}R$, serotonin receptor 1A; B2M, beta-2-microglobulin; HSD11β2, 11β-hydroxysteroid dehydrogenase type 2; CRHR1, corticotropin releasing hormone receptor 1; BDNF I, brain-derived neurotrophic factor exon I; BDNF IV, brain-derived neurotrophic factor exon IV, GR, glucocorticoid receptor; MR, mineralocorticoid receptor; OXTr1, oxytocin receptor type 1; TRκB, tropomyosin receptor kinase B.

3.3. Prenatal stress and maternal exercise alters gene expression in fetal brain

Considering the results found for the placenta, we have evaluated the expression of genes related to stress response and neurodevelopment in the fetal (PND0) brain (Fig. 3). When MR was evaluated ($F_{(4,21)} = 3.434$, $p = 0.02$), both PNS + EX2 ($p = 0.04$) and PNS + EX3 ($p = 0.02$) showed a decreased gene expression when compared to PNS mice (Fig. 3C). For CRHR1 ($F_{(4,17)} = 6.228$, $p = 0.002$), prenatal stress ($p = 0.04$) significantly decreased the mRNA expression when compared to CON mice, and exercise before pregnancy prevented this effect ($p = 0.002$) (Fig. 3D). Similar to PNS mice, exercise before and during pregnancy significantly decreased the CRHR1 gene expression when compared to the CON group ($p = 0.007$) (Fig. 3D). Regarding $5HT_{1A}R$ analyzes ($F_{(4,24)} = 4.857$, $p = 0.005$), PNS ($p = 0.002$), PNS + EX1 ($p = 0.002$), PNS + EX2 ($p = 0.002$) and PNS + EX3 ($p = 0.001$) showed a reduced gene expression when compared to CON mice (Fig. 3E). The TRκB mRNA evaluation ($F_{(4,17)} = 3.262$, $p = 0.03$) showed that exercise before and during pregnancy significantly increased gene expression when compared to CON ($p = 0.01$) and PNS ($p = 0.03$) groups (Fig. 3G). For BDNF I mRNA analyzes ($F_{(4,22)} = 4.447$, $p = 0.008$), we found a decreased gene expression in the PNS ($p = 0.02$), PNS + EX2 ($p = 0.01$) and PNS + EX3 ($p = 0.001$) when compared to CON mice (Fig. 3H). No other significant differences were found.

3.4. Exercise before and during pregnancy increases female body weight at PND10

At PND10 ($F_{(4,113)} = 2.695$, $p = 0.03$), we observed a significant increase in female body weight (Fig. 4D) from PNS + EX3 and PNS + EX1 groups when compared to the CON ($p = 0.03$) and PNS ($p = 0.04$) groups, respectively. No other significant differences for both dams and neonates were found.

3.5. Gestational exercise alters the effects of prenatal stress on maternal behavior

In order to assess the effects of prenatal stress and gestational physical exercise, maternal behavior was monitored at PND3, PND4 and PND5 (Fig. 5). Therefore, the percentage of breastfeeding (PND3: $F_{(4,26)} = 0.726$, $p = 0.58$; PND4: $F_{(4,26)} = 1.696$, $p = 0.18$; PND5: $F_{(4,25)} = 1.328$, $p = 0.28$), maternal self-maintenance (PND3: $F_{(4,26)} = 1.582$, $p = 0.20$; PND4: $F_{(4,26)} = 3.103$, $p = 0.03$; PND5: $F_{(4,25)} = 1.994$, $p = 0.12$) and time of no interaction with pups (PND3: $F_{(4,26)} = 0.662$, $p = 0.62$; PND4: $F_{(4,26)} = 1.625$, $p = 0.19$; PND5: $F_{(4,25)} = 3.184$; $p = 0.03$) was evaluated. At PND4, data showed that PNS dams spent significantly more time in self-maintenance ($p = 0.008$) when compared to the CON group (Fig. 5E). However, also at PND4, maternal exercise decreased (PNS + EX1 $p = 0.02$; PNS + EX2 $p = 0.01$; PNS + EX3 $p = 0.009$) the percentage of maternal self-maintenance when compared to the PNS group (Fig. 5E). Moreover, at PND5, maternal exercise decreased (PNS + EX1 $p = 0.004$; PNS + EX2 $p = 0.009$; PNS + EX3 $p = 0.01$) the percentage of no interaction with pups when compared to PNS group (Fig. 5I). No other significant differences were found.

3.6. Prenatal stress and maternal exercise alters gene expression in the hippocampus of neonatal male and female mice

PND10 male and female gene expression in the hippocampus was evaluated (Fig. 6 and Fig. 7). Different responses for GR gene expression in males ($F_{(4,25)} = 5.818$, $p = 0.001$) (Fig. 6B) and females ($F_{(4,27)} = 2.787$, $p = 0.04$) (Fig. 7B) were found. In males, we observed that exercise before pregnancy prevented ($p = 0.003$) the decrease in GR mRNA expression promoted by PNS when compared to the CON group ($p = 0.0005$) (Fig. 6B). Similar to the PNS group, PNS + EX2 males showed a decreased ($p = 0.001$) GR gene expression when compared to the CON

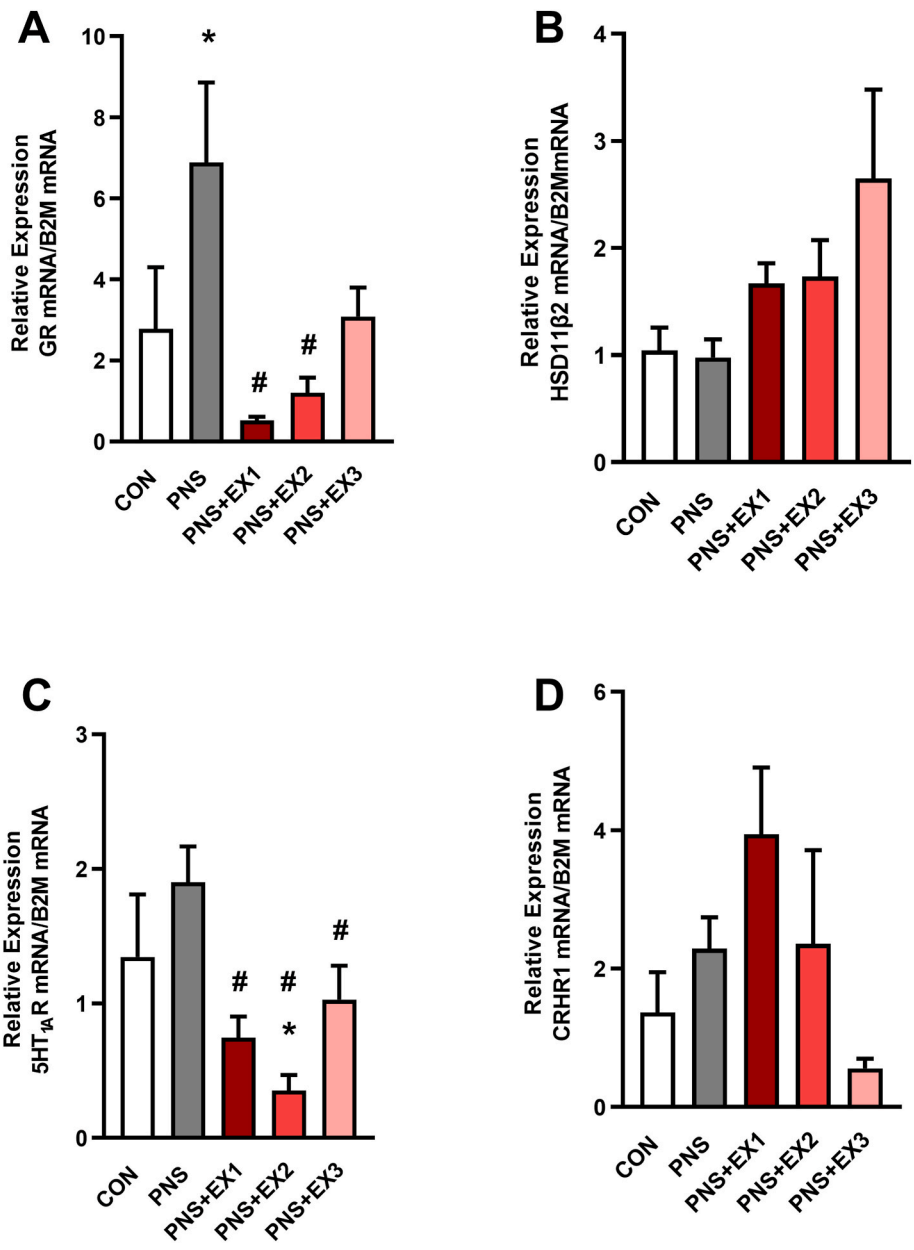


Fig. 2. Placental gene expression. Glucocorticoid receptor (A), 11β-hydroxysteroid dehydrogenase isoform 2 (B), serotonin receptor 1A (C), and corticotropin releasing hormone receptor 1 (D) mRNA expression were evaluated in the placenta. Data are shown as mean and standard error of the mean (SEM), and groups were compared by one-way ANOVA followed by the LSD post-hoc test. $^{*}p < 0.05$ denotes significant differences compared to the CON group; $^{\#}p < 0.05$ denotes significant differences compared to the PNS group. B2M: beta-2-microglobulin; GR: glucocorticoid receptor; HSD11β2: 11β-hydroxysteroid dehydrogenase isoform 2; $5HT_{1A}R$: serotonin receptor 1A; CRHR1: corticotropin releasing hormone receptor 1; CON: control; PNS: prenatal restraint stress; PNS + EX1: prenatal restraint stress and physical exercise before pregnancy; PNS + EX2: prenatal restraint stress and physical exercise during pregnancy; PNS + EX3: prenatal restraint stress and physical exercise before and during pregnancy.

group (Fig. 6B). Conversely, PNS + EX2 females showed an increased ($p = 0.009$) GR gene expression when compared to the CON group (Fig. 7B). In females, ($F_{(4,23)} = 4.141$, $p = 0.01$), we observed an increased MR gene expression in the PNS group ($p = 0.008$) when compared to the CON group, and this effect was prevented by maternal exercise (PNS + EX1: $p = 0.01$; PNS + EX2: $p = 0.001$; PNS + EX3: $p = 0.003$) (Fig. 7C). Regarding female OXTr1 gene expression ($F_{(4,22)} = 2.901$, $p = 0.04$), results showed that exercise before and during pregnancy decreased ($p = 0.04$) mRNA levels when compared to the CON group (Fig. 7F). When BDNF I ($F_{(4,20)} = 4.440$, $p = 0.009$) was evaluated in males, data showed that exercise during pregnancy increased the gene expression when compared to the CON ($p = 0.008$) and PNS ($p = 0.009$) groups (Fig. 6H). Finally, the analyzes of BDNF IV in females ($F_{(4,22)} = 4.323$, $p = 0.009$) showed an increased gene expression in the PNS + EX1 group ($p = 0.03$) when compared to the CON group (Fig. 7I). Also in females, we observed a decreased BDNF IV gene expression in the PNS + EX3 group ($p = 0.03$) when compared to the PNS group (Fig. 7I).

4. Discussion

The present study demonstrates that maternal exercise induced some protective effects on alterations produced by prenatal stress in neonatal mice. Our main findings have shown brain and placental preventive effects that are related to neurodevelopmental programming induced by maternal stress and to mechanisms of stress response in the offspring. Furthermore, the effects of prenatal stress and maternal exercise seem to have different outcomes for males and females.

Several studies have proposed different mechanisms that could explain the increase in fetal corticosteroid levels during maternal exposure to stress. The placental enzyme 11β-hydroxysteroid dehydrogenase (HSD) has the 11β-HSD2 isoform, with only dehydrogenase activity, responsible for the conversion of maternal cortisol (corticosterone in rodents) into inactive cortisone (Chapman et al., 2013). The 11β-HSD2 enzyme has been widely studied as a possible altered marker in the placenta from individuals stressed *in utero*, considering its role in inactivating circulating glucocorticoids (Chapman et al., 2013). Thus, a reduced expression of this enzyme could be responsible for the increase

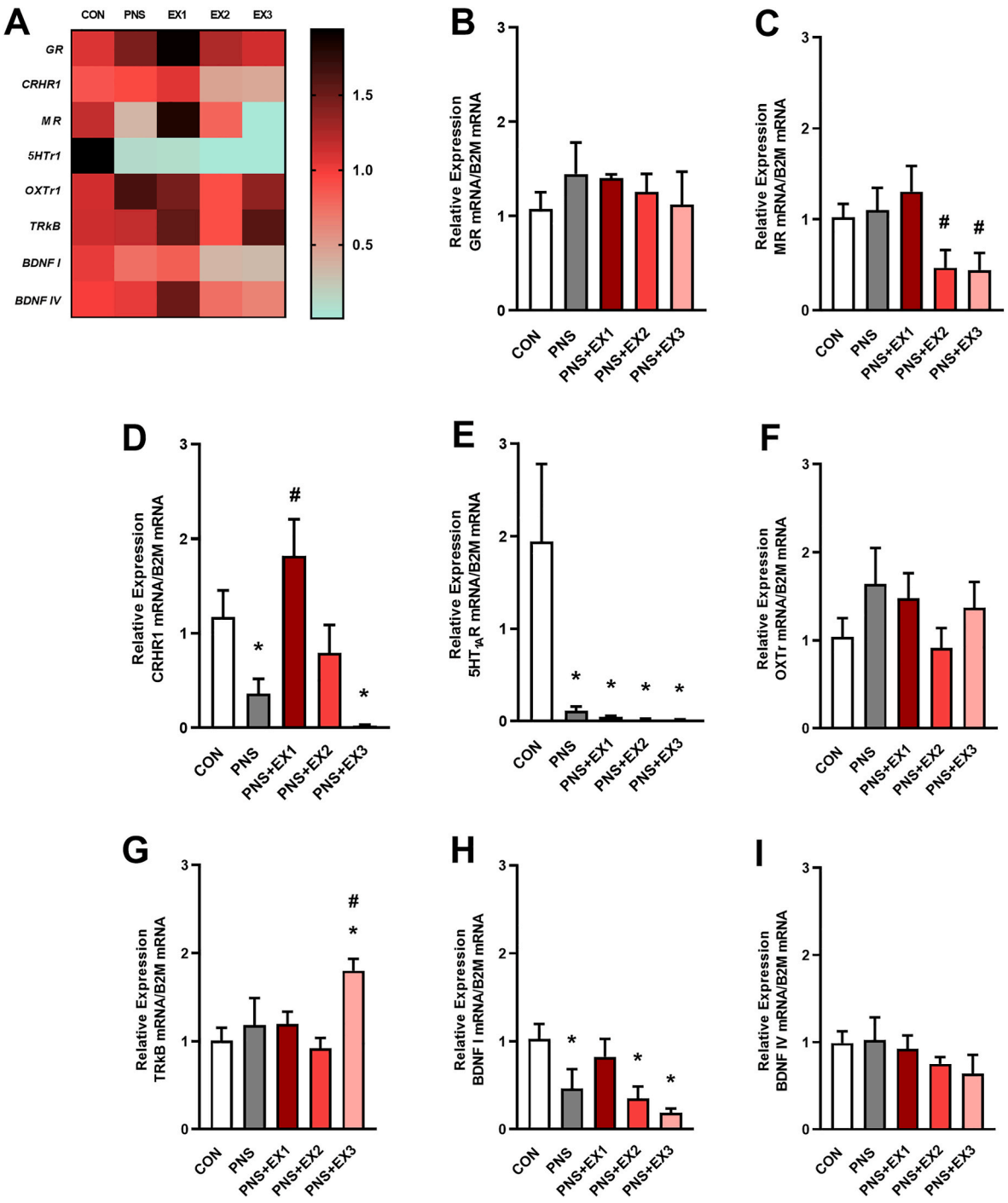


Fig. 3. Fetal brain gene expression. Heat map plot of gene expression (A), glucocorticoid receptor (B), mineralocorticoid (C), corticotropin releasing hormone receptor 1 (D), serotonin receptor 1A (E), oxytocin receptor type 1 (F), tropomyosin receptor kinase B (G), brain-derived neurotrophic factor exon I (H), and brain-derived neurotrophic factor exon IV (I) mRNA expression were evaluated in the fetal brain. Data are shown as mean and standard error of the mean (SEM), and groups were compared by one-way ANOVA followed by the LSD post-hoc test. $^{*}p < 0.05$ denotes significant differences compared to the CON group; $^{\#}p < 0.05$ denotes significant differences compared to the PNS group. B2M: beta-2-microglobulin; GR: glucocorticoid receptor; MR: mineralocorticoid receptor; CRHR1: corticotropin releasing hormone receptor 1; $5HT_{1A}R$: serotonin receptor 1A; OXTr1: oxytocin receptor type 1; TRκB, tropomyosin receptor kinase B; BDNF I, brain-derived neurotrophic factor exon I; BDNF IV, brain-derived neurotrophic factor exon IV; CON: control; PNS: prenatal restraint stress; PNS + EX1: prenatal restraint stress and physical exercise before pregnancy; PNS + EX2: prenatal restraint stress and physical exercise during pregnancy; PNS + EX3: prenatal restraint stress and physical exercise before and during pregnancy.

in fetal corticosteroid levels (Challis et al., 2000). Our data did not show differences on placental 11β-HSD2 gene expression. It is important to mention that the lack of analysis of the HSD enzymatic activity is a limitation of this study. Thus, although we did not find differences on gene expression, different placental enzymatic responses could not be ruled out. The exposure to glucocorticoids could be also controlled by placental GR (Bivol et al., 2016). Therefore, we are hypothesizing that the PNS placental increase in GR would be related to an increased tissue corticosteroid promoted by restraint stress and that exercise may have acted protecting mice from this effect. Interestingly, placentas from EX1

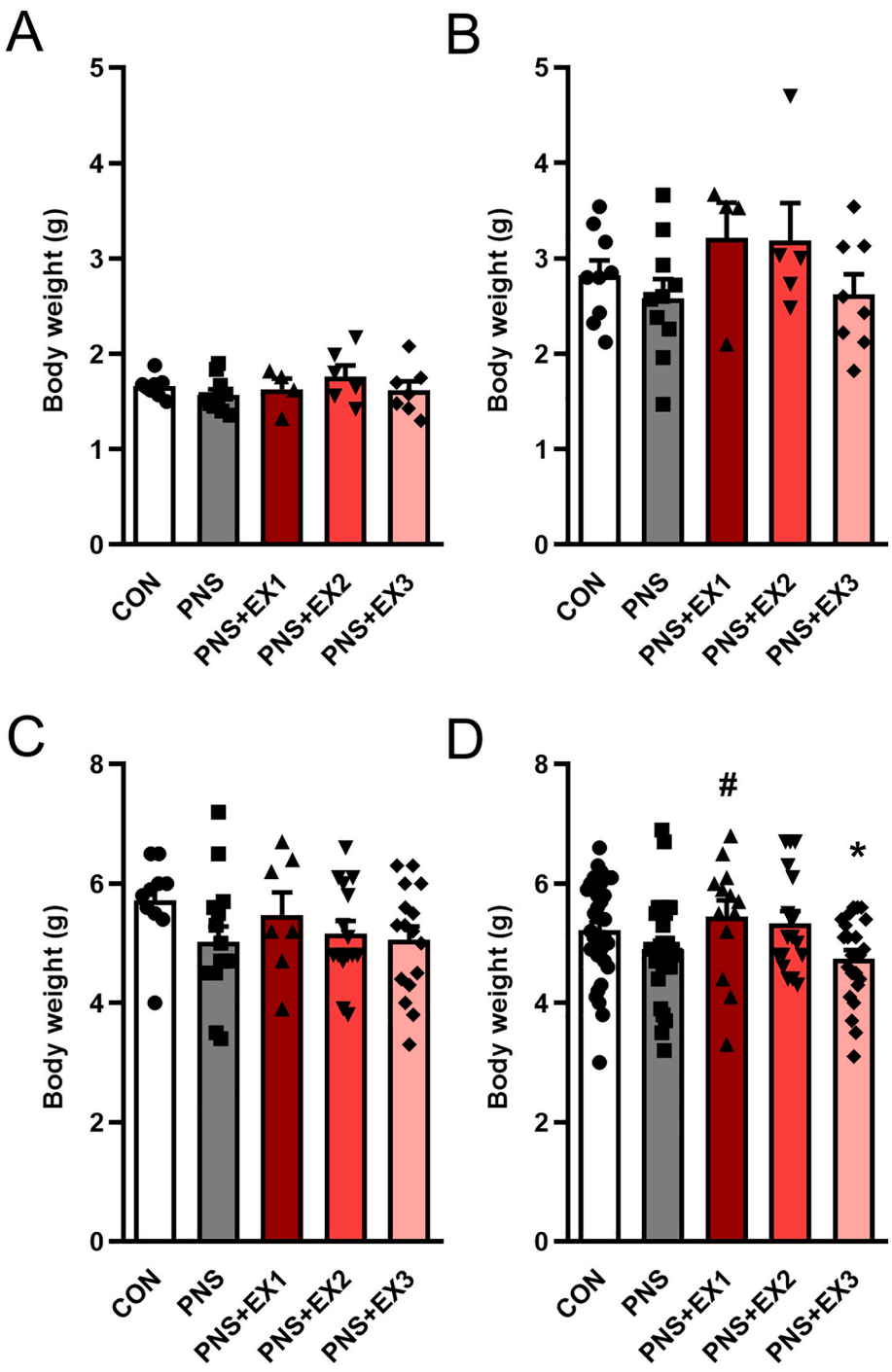


Fig. 4. Offspring body weight. The offspring body weight at PND1 (A), PND5 (B), males PND10 (C), and females PND10 (D) was evaluated. Data are shown as mean and standard error of the mean (SEM), and groups were compared by one-way ANOVA followed by the LSD post-hoc test. $^{*}p < 0.05$ denotes significant differences compared to the CON group; $^{\#}p < 0.05$ denotes significant differences compared to the PNS group. CON: control; PNS: prenatal restraint stress; PNS + EX1: prenatal restraint stress and physical exercise before pregnancy; PNS + EX2: prenatal restraint stress and physical exercise during pregnancy; PNS + EX3: prenatal restraint stress and physical exercise before and during pregnancy.

and EX2 groups showed an attenuated stress-induced increase in GR gene expression. The modulation of glucocorticoid secretion in exercised animals has been suggested as one of the factors responsible for the mechanism of action of exercise (Sampedro-Piquero and Moreno-Fernandez, 2021).

Glucocorticoid levels dramatically increase during the gestational period and are critical for fetal growth and maintenance of pregnancy (Solano and Arck, 2019). In this period, there is an increase in placental corticotrophin-releasing hormone (CRH) secretion, which stimulates the pituitary to raise adrenocorticotropic hormone (ACTH) and cortisol/corticosterone levels (Brunton and Russell, 2008). Studies in rodents have already shown that prenatal stress is capable of promoting an increase in the expression of CRH, which has been associated with higher levels of corticosterone and anxiety (Creutzberg et al., 2021). Our prenatal stress model did not alter placental CRH receptor type 1 (CRHR1) gene expression and no effects of maternal exercise were observed. The presence of CRHR1 in the human placental tissue has already been demonstrated (Petraglia et al., 1987), although there are no studies associating stress and exercise with this receptor in the placenta of rodents. Moreover, studies showed that prenatal exposure to glucocorticoids promotes changes in CRH, a potent modulator of long-term effects resulting from stress, since CRHR1 expression in the hippocampus is associated with behavioral and cognitive alterations. We have previously demonstrated that decreased expression of CRHR1 in the hippocampus promote memory impairment in adult rodents (Luft et al., 2021b). Conversely, another study showed that prenatal stress promotes

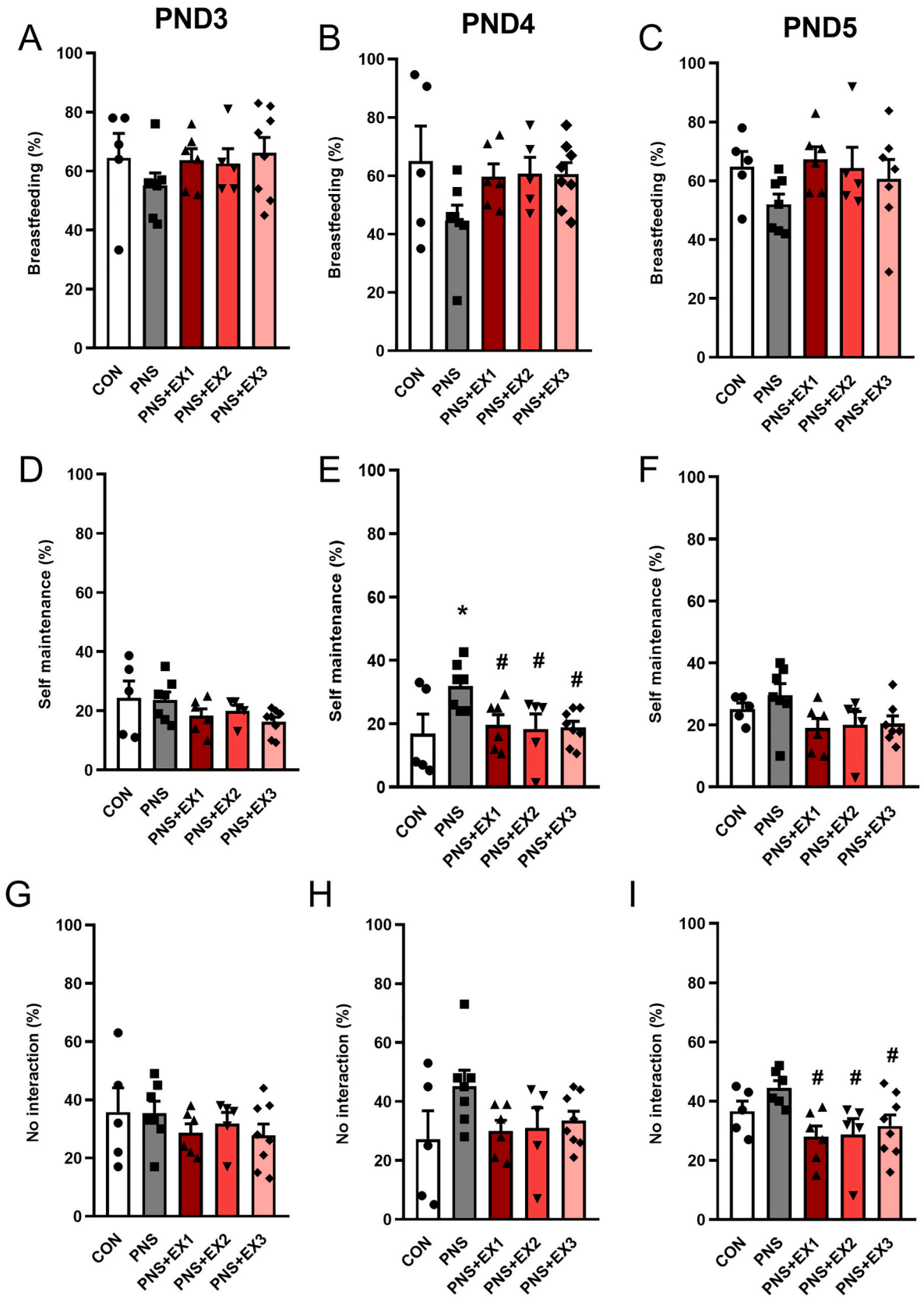


Fig. 5. Maternal behavior. Breastfeeding (A, B, and C), self-maintenance (D, E, and F), and no interaction (G, H, and I) behaviors were evaluated at PND3, PND4, and PND5. Data are shown as mean and standard error of the mean (SEM), and groups were compared by one-way ANOVA followed by the LSD post-hoc test. $^{*}p < 0.05$ denotes significant differences compared to the CON group; $^{\#}p < 0.05$ denotes significant differences compared to the PNS group. PND 3: post-natal day 3; PND 4: post-natal day 4; PND 5: post-natal day 5; CON: control; PNS: prenatal restraint stress; PNS + EX1: prenatal restraint stress and physical exercise before pregnancy; PNS + EX2: prenatal restraint stress and physical exercise during pregnancy; PNS + EX3: prenatal restraint stress and physical exercise before and during pregnancy.

Males

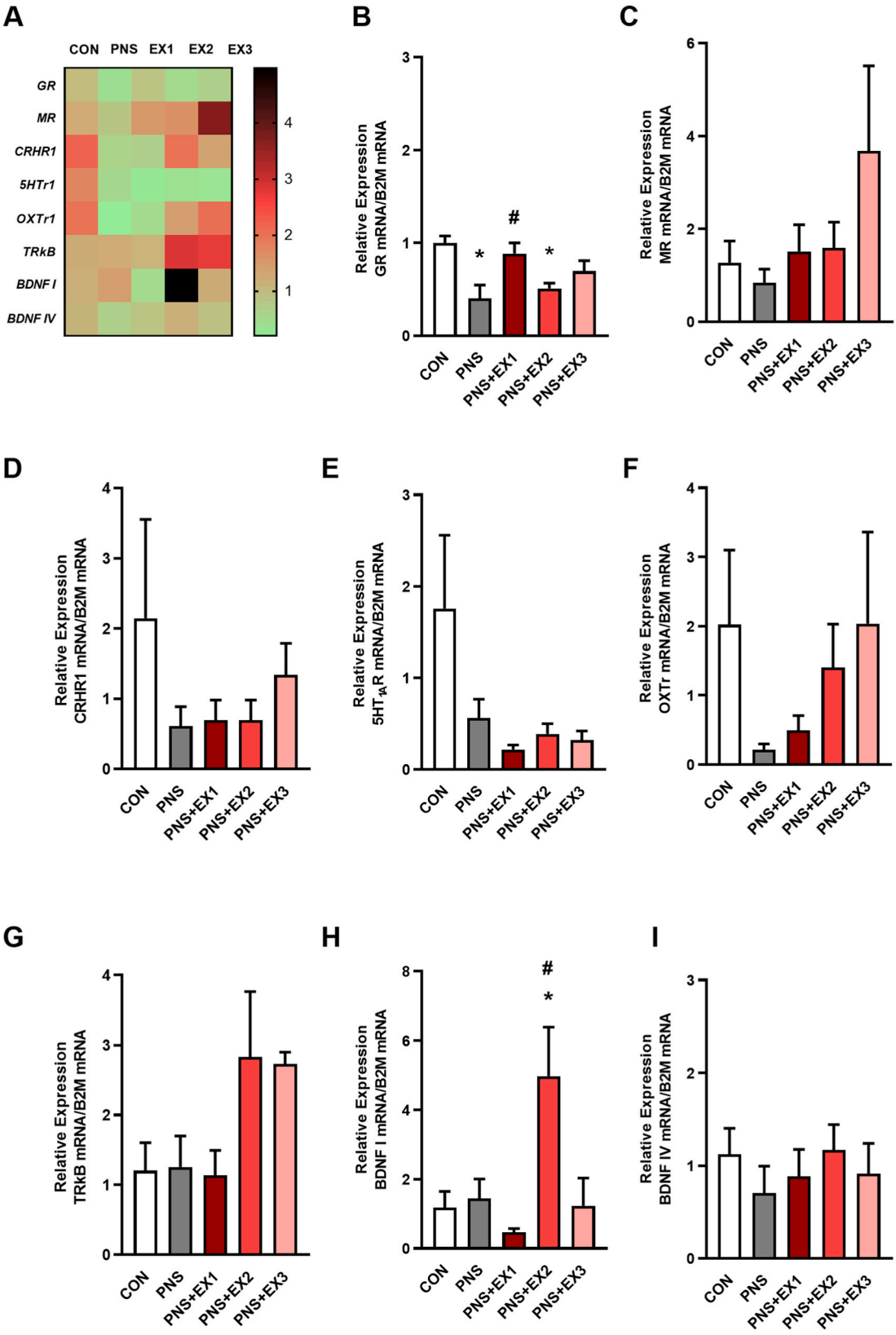


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Fig. 6. Male hippocampal gene expression. Heat map plot of gene expression (A). Glucocorticoid receptor (B), mineralocorticoid (C), corticotropin releasing hormone receptor 1 (D), serotonin receptor 1A (E), oxytocin receptor type 1 (F), tropomyosin receptor kinase B (G), brain-derived neurotrophic factor exon I (H), and brain-derived neurotrophic factor exon IV (I) mRNA expression were evaluated in the hippocampus at PND10. Data are shown as mean and standard error of the mean (SEM), and groups were compared by one-way ANOVA followed by the LSD post-hoc test. $^{*}p < 0.05$ denotes significant differences compared to the CON group; $^{\#}p < 0.05$ denotes significant differences compared to the PNS group. B2M: beta-2-microglobulin; GR: glucocorticoid receptor; MR: mineralocorticoid receptor; CRHR1: corticotropin releasing hormone receptor 1; $5HT_{1A}R$: serotonin receptor 1A; OXTr1: oxytocin receptor type 1; TRκB, tropomyosin receptor kinase B; BDNF I, brain-derived neurotrophic factor exon I; BDNF IV, brain-derived neurotrophic factor exon IV; CON: control; PNS: prenatal restraint stress; PNS + EX1: prenatal restraint stress and physical exercise before pregnancy; PNS + EX2: prenatal restraint stress and physical exercise during pregnancy; PNS + EX3: prenatal restraint stress and physical exercise before and during pregnancy.

a decrease in CRHR1 expression in the hippocampus, associated with a decrease anxiety-like behavior in adult male mice (Lian et al., 2019). In the fetal brain, we have found that prenatal stress decreased CRHR1 gene expression and exercise before pregnancy prevented this effect. To the best of our knowledge, this is the first study to report the effects of prenatal stress and exercise on CRHR1 expression in the mice fetal brain. We hypothesized that the beneficial effects of pre-pregnancy exercise may be acting on a region of fetal brain tissue other than the hippocampus, since these changes were not observed in PND10 mice. Furthermore, the first days of life are marked by high brain plasticity, which may influence the trajectories of programming effects after prenatal stress (Kolb and Gibb, 2011).

The $5HT_{1A}R$ is one of the main targets in studies involving neurological diseases due to its important role in the regulation of alterations related to stress and mood. $5HT_{1A}R$ is widely expressed in regulatory regions, such as hippocampus, amygdala, and prefrontal cortex (Garcia-Garcia et al., 2014). Moreover, placenta from both rodents and humans synthesizes 5HT and has already been reported that its expression can influence fetal brain development (Velasquez et al., 2013). Our findings did not demonstrate an effect of prenatal stress on placental $5HT_{1A}R$ gene expression. However, maternal physical exercise models decreased the expression of this receptor. It is well established that physical exercise collaborates in the maintenance of serotonergic metabolism (Greenwood and Fleshner, 2011). Furthermore, an increase in 5HT secretion activates a negative feedback, which inhibits 5HT by decreasing the expression of its receptors (Carhart-Harris and Nutt, 2017). Although we did not measure peripheral or central levels of 5HT, we are surmising here that our exercise protocol increased 5HT secretion and activated this physiological mechanism, decreasing $5HT_{1A}R$ gene expression. Similarly, our study found that maternal exercise, in addition to prenatal stress, also decreased $5HT_{1A}R$ gene expression in fetal brain. A recent study showed that prenatally stressed male rats presented decreased 5HT in the whole brain and impaired anxiety and depressive behavior (Amani et al., 2021). Our group has previously demonstrated that prenatal stress reduces $5HT_{1A}R$ in primary cortical neurons from fetal mice (Luft et al., 2021a).

An adverse intrauterine environment is associated to decreased birth weight and size, in addition to increased offspring mortality (Balasubramanian et al., 2015; Class et al., 2013). Although studies support the effects of prenatal stress in these alterations, we have only observed a decreased body weight in prenatally stressed female mice from mothers who performed physical exercise before and during pregnancy. We believe the period and frequency of the stressful event are determining factors for the outcomes observed in early life stress models. In a previous study, using the same stress model during pregnancy, we did not find significant differences for body weight in PNS mice during the neonatal period (Luft et al., 2021c). Although little is known on body weight changes in models of maternal exercise, a study showed that gestational exercise during pregnancy prevents fetal overgrowth in association with improved metabolic health (Son et al., 2019).

The postnatal period is essential for the development and integration of several peripheral and central systems related to the control of offspring homeostasis. During lactation, maternal stress has been associated with morphological and functional changes, such as decreased neural plasticity and altered neuroendocrine responses, contributing to increased corticosterone secretion and the development of depressive and anxious behaviors (Bosch et al., 2007; Haim et al., 2014; Zoubovsky et al., 2020). Our results on maternal behavior reveal that stress during pregnancy may alter important behaviors, such as the time of maternal self-maintenance. Together, our findings indicate that maternal exercise can ameliorate maternal care. Although the importance of physical exercise on different behavioral changes is known (Cao et al., 2021; Tomiga et al., 2021), this is the first study evaluating the effects of maternal exercise on maternal care.

At the molecular level, the stress response is mainly mediated by the regulation of the expression of key genes. In order to modulate the HPA axis activity, glucocorticoids provide feedback to the hippocampus via binding to both GR and MR. GR is distributed throughout the brain tissue, and the increase in cortisol/corticosterone concentrations primarily saturates MR and stimulates GR activation (De Kloet et al., 1998). Our results did not indicate differences on fetal brain GR gene expression. It is already well established that prenatal stress promotes a decrease in the expression of GR in the hippocampus of rodents (Cottrell and Seckl, 2009; Seckl, 2004). Thus, as expected, in the present study prenatal stress decreased the expression of GR in the hippocampus from PND10 males and maternal exercise before pregnancy prevented this effect. It is already known that males have increased expression of GR in the hippocampus, suggesting an increase in the negative feedback mechanism in a sex-dependent manner (Heck and Handa, 2019). In addition, a study has demonstrated that forced exercise increases GR protein expression in the hippocampus (Kim et al., 2019). Regarding MR, our data showed decreased gene expression in fetal brains from EX2 and EX3 groups. Forced exercise in the treadmill has already been associated with a decreased MR expression in the hippocampus of adult male mice (Chang et al., 2008). Considering that exercise can stimulate the HPA axis activation, MR downregulation in the brain from fetuses of exercised dams may be due to a feedback mechanism to reduce circulating corticosterone levels. We also found that prenatal stress increases MR mRNA expression in the hippocampus of female mice at PND10. This finding is consistent with other studies demonstrating an increase in this marker in neonatal rats stressed *in utero* (Lan et al., 2017), indicating an attenuated HPA axis response, as also observed in a previous study (Mairesse et al., 2007).

We have also evaluated OXTr1 gene expression, considering the participation of oxytocin in the modulation of different physiological and behavioral alterations in order to attenuate the stress response (Sippel et al., 2017). Exposure to prenatal stress has been already associated with a reduction on its receptor (OXTr) mRNA in the cortex of adult male mice (Gur et al., 2019). In the hippocampus, oxytocin plays a regulatory role in several behavioral functions, such as social attachment (Feldman et al., 2016). We did not observe effects of prenatal stress on OXTr1 expression in the whole brain and hippocampus from offspring. Similarly, a previous study did not find effects of gestational stress on this receptor in the hippocampus of adult mice (Schmidt et al., 2018). Surprisingly, our data showed that exercise before and during pregnancy decreased OXTr1 gene expression in the hippocampus of PND10 females. Few studies have associated physical exercise with the expression of oxytocin and mental health. However, voluntary aerobic exercise has recently been shown to decrease anxiety and increase both brain and serum oxytocin levels in female mice (Yuksel et al., 2019). Additional studies are needed for a better understanding of the relationship between maternal physical exercise and the expression of OXT

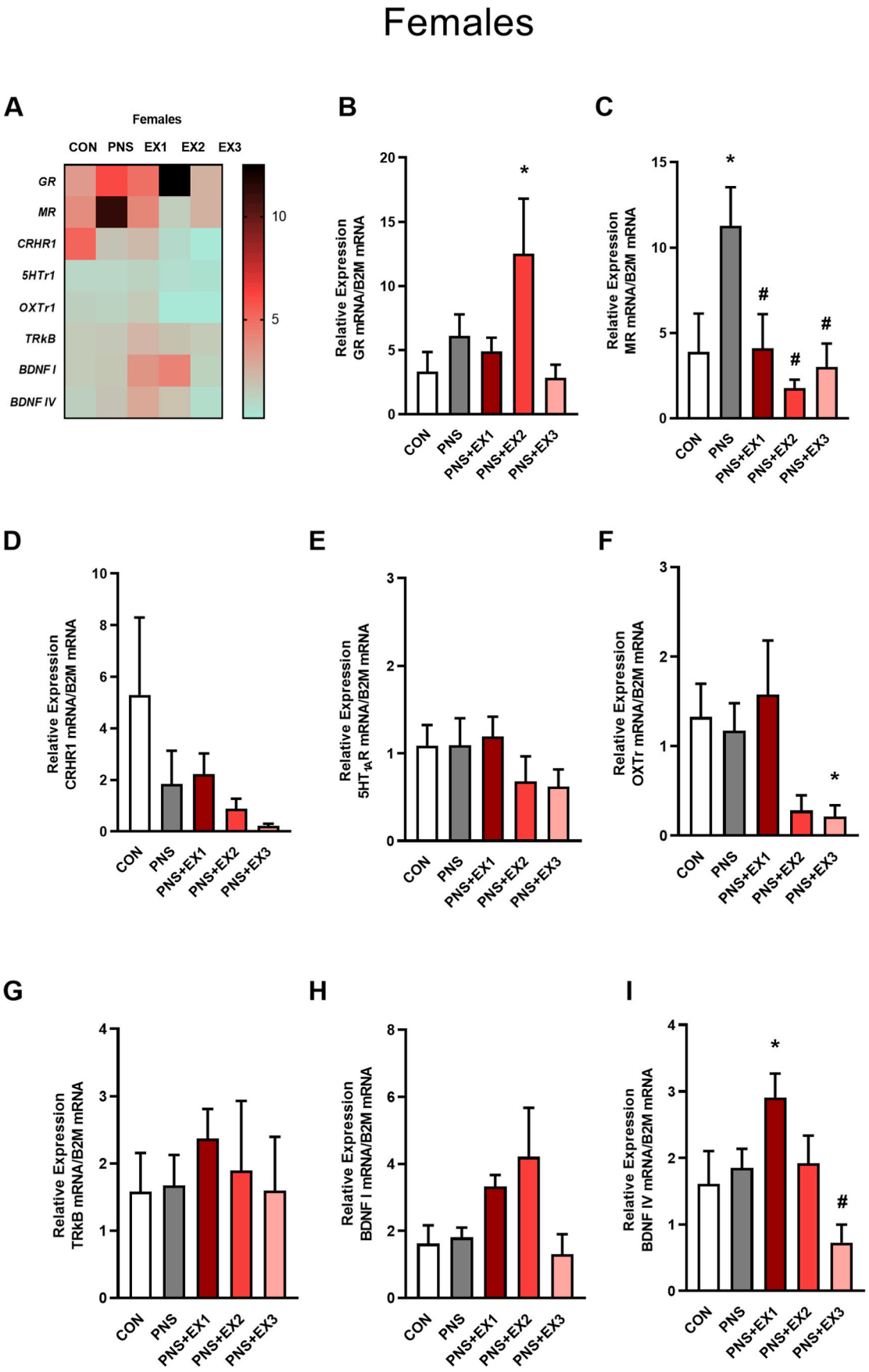

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Fig. 7. Female hippocampal gene expression. Heat map plot of gene expression (A). Glucocorticoid receptor (B), mineralocorticoid (C), corticotropin releasing hormone receptor 1 (D), serotonin receptor 1A (E), oxytocin receptor type 1 (F), tropomyosin receptor kinase B (G), brain-derived neurotrophic factor exon I (H), and brain-derived neurotrophic factor exon IV (I) mRNA expression were evaluated in the hippocampus at PND10. Data are shown as mean and standard error of the mean (SEM), and groups were compared by one-way ANOVA followed by the LSD post-hoc test. $^{*}p < 0.05$ denotes significant differences compared to the CON group; $^{\#}p < 0.05$ denotes significant differences compared to the PNS group. B2M: beta-2-microglobulin; GR: glucocorticoid receptor; MR: mineralocorticoid receptor; CRHR1: corticotropin releasing hormone receptor 1; $5HT_{1A}R$: serotonin receptor 1A; OXTr1: oxytocin receptor type 1; TRκB, tropomyosin receptor kinase B; BDNF I, brain-derived neurotrophic factor exon I; BDNF IV, brain-derived neurotrophic factor exon IV; CON: control; PNS: prenatal restraint stress; PNS + EX1: prenatal restraint stress and physical exercise before pregnancy; PNS + EX2: prenatal restraint stress and physical exercise during pregnancy; PNS + EX3: prenatal restraint stress and physical exercise before and during pregnancy.

receptors.

BDNF plays a key role in cell survival, synaptic plasticity, and cognitive processes, acting mainly by binding to tropomyosin receptor kinase B (TRκB) (Aid et al., 2007). Particularly, the BDNF exons I and IV appear to be the most sensitive regions to environmental stimuli, such as early-life stress (Dong et al., 2015). In a similar model of prenatal restraint stress, a decreased gene expression of BDNF exon I in the frontal cortex and hippocampus, and BDNF exon IV in the hippocampus of adult mice was observed (Dong et al., 2015). In the present study, we observed a decrease in BDNF exon I in the brain of PNS fetuses, which was prevented by maternal physical exercise before pregnancy. This is the first study evaluating the effects of maternal exercise on the expression of BDNF I and BDNF IV in fetuses and neonates. However, a study has associated exercise during pregnancy and increased expression of TrkB and BDNF in the hippocampus of adult offspring (Rahimi et al., 2018). Although no effects of prenatal stress were found, our findings demonstrate that physical exercise during pregnancy increased BDNF exon I gene expression in the hippocampus of males on PND10. In adult rodents, there is also evidence supporting the effects of physical exercise on the increased expression of BDNF I in the hippocampus (Boschen et al., 2017; Wearick-Silva et al., 2017).

5. Conclusion

Our results indicate that maternal physical exercise may play a role in modulating maternal-fetal health and can contribute to preventing neurodevelopmental changes induced by prenatal stress. The protective effects of physical exercise include improved maternal behavior and the regulation of markers responsible for the stress response in the placenta and brain of neonatal mice prenatally stressed. Although each physical exercise protocol studied showed specific effects on the measured outcomes, data presented here indicate that physical exercise before pregnancy seems to have the most pronounced protective role. These findings may represent an additional step towards a better understanding of prenatal stress repercussions during the neonatal period.

Author contributions

Carolina Luft conceived the work, acquired data, drafted the paper, performed data analysis, and approved the final version. Mariana Severo da Costa and Géssica Luana Antunes acquired data, revised the article and approved the final version. Jarbas Rodrigues de Oliveira conceived the work, revised the paper, and approved the final version. Márcio Vinícius Fagundes Donadio conceived the work, acquired funding, performed data analysis, revised the article, and approved the final version.

Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request.

Declaration of competing interest

The authors have no conflict of interests related to the present study to declare.

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