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ORIGINAL RESEARCH REPORT

Parental pre-conception stress status and risk for anxiety in rat offspring: specific and sex-dependent maternal and paternal effects

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ABSTRACT

Prenatal stressful events have long-lasting consequences on behavioral responses of offspring. While the effects of gestational and maternal stress have been extensively studied on psychological alterations in the progeny, little is known about effects of each parent's pre-conception life events on emotional responses in offspring. Here, the effect of maternal and/or paternal pre-conception stress was investigated on anxiogenic responses of offspring. Male and female adult rats were subjected to predatory stress (contactless exposure to a cat for 1 + 1 h per day) for 50 (male, *n*: 12) and 15 (female, *n*: 24) consecutive days; controls were not exposed. After the stress procedure, the control and stressed rats were mated to create four types of breeding pairs: control female/control male, stressed female/control male, control female/stressed male, and stressed female/stressed male. On postnatal days 30–31, the offspring were tested on the elevated plus maze and plasma corticosterone concentration was measured. Half of the pups were exposed to acute predatory stress before the elevated plus maze test. In most subgroups, corticosterone and anxiety-like behaviors in the offspring with both or only one parent exposed to pre-gestational stress increased compared to their control counterparts. However, under acute stress conditions, a different sex-dependent pattern of anxiety responses emerged. The combined effects of maternal and paternal stress were not additive. Hence, individual offspring behaviors can be influenced by the former life stress experiences of either parent. Incorporation of genetic and epigenetic aspects in development of neurobehavioral abnormalities and reprograming of the hypothalamic-pituitary-adrenal axis may contribute to this phenomenon.

LAY SUMMARY

Early life stress (including during pregnancy) is known to have long-lasting effects on offspring, including emotional behaviors. Whether individual anxiety behaviors can be influenced by stress experiences of each parent even before a pregnancy is less well-understood. Our findings from this study on rats exposed to predator stress before mating suggest that maternal or paternal adult life events prior to pregnancy can lead to maladaptive behavior in their offspring later in life.

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Anxious behavior; corticosterone; elevated plus maze; maternal stress; paternal stress; pre-conception predator stress

Introduction

Chronic activation of the stress axis in response to prolonged and unpredictable factors in the environment has been regarded as causing or contributing to pathological effects (Cottrell & Seckl, 2009). Stress activates the autonomic nervous system and the hypothalamus, which is responsible for the increased release of corticotropin-releasing hormone (CRH), in response to input from extra-hypothalamic sources. This release, in turn, increases the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which subsequently leads to the release of corticosterone from the adrenal cortex. Elevated blood concentrations of corticosterone following exposure to stressful events improve the restorative capacity of the body and prepare the organism for future challenges (De Kloet, Joëls, & Holsboer, 2005; Joëls, 2009). Previous studies have demonstrated that prenatal environmental factors including stressful conditions can also lead to prolonged enhancement of corticosterone levels and exert severe and significant influences on the offspring postnatal development, leading to various behavioral abnormalities. In children, prenatal stress is linked to problems including preterm birth, fetal growth retardation, and an increased vulnerability for developing various psychosocial, cognitive, behavioral, physical, and emotional problems, with delays in motor development, impaired memory and language development, autism, attention deficits, and anxiety disorder (van den Bergh, Dahnke, & Mennes, 2018). In adults, prenatal stress is associated with an increased rate of depression, schizophrenia and learning disabilities (van den Bergh et al., 2018). The effects of prenatal stress are also observed

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in animals, seen as increased behavioral abnormalities, anxiety, and attention deficits as well as decreased learning and memory ability (Coe et al., 2003; Fride & Weinstock, 1988; Takahashi, Haglin, & Kalin, 1992). In this respect, our team has previously shown that stress during gestation can cause early and long-lasting effects on neurobehavioral development in both humans (Gholipoor et al., 2017) and animal offspring (Ebrahimi, Saboory, Roshan-Milani, & Hashemi, 2014; Hashemi, Ebrahimi, Saboory, & Roshan-Milani, 2013; Hashemi, Roshan-Milani, Saboory, Ebrahimi, & Soltanineghad, 2016; Heshmatian, Roshan-Milani, & Saboory, 2010; Saboory, Ebrahimi, Roshan-Milani, & Hashemi, 2015).

While the effects of gestational and neonatal stress on psychological alterations in offspring have been widely studied, much less is known regarding the effects of parental pre-gestational life events on offspring behavior. Humans and animals may be challenged with a wide range of stressors every day during their routine life and may therefore experience different cumulative life histories prior to the beginning of each pregnancy and not only during the perinatal period. Whether maternal or paternal adult life events prior to pregnancy can lead to maladaptive behavior in the offspring later in life is less well understood (Bale et al., 2010). The epigenetic effect of parental life events prior to pregnancy on inter-individual difference among offspring has also yet to be examined. Stressors that activate the hypothalamic-pituitary-adrenal (HPA) axis are found to affect oocytes (Zhang et al., 2011; Zhou et al., 2012) and spermatocytes (Yazawa, Sasagawa, Ishigooka, & Nakada, 1999) in adult animals. It is suggested that such stressors might "program" persistent behavioral changes in offspring by their direct effects on developing gametes (He et al., 2016), as well as on stress-induced phenotypes that may be transmitted to offspring. It has also been reported that chronic activation of the stress axis before pregnancy causes long-lasting changes in hippocampal mechanisms and reduces the expression of brain receptors and neurotrophic factors associated with impairment of memory in offspring rats (Huang et al., 2010). These data suggest that stress before pregnancy appears to cause changes in brain mechanisms and might have a profound influence on brain development of offspring.

The predatory stress experimental protocol is considered as a pure psychological stressor, and has been widely used to study the effects of psychological stress. Predator stimuli are obviously stressful for rodents (Liu et al., 2012), and when the predator is in contact with the subject, the animal escapes whenever possible or attacks the predator in a defensive manner (Blanchard & Blanchard, 1988, 2008). It has been reported that exposure of rats and mice to natural predators or even to their odors causes anxiety-like behaviors when tested in the social interaction and elevated plus maze (EPM) test (Adamec & Shallow, 1993; Berton, Vogel, & Belzung, 1998; Dielenberg & McGregor, 2001; Zangrossi & File, 1992b). Prenatal exposure to predator stress also leads to anxiety and depressive behaviors in offspring (Green, Esser, & Perrot, 2018; Korgan et al., 2016). Exposure to natural predators and their odors induces a pattern of increased stress hormone secretion in rats and mice (Belzung, El Hage, Moindrot, & Griebel, 2001; Hayley, Borowski, Merali, & Anisman, 2001), which raises the possibility that prenatal exposure to elevated glucocorticoids may increase the risk for expressing anxiety responses among offspring (Korgan et al., 2016; McGowan & Matthews, 2018).

As mentioned above, most recent studies on prenatal stress have focused on the impact of stress during the gestational period. However, stressors present early in pregnancy are often present before conception as well (Hobel & Culhane, 2003). It has also been reported that the effects of a hostile environment prior to pregnancy or in early life can be transmitted to the next generations, apparently via genomic or non-genomic mechanisms (Bale, 2015; Grundwald & Brunton, 2015; He et al., 2016; Mahmoodkhani et al., 2018). Moreover, most of the previous studies have focused on the effect of one parent's stress (especially maternal stress) on offspring postnatal development. To our knowledge, few studies have examined the modulation of behavioral disturbance by pre-gestational stress involving both parents. With respect to anxiety behavior, the present study is also the first to establish a link between predatory pre-gestational stress in both parents and anxiety behavior of offspring. This study was aimed to investigate the effect of maternal and/or paternal pre-pregnancy stress on anxiogenic responses in rat offspring to address the hypothesis that pre-gestational stress (i.e. during parental spermatogenesis and oogenesis) may increase vulnerability of the offspring brain to develop neurobehavioral abnormalities.

Methods

Animals

Male and female Wistar rats (200–250 g) were obtained from the animal facility at the Urmia University of Medical Sciences, Urmia, Iran. They were 12 weeks old on delivery. The rats were housed in single-sex groups of four per cage (standard polycarbonate box 42 cm × 21 cm × 20 cm) and kept in standard conditions as follows: standard 12 h light–dark cycle (light from 07 am, dark from 07 pm), environmental temperature 22 ± 2 °C, and food and water accessible *ad libitum*. A conscious effort was made to minimize the number and suffering of animals used in this study. After 15 days of adaptation, the male and female rats were randomly divided into four groups to form a combination of control and stressed groups for each sex [$n = 24$ male (12 control and 12 stressed rats) and $n = 48$ female (24 control and 24 stressed rats)]. In the stressed group, the female and male rats were exposed to a predatory stressor for 15 and 50 consecutive days, respectively. "Fifteen days" include three estrous cycles in the female rats and "50-day period" is the time needed for a complete spermatogenesis cycle in the male rats. A total of 48 female and 24 male rats were then allowed to mate in stainless steel cages with two females and one male per cage (see "types of breeding pairs" below). Moreover, 12 additional female rats (6 control and 6 stressed rats) were obtained using the same stress protocol, for blood sampling at the end of the stress procedure, except that they were not mated.

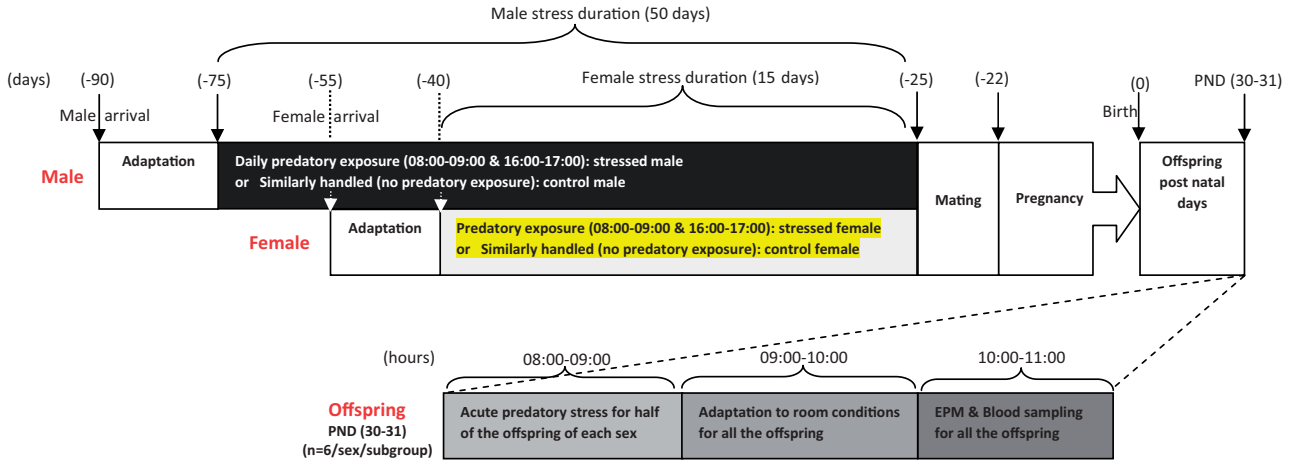

Figure 1. Timeline of the experimental procedures.

The timeline of the described experimental procedures is portrayed in Figure 1.

Predatory stress procedure

A healthy adult feral cat, received from the Faculty of Veterinary Medicine, the Urmia University, was used as the predator for the rats. The cat was placed in a stainless steel holding cage (72 cm × 72 cm × 63 cm) consisting of a solid metal floor with a hinged, metal rod door with air holes in the side so that the cat could be observed. To produce predator stress, the rats were first placed in boxes (20 cm × 22 cm × 22 cm) with multiple air holes in the side walls. The boxes were then immediately moved to close to the cage of the hungry cat. In this situation, the stressed rats could not be physically harmed but experienced fear and were exposed to all sensory stimuli such as the sight, odor, and sounds produced by the cat (Ahmadzadeh, Saboory, Roshan-Milani, & Pilehvarian, 2011; Wilson, Ebenezer, McLaughlin, & Francis, 2014). The rats were subjected to predatory stress twice per day (for a period of one hour each, between 08 am–09 am and 4 pm–5 pm) for 15 consecutive days for the female rats and 50 consecutive days for the male rats. After each stress session, the rats were returned to their home cage. During these periods, the control rats were also transported to another experimental room and handled similarly to the stressed rats, but were not exposed to the cat.

Breeding procedure

At the end of the stress protocol, the control and stressed female and male rats were mated with other stressed or control rats for 72 h (two females and one male per cage). In total, 4 types of breeding pairs were created as follows (Figure 2): (1) pair of both control rats (CC), (2) pair of stressed females and control males (SC), (3) pair of control females and stressed males (CS), and (4) pair of both stressed females and males (SS). After a 72 h coupling period, the female rats were moved to new cages, in which they were housed three per cage for the entire pregnancy and kept in normal conditions. After parturition, the dam and offspring were transferred to a new cage and the pups were counted. Thus the pups were placed into four main groups based on their parents' grouping as follows: C-C: none of the parents was stressed; S-C: only the mother was stressed; C-S: only the father was stressed; S-S: both the parents were stressed.

At postnatal days 30–31 (pre-pubertal period), before behavioral assessment and blood sample collection, half of the female and male pups in each group were exposed to acute predatory stress, as described above. The remaining pups were not exposed to acute predatory stress and were considered as non-acute stressed subgroups. In total, 16 subgroups of the pups were formed and studied as follows:

C-C(female): The female non-acute stressed offspring of the CC parents;
C-C(female-s): The female acute stressed offspring of the CC parents;
C-C(male): The male non-acute stressed offspring of the CC parents;
C-C(male-s): The male acute stressed offspring of the CC parents;
S-C(female): The female non-acute stressed offspring of the SC parents;
S-C(female-s): The female acute stressed offspring of the SC parents;
S-C(male): The male non-acute stressed offspring of the SC parents;
S-C(male-s): The male acute stressed offspring of the SC parents;
C-S(female): The female non-acute stressed offspring of the CS parents;
C-S(female-s): The female acute stressed offspring of the CS parents;
C-S(male): The male non-acute stressed offspring of the CS parents;
C-S(male-s): The male acute stressed offspring of the CS parents;
S-S(female): The female non-acute stressed offspring of the SS parents;

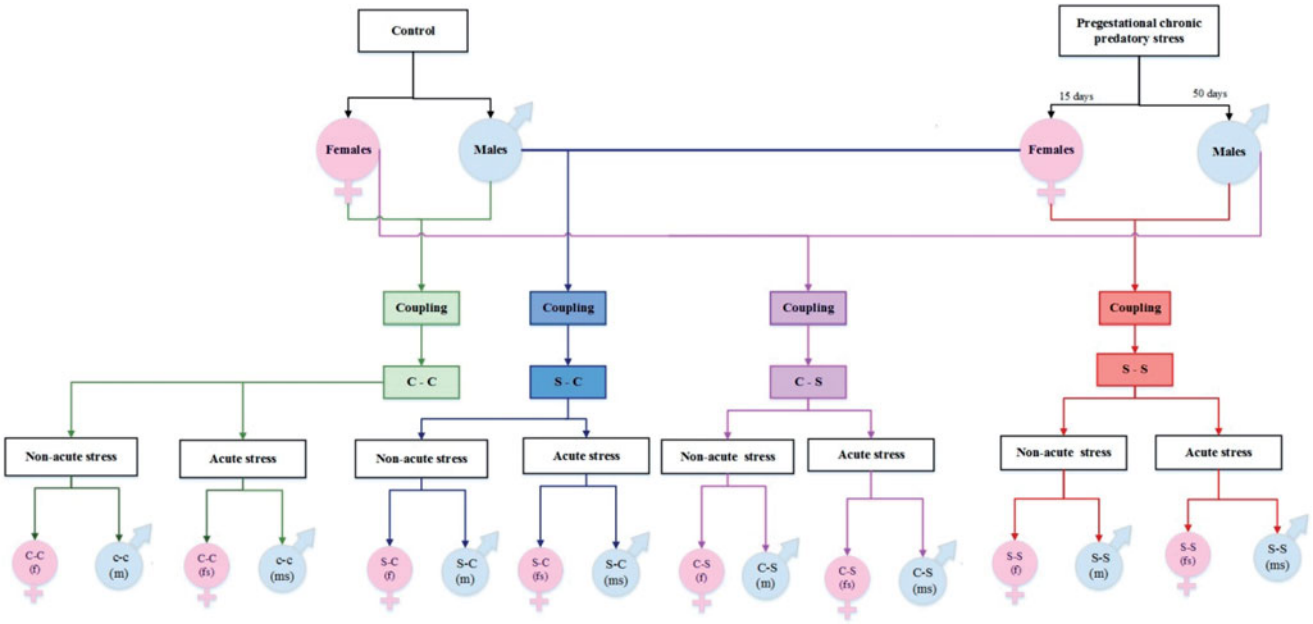

Figure 2. A coupling diagram of the breeding pairs and the subgroups of the offspring. f: female; m: male; fs: female acute-stressed; ms: male acute-stressed; C-C: none of the parents was stressed; S-C: only the mother was stressed; C-S: only the father was stressed; S-S: both parents were stressed.

S-S(female-s): The female acute stressed offspring of the SS parents;
S-S(male): The male non-acute stressed offspring of the SS parents;
S-S(male-s): The male acute stressed offspring of the SS parents;
Coupling of the breeding pairs and the subgroups of the offspring are presented in Figure 2.

Behavioral assessment

At postnatal days 30–31 (PND30–31), the elevated plus maze (EPM) test was performed for all the subgroups of the rat offspring, including the control pups (maximum 1 male and female per litter per group) as described previously (Gholami, Saboory, & Khalkhali, 2014; Nakhjiri, Saboory, Roshan-Milani, Rasmi, & Khalafkhani, 2017; Rodgers & Dalvi, 1997). The EPM test is a commonly used behavioral assay for evaluating baseline anxiety-like behavior in rodents. As PND30–31 in rodents corresponds to the age of ~9 years in humans, we chose this pre-pubertal period for behavioral assessment in order to both provide neurodevelopment insights for an equivalent challenge at the end of childhood, before puberty onset in humans, (preadolescence), and to remove the impact of the estrous cycle on the EPM performance. PND30–35 clearly represents a period of intense physical, cognitive, emotional, and social development (Semple, Blomgren, Gimlin, Ferriero, & Noble-Haeusslein, 2013). In the present work, half of the female and male pups in each group were exposed to acute predatory stress (by placing the cage of the pups close to the cage of the predator cat for 1 h), before the EPM test (the acute stressed subgroups). The remaining pups were not exposed to acute predatory stress and directly tested in the EPM task (the non-acute stress subgroups). All the rat pups were placed in a behavioral testing room at least 1 h before testing. The timeline of the described experimental procedures is portrayed in Figure 1.

As the behavior of the rats on the EPM can be affected by the light cycle and circadian rhythms, all the pups were tested between 10 am and 11 am, in the light phase of their day–night cycle. An EPM constructed specifically for rats was used. The maze was made of plexiglas and consisted of a central square (15 × 15 cm), two open arms without walls (15 × 45 cm) and two closed arms with plexiglas walls (15 × 40 × 30 cm). The maze was elevated 50 cm above the floor. The apparatus was placed in a soundproof room illuminated by a 100 W red light bulb. For the EPM test, the rat pups of each subgroup were placed at the junction of the four arms of the maze (facing an open arm) and the entry into and time spent in each arm were recorded on videotape and observed for a duration of 5 min. After each test, the EPM was cleaned before testing a new rat to avoid any odorant undermining the test results. The percent of open arm time and entry (open arm activity), percent of closed arm time and entry (closed arm activity) and index of open arm avoidance (IOAA) were calculated for each offspring sex separately and considered as anxiety parameters. The percent of open arm time (OAT%) and closed arm time (CAT%) was used to represent the time spent by a rat in the open arms and closed arms, respectively, as a percentage of the total observation period of 5 min (300 s). The percent of open arm entries (OAE%) was used to represent the number of entries into the open arms and calculated as: $OAE\% = OAE/OAE + CAE \times 100$, where OAE is the number of entries into the open arms and CAE is the number of entries into the closed arms. The percent of closed arm entries (CAE%) was used to represent the number of entries into the closed arms and calculated as: $CAE\% = CAE/OAE + CAE \times 100$. IOAA was calculated as: $100 - [(OAT\% + OAE\%)/2]$.

Blood sample collection

Immediately following the EPM test, blood samples were collected by cardiac puncture under isoflurane anesthesia from all the subgroups of the rat offspring (maximum 1 male and female per litter per group; 5 per sex per sub-group). As ether inhalation might lead to an increase in ACTH and corticosterone secretion in rats (Laczi et al., 1994; Zelena, Domokos, Jain, Jankord, & Filaretova, 2009), we used isoflurane for induction of anesthesia. Blood was collected in 1.5-ml EDTA-coated micro-centrifuge tubes, kept on ice and then centrifuged for 15 min at 1000 g, at 4°C. Plasma was transferred to clean 1.5-ml microcentrifuge tubes and stored frozen at −80 °C until corticosterone was determined. Corticosterone was measured using a commercial ELISA kit (Rat corticosterone ELISA Kit, Elabscience Biotechnology Co. China). The inter- and intra-assay coefficients of variation for the corticosterone assay were <10%. The sensitivity of the kit was 1.88 ng/ml. The detection range was between 3.13 and 200 ng/ml, which is consistent with the obtained standard curve.

Moreover, to confirm stress induction in the adult rats, plasma corticosterone concentration was measured in the adult female rats. Twelve female adult rats (six control and six stressed rats) were anesthetized with isoflurane at the end of the predatory stress procedure; blood samples were collected and corticosterone blood concentrations were measured using the same procedure as described above.

Ethical approval

All the experimental protocols and procedures were conducted according to the guidelines of the 1975 Declaration of Helsinki, as reflected in the guidelines of the Medical Ethics Committee, the Ministry of Health, Iran. In addition, the Regional Medical Ethics Committee in the West Azarbayjan province of I.R. of Iran approved this study.

Statistical analysis

Statistical analysis was performed using SPSS 16 (SPSS/PC-16, SPSS Inc, USA). Data distribution was controlled using the Kolmogorov–Smirnov test. The data were normally distributed and analyzed using parametric techniques. Three-way ANOVA was performed to analyze the data related to plasma corticosterone concentrations and anxiety behaviors for three factors of "maternal condition (stress/control)", "paternal condition (stress/control)," and "offspring condition (stress/control)". Interactions were analyzed *post hoc* with simple effects analyses, using LSD correction. Unpaired samples *T*-Test was performed to compare corticosterone blood concentrations between the control and stressed adult rats. The results are expressed as mean ± SEM, and the differences were considered significant at $p \leq .05$.

Results

Three-way ANOVA was performed for each offspring sex separately to analyze the data related to plasma corticosterone concentration and anxiety behaviors for the three factors of "maternal condition (stress/control)", "paternal condition (stress/control)," and "offspring condition (stress/control)". Interactions were analyzed *post hoc* with simple effects analyses, using LSD correction (Figure 3). The LSD test for multiple comparisons of plasma corticosterone concentration and anxiety parameters in the female and male pups was based on three main comparisons: (A) comparison among the non-acute stressed pups of the C-C, S-C, C-S, and S-S groups; (B) comparison among the acute stressed pups of the C-C, S-C, C-S, and S-S groups and (C) comparison between the non-acute stressed and acute stressed pups within the C-C, S-C, C-S, and S-S groups. A summary of these results is presented in Figure 3.

Effects of pre-gestational stress on plasma concentrations of corticosterone in female and male rat offspring

The results of three-way ANOVA indicated a significant interaction among "maternal condition" × "paternal condition" × "offspring condition" on corticosterone blood concentration in the female pups ($F = 5.99$, $p = .02$). The results also indicated a significant interaction among "maternal condition" × "paternal condition" × "offspring condition" in the male pups ($F = 3.74$, $p = .05$). In the male pups, the interaction of "maternal condition" × "paternal condition" ($p = .03$) as well as the interaction of "paternal condition" × "offspring condition" ($p = .05$) was also significant.

According to the simple effects analysis, by using LSD correction, plasma corticosterone concentration was observed to be significantly different among the experimental groups ($p < .001$, Figure 3). Overall, pre-gestational stress was associated with an increase in plasma corticosterone concentration in both the female and male offspring. In the non-acute stressed pups in both the female and male offspring, plasma corticosterone concentration increased overall in both the "only mother" and "only father" stressed offspring, but not in the "both parents" stressed pups. In the acute stressed pups in the female offspring, plasma corticosterone concentration increased in "both parents" stressed pups; however, the increase in the "only mother" and "only father" stressed pups was not significant. In the male acute stressed offspring, plasma corticosterone concentration increased in the "only father" and "both parents" stressed pups. However, the increase in the "only mother" stressed offspring was not significant.

In the adult female rats, plasma corticosterone concentration increased from 43.4 ± 5.2 ng/ml in the control rats ($n = 6$) to 58.7 ± 4.5 ng/ml in the stressed rats ($n = 6$, unpaired samples *T*-Test, $p \leq .05$), which confirms stress induction in the adult stressed rats.

Effects of pre-gestational stress on anxiety behaviors in female and male rat offspring

The elevated plus-maze (EPM) test was performed to assess anxiety-like behavior for each offspring sex separately. As described in the "Methods" section, the open arm activity

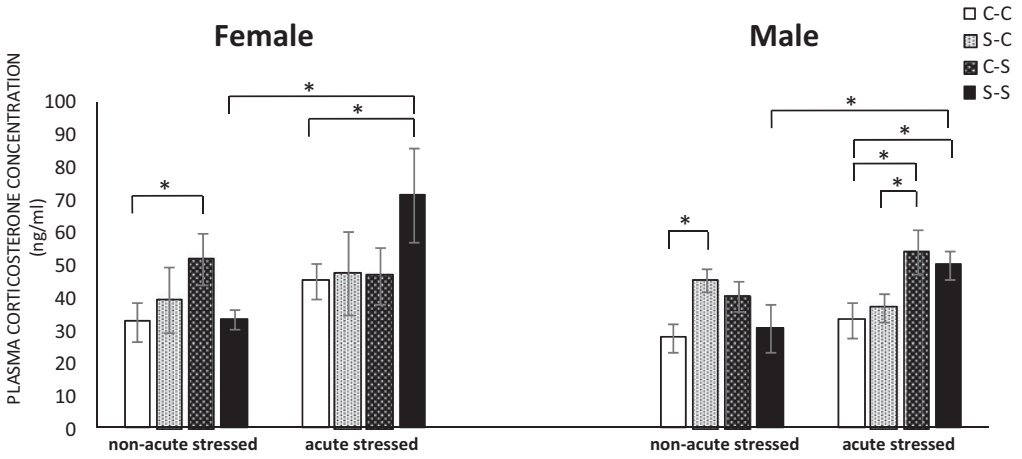

Figure 3. Comparison of the pups' plasma corticosterone concentrations among the experimental groups. Data are expressed as mean ± SEM ($n = 5$ for each subgroup, except the S-S subgroups). For clarity and in order to show the most relevant results, not all the significant changes are shown. Difference between the indicated groups: $^{*}p \leq .05$ (LSD test). C-C: none of the parents was stressed; S-C: only the mother was stressed; C-S: only the father was stressed; S-S: both parents were stressed.

[(open arm time (OAT) and open arm entry (OAE)], closed arm activity [closed arm time (CAT) and closed arm entry (CAE)] and index of open arm avoidance (IOAA) were calculated and considered as anxiety parameters. Anxiety behaviors of the female and male offspring were markedly affected by pregestational stress in this study.

The open arm activity in the EPM test

The three-way ANOVA analysis of OAT% for the entire 5-min trial revealed a significant interaction among "maternal condition" × "paternal condition" × "offspring condition" in both the female ($F = 18.2$, $p \leq .001$) and male pups ($F = 59.76$, $p \leq .001$). In both the female and male pups, the interactions of "maternal condition" × "paternal condition," "maternal condition" × "offspring condition" and "paternal condition" × "offspring condition" were all significant (all $p \leq .001$).

The results related to the simple effects analyses of OAT% and OAE% (the LSD test) are presented in Figure 4. Overall, pre-gestational stress was associated with a decrease in the open arm activity (decreased OAT% and OAE%, indicative of an increased anxiety-like behavior) in both the female and male offspring but more specifically, when they were exposed to stressful conditions. In the non-acute stressed pups, in the female offspring, the open arm activity decreased in all the pre-gestationally stressed offspring, but more significantly in the "only father" stressed offspring. In the male non-acute stressed offspring, the open arm activity decreased in all the pre-gestationally stressed offspring, but more significantly in the "only mother" stressed offspring. In the acute stressed pups, in female offspring, OAT% decreased significantly in the "only mother" and "both parents" stressed pups; however, the decrease was not significant in the "only father" stressed pups. In the male acute stressed offspring, OAT% decreased significantly in the "only father" and "both parents" stressed pups, but the decrease was not significant in the "only mother" stressed pups.

The closed arm activity in the EPM test

In a similar manner to OAT, the three-way ANOVA analysis of CAT% for the entire 5-min period indicated a significant interaction among "maternal condition" × "paternal condition" × "offspring condition" in both the female ($F = 18.32$, $p \leq .001$) and male pups ($F = 42.59$, $p \leq .001$). In both the female and male pups, the interactions of "maternal condition" × "paternal condition," "maternal condition" × "offspring condition" and "paternal condition" × "offspring condition" were all significant (except the interaction of "maternal condition" × "paternal condition" in the female pups, all $p \leq .001$).

The results related to the simple effects analyses of CAT% and CAE% (the LSD test) are presented in Figure 5. Overall, pre-gestational stress was associated with an increase in the closed arm activity (increased CAT% and CAE%, indicative of an increased anxiety-like behavior) in both the female and male offspring but more specifically, when they were exposed to stressful conditions. In the female non-acute stressed offspring, the closed arm activity increased in all the pre-gestationally stressed offspring, but more significantly, in the "only father" stressed offspring. In the male non-acute stressed offspring, the closed arm activity increased in all the pre-gestationally stressed offspring, but more significantly, in the "only mother" stressed offspring. Moreover, in the female acute stressed offspring, CAT% increased in the "only mother" and "both parents" stressed pups; however, the increase was not significant in the "only father" stressed pups. In the male acute stressed offspring, CAT% increased significantly in the "only father" and "both parents" stressed pups, but the increase was not significant in the "only mother" stressed pups.

"The index of open arm avoidance (IOAA)" in the EPM test

The three-way ANOVA analysis of IOAA revealed a significant interaction among "maternal condition" × "paternal

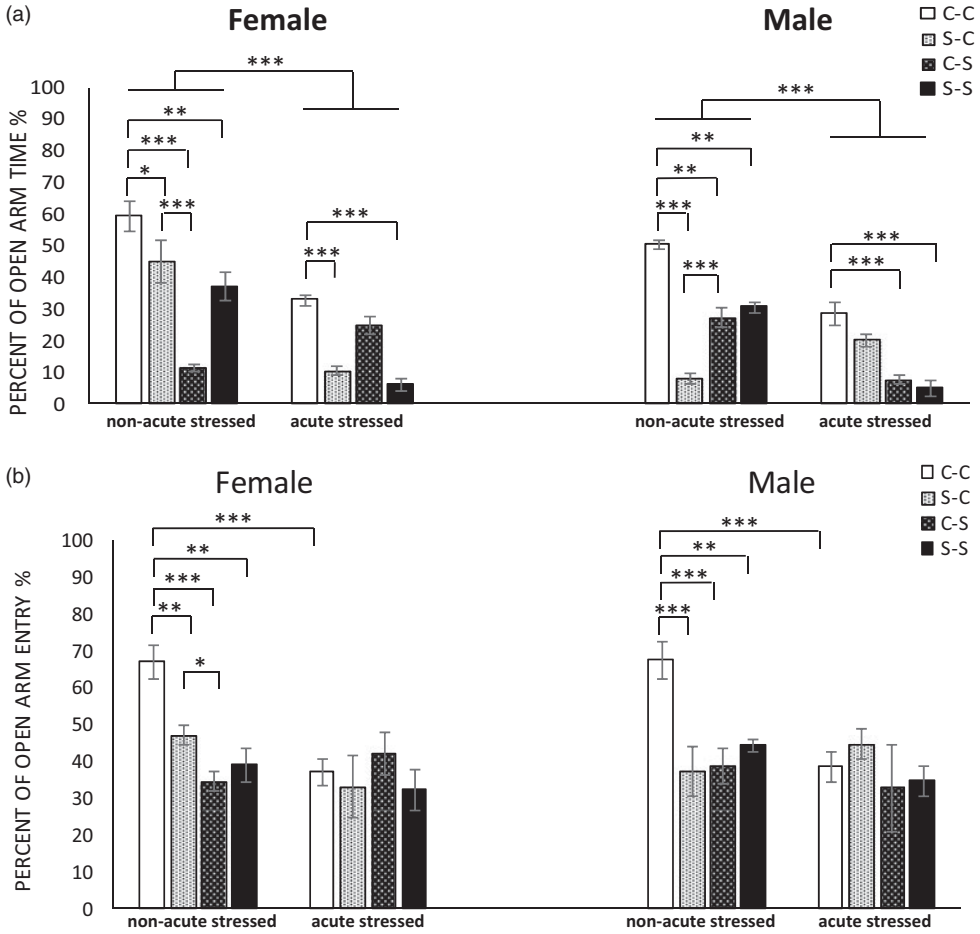

Figure 4. Comparison of the pups' "open arm activity" among the experimental groups. (A) The percent of open arm time (OAT%) in the female (left) and male (right) rat pups. (B) The percent of open arm entry (OAE%) in the female (left) and male (right) rat pups. Data are expressed as mean ± SEM ($n = 6$ for each subgroup). For clarity and in order to show the most relevant results, not all the significant changes are shown. Difference between the indicated groups: $^{*}p \leq .05$; $^{**}p \leq .005$, $^{***}p \leq .001$ (LSD test). C-C: none of the parents was stressed; S-C: only the mother was stressed; C-S: only the father was stressed; S-S: both parents were stressed.

condition" × "offspring condition" in both the female ($F = 13.77$, $p \leq .001$) and male pups ($F = 14.88$, $p \leq .001$). In both the female and male pups, the interactions of "maternal condition" × "paternal condition," "maternal condition" × "offspring condition" and "paternal condition" × "offspring condition" were all significant (except the interaction of "paternal condition" × "offspring condition" in the male pups, all $p \leq .001$).

The results related to the simple effects analyses of IOAA (the LSD test) are shown in Figure 6. Overall, pre-gestational stress was associated with an increase in IOAA (indicative of an increased anxiety-like behavior) in both the female and male offspring but more specifically, when they were exposed to stressful conditions. In the female non-acute stressed offspring, IOAA increased in all the pre-gestationally stressed offspring, but more significantly, in the "only father" stressed offspring. In the male non-acute stressed offspring, IOAA increased in all the pre-gestationally stressed offspring, but more significantly, in the "only mother" stressed offspring. In the female acute stressed offspring, IOAA increased in the "only mother" and "both parents" stressed pups; however, the "only father" stressed pups were not significantly affected. In the male acute stressed offspring, IOAA increased significantly in the "only father" and "both parents" stressed pups, but the "only mother" stressed pups were not significantly affected.

Discussion

In the present study, the impact of predatory stress in pre-gestational period, involving both or only one parent rats, was investigated on plasma corticosterone concentrations and anxiety behaviors of their offspring. The main finding was that maternal and/or paternal pre-pregnancy stresses led to a significant increase in anxiety-like behaviors of offspring, while plasma corticosterone concentrations increased only in pups whose only one parent was exposed to predatory stress before pregnancy. However, when offspring were exposed to the same threat experienced by the parents, a significant and sex-dependent increase in anxiety-like behaviors and plasma corticosterone concentrations was found in most of the subgroups. In addition, in most of the experimental subgroups, acute stressed pups showed an additional increase in anxiety behaviors in comparison to non-acute stressed pups.

The predator stimulus has been considered as a stressful and fear arousing model of psychological stress which leads

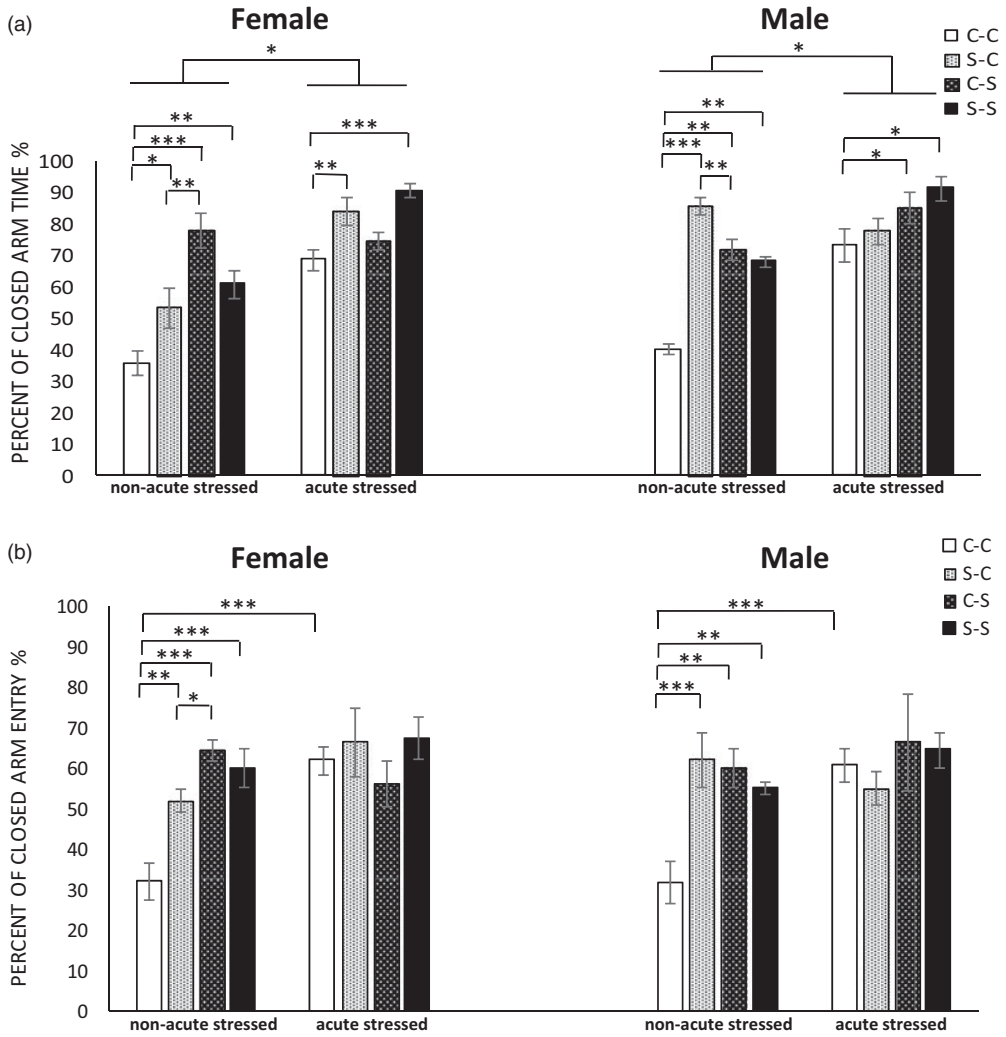

Figure 5. Comparison of the pups' "closed arm activity" among the experimental groups. (A) The percent of closed arm time (CAT%) in the female (left) and male (right) rat pups. (B) The percent of closed arm entry (CAE%) in the female (left) and male (right) rat pups. Data are expressed as mean ± SEM ($n = 6$ for each subgroup). For clarity and in order to show the most relevant results, not all the significant changes are shown. Difference between indicated groups: $^{*}p \leq .05$; $^{**}p \leq .005$, $^{***}p \leq .001$ (LSD test). C-C: none of the parents was stressed; S-C: only the mother was stressed; C-S: only the father was stressed; S-S: both parents were stressed.

to more natural reactions and a wider range of defensive responses (Blanchard et al., 1998; Dielenberg, Carrive, & McGregor, 2001; Kavaliers & Choleris, 2001). When rodents are exposed to predator stimuli or even their odors, they do not show habituation in defensive responses, which eliminates the possibility of rats adapting to the predatory stress (Blanchard et al., 1998; Zangrossi & File, 1992a). In particular, exposure to a cat creates long-lasting increases in rat anxiety-like behavior (Adamec & Shallow, 1993; Berton et al., 1998). Prenatal exposure to predator stress also leads to anxiety and depressive behaviors in offspring (Green et al., 2018; Korgan et al., 2016). In the present study, we applied predatory stress in the pre-gestational period to create a distinctive study feature providing a stressed status for both parents, along with a single maternal or paternal stress condition to test whether stress-induced phenotypes may be disseminated to offspring and whether male and female parents may differ in this phenomenon and in their inheritance criteria in reprograming HPA stress axis regulation in offspring.

The effects of prenatal stress on fetal brain development and programing of the HPA axis function have been well characterized (Coe et al., 2003; Cottrell & Seckl, 2009; Radley et al., 2008; van Hasselt et al., 2012). Prenatal stresses cause high levels of glucocorticoid exposure to the fetus (Fietta, Fietta, & Delsante, 2009) and also alter endocrine function of the feto-placental unit and fetal corticosterone and corticotropin hormone levels (Lesage et al., 2002; Mairesse et al., 2007), which induce life-long changes in stress responsiveness (Meaney et al., 1996). In the present study, to confirm predatory stress induction in adult rats before breeding, plasma corticosterone concentrations in adult female stressed rats were measured and compared with non-stressed rats. A significant increase was observed in plasma corticosterone concentrations of adult female stressed rats in comparison to non-stressed control rats. This finding is consistent with previous studies, and in particular with that of Huang et al. (2012) that reported a high serum corticosterone concentration in female rats with chronic unpredictable stress in the pre-gestational period and in their fetuses, which was related to the dysregulation of the HPA axis after exposure to chronic stress (Huang et al., 2012). According to our findings, plasma corticosterone concentrations were also significantly

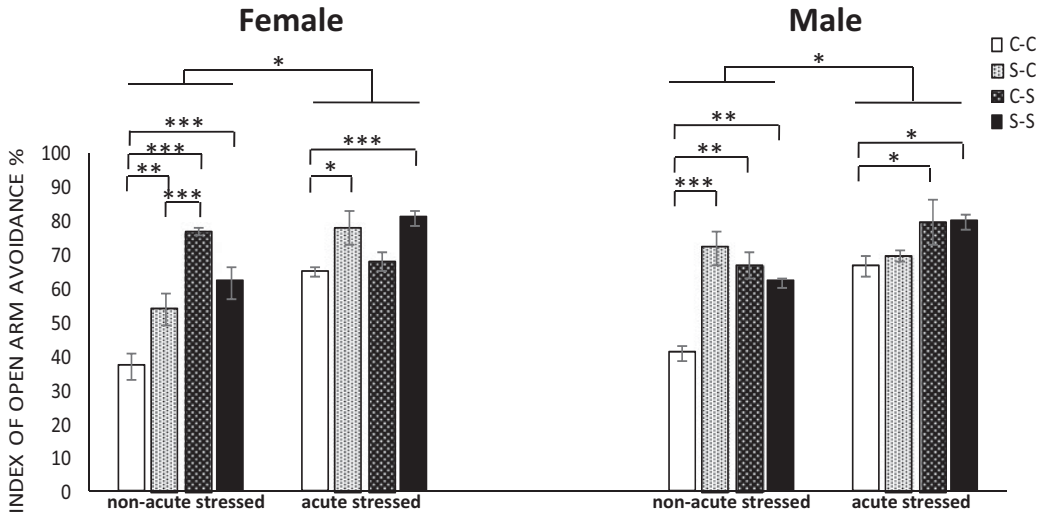

Figure 6. Comparison of the "index of open arm avoidance" (IOAA) among the experimental groups. Data are expressed as mean ± SEM ($n = 6$ for each subgroup). For clarity and in order to show the most relevant results, not all the significant changes are shown. Difference between the indicated groups: $^{*}p \leq .05$; $^{**}p \leq .005$, $^{***}p \leq .001$ (LSD test). C-C: none of the parents was stressed; S-C: only the mother was stressed; C-S: only the father was stressed; S-S: both parents were stressed.

higher in pups, whose only mother or only father was under pre-gestational stress (S-C and C-S groups), in comparison with pups of the control, C-C group. Unexpectedly, no significant changes in plasma corticosterone concentrations were found in pups with both parents prenatally stressed (S-S group, Figure 3). This negative finding may be attributed to the small sample size of pups in the S-S experimental group. According to our recent study, fertility rate significantly declines in stressed adult rats, which was particularly evident in the breeding of pairs with both stressed (Mahmoodkhani et al., 2018). Thus, the number of pups in these litters was less than others, which may explain this unexpected result with the low sample size. However, plasma corticosterone concentrations in acutely stressed pups of the S-S group were significantly increased compared with acutely stressed pups of the control C-C group, which indicates a greater increase in corticosterone concentrations when exposed to acute stress in offspring of pre-gestationally stressed parents.

Prolonged activation of the HPA axis leads to structural changes in the central nervous system (Coe et al., 2003; Cottrell & Seckl, 2009; Radley et al., 2008; van Hasselt et al., 2012) and raises vulnerability to behavioral and psychological abnormalities, including depression, bipolar disorder, schizophrenia, and anxiety (Corcoran et al., 2009; Fride & Weinstock, 1988; Gholipoor et al., 2017; Vallee et al., 1997). In this respect, prenatal stress causes alterations of the HPA axis and brain neurotransmitter systems in the offspring (Kofman, 2002; Maccari et al., 2003) which, in turn, increases anxiety and emotionality and decreases motor development and learning abilities (Huizink, Mulder, & Buitelaar, 2004; Maccari et al., 2003; Nakhjiri et al., 2017; Vallee et al., 1997). The HPA hyper-reactivity also induces anxiety-like behaviors and triggers heightened stress responses, including increased freezing and decreased exploratory behaviors in the adult offspring prenatally exposed to stress (Nakhjiri et al., 2017; Rosecrans, Johnson, Tilson, & Hong, 1984; Szuran, Zimmerman, Pliska, Pfister, & Welzl, 1991). Parental exposure to prominent environmental stimuli before the conception also influences neural structure and neurobehavioral development (Dias & Ressler, 2014; Grundwald & Brunton, 2015; Haloui, Djouini, Benkermiche, Bououza, & Tahraoui, 2017; Li et al., 2010; Mahmoodkhani et al., 2018) and is associated with high risk of anxiety (Grundwald & Brunton, 2015; Korgan et al., 2016), impairment of memory (Huang et al., 2010) and depression in the offspring (Li et al., 2010).

In the present study, three-way ANOVA was performed for each offspring sex separately. The results showed that, in both the female and male offspring, our three main factors and their interactions were all statistically significant, for most experimental parameters, indicating that two-way interactions differed as a function of the level of the third variable. A significant three-way interaction involves a difference between differences of differences. Accordingly, our three-way analysis regarding anxiety responses showed that, in the female non-acute stressed offspring, the effect of paternal stress was greater when present alone than when combined with maternal stress. For the acutely stressed pups, the effect of maternal stress when present along with paternal stress was approximately the same as its effect when present without paternal stress. In the male non-acute stressed offspring, the effect of paternal stress when present along with maternal stress was approximately the same as its effect when present without maternal stress. For the acutely stressed pups, however, the effect of maternal stress was greater when present along with paternal stress than when present alone. The relative influence of paternal versus maternal conditions on offspring behavior has not been previously studied. Our three-way analysis regarding plasma corticosterone concentration was mostly in line with those of anxiety behaviors.

Moreover, the result of the simple effects analyses (the LSD test) indicated that, overall, pre-gestational stress, regardless of which parent was exposed to stress, was associated with an increase in corticosterone and a marked decrease in the EPM open arm activity (decreased time spent and frequency of entry in the open arms), increase in the closed arm activity (increased time spent and frequency of

entry in the closed arms) and increase in IOAA. This contributes to an overall picture that pre-gestational stress promotes anxiety-like behavior in offspring. Plasma corticosterone concentration and anxiety behaviors were mainly not affected by sex of the offspring; however, some sex differences were observed. Furthermore, in both offspring sexes, associations between pre-conception stress and anxiety behavior were observed not only following acute stress exposure, but also under basal conditions. However, the offspring responses were more affected when they were under stressful conditions. This might suggest that both maternal and paternal stress experiences are priming offspring for a more direct response to potentially threatening stimuli.

Our analysis also showed that, in the non-acute stressed pups, plasma corticosterone concentration, and anxiety-like behaviors increased more significantly in pups with only one parent exposed to chronic pre-gestational stress. More specifically, considerable increases were observed in female offspring whose father was only exposed to stress and in male offspring whose mother was only exposed to stress. This finding is consistent with the results of a previous study which showed that chronic unpredictable stress of female rats before pregnancy was associated with high risk of depression in male offspring. Unexpectedly, the effect of maternal stress in combination with paternal stress (in the group with both parents stressed) was not additive on anxious behaviors or corticosterone concentrations in most of the experimental subgroups, based on our data. This suggests that the combined effects of these pre-gestational manipulations do not produce greater effects than either maternal or paternal stress alone.

In spite of consistent findings revealed by previous research that prenatal stresses cause HPA hyperactivity and neurobehavioral abnormalities, inconsistent results have also been reported. He et al. (2016) found that adult pre-gestational restraint stress reduced anxiety responses across generations. According to this study, both female and male mice from restrained mothers and/or fathers showed significantly reduced anxiety and serum cortisol compared to control offspring from unstressed parents (He et al., 2016). This inconsistency shows that differences in animal species and age, experimental design, and conditions as well as type, duration, and time of stress may cause different phenotypes and behavioral responses in offspring. Findings are also discussed within the context of potential differences in effects of repeated physical versus psychological stressors suggesting that habituation/sensitization processes in response to repeated stressor exposure might be influenced by the nature of the stressor (Mashoodh, Wright, Hebert, & Perrot-Sinal, 2008). Chronic and repeated exposure to a restraint stressor may cause a reduction in behavioral responses to a restrainer, but not to a predator, which eliminates the possibility of habituation in defensive responses when a predator is present (Blanchard et al., 1998; Zangrossi & File, 1992a). Moreover, as corticosterone is the main regulator of the stress-induced HPA axis activity in rodents, its measurement can be more acceptable and reliable compared to cortisol in mediating stress responses.

In the present study, under acute stress conditions, a different pattern of anxiety responses emerged in the acutely stressed pups. Pre-gestational stress differentially increased plasma corticosterone concentration and anxiety-like behaviors in the acutely stressed males and females compared to their control counterparts. Under acute stress conditions, anxiety-like behaviors increased more significantly in females with maternal pre-gestational stress and in males with paternal pre-gestational stress. This finding is in line with a previous study reported by Huang et al. who showed that maternal pre-gestational stress affected dopaminergic activity in response to acute stress more in female rat offspring than in male counterparts (Huang et al., 2013). These data suggest again a sex-dependent anxiogenic effect in exposure to acute stressful experiences. Sex differences in vulnerability to prenatal stress have been defined by a search of the world's literature (Sutherland & Brunwasser, 2018). This research adds to an emerging body of literature, which suggests that fetal reprograming of HPA stress axis regulation may be sex-dependent and gonadal steroids affect the way that the HPA axis responds to stress. While many studies demonstrate that differences in neurobiological stress response favor females, there have been some reports indicating the opposite effect and some indicating no differences between the sexes (Sutherland & Brunwasser, 2018). Although these sex differences in corticosterone and anxiety appear to vary with type and duration of prenatal stress exposure and/or age and developmental stages of offspring, sex-dependent differential expression of stress-related receptors may underlie these differences. Emerging research suggests that sex differences in expression of receptors for stress hormones, CRH, corticotrophin and glucocorticoids, contribute to this disparity, which links ideas of sex differences in receptor expression patterns with the development of anxiety disorders (Bangasser, 2013). Similar to what was explained in the non-acutely stressed pups, we found that, overall, in stressed pups with both parents prenatally stressed, most anxiety responses were not additive, when the pups were under the acute stress condition. This suggests that maternal condition and paternal condition independently affected stress responses in the offspring, and as such, the combined effects of maternal and paternal stress did not produce greater effects than either alone.

Previous studies reported that parental pre-reproductive stress may affect susceptibility to reprograming across generations by affecting germ cells of either parent. Pre-conception stress in female rats alters corticotropin releasing factor type 1 expression in ova (Zaidan, Leshem, & Gaisler-Salomon, 2013). It has also been reported that pre-conception stress in males alters cells in the epididymis (critical for sperm maturation), alters sperm microRNA content in mice and reprograms the HPA stress axis regulation in offspring (Rodgers, Morgan, Bronson, Revello, & Bale, 2013). Alteration in microRNA expression in stressed male mice also alters behavioral responses in offspring, suggesting the role of sperm RNAs in trans-generational inheritance of effects of early stressors. MicroRNAs are thus potential vectors at the interface between genes and environment (Gapp et al., 2014). Stress-induced alterations of maternal and paternal germ cell

susceptibility to reprograming across generations provide strong support for the importance of oocyte and sperm contributions to epigenetic inheritance. Our findings provided the first evidence that variation among the male and female parents in their predator encounters may contribute to stable behavioral variation among offspring, even when they were not directly exposed to predator threats. However, overall, the offspring responses were more significant when they were exposed to the same threat experienced by the parents. We showed an interaction between maternal and paternal conditions on one measure of offspring behavior, and as such, future studies are needed to explore mechanisms, by which parental behavior alters anxiety behavior and impacts the epigenetic status of offspring. Clearly, behavioral and endocrine measurements are limited in scope. However, the predictive validity for such measurements is well-established, and they can consequently serve as a valuable tool to study the effect of many different neurological conditions on key parameters of offspring stress responses. Obviously, the gene-environment interaction is complex, especially when it is related to early life programing and the intergenerational transmission of stress-induced characteristics. Hence, other markers such as levels of stress-related receptor expression and more behavior tests should also be investigated to obtain firm conclusions.

In conclusion, we demonstrated that only one or both parents' exposure to a chronic psychological stressor in the pre-conception period induced corticosterone increases as well as anxiogenic responses in male and female rat offspring, with some evidence for sex differences in hormonal and behavioral responses. Associations between pre-conception stress and infant negative emotionality were observed not only following acute stress exposure, but also under basal conditions. However, offspring responses were more affected when they were under stressful conditions. Moreover, different sex-dependent patterns were revealed in anxiogenic responses between acute and non-acute stressed pups. These data suggest interactive effects of pre-conception paternal and maternal stressful experience on anxiety-like phenotypes and associated stress-related hormones in offspring. Stress-induced alterations of maternal and paternal germ cell susceptibility to contribute to epigenetic inheritance revealed stress-induced phenotypes that may be disseminated to offspring and provide strong support for incorporation of genetic and epigenetic aspects in development of neurobehavioral abnormalities and reprograming of the HPA axis responses to stress.

Disclosure statement

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