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Multiple prenatal stresses increase sexual dimorphism in adult offspring behavior

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ABSTRACT

Introduction: Maternal gestational stress and immune activation have independently been associated with affective and neurodevelopmental disorders across the lifespan. We investigated whether rats exposed to prenatal maternal stressors (PNMS) consisting of psychological stress, interleukin (IL)-1β or both (two-hit stress) during critical developmental windows displayed a behavioral phenotype representative of these conditions.

Methods: Long-Evans dams were exposed to psychological stressors consisting of restraint stress and forced swimming from gestational day (GD)12 to 18 or to no stress (controls). From GD17 until day of delivery, these same animals were injected with saline or IL-1β as a second hit and immune stressor (5 μg/day, intraperitoneally). The behavior of F1 offspring adults was tested on the open field test, elevated plus maze and affective exploration task on postnatal days (P)90, 100 and 110 respectively.

Results: The effects of PNMS differed depending on the specific testing environment and potentially the age at assessment, especially in female offspring. Both locomotion and anxiety-like behavioral measures were susceptible to PNMS effects. In females, psychological stress increased anxiety-like behavior, whereas IL-1β had an opposite effect, inducing exploration and risk-taking behavior on the open field test and the elevated plus maze. When present, interactions between both stressors limited the anxiogenic effect of psychological stress on its own. In contrast, prenatal psychological stress increased anxiety-like behavior in adult males overall. A similar anxiogenic effect of IL-1β was only found on the open field test while the Stress*IL-1β interaction appeared to limit the effect of either alone. Contrarily, the PNMS effects on anxiety-like behavior on the affective exploration task were highly similar between both sexes. Analysis of males and females together revealed an additive effect of Stress and IL-1β on the number of exits from the refuge, a measure of risk assessment and thus correlated with anxiety.

Conclusion: PNMS affected offspring adult behavior in a sex-dependent manner. Effects on females were more variable, whereas psychological stress mostly induced anxiety-like behavior in males. These data highlight the sexual dimorphism in vulnerability to prenatal stressors. Maternal or stress-induced programming of the stress response and neuroinflammation may play an important role in mediating stress effects on offspring adult behavior.

1. Introduction

Exposure to stress during pregnancy and in early life is known to be associated with adverse pregnancy outcomes including preterm birth, as well as with long-term consequences for the offspring, both in humans and animals. Indeed, it has become abundantly clear in animal models that maternal and neonatal stress can have far-reaching effects on offspring development of a wide range of organ systems, including metabolism (Yao et al., 2014; Wu et al., 2018), the (neuro)endocrine (Richardson et al., 2006; Brunton and Russell, 2010) and immune systems (Vanbesien-Mailliot et al., 2007) whereas it also induces behavioral alterations (Weinstock, 2017). Similar associations have been

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observed in epidemiological human studies (Roseboom et al., 2011; Beijers et al., 2014; Veru et al., 2015). More specifically, maternal stress has been linked to an increased risk for neuropsychiatric diseases (e.g. anxiety, depression) and neurodevelopmental disorders as well as schizophrenia (Jiang et al., 2016; Say et al., 2016; Murphy et al., 2017). Prenatal maternal stress (PNMS) describes different types of stress a pregnant mother can be exposed to and can affect the development and lifelong health of her offspring. As inflammation or infections activate not only the inflammatory but also the stress response, they can be viewed as stressors (Schiltz and Sawchenko, 2003). Conversely, stress has been shown to increase the susceptibility to infections and to be associated with a pro-inflammatory profile (Coussons-Read et al., 2005; Glaser and Kiecolt-Glaser, 2005; Hueston and Deak, 2014).

A large body of literature indicates that PNMS influences offspring (neuro)development (Knuesel et al., 2014; Schaafsma et al., 2017). Subsequently, aberrant development of the brain and hypothalamic-pituitary adrenal (HPA) axis have been linked to phenotypes representative of a variety of neuropsychiatric diseases including anxiety and depression (Van den Bergh et al., 2008; Slykerman et al., 2015), schizophrenia (Garay et al., 2013; Van den Eynde et al., 2014; Fineberg et al., 2016) and neurodevelopmental disorders including autism spectrum disorder (ASD)(Jiang et al., 2016; Bilbo et al., 2018) and attention deficit hyperactivity disorder (ADHD)(Bock et al., 2017). Increased levels of maternal perceived stress and anxiety as well as bacterial, viral and other infections have been associated with these disorders in humans (Gardener et al., 2009; Slykerman et al., 2015). Moreover, it was recently demonstrated that the risk of depression is increased in adolescent children only when their mothers were exposed to both stress and infections during pregnancy, as compared to infection alone if it occurred during the second trimester (Murphy et al., 2017). These data suggest that the presence of multiple stressors during the same pregnancy do not simply have an additive effect but that interactions occur between prenatal stress and inflammatory processes, in line with our findings with regard to pregnancy outcomes (Verstraeten et al., 2019).

Animal models for neuropsychiatric disorders have used either prenatal maternal (mostly) psychological stress, such as restraint or continuous variable stressors, or maternal immune activation (MIA) to mimic PNMS (Richardson et al., 2006; Baker et al., 2009; Van den Hove et al., 2013; Knuesel et al., 2014; Schaafsma et al., 2017). In light of the well-described bidirectional neuroimmune interactions in the functioning and regulation of the brain and stress response system with an important role for interleukin (IL)-1β (Goshen and Yirmiya, 2009; Dantzer, 2018), it is of interest to know what the long-term effects are of exposure to both prenatal psychological and immune stressors during the same pregnancy in an animal model.

In our previous studies, we analyzed the effects of multi- and transgenerational psychological stress on rat pregnancy and offspring outcomes as well as maternal behavior (Ward et al., 2013; Yao et al., 2014). In both models we observed cumulative effects of additional stress in the maternal lineage, be it repeated in each generation or as a consequence of effects on maternal behavior transmitted across generations. Indeed, maternal behavior is known to be greatly influenced by maternal prenatal stressors (Baker et al., 2009; Ronovsky et al., 2017). Given the increase in negative consequences such as shorter gestational length and lower offspring birth weight (Yao et al., 2014), we developed the two-hit paradigm used in the present study, subjecting dams to psychological stress and MIA with IL-1β during the same gestation. Using this model, we previously demonstrated an increase in adverse pregnancy outcomes including reduced maternal gestational weight gain as well as low offspring birth weight. We also showed generation-dependent effects of PNMS on uterine gene expression (Verstraeten et al., 2019).

Here, we address the effects of prenatal maternal psychological stress and activation of the immune response in parental generation (F0) dams on the behavior of male and female F1 adult offspring. Correspondingly, we hypothesized that sequential exposure to both stressors during the F0 gestation affects offspring behavior differently than each on its own in a sex-dependent manner. More specifically, we posit that prenatal exposure to multiple stressors would have a cumulative effect on anxiety-like behavior. The data presented here add to the current knowledge regarding PNMS and are of particular importance since life is complicated by the accumulation of stressors rather than a single stress hit.

2. Materials and methods

2.1. Animals

A total of 115 Long-Evans hooded rats (*Rattus Norvegicus*) were used for this study. As previously described (Verstraeten et al., 2019), nulliparous female rats (n = 25, 110–150 days of age) raised at the vivarium of the Canadian Centre for Behavioural Neuroscience (University of Lethbridge, AB, Canada) were mated with stress-naïve males (n = 10) forming the F0 parental generation for this project. Males were purchased from Charles River Laboratories Inc. (Saint-Constant, QC, Canada). Pregnant dams were housed in pairs until gestational day (GD)19 and then moved to a single-housed cage. Pups were weaned on postnatal day (P)21 after which they were housed with siblings by sex. For behavioral analyses, male and female offspring were randomly selected from 4 to 5 different litters. The rats were housed under a 12 h light/dark cycle with lights on at 7:30 AM, room temperature at 20 °C and relative humidity of 30%, with food and water *ad libitum*. All procedures were in accordance with the guidelines of the Canadian Council for Animal Care and were approved by the Animal Welfare Committee of the University of Lethbridge, AB, Canada.

2.2. Experimental design and stress procedures (Fig. 1)

The design of the study and the procedures for stress exposure used were published previously (Verstraeten et al., 2019). Briefly, F0 pregnant dams were allocated to either exposure to daily psychological stress (denoted as **S**tress or S) from GD12 to 18, using restraint (20 min/day) and forced swimming (5 min/day) administered alternatingly to minimize habituation, or left alone (non-Stress or N). For restraint, dams were confined in a customized plastic cylinder adaptable to the size of the animal with holes for breeding at one end. Dams were forced to stand and unable to turn around but allowed limited movement while avoiding compression of the body. Forced swimming occurred in a cylindrical bath (45 × 77 cm) filled up to 50 cm with water at room temperature (Yao et al., 2014; Verstraeten et al., 2019). As of GD17 until delivery, each mother received a daily intraperitoneal (i.p.) injection with either IL-1β as immune stressor (5 μg/day; rat recombinant, ProSpec, Rehovot, Israel, catalogue number CYT-394) or saline for controls, alternatingly on the left and right side of the body. The dose of IL-1β was deducted from a comprehensive dose-response curve. Although IL-1β is clearly effective in inducing adverse pregnancy outcomes in mice, the rat appeared unresponsive to it, regardless of the dose, therefore the lowest dose of 5 μg/day was used (Ishiguro et al., 2016). In this manner, four groups of F0 mothers (n = 6–7 dams/group) and their F1 offspring were formed: no Stress/saline (N/Sa), no Stress/IL-1β (N/IL-1β; 7 dams), Stress/saline (S/Sa) and Stress/IL-1β (S/IL-1β) (Verstraeten et al., 2019). As all treatments were administered to the pregnant dams, all references to Stress and IL-1β are to the *in utero* exposure of the F1 offspring. The MIA model reporting guidelines checklist is available as Supplemental Table 1 (Kentner et al., 2019).

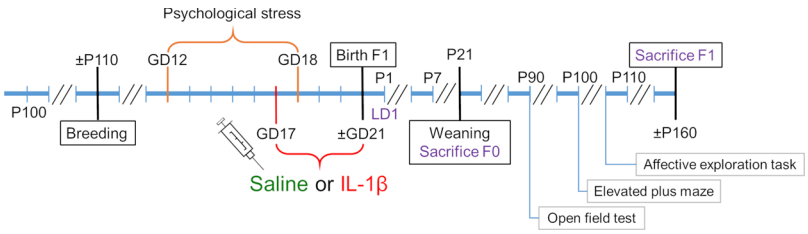

Fig. 1. Experimental design of the Stress/IL-1β cohort. F0 dams were exposed to psychological stress from GD12-18 (restraint 20 min/day and forced swimming 5 min/day) and daily intraperitoneal injections with saline or IL-1β (5 µg/mL) from GD17-delivery. Male and female offspring were evaluated using the open field test, elevated plus maze and the affective exploration task on P90, P100 and P110 respectively. Dams were sacrificed on LD21, their virgin daughters on P160. F: filial generation, GD: gestational day, IL: interleukin; LD: lactational day, P: postnatal day.

2.3. Behavior

F1 offspring were evaluated for stress-related behavioral changes using various tests. They were assessed on P90 with the open field test (OFT)(McCreary et al., 2016), the elevated plus maze on P100 (EPM) (Lister, 1987; McCreary et al., 2016) and on P110 using the affective exploration task (AET)(Erickson et al., 2014). All behavioral tests were performed by experienced experimenters blinded to the stress conditions with n = 10 animals/sex/treatment group for each test except n = 9 females in the N/Sa and N/IL-1β groups for the AET. The testing environments were cleaned thoroughly after each session to remove olfactory clues.

2.3.1. Open field test (OFT)

The open field test was designed to quantify exploration of a threatening open arena as well as motor activity and can be used as a mild test of anxiety-like behaviors (McCreary et al., 2016). It was conducted using the VersaMax Legacy Open Field system (Omnitech Electronics, Inc., Dartmouth, NS, Canada), which was placed with the rear side facing a wall. For a period of 10 min after placing the animal in the center of the box, activity was recorded using a grid of infrared sensors connected to a computer under 580–620 lux overhead lights. Behavioral measures collected included the total distance travelled as well as the time spent along the walls of the box (margin time).

2.3.2. Elevated plus maze (EPM)

The EPM assesses anxiety-like behavior in an aversive environment by exploiting the conflict between the animal's innate desire to explore novel environments and fear of open spaces (Lister, 1987). The maze was an opaque black Plexiglas structure built up of two arms enclosed by 40 cm Plexiglas walls and two arms without walls, or closed and open arms respectively, with arms measuring 50 cm x 10 cm. It was suspended 50 cm above the ground. After placing rats in the center of the maze in a room with lighting of 540 lux (center square), they were allowed to roam freely for 5 min. Behavioral measures assessed were the total number of entries into the arms, as well as those in the open and closed arms separately, the time spent in the different arms, the time in the center square, the time spent risk assessing and the latency until they entered a closed arm for the first time. The latter is considered a defensive strategy as escape from an aversive environment and can thus be used as an index of anxiety-like behavior in addition to the conventional spatiotemporal measures (Rodgers and Cole, 1993a,b; Estanislau and Morato, 2005).

2.3.3. Affective exploration task (AET)

We previously designed the affective exploration task, alternatively called the novel emergence test, to evaluate affective state and emotion (Erickson et al., 2014). It is similar to other emergence tests published previously (Baker et al., 2009; Van den Hove et al., 2013) and combines elements of the light/dark test with an open field arena. For this test F1 offspring at an age of 110 days were placed inside a refuge on top of a table (75 cm × 100 cm) and left to explore for 5 min (610 lux). The refuge was a 10 cm long plastic tube attached to a platform, as described previously (Erickson et al., 2014). Behavioral measures recorded were time before the first exit from the refuge (initial emergence), number of exits from and time spent in the refuge. The number of exits can be considered a measure of risk assessment behavior, whereas time in the refuge as avoidance mechanism fits within defensive behavior similarly to the initial emergence or emergence latency, such that they can be regarded as proxies for anxiety-related behavior (Erickson et al., 2014; Lezak et al., 2017).

2.4. Statistical analyses

All analyses were performed using IBM SPSS Statistics, Version 24.0 (IBM Corp, Armonk, NY, USA). Data were transformed (logarithmic, square root, reciprocal transformations) to reach normal distributions and homogeneity of variances, with $p > 0.1$ accepted as the level at which assumptions were met. Data were analyzed using three-way analysis of variance (ANOVA) with Stress, IL-1β and Sex as factors. Subsequently, we performed two-way ANOVA for each sex separately. For specific comparisons between two groups (all females *versus* males) Student's *t*-test was used. *P*-values of ≤ 0.05, achieved after Bonferroni correction for multiple comparisons, were deemed significant, with *p*-values of $0.05 < p \leq 0.1$ considered as trends or tendencies. Bonferroni adjustment of statistical significance was performed by SPSS by multiplying the observed *p*-value with the number of comparisons made. If the resulting values were less than 0.05, this was considered significant, as per the calculations by SPSS. Specific *p*-values are indicated with asterisks on the graphs and explained in the figure legends. Data are presented as mean ± standard error of the mean (SEM). Graphs show untransformed data and are marked in the top left corner to indicate the generation (F1) and sex of the animals (♀ or ♂). All behavioral data concern the male and female adult F1 generation. Results for male offspring are shown in blue while those regarding F1 females are presented in red (grey in print). Behavioral data for males and females combined in one graph are depicted in green (grey in print) and marked with the sex symbols superimposed. Significant results are indicated below the graphs for a main effect of Stress and next to the legend for a main effect of IL-1β, whereas the *post hoc* tests in the event of an interaction effect are shown directly above the different columns of the graphs. N-numbers per analysis are presented in figure captions as n = a-b-c-d/treatment group with letters corresponding to the bars on the graph, i.e. N/Sa – N/IL-1β – S/Sa – S/IL-1β respectively.

3. Results

3.1. Prenatal stress affects offspring behavior in a sex-specific way

3.1.1. P90 Open field test (OFT)

The total distance travelled in the open field was not different between males and females (Fig. 2A) or between any of the groups for either sex in separate analyses (Fig. 2B–C). For the time spent in the margin of the box, a three-way interaction was present (Stress*IL-1β*Sex $F(1,72) = 6.37$, $p < 0.05$). As the time spent in the center square is the reverse of the margin time, a similar interaction was found for this measure. The effect of Sex was mostly visible in the control groups. Female N/Sa offspring spent more time in the margins of the field as compared to their male N/Sa counterparts, a difference of 177.66 s, 95% CI [47.45–187.87] (Fig. 2D).

Females did not show any significant differences on the OFT as a result of PNMS (Fig. 2E). Male behavior on the other hand was

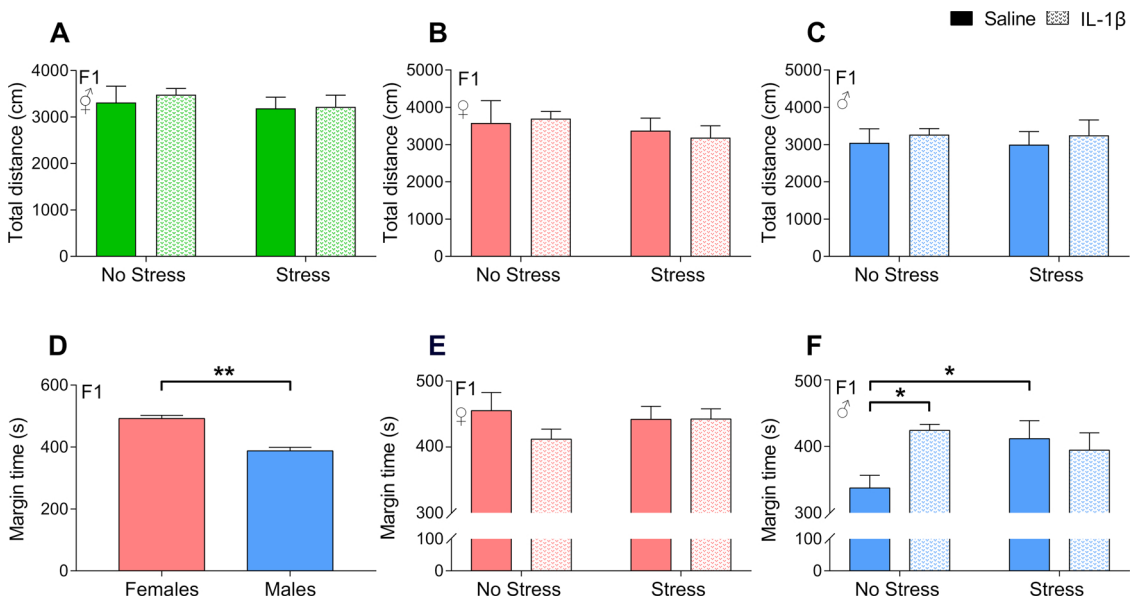

Fig. 2. Prenatal stressors increase the time male offspring spend in the margins on the OFT and thus anxiety-like behavior. (A) There was no effect of Sex on the total distance travelled. (B–C) PNMS did not affect the total distance travelled by females or males. (D) N/Sa females displayed increased thigmotaxis over their male counterparts. (E) No effects were visible in female offspring. (F) Males exposed to either Stress or IL-1β spent significantly more time in the margins than their N/Sa peers, with the interaction between both stressors reducing this effect. Asterisks indicate significant differences: $*p < 0.05$, $**p < 0.01$; n = 10 animals/group/sex. ♀: females; ♂: males; F: filial generation.

influenced by both factors with a Stress*IL-1β interaction present for the margin time ($F(1,36) = 5.821$, $p < 0.05$). Male F1 animals prenatally exposed to Stress ($F(1,36) = 6.69$, $p < 0.05$) or IL-1β ($F(1,36) = 7.14$, $p < 0.05$) spent more time along the walls as compared to N/Sa animals, whereas the interaction of both stressors slightly reduced the effect of each separately since a significant difference was no longer present ($p = 0.073$; Fig. 2F).

3.1.2. P100 elevated plus maze (EPM)

All measures evaluated showed a significant effect of Sex whether or not in interaction with PNMS when analyzed using a three-way model ($p < 0.01 - 0.05$). No differences were found in the time spent risk assessing for either sex in separate analyses.

Stress, IL-1β or the combination of both affected F1 female behavior on the EPM. Each factor independently impacted the total number of entries into any of the arms by F1 daughters (Fig. 3A). Whereas Stress had a reducing effect ($F(1,36) = 5.54$, $p < 0.05$), IL-1β increased their entrances into the arms ($F(1,36) = 6.02$, $p < 0.05$). The interaction did not have a significant effect. The same effect of IL-1β was seen when evaluating just the entries into the closed arms ($F(1,36) = 4.52$, $p < 0.05$; Fig. 3B). An interaction between Stress and IL-1β was found for the percentage of entries into ($F(1,36) = 4.57$, $p < 0.05$; Fig. 3C) and time spent in the open arms ($F(1,36) = 4.21$, $p < 0.05$; Fig. 3D), with S/Sa females having the lowest scores. The raw number of entries into the open arms was not significantly altered by Stress nor the Stress*IL-1β interaction ($p = 0.088$ for both; Supplemental Fig. 1A). Moreover, the time spent in the center square (Fig. 3E) and in the closed arms (Supplemental Fig. 1B) was not changed by either stressor. Stress and IL-1β separately affected the latency to enter a closed arm of the EPM, with Stress causing the females to find shelter in the closed arm faster ($F(1,36) = 5.51$, $p < 0.05$) while offspring exposed to IL-1β took longer ($F(1,36) = 6.02$, $p < 0.05$; Fig. 3F).

Male F1 offspring showed an increase in anxiety-like behavior after exposure to Stress, regardless of IL-1β treatments. The total number of entries was significantly decreased ($F(1,36) = 12.61$; $p = 0.001$; Fig. 3G) similarly to the entries into the closed arms ($F(1,36) = 8.42$, $p < 0.01$; Fig. 3H). The percentage of open arm entries did not differ significantly, although a trend towards reduction was observed ($p = 0.081$; Fig. 3I). However, there was a lower raw number of entries into the open arms of the maze ($F(1,36) = 4.99$, $p < 0.05$; Supplemental Fig. 1C). Accordingly, the percentage of time spent in the open arms was not significantly altered by either stressor (Fig. 3J). Although Stress-exposed males entered the closed arms less often, they spent more than 90% of their time there as compared to 85% by their non-Stressed counterparts ($F(1, 36) = 6.92$, $p < 0.05$; Supplemental Fig. 1D). The effect of Stress was also clear from the small amount of time spent in the center ($F(1,36) = 17.26$, $p < 0.001$; Fig. 3K), as well as the shorter latency to enter one of the closed arms ($F(1,36) = 12.61$, $p = 0.001$; Fig. 3L).

3.1.3. P110 affective exploration task (AET)

The effects of prenatal stressors on behavior on the AET were more similar for males and females, with the exception of the emergence latency. F1 females exposed to Stress, i.e. S/Sa and S/IL-1β animals, took 60% longer to emerge from the refuge for the first time ($F(1,34) = 8.86$, $p < 0.01$; Fig. 4A). No significant differences were found for male progeny exposed to Stress or IL-1β (Fig. 4B).

As no Sex differences were found on three-way ANOVA for the other measures evaluated, these are reported for females and males combined as well as individually. When evaluating male and female offspring together, prenatal Stress and IL-1β each augmented the number of exits from the refuge (Stress $F(1,72) = 12.33$, $p = 0.001$; IL-1β $F(1,72) = 4.25$, $p < 0.05$; Fig. 4C), representative of risk assessment behavior. Correspondingly, this number was higher in females and males exposed to Stress ($F(1,34) = 3.30$; $p < 0.05$ and $F(1,36) = 7.76$, $p = 0.01$ respectively; Fig. 4D–E). In females there was a trend towards a main effect of IL-1β as well ($p = 0.078$). Offspring of mothers exposed to Stress also spent more time in the refuge ($F(1,72) = 6.92$, $p = 0.01$; Fig. 4F). Analyses of the sexes separately did not yield a significant main effect of Stress for females ($p = 0.077$; Fig. 4G) or males ($p = 0.066$; Fig. 4H). There were no interaction effects observed for any of the AET measures.

4. Discussion

It is well established that early life adversity can affect offspring

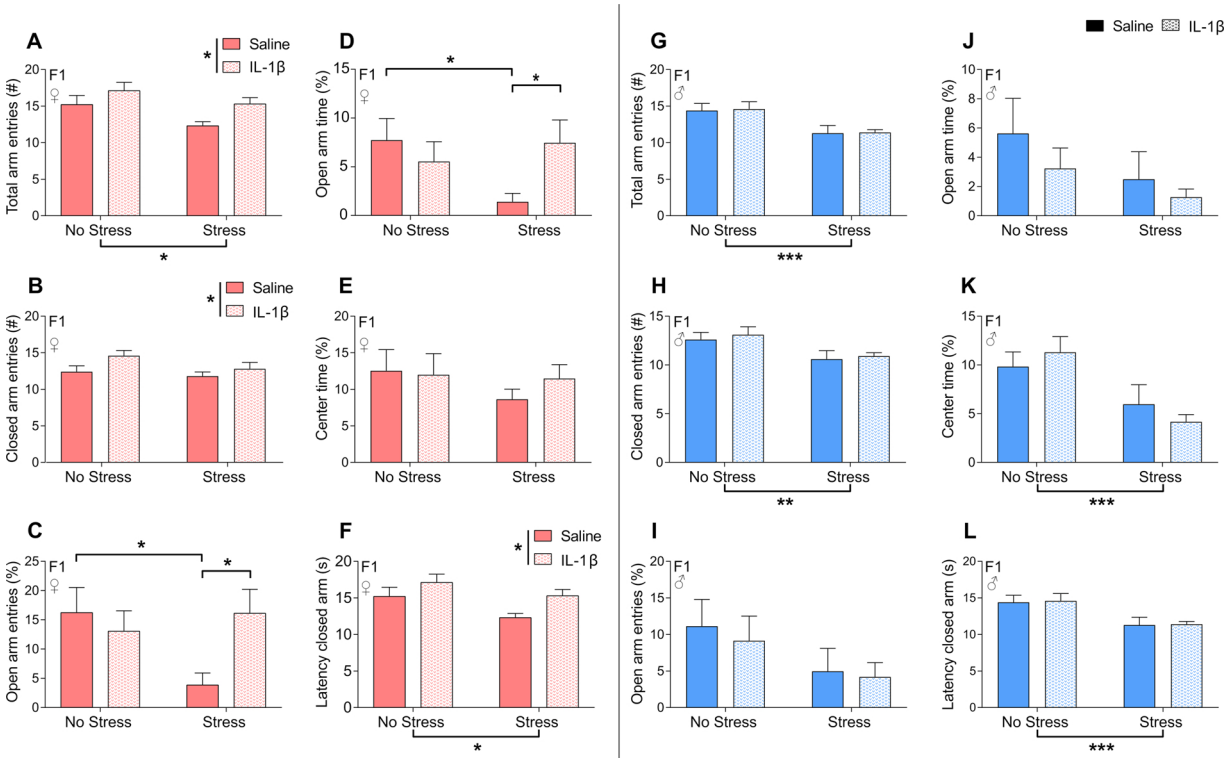

Fig. 3. Performance of F1 adult females on the EPM on P100 is affected by *in utero* IL-1β exposure, whereas males are influenced by Stress, but not IL-1β. (A) Prenatal exposure to Stress reduced whereas IL-1β increased the total number of entries by females. (B) The same IL-1β effect was seen for the number of entries into the closed arms. (C) S/Sa females hardly entered the open arms, with the Stress*IL-1β interaction normalizing this behavior with regards to percentage of open arm entries. (D) This was paralleled by the percentage of time in the open arms. (E) The time spent in the center square was not significantly changed by PNMS. (F) Individually, Stress reduced and IL-1β increased the latency to enter a closed arm. (G) The total number of arm entries by males was reduced by Stress. (H) The same was true for the number of entries into the closed arms. (I–J) Neither the percentage of entries into or time in the open arms was significantly affected by PNMS. (K) The increase in anxiety-like behavior was evident from the reduction in time spent in the center square by Stress. (I) This corresponded with a decrease in latency to enter a closed arm by the same animals. Asterisks indicate significant differences: $*p < 0.05$, $**p < 0.01$, $***p = 0.001$ except for center time $p < 0.001$; n = 10 animals/group/sex. ♀: females; ♂: males; ♀ + ♂: females and males combined (n = 20 animals/group); F: filial generation.

development and behavior at all ages. In the present study we demonstrate that prenatal exposure to distinct maternal stressors had differential effects on adult F1 offspring behavior and that diverse prenatal stress types introduced separately during gestation impact offspring in a sex-dependent manner, either separately or by interacting.

Numerous studies in animal models as well as human data point towards alterations in the reactivity of the HPA axis and shifts in neuroimmune interactions in the offspring brain leading to neurodevelopmental changes as two of the many factors associated with neuropsychiatric diseases (Knuesel et al., 2014; Mattei et al., 2017; McGowan and Matthews, 2018). One of the brain areas with an important role in regulating the HPA axis and shown to be affected by PNMS is the hippocampus (Richardson et al., 2006; Schaafsma et al., 2017; Weinstock, 2017). Taken together, this implies that for normal brain development and functioning, it is paramount that adequate levels of neuroinflammatory mediators are present, i.e. levels that are neither too high nor too low, including cytokines and glucocorticoids (GCs) (Reemst et al., 2016; Gray et al., 2017). Conversely, disruptions of the normal stress response system and its feedback mechanisms are characterized by increased levels of neuroinflammation (Garay et al., 2013; Uchoa et al., 2014; Réus et al., 2017).

The perturbations observed at the level of the stress response and the neuroimmune system as indicated above are considered to be, at least in part, the result of programming effects of PNMS through alterations of the epigenome. This is thought to occur secondary to exposure to excess GCs from maternal or fetal origin (Kundakovic and Jaric, 2017; McGowan and Matthews, 2018). Correspondingly, the consequences of PNMS programming were shown to be transferrable to future generations in an inter- and transgenerational way, suggesting epigenetic inheritance, as well as in a non-genomic behavioral manner through effects of PNMS on maternal behavior (Weaver et al., 2004; Ward et al., 2013; Erickson et al., 2014; Ronovsky et al., 2017).

4.1. Effect of gestational stressors on offspring behavior is sex-dependent

The findings presented herein support existing evidence that exposure to PNMS can have differential effects on the behavior of male and female F1 offspring (Baker et al., 2009; Brunton and Russell, 2010; Van den Hove et al., 2013, 2014). At the same time our findings strengthen those from other studies showing similar behavioral changes, i.e. anxiogenic effects of Stress on some measures collected on the EPM in males and females (Van den Hove et al., 2014). Sexual dimorphisms in brain development have been amply described and are suggested to underlie variations in HPA axis gene expression and the stress response, as well as the sex-dependent effects of PNMS (Li et al., 2017). In addition, brain functioning and stress-related psychopathology have been designated as sex-dependent as well (Li et al., 2017; McCarthy et al., 2017).

With regards to male F1 offspring, our behavioral observations corroborate previous reports of increased anxiety-related behavior (hereafter referred to as anxiety) since PNMS induced an anxiety-prone phenotype across the line (Van den Hove et al., 2013). The total number of entries into the arms and especially those into the closed arms of the EPM as well as the total distance travelled on the OFT are generally regarded as a display of locomotor activity, although the total number

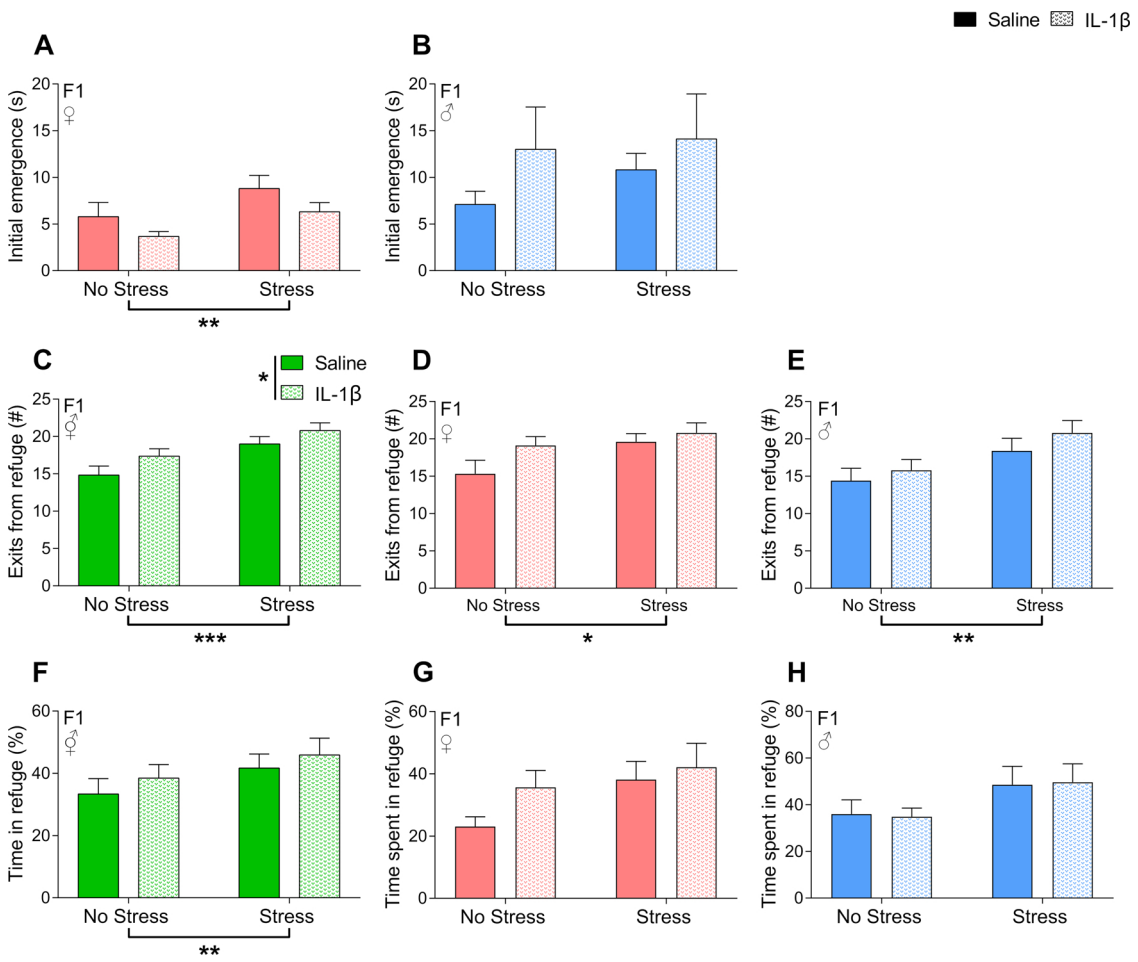

Fig. 4. Performance of F1 progeny on the affective exploration task is altered by prenatal exposure to Stress or IL-1β. (A) Stress increased the time until first emergence of F1 females from the safety of the refuge. (B) No significant differences were found in males. No Sex effects were found in any other measures. Therefore, males and females were first analyzed together for those parameters. (C) Stress and IL-1β both increased the activity level as measured by the number of times the animals exited the refuge. This was reflected in a main effect of Stress in both (D) females and (E) males. (F) Prenatally Stressed progeny spent more time in the refuge. This was no longer the case when assessing (G) females and (H) males separately. Asterisks indicate significant differences: $*p < 0.05$, $**p < 0.01$; $***p = 0.001$; ♀: females; ♂: males (n = 9–10 animals/group/sex); ♀ + ♂: females and males combined (n = 19–20 animals/group); F: filial generation.

of entries was also shown to be associated with anxiety (Rodgers and Dalvi, 1997; Richardson et al., 2006). However, it was suggested that reduced locomotion as observed on the EPM may also indicate avoidance behavior as a reactive strategy, i.e. withdrawing from the threatening environment (Qi et al., 2010; Van den Eynde et al., 2014). This withdrawal can be seen as a consequence of the activation of the behavioral inhibition system (Klackl et al., 2018), which is triggered in situations where an internal approach-avoidance conflict exists with regard to the threatening environment. In fact, the EPM was suggested to test for behavioral inhibition which is characterized by threat-induced freezing and hypervigilance rather than anxiety (Qi et al., 2010; Hoffman, 2016). The available literature on female F1 animals is more controversial. Indeed, PNMS has been demonstrated to be anxiogenic, anxiolytic, or having no effect on female anxiety levels in several studies (Richardson et al., 2006; Mueller and Bale, 2008; Van den Hove et al., 2013, 2014). In the current study, the effect of Stress on female behavior differed depending on the task performed. Indeed, while no effects could be demonstrated on the OFT, Stress had similar anxiogenic or threat-avoiding effects as in males on S/Sa daughters on the EPM and all stress-exposed females on the AET.

The affective exploration task was previously designed as an alternative measure of affective state and exploration, exploiting the approach-avoidance conflict by combining features of the light-dark test with those of an open field (Erickson et al., 2014). In the latter study, we demonstrated a negative correlation between the time in the center of the open field and the latency to emerge from the refuge, validating it for the evaluation of anxiety-related and risk-taking behaviors, when performed on the same day. The exits and time can be interpreted in light of risk assessment and anxiety-related behavior, in parallel with closed arm returns on the EPM and time in the dark compartment of the light-dark test respectively. The emergence latency can potentially be explained in like manner as the latency to emerge from the dark compartment or as the reverse of the latency to enter a closed arm (Rodgers and Cole, 1993b; Arrant et al., 2013).

In the current study, there was no correlation between the center time on the OFT, as the reverse of the time spent in the margins, and the latency to emerge from the refuge. Indeed, the margin time was not different in females as a result of PNMS, whereas on the AET, Stress clearly increased the time until emergence from the refuge. In males, their performance on the OFT was significantly affected whereas no significance was found for the emergence latency.

Remarkably, the effects of Stress and IL-1β on the number of exits from and time in the refuge on the AET were similar in males and females, in contrast to most of the observations on the OFT and EPM. Moreover, and especially for the exits from the refuge, the effects of both stressors appeared additive when evaluating both sexes together, although in separate analyses a main effect of IL-1β was no longer present. Notably, IL-1β induced anxiety-like behavior in males and

females combined, with a trend towards significance in females, for the exits from the refuge. This is in contrast to several other measures evaluated in this study, in which immune stress seemed to have an effect opposite to that of Stress in female animals.

Taken together, these data can be interpreted in several ways. First, performance on behavioral tests are snapshots of the emotional state of the animal at the time they are tested (Ramos et al., 2008). From that perspective, it is possible that the results in the females differ by day. Given that the tests were performed with an interval of 10 days each, testing occurred at similar phases of the estrous cycle, although this was not explicitly confirmed. On the other hand, effects of exposure to stressors on anxiety-like behavior have been demonstrated to vary across the female lifespan, whereas males show increased anxiety-related behaviors at different ages (Bowman, 2005; Bowman et al., 2006). Secondly, these results may indicate that the AET requires additional validation, especially since it has not undergone pharmacological validation. Nevertheless, other studies have shown that anxiety-related behaviors evaluated using different tests often show little correlation, either due to time-dependent variations in the emotional state of the animals or to variability in the construct of the tests (Ramos et al., 2008). This also suggests that each test may measure separate aspects of emotionality.

For the MIA in our model, we administered IL-1β in late gestation, coinciding with the developmental window of the hippocampus (Semple et al., 2013), which has been recognized as the main target of perinatal stressors. Given its role in modulating the HPA axis as wells as emotions and emotionally-laden motivational behavior together with the amygdala and the medial prefrontal cortex, it should come as no surprise that fetal exposure to stressors during this vulnerable window can be linked to alterations in behavior (Padilla-Coreano et al., 2016; Qi et al., 2018). MIA-induced hippocampal alterations have been shown to be characterized by structural and functional changes as well as immune alterations, including disturbed microglial functioning among others, and to depend on the specific timing of the immune stimulus (Bergdolt and Dunaevsky, 2019; Zhao et al., 2019). However, research using a single, downstream cytokine to induce immune activation is rather limited (Nadeau-Vallée et al., 2017; Bittle and Stevens, 2018). Notably, administration of e.g. IL-1β or IL-6 undoubtedly induces a vast array of immune changes comparable to those after exposure to pathogens such as lipopolysaccharide (Basta-Kaim et al., 2015; Ronovsky et al., 2017; Schaafsma et al., 2017) as they are the downstream effectors of Toll-like receptor activation (Wahid et al., 2015). Because IL-1β sits at the apex of the inflammatory cascade, is heavily involved in neuroimmune responses to stress and is associated with inflammation-induced fetal injury as well, it was chosen as the agent to induce MIA in this model (Goshen and Yirmiya, 2009; Nadeau-Vallée et al., 2017).

In the current study, MIA appeared to have a greater effect on female progeny as IL-1β only had a significant effect on margin time on the OFT in males. The behavioral alterations on the EPM observed in IL-1β-exposed F1 females, with or without Stress, were not associated with increased anxiety, but rather suggestive of exploratory and risk-taking behavior. These data corroborate several findings on the OFT regarding reduced anxiety in and increased exploration by F1 females although we did not find any differences in females on the OFT (Paris et al., 2011; Basta-Kaim et al., 2015). On the other hand, they also support the anxiogenic effect of MIA on males on the same test (Lin et al., 2012). The increased exploratory and risk-taking behavior in females can be interpreted as representative of novelty seeking and low harm avoidance, with the former associated with impulsivity (Mann et al., 2018). Notably, female rats have been found to display more exploratory activity than males (Lynn and Brown, 2009). Interestingly in males, both increased and reduced anxiety have been reported after MIA (Basta-Kaim et al., 2015; Schaafsma et al., 2017), in addition to lower and higher levels of locomotion (Van den Eynde et al., 2014; Basta-Kaim et al., 2015). Remarkably, few Stress*IL-1β interaction effects were present on F1 behavior although we previously demonstrated significant effects of the interaction on pregnancy outcomes (Verstraeten et al., 2019). In females, two-hit stress had a different effect than either stressor on its own on the percentage of entries and time in the open arms, which is in line with our hypothesis. Antenatal exposure to IL-1β after Stress reduced the effects of the latter or each stressor on its own. The only interaction found on male F1 behavior was found on OFT measures, where sequential exposure to both stressors seemed to reduce the individual effects of Stress and IL-1β as no significant difference was present between the N/Sa and S/IL-1β. The limited effect of IL-1β on males corresponds with our previous findings that females appear more sensitive to MIA and Stress*IL-1β interactions than their male counterparts with the opposite being true for Stress (Verstraeten et al., 2019).

In humans, PNMS has been linked with a predisposition for stress vulnerability as well as neurodevelopmental and other psychiatric disorders, albeit with small effect sizes (Kundakovic and Jaric, 2017; Bilbo et al., 2018). In fact, the effects of psychological stressors were shown to be associated with mood and cognitive disorders (Slykerman et al., 2015). MIA on the other hand has primarily been linked with schizophrenia (Fineberg and Ellman, 2013; Fineberg et al., 2016) and ASD (Jiang et al., 2016; Bock et al., 2017; Bilbo et al., 2018), although considerable overlap exists between the risk factors and disorders. Indeed, both stress (Fineberg et al., 2016) and infections or immune activation in early life (Knuesel et al., 2014) have been associated with several of these disorders (Kundakovic and Jaric, 2017). However, epidemiological studies indicate that in humans anxiety and depression occur with a female bias, whereas neurodevelopmental disorders are more often diagnosed in men, which is in contrast with most animal studies (Bekhbat and Neigh, 2018).

Increased novelty seeking and low harm avoidance as observed here in F1 females are linked in humans with reduced conscientiousness and increased extraversion respectively (Mann et al., 2018). High levels of sensation seeking and impulsivity have been suggested to underlie risk-taking behaviors typically observed in antisocial behavior (Quinn and Harden, 2013; Mann et al., 2018). This also translates into individual differences in the susceptibility to substance (ab)use (Quinn and Harden, 2013). Moreover, these behavioral elements have been associated with ADHD, as well as other cluster B personality disorders (van Dijk et al., 2012). On the contrary, high levels of harm avoidance, as seen in the Stress-exposed male and female F1 animals, are linked with obsessive-compulsive disorder as well as anxiety and psychotic disorders in humans (Pietrefesa and Coles, 2009).

4.2. Limitations

This study has several limitations. First, the behavioral tests employed here are traditionally used to evaluate anxiety in rodents. Whereas psychological stress is most often linked to anxiety- and depression-related behavior in animal models, MIA has mostly been evaluated in relation to neurodevelopmental issues and schizophrenia using specific tests (e.g. prepulse inhibition) (Boksa, 2010). However, symptoms of mood disorders may present as a component of or co-occur with these disorders (Zaboski and Storch, 2018). Therefore, tests aimed at investigating anxiety- and depression-related behaviors as in the current study may be used in models of neurodevelopmental disorders and schizophrenia as well. In addition, they allow for the comparison between the effects of Stress and IL-1β. Evidently, to assess the usefulness of the two-hit paradigm as a model for neurodevelopmental disorders or schizophrenia, additional tests need to be performed.

4.3. Summary and considerations

In summary, we demonstrated that exposure to prenatal two-hit stress had highly sex-specific consequences on offspring adult behavior. Psychological Stress induced anxiety in F1 male and female adults, whereas IL-1β increased exploratory and risk-taking behavior only in

females. MIA had little effect on males. In females, the Stress*IL-1β interaction appeared to mitigate the impact of each stressor on its own.

The behavioral data presented here can be interpreted in accordance with the cumulative stress as well as the match/mismatch hypotheses of perinatal programming (Santarelli et al., 2017; Verstraeten et al., 2019). Increased anxiety or harm avoidance induced by stress can indicate a hypersensitivity of the HPA axis to minor stressors, with each stressor adding to the cumulative stress load. The match/mismatch theory on the other hand postulates that behavioral abnormalities occur when the current circumstances, *i.e.* stress levels in adult life, do not match those expected based on perinatal experiences. The observed behavior in F1 offspring can in this context be conceptualized as the two opposite sides of an anxiety or harm avoidance spectrum. On the one hand, there are the anxiogenic effects of stress in males, corresponding with high harm avoidance, and on the other hand the reduced anxiety in female IL-1β-offspring as evident from the impulsivity and risk-taking. Both extremes may indicate mismatch and thus maladaptations when life in adulthood turns out to be less stressful than the animal was epigenetically prepared for. More specifically, the stress response system in the male F1s appears activated easily by mild stressors, corresponding with a propensity for anxiety-related disorders. Contrarily, female F1s seem relatively unaffected by danger and stressful situations, which has been associated with antisocial behavior as mentioned previously. Both scenarios are inadequate responses to the situation at hand, and thus a mismatch (Santarelli et al., 2017).

Notwithstanding these findings, it is important to note that data from animal models of PNMS are largely inconsistent, which significantly hamper the comparability of the available data. Although PNMS and its downstream effects have been evaluated extensively in animal models, the evidence available in the literature is rather conflicting with regards to the specific effects, mechanisms and transmission to future generations. These differences may be explained, at least in part, by the wide variation in methodology, including differences in the stress paradigm as well as the timing, duration and intensity of the stressors used. Besides the modalities of the stressors, the age at assessment and sex of the offspring as well as other potential confounders, e.g. maternal behavior (Curley and Champagne, 2016), are among the factors that cannot be disregarded. Furthermore, the timing of the gestational exposure in relation to the organ- and region-specific developmental windows of vulnerability should be taken into account as well (Kapoor et al., 2009). Overall, these are arguments indicating that results from models using perinatal stress need to be interpreted with a considerable amount of caution.

5. Conclusion

It is undeniable that stress affects all aspects of life whenever it occurs and the awareness of programming effects by early life adversity has exploded in recent years. The present study focused on the direct and indirect effects of maternal gestational stressors but did not evaluate the potential impact of pre-conceptional parental stress or correct for maternal care effects. While these are definite options for future research, they also highlight the difficulties in using animal models when studying the programming effects of PNMS. In addition, these individual models are hardly representative of the all-in-one human situation, in which cognitive appraisal plays an important role. In that regard, it may be more valuable to consider using animal models to reveal overall programming mechanisms instead of attempting to elucidate the precise molecular and epigenetic machinery underlying the developmental origins of diseases. This strategy would be more geared towards finding mediating factors or therapies. Of note, it increasingly appears that these prevention or mitigation efforts may not be of pharmacological nature. On the other hand, our data emphasize the importance of including both sexes in perinatal stress research, given the extensive sexual dimorphism. Going forward, it will therefore be key to develop therapeutic strategies in a sex-dependent manner. Indisputably, several questions remain to be answered to advance the field of early life programming of health and disease.

Declaration of interests

None.

CRediT authorship contribution statement

Barbara S.E. Verstraeten: Conceptualization, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Funding acquisition. **J. Keiko McCreary:** Investigation, Writing - review & editing. **Erin A. Falkenberg:** Investigation. **Xin Fang:** Supervision. **Steven Weyers:** Writing - review & editing, Supervision. **Gerlinde A.S. Metz:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition. **David M. Olson:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2019.05.003.

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