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Postnatal stress is associated with impaired fear conditioning and extinction, and heightened hippocampal fibroblast growth factor 2, in mother rats

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ABSTRACT

Rats exposed to early-life maternal separation (MS) exhibit later alterations in fear conditioning and impairments in fear extinction. As MS creates long-lasting anxiety in the mother, the present study assessed the influence of MS on fear conditioning and extinction in mother rats. It also examined whether estrous cycle effects on extinction, which are robust in nulliparous rats, but abolished in primiparous rats, re-emerge after MS. Following parturition, pups were removed from their mothers for 3 h daily from postpartum day 2–14 (MS), or remained housed with their mothers (standard reared condition, SR). Pups were weaned at postpartum day 24, and three months later, mothers received fear conditioning, extinction training, and test for extinction recall over three days. Extinction training took place during Proestrus (high estradiol and progesterone) or Metestrus (low estradiol and progesterone). Similar to past findings in non-stressed mothers, estrous cycle was not associated with conditioned fear expression (indexed by fear responses at the start of extinction training) or extinction recall in either MS or SR mothers. However, MS mothers exhibited weaker conditioned fear expression and impaired extinction recall, relative to SR mothers. Hippocampal fibroblast growth factor-2, a neurotrophin involved in stress regulation and fear expression, was elevated in MS relative to SR mothers. These results indicate that postnatal stress has long-lasting consequences for neural and behavioral systems involved in fear learning and inhibition without altering the involvement of ovarian hormones in these processes.

1. Introduction

Fear extinction is the reduction in fear that results from exposure to a threatening conditioned stimulus (CS, a stimulus that was previously paired with an aversive outcome) without negative consequence. This process underlies exposure therapy for anxiety and trauma disorders, like phobias and PTSD. Stress, which often accompanies anxiety disorders, impairs extinction and causes abnormalities in underlying neural pathways, which poses serious problems for exposure-based treatments that ostensibly depend on a functioning extinction system (reviewed in Maren and Holmes, 2016).

In particular, early-life stress alters extinction and increases susceptibility to anxiety and trauma disorders (Maren and Holmes, 2016). Animal models of early-life stress frequently entail maternal separation (MS), in which rat pups are separated from their mother for several hours daily during the first weeks of life, leading to a host of long-lasting neurobehavioral changes in emotionality and stress regulation in the offspring (Meaney, 2001). Surprisingly, the impact of MS on the mother has received little attention. A small body of work has demonstrated that MS alters maternal behavior and increases anxiety and depressive-like behavior in the mother, prompting suggestions that MS might be a useful model of postnatal mental illness (Aguggia et al., 2013; Boccia et al., 2007; Maniam and Morris, 2010). However, the impact of MS on fear conditioning and extinction, which have relevance to the development and treatment of postnatal anxiety, has never been examined in mothers.

The present study assessed whether mothers exposed to MS exhibit altered fear conditioning and extinction. Typically, females exhibit weak extinction recall when extinguished during estrous/menstrual phases when sex hormones are low, and strong extinction recall when extinguished during phases of high sex hormones (reviewed in Li and Graham, 2017). We have demonstrated that this relationship between cycle phase and extinction is absent following motherhood, even when rats were tested 3 months post weaning (Milligan-Saville and Graham, 2016). As other consequences of motherhood (enhanced spatial learning, altered associative learning) are prevented by gestational stress (Lemaire et al., 2006) or pup removal (Leuner and Shors, 2006), the potential impact of MS on estrous cycle effects on extinction was also assessed. Finally, hippocampal fibroblast growth factor-2 (FGF2), a neuroprotective protein, increases after acute or controllable stress, and

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decreases after chronic uncontrollable stress (reviewed in Callaghan et al., 2013). Conditioned fear expression negatively correlates with hippocampal and salivary FGF2 (Graham and Richardson, 2016; Walters et al., 2016; Graham et al., 2017). Given the link between FGF2, stress, and conditioned fear, the present study assessed whether hippocampal FGF2 differs between mothers subjected to different postpartum experiences.

2. Methods

2.1. Subjects

30 Sprague Dawley-derived female rats, aged 10–12 weeks, obtained from the Animal Resources Centre (Australia) were used. Procedures were approved by the Animal Care and Ethics Committee at UNSW Australia and followed guidelines in *The Australian Code Of Practice For The Care And Use Of Animals For Scientific Purposes* (8th edition, 2013).

2.2. Breeding, maternal separation, and estrous cycling

Breeding occurred as previously described (Milligan-Saville and Graham, 2016). After pregnant rats gave birth, MS rats (n = 16) had pups removed for 3 h daily from postpartum day (PPD) 2–14. Standard reared (SR) rats (n = 14) had their pups removed and weighed, and then immediately returned. Rats then remained with their pups until weaning (PPD 24), after which they were housed in groups of eight. Behavioral procedures occurred three months later, with MS and SR rats from the same cohort being tested over the same days. Vaginal smears were conducted daily, commencing four days prior to conditioning, as previously described (Milligan-Saville and Graham, 2016).

2.3. Apparatus

Two sets of Med Associates experimental chambers were used, designated Context A and B, differing in visual and tactile features, as previously described (Milligan-Saville and Graham, 2016).

2.4. Procedure

2.4.1. Handling and context pre-exposure

Rats were handled for 4–5 min for 3 consecutive days. After handling rats were placed in Context A for 10 min.

2.4.2. Fear conditioning

Rats were placed in Context A and after 2-min received 2 × 10 s white-noise CS (4 dB above background noise), coterminating with 1 s, 0.6 mA, shock (intertrial interval 135 s).

2.4.3. Extinction training

24 h after conditioning, rats were placed in Context B and after 2-min received 30 × 10 s CS (intertrial interval 10 s). Extinction training occurred during Proestrus or Metestrus.

2.4.4. Extinction recall

24 h after extinction training, rats were placed in Context B, and after 1-min received a single 2-min CS. This design varies the estrous phase during extinction training while keeping the interval between the experimental phases constant; rats are necessarily in different estrous phases during conditioning and recall. Previous research has shown that the difference in extinction recall between Proestrus and Metestrus rats (based on phase during extinction training) is evident irrespective of whether estrous phase during conditioning and extinction recall is held constant or allowed to vary (Graham and Daher, 2016; Milad et al., 2009).

2.4.5. FGF2 quantification

3 days following extinction recall, when rats were in the same estrous phase as during extinction training, a subset of rats (n = 16) were sacrificed and the hippocampus was gross dissected and homogenized in lysis buffer (50 µg/µl). 2.6 µg protein/sample was analyzed for FGF2 using a commercially available ELISA kit, following manufacturer instructions (R&D Systems, USA). This kit has a sensitivity of 3.68 pg/mL, < 0.5% cross-reactivity, and an intra-assay precision CV of 2.2–3.1%.

2.5. Scoring

2.5.1. Behavioral data

Rats were scored for “freezing” (the absence of movement except that required for respiration; Fanselow, 1980) or “not freezing” every 3 s. A percentage score was calculated to determine the proportion of total observations spent freezing.

2.5.2. ELISA

Integrated optical density was measured using an iMARK plate reader at 450 nm.

2.6. Statistical analyses

A univariate Analysis of Variance (ANOVA) with the between subject factors of estrous phase (Proestrus or Metestrus, based on estrous phase during extinction training) and stress (MS or SR) was used to analyze pre-CS freezing and CS-elicited freezing during extinction recall. A mixed-model ANOVA with the same between subject factors as above, and the within-subjects factor of conditioning trial or extinction block, was used to analyze CS-elicited freezing during conditioning and extinction training. Main effects and interactions were investigated with Tukey's Honestly Significant Differences test. Group differences in FGF2 were analyzed using an independent samples *t*-test. Partial correlations were conducted between FGF2 and behavior, covarying for estrous phase.

3. Results

Fig. 1A depicts pre-CS and CS-elicited freezing during conditioning. There were no group differences in pre-CS freezing (no main effects or interactions; largest $F = 2.46$). During conditioning, CS-elicited freezing increased (significant effect of conditioning trial; [$F(1,26) = 178.79$, $P < 0.0001$, $\eta^2 = 0.85$]). There was no main effect of stress or estrous phase, or interaction between factors (largest $F = 3.87$), but there was an interaction between conditioning trial and estrous phase ($F(1,26) = 4.51$, $P = 0.043$, $\eta^2 = 0.02$). Post-hoc tests revealed no group differences at either conditioning trial (smallest $P = 0.07$).

Fig. 1B depicts pre-CS and CS-elicited freezing during extinction training. There was no main effect of stress or estrous phase on pre-CS freezing prior to extinction training (Fs < 1), but there was a significant interaction between stress and estrous phase ($F(1,26) = 5.28$, $P = 0.03$, $\eta^2 = 0.1$). Post-hoc tests revealed no group differences underlying this effect (smallest $P = 0.14$). During extinction training, CS-elicited freezing decreased (significant effect of extinction block; [$F(9,234) = 331.82$, $P < 0.0001$; $\eta^2 = 0.51$]). There were no main effects of stress or estrous phase, or interaction between factors, and there was no interaction between these factors and extinction block (largest $F = 1.82$). To determine whether groups showed comparable conditioned fear expression, the first extinction block was analyzed. This revealed a main effect of stress ($F(1,26) = 9.06$, $P = 0.01$, $\eta^2 = 0.24$) due to MS mothers freezing less than SR mothers, but no main effect of estrous cycle or interaction between factors (largest $F = 0.76$). There were no group differences by the final extinction block ($F = 0.11$).

Fig. 1C depicts pre-CS and CS-elicited freezing during extinction