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Social defeat affects inflammatory signaling and exploratory behavior in mice in a sex-dependent manner

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Abstract

Aim: Psychosocial stress negatively affects both physical and mental health; and stress-related psychiatric disorders are more common in women. Interestingly, preclinical studies have predominately investigated the effects of psychosocial stress on male mice. These studies suggest that adverse effects of psychosocial stress are due in part to the disruption of inflammatory signaling; however, the extent to which these findings translate to females remains unclear, particularly in the context of female-mediated aggression.

Methods: We investigated the effects of acute (2 h) and repeated social defeat (RSD; 2 h/day × 6 days) on proinflammatory cytokine/chemokine expression in male and female C57BL/6J mice: importantly, the CD-1 aggressor mice were the same sex as the subject mice.

Results: The effects on these inflammatory factors were dependent on the duration of social defeat, sex, and tissue. A single bout of social defeat reduced brain IL-1β levels in females only, whereas liver IL-1β and CXCL10 levels increased only in males. RSD decreased brain IL-1β levels in males only; while liver IL-1β and CCL2 levels decreased only in females. RSD led to increased exploratory activity in females; behavioral changes were not apparent in males.

Conclusion: The observed effects of social defeat do not appear to be directly related to stress per se. These novel insights into sex-dependent effects of psychosocial stress on inflammatory signaling and behavior warrant further investigation.

Keywords: Psychosocial stress, repeated social defeat, brain, liver, chemokines, cytokines

INTRODUCTION

Psychosocial stress encompasses a variety of stressors, including trauma, emotional/physical abuse, and bullying to name a few. These forms of stress can be acute or chronic and have far-reaching consequences on health. Indeed, irritable bowel syndrome[1], metabolic syndrome[2], cardiovascular disease[3], and cancer[4] are all exacerbated by chronic psychosocial stress. Additionally, psychosocial stress is instrumental in the development and exacerbation of a range of psychiatric disorders, including anxiety disorder[5] and post-traumatic stress disorder (PTSD)[6]. While there is still much to learn about the pathogenesis of these psychiatric disorders, increasing evidence suggests that central-peripheral crosstalk involving inflammatory signaling between the brain and peripheral tissues (e.g., gut, liver, and spleen) is critical[7-11]. The proinflammatory cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF); and chemokine monocyte chemoattractant protein-1 (CCL2) are all inflammatory mediators implicated in psychiatric disorders[12]. Indeed, emerging evidence suggests that inflammatory dysregulation is involved in anxiety disorders[13,14]. For instance, a recent meta-analysis revealed that circulating levels of proinflammatory cytokines (i.e., IL-1β, IL-6, and TNF) in patients with anxiety disorders, including PTSD, were increased compared to healthy controls[15]. Among chemokines, CCL2 has received considerable attention as a relevant biomarker and therapeutic target for the treatment of psychiatric disorders, including PTSD[16,17]. Furthermore, preclinical and clinical studies suggest that inflammatory factors are sensitive to psychosocial stress[18,19]; and anti-inflammatory agents provide therapeutic benefits in preclinical rodent models of psychiatric disease, including mood and anxiety disorders[20,21].

Interestingly, women are more prone to stress-related psychiatric disorders than are men[22-24]; yet, the overwhelming majority of preclinical studies assessing psychosocial stress have been performed in male rodents, primarily mice. Thus, there remains much to learn about the effects of psychosocial stress on females and the importance of inflammatory signaling. New insights into sex-dependent effects of psychosocial stress on inflammatory signaling and behavior will advance the development of novel therapeutic strategies.

Repeated social defeat (RSD) is a well-established preclinical model of psychosocial stress in male mice, but the effects of RSD on female mice are less clear. While there are several variations of RSD models used for male mice, the method described by Wohleb *et al.*[25] is commonly used. Briefly, a retired male breeder CD-1 mouse (aggressor) is placed in a cage containing three C57BL/6J mice (experimental subjects) for 2 h daily for 6 consecutive days using a different CD-1 aggressor each day. Another frequently used model involves a 10-min exposure to the male aggressor followed by a 24 h period in which the aggressor and test mouse are separated by a perforated Plexiglass panel that allows for visual and olfactory stimulation without physical contact; this cycle is then repeated daily for 10-15 days[26,27].

Although repeated psychosocial stress has often been used to test male responses, there have been relatively few studies assessing the effects of psychosocial stress on female mice, and even those studies used male aggressors[28,29]. We, however, were interested in utilizing a female mouse as the "aggressor". Therefore, in this study, using same-sex aggressors, we investigated the effects of acute and repeated social defeat on exploratory behavior and proinflammatory cytokine/chemokine expression in male and female C57BL/6J mice.

METHODS

Animals

Seven-week-old male and female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME), and housed in USDA-approved facilities for 1-2 weeks prior to experimental treatment. These mice

were housed in same-sex trios in plastic cages (10 cm × 17 cm × 28 cm) containing corncob bedding with paper towel tubes to provide environmental enrichment. Retired breeder male and newly-mated female CD-1 mice were purchased (Jackson Laboratories) for use as aggressors and, aside from being housed singly, were maintained in the same conditions as were the C57BL/6J mice. Mice were housed at 21 °C with a 12:12 light:dark cycle (lights on at 400 h and off at 1600 h) and had *ad libitum* access to food and water. C57BL/6J and CD-1 mice were housed in separate rooms and male and females were never housed together. All experiments were approved by the Institutional Animal Care and Use Committee at Oklahoma State University - Center for Health Sciences.

Social defeat

Retired male CD-1 breeders and primiparous CD-1 females (7-14 days post-partum) were used as aggressors. Females were housed with their pups except when used as aggressors, after which they were returned to their cages. Mouse litters greater than about 7 days of age are capable of thermoregulation[30], so they remained in their respective nests. Pups were in the same room where social aggression occurred and thus the females could hear both audible and ultrasonic vocalizations, if any.

Social defeat occurred in a testing room, separate from the housing room and followed the basic protocol described by Wohleb *et al.*[25], except that they used a male aggressor (CD-1) and male experimental (C57BL/6J) mice, whereas we used males and females (same sex in each cross). Briefly, a CD-1 aggressor was placed in a clean cage (10 cm × 17 cm × 28 cm) containing three C57BL/6J mice for 2 h (1,500 h-1,700 h) followed by immediate euthanasia of the three experimental mice to examine the acute effects of psychosocial stress, or for 2 h/day for 6 consecutive days to examine the effects of chronic stress. In the chronic stress model, the mice remained in the testing room for the remainder of the 6-day period. In terms of aggressive behavior, the aggressive CD-1 mouse confronted/chased the experimental, C57BL/6J mice and repeatedly nipped at their hind quarters. After the first 5 min, this aggression largely subsided (with intermittent confrontations), and the C57BL/6J mice retreated to the corner of the cage and huddled in a submissive manner for the remainder of the 2-h bout. There were intermittent, brief aggressive encounters after the initial 5 min. In the few instances that CD-1 mice did not show this aggression within the first 5 min, they were replaced. There was no evidence of excessive aggression (e.g., biting that resulted in bleeding). Different aggressor/experimental trio pairings were used each day. After the 2-h bout, the aggressor was removed and the experimental mice left undisturbed until the next day when the procedure was repeated.

The handling and housing procedures for control mice were identical to those used for the mice exposed to social defeat, with one exception, control mice were not exposed to a CD-1 aggressor. To control for the possibility that an unfamiliar mouse in the experimental subjects' cage could induce stress, we performed an initial experiment in which a novel, same-sex C57BL/6J mouse was placed in the cage with 3 experimental C57BL/6J mice. Following the 2-h exposure period, plasma corticosterone (CORT) levels were measured as described below. We found that plasma CORT levels in mice exposed to a novel intruder were not significantly different (data not shown) from those in control mice (not exposed to an intruder). Therefore, to reduce animal usage, subsequent experiments only included control mice and mice exposed to a CD-1 aggressor.

Behavioral procedures

Twelve hours after the final bout of social defeat, exploratory locomotor activity and anxiety-like ("wall-hugging") behavior were assessed using a 10-min open-field test (OFT) as previously described[31]; and a 5-min elevated plus maze (EPM) was then used to further assess anxiety-like behavior[32]. Both tests were digitally recorded for subsequent scoring using Ethovision software. To ensure that any changes in behavior were due to RSD as opposed to the acute changes associated with the most recent bout of social defeat, we

delayed behavioral testing until 12 h after the last social defeat exposure. Also, at least in male C57BL/6J mice, the literature suggests that proinflammatory factors are affected in this timeframe.

Tissue collection

For single bout of social defeat (SSD) mice, tissue/blood was collected immediately (within 30 min) after the final 2-h bout of social defeat. For RSD mice, tissue/blood was collected after behavioral testing (which occurred 12 h after the final bout of social defeat). In both experiments, mice were euthanized by CO_2 inhalation, followed by decapitation and collection of trunk blood, brain, and liver on water-ice. Plasma was immediately separated by centrifugation and stored at -80 °C. Both brain and liver tissues were collected and prepared using the method previously described for whole brain[31]. Briefly, tissue was homogenized in lysis buffer (100 mg tissue/1 mL) using a sonic dismembrator. The homogenate was then centrifuged for 20 min at 20,000 × *g* at 4 ˚C. Supernatant was then stored at -80 ˚C.

Plasma corticosterone levels

CORT levels in the plasma were determined by ELISA according to the manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY).

Chemokine and cytokine expression in liver and brain

Standard dual-antibody solid-phase immunoassays (ELISA Development Kit, Peprotech) were used for quantitation of cytokines/chemokines in tissues as previously described[33]. Values were normalized to total protein content, which was determined using the bicinchoninic acid protein assay as described previously[34].

Statistical analysis

PrismTM version 7.04 (GraphPad Inc., San Diego, CA) was used for statistical analysis and figure presentation. Overall analysis of dependent measures was performed using two-way analysis of variance (ANOVA) with sex and treatment (presence/absence of CD-1 aggressor) as grouping variables. Data that were 2 S.D. ± the mean were considered outliers and removed from the analyses. When ANOVA revealed a statistically significant interaction, data were further assessed using a Fisher's LSD test. All data are presented as mean ± S.E.M.

RESULTS

Effect of social defeat on plasma corticosterone levels

Plasma CORT levels were used as an indicator of stress. Two-way ANOVA indicated that there was a significant main effect of sex on CORT levels following 2-h SSD (males, 161.2 ± 21.4; females 281.7 ± 29.8; $F_{1,31} = 14.76$, $P < 0.001$), but no main effect of treatment (i.e., presence/absence of CD-1 aggressor; controls, 202.1 ± 36.2; defeated 243.1 ± 22.9; $F_{1,31} = 2.16$, $P = 0.150$). However, there was a significant interaction between sex and treatment ($F_{1,31} = 10.07$, $P < 0.005$). Pairwise comparisons showed a significant ($P < 0.005$) increase in CORT in males that were subjected to social defeat, whereas CORT levels in females were unaffected by social defeat. In males, plasma CORT was 81.3 ± 15.0 ng/mL in controls *vs.* 232.3 ± 14.6 ng/mL following 2-h SSD. In contrast, plasma CORT in females was 309.4 ± 41.5 ng/mL in controls *vs.* 254.0 ± 43.1 ng/mL following 2-h SSD.

Plasma CORT levels were similar between all groups 12 h following the final bout of RSD. Two-way ANOVA indicated that there were no significant main effects of sex ($F_{1,37} = 4.06$; $P = 0.051$) or treatment ($F_{1,37} = 2.01$, $P = 0.17$) on plasma CORT levels at this point following RSD.

Effect of acute social defeat on cytokine and chemokine expression

To determine the impact of social defeat on proinflammatory cytokine/chemokine expression, we assessed IL-1β, CCL2, and CXCL10 protein expression in brain, liver, and plasma following 2-h SSD, and 12 h after

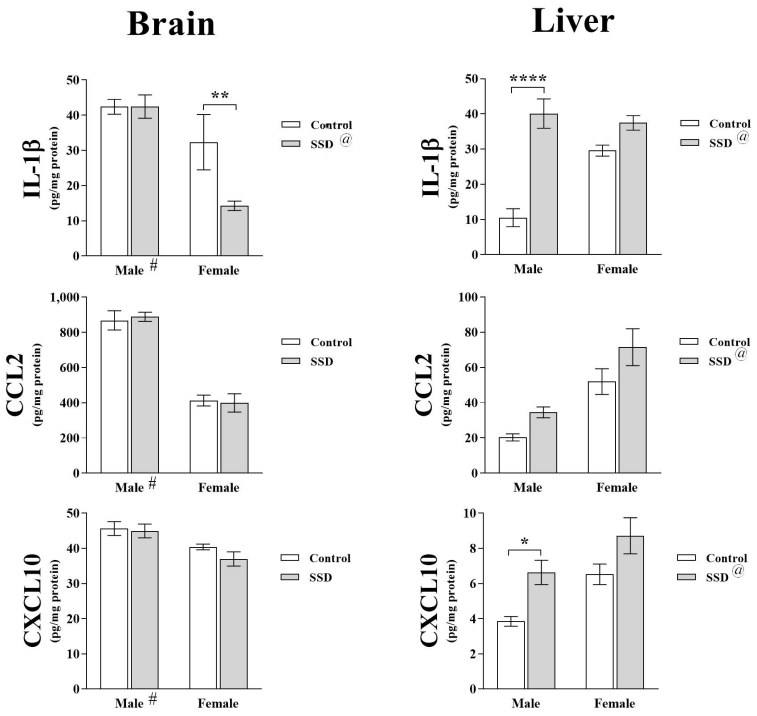

Figure 1. Effect of a single bout of social defeat (SSD) on IL-1β, CCL2, and CXCL10 protein expression in the brain and liver. C57BL/6J mice (n = 7-9 per group) were subjected to social defeat (exposure to a same-sex CD-1 aggressor mouse) for 2 h; immediately thereafter, subject mice were euthanized and tissues were collected and homogenized. Cytokine/chemokine protein expression was measured by ELISA. Data were analyzed by two-way ANOVA followed by pair-wise comparisons and represent mean ± S.E.M.; #main effect of sex, $P < 0.01$; @main effect of treatment, $P < 0.05$; *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$

the final bout of RSD. Due to the limited amount of plasma obtained, we were unable to measure levels of all three of these cytokines/chemokines of interest, and thus, we focused on CCL2.

Expression of the three immune factors in the brain [Figure 1] following SSD was significantly influenced by sex only (IL-1β, $F_{1,31} = 17.67$, $P < 0.001$; CCL2, $F_{1,31} = 14.29$, $P < 0.001$; CXCL10, $F_{1,31} = 126.30$, $P < 0.0001$, respectively).

There was no main effect of treatment ($F_{1,31} = 3.901$, $P = 0.06$), nor was there a sex x treatment interaction ($F_{1,31} = 3.961$, $P = 0.06$) for IL-1β expression in the brain following SSD. However, *a posteriori* pairwise comparison indicated that, in females, IL-1β levels following SSD were significantly reduced compared to those of control females ($P < 0.01$).

As shown in Figure 1, the expression of IL-1β, CCL2, and CXCL10 in the liver following SSD was significantly influenced by both sex ($F_{1,29} = 8.23$, $P < 0.01$; $F_{1,29} = 10.13$, $P < 0.005$; $F_{1,29} = 23.77$, $P < 0.001$, respectively) and treatment ($F_{1,29} = 42.78$, $P < 0.001$; $F_{1,29} = 11.06$, $P < 0.005$; $F_{1,29} = 5.73$, $P < 0.05$, respectively). IL-1β expression in the liver was influenced significantly by the interaction of sex and treatment ($F_{1,29} = 14.36$, $P < 0.001$). In addition, pairwise analysis revealed that following SSD, the expression of both IL-1β and CXCL10 was significantly ($P < 0.001$; $P < 0.05$, respectively) elevated in the liver of males, compared to females.

The levels of CCL2 in the plasma were strongly affected by SSD. In males, plasma CCL2 was 3.3 ± 1.5 pg/mL in controls *vs.* 60.5 ± 7.5 pg/mL following 2-h SSD. In contrast, plasma CCL2 in females was 3.5 ± 2.1 pg/mL in controls *vs.* 3.0 ± 0.9 pg/mL following 2-h SSD. Two-way ANOVA indicated significant main effects of sex ($F_{1,30} = 46.71$, $P < 0.001$), treatment ($F_{1,30} = 45.55$, $P < 0.001$), and the interaction ($F_{1,30} = 47.36$, $P < 0.001$)

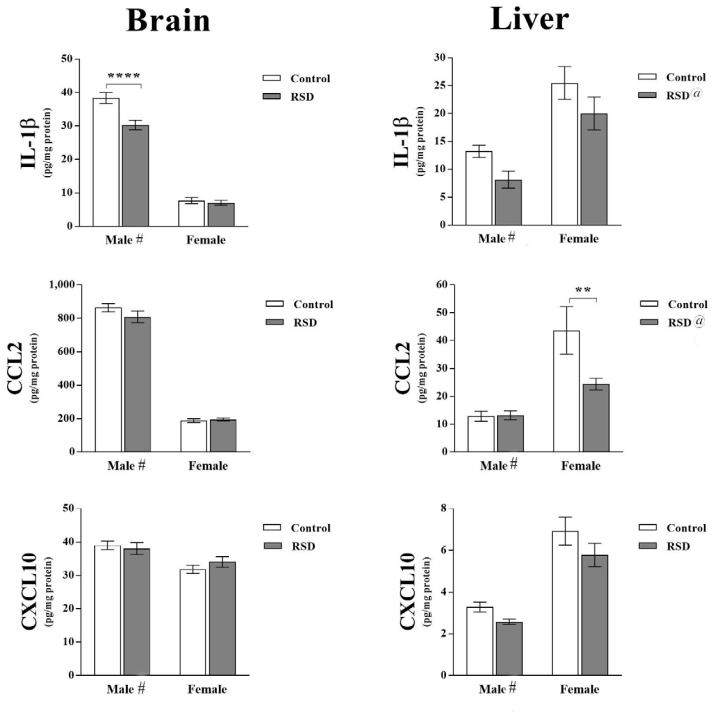

Figure 2. Effect of repeated social defeat (RSD) on IL-1β, CCL2, and CXCL10 protein expression in the brain and liver. C57BL/6J mice (n = 7-12 per group) were subjected to RSD (exposure to a same-sex CD-1 aggressor mouse for 2 h/day for 6 consecutive days); 12 h after the final bout of social defeat, subject mice were then euthanized and tissues were collected and homogenized. Cytokine/chemokine protein expression was measured by ELISA. Data were analyzed by two-way ANOVA followed by pair-wise comparisons and represent mean ± S.E.M.; #main effect of sex, $p < 0.001$; @main effect of treatment, $P < 0.05$; **$P < 0.01$; ****$P < 0.0001$

of these main effects. Additionally, pairwise analysis revealed that plasma CCL2 levels were significantly ($P < 0.001$) increased in males subjected to SSD, compared to controls.

Effect of RSD on cytokine and chemokine expression

The expression of IL-1β in the brain following RSD was significantly affected by both sex ($F_{1,38} = 445.5$, $P < 0.0001$) and treatment ($F_{1,38} = 11.65$, $P < 0.005$); and there was a significant interaction between sex and treatment ($F_{1,38} = 8.47$, $P < 0.01$) [Figure 2]. Subsequent pairwise analysis indicated that IL-1β was significantly ($P < 0.0001$) lower in the brain of RSD males, compared to male controls. The expression of both CXCL10 and CCL2 in the brain 12 h after the last bout of RSD was significantly influenced by sex ($F_{1,37} = 13.54$, $P < 0.001$ and $F_{1,37} = 798.0$, $P < 0.0001$, respectively), but unaffected by treatment ($F_{1,37} = 0.1595$, $P = 0.69$ and $F_{1,37} = 1.13$, $P = 0.29$, respectively) [Figure 2].

Cytokine/chemokine expression in the liver also responded to RSD [Figure 2]. Expression of CXCL10 in the liver 12 h after the last bout of RSD was significantly influenced by sex ($F_{1,34} = 49.91$, $P < 0.001$). The expression of IL-1β and CCL2 in the liver following RSD was significantly affected by both sex ($F_{1,36} = 27.17$, $P < 0.0001$ and $F_{1,36} = 24.69$, $P < 0.001$, respectively) and treatment ($F_{1,36} = 5.24$, $P < 0.05$ and $F_{1,36} = 4.99$, $P < 0.05$, respectively). Also, with respect to CCL2, there was a significant interaction ($F_{1,36} = 5.40$, $P < 0.05$) between sex and treatment. Follow-up pairwise analysis indicated that following RSD, both IL-1β and CCL2 expression was significantly ($P < 0.05$ and $P < 0.01$, respectively) lower in the liver of RSD females, compared to female controls.

RSD also affected the levels of CCL2 in the plasma. Plasma CCL2 was 1.2 ± 0.7 pg/mL in control males *vs.* 33.8 ± 15.4 pg/mL in males following RSD. However, plasma CCL2 in females was 4.0 ± 1.7 pg/mL in

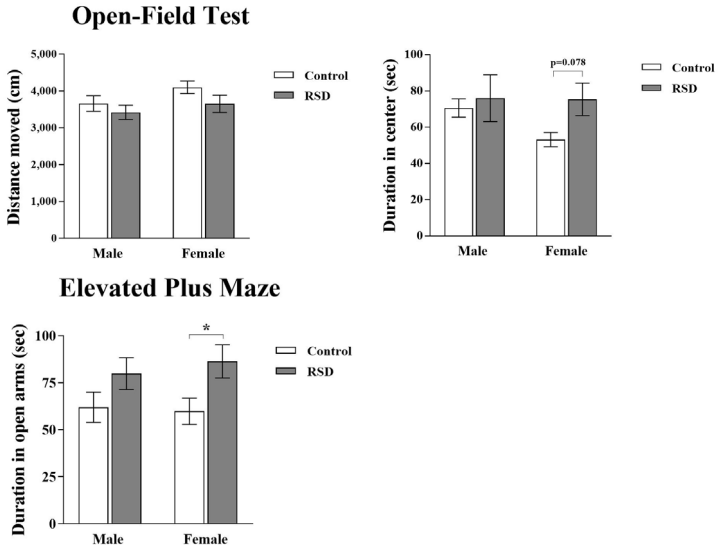

Figure 3. Effect of repeated social defeat (RSD) on locomotor activity, exploratory behavior, and anxiety-like behavior. C57BL/6J mice (n = 8-12 per group) were subjected to RSD (exposure to a same-sex CD-1 aggressor mouse for 2 h/day for 6 consecutive days). At 12 h after the final bout of social defeat, subjects were then subjected to behavioral testing. Behavioral tests included a 10-min open-field test, followed by a 5-min elevated plus maze. Data were analyzed by two-way ANOVA followed by pair-wise comparisons and represent mean ± S.E.M. *P < 0.05

controls *vs.* 1.6 ± 0.8 pg/mL after RSD. Two-way ANOVA indicated significant main effects of sex ($F_{1,37}$ = 4.58, P < 0.05), treatment ($F_{1,37}$ = 4.86, P < 0.05), and an interaction ($F_{1,37}$ = 6.54, P < 0.05) of these main effects. Subsequent pairwise analysis revealed that following RSD, plasma CCL2 levels were significantly (P < 0.005) increased in males exposed to RSD, compared to control males.

Effect of repeated social defeat on locomotor activity, exploratory behavior, and anxiety-like behavior

Twelve hours following the final bout of RSD, locomotor activity and exploratory behavior were assessed in an OFT [Figure 3]. There were no significant main effects of sex ($F_{1,37}$ = 2.50, P = 0.12) or treatment ($F_{1,37}$ = 2.62, P = 0.11) on distance moved. Exploratory behavior, as indicated by duration of time spent in the center zone during the OFT, was not significantly affected by sex ($F_{1,37}$ = 1.11, P = 0.30) or treatment ($F_{1,37}$ = 2.62, P = 0.11). Pairwise comparison (Fisher's LSD) suggested that females subjected to RSD tended to explore the center zone more than female controls, although not quite to the level of significance (P = 0.08).

The EPM was used to assess anxiety-like behavior [Figure 3]. There was a significant main effect of treatment ($F_{1,37}$ = 7.10, P < 0.01) on time spent in the open arms of the EPM. There was neither a main effect of sex ($F_{1,37}$ = 0.07, P = 0.79) nor an interaction between sex and treatment ($F_{1,37}$ = 0.27, P = 0.61) on time spent in the open arms.

DISCUSSION

RSD is commonly used in preclinical studies of psychosocial stress; and results suggest that altered inflammatory signaling induced by RSD adversely contributes to liver injury, increased pain sensitivity, increased susceptibility to endotoxic shock, and behavioral deficits[25,35-38]. However, these findings stem primarily from experiments with male mice; and in the relatively few studies of RSD in female mice, the aggressor was a male[28,29,39]. While such studies have provided important information about male-mediated social defeat on females, they do not necessarily inform on the effects of a female-mediated social defeat on females.

To our knowledge, this is the first study to use post-partum female CD-1 mice as intruders in a social defeat model. The only observable difference between male and female CD-1 aggressors was that the female aggressor had to be replaced more frequently than did male aggressor, due to insufficient aggression. Importantly though, regardless of sex, successful aggressors displayed similar levels of aggression. We were intrigued that the single bout of aggression was not sufficient to increase plasma CORT in the female C57B/6J mice. It should be noted however that our criterion for a successful CD-1 intruder-encounter included the display of submissive behavior on the part of the subjects. The fact that all encounters included such displays suggests that the subject females were experiencing social stress, despite the absence of the expected increase in circulating CORT. It is also worth noting that the baseline CORT levels were higher in females than in males, which is consistent with previous reports[40,41]. Conversely, in males, SSD induced a stress response in male C57BL/6J mice as indicated by elevated plasma CORT, and this is similar to the findings of McQuaid *et al.*[42]. The specific factors responsible for these sex differences in CORT responses remain to be determined.

RSD did not affect CORT levels at 12 h after the final bout of social defeat, which is consistent with previous reports. For instance, Niraula *et al.*[43] measured CORT immediately after the final bout and observed increased CORT levels. However, Zhu *et al.*[44] found that CORT levels increased after acute social defeat, and found that RSD did not affect CORT levels. Furthermore, circulating CORT levels peak 30 min after social defeat, and then begin to decline toward baseline by 60 min post-defeat[45].

In males, SSD resulted in increased expression of proinflammatory factors in the plasma (CCL2) and liver (IL-1β and CXCL10), whereas the factors were unaffected in the female liver. Acute social defeat did not affect cytokine/chemokine expression in the male brain; and reduced IL-1β levels in the female brain. The change in IL-1β expression in females was likely not stress-induced per se, given that CORT levels were normal in females.

Similar to SSD, RSD increased plasma CCL2 in males; however, the expression of proinflammatory factors was not affected in females. Interestingly, while the male liver was affected by SSD (increased IL-1β and CXCL10), the female liver was more sensitive to RSD; and the direction of change was different, with decreased levels of IL-1β and CXCL10 in the female liver. In the brain, IL-1β was the most sensitive to RSD, as indicated by reduced expression, but in males only.

These findings highlight the differential effects of both sex and duration of social stress, on inflammatory cytokine/chemokine expression. Furthermore, there are tissue-specific effects to consider. The RSD-mediated increase in circulating CCL2 in males is consistent with the elevated levels of CCL2 observed in patients following psychosocial stress[46]. CCL2 is instrumental in monocyte recruitment and response to injury and infection, and thus, dysregulation of CCL2 signaling could certainly have adverse effects. For instance, we previously reported that LPS-induced sickness behavior in C57BL/6J mice is positively correlated with increased CCL2[31]. Similarly, bacterial-induced colitis and the increased severity of the disease following social stress is CCL2-dependent[47].

The differences in cytokine/chemokine expression in the liver following single or repeated bouts of social defeat, and between sexes are intriguing. Overall, inflammatory factors in the male liver were more sensitive to acute social stress, whereas RSD had a more profound effect on the liver in females. The biological relevance of these stress-induced effects remains to be determined, but it is critical to understand given the importance of inflammatory signaling in liver function. Indeed, mounting data indicate that psychosocial stress leads to liver pathogenesis through an inflammatory-mediated mechanism[48]. For instance, Sanchez *et al.*[49] reported that a single aggressive encounter was sufficient to induce liver injury, and IL-1β has been implicated in stress-induced liver insult[50]. There is also evidence that susceptibility to endotoxic

shock is increased by social stress, and mechanistically, increased proinflammatory cytokine levels are involved[37].

We found that IL-1β protein levels in the brain of male mice decreased following RSD. This finding is similar to a previous report in which RSD resulted in reduced IL-1β mRNA expression in the hippocampus[51]. Whereas Szyszkowicz *et al.*[52] observed no effect of RSD on IL-1β mRNA expression in either the hippocampus or prefrontal cortex, others found that IL-1β mRNA expression in the male brain was increased after RSD[35]. However, IL-1β was specifically measured in macrophages that accumulated in the brain[35]. More recently, this same group found that IL-1β mRNA expression also increased in the female brain (coronal slice that included hippocampus and amygdala) following RSD[39]. However, in contrast to our model, the recent study by Yin *et al.*[39] used male aggressors.

Compared to the reported effects of RSD on IL-1β in the brain, there has been considerably less reported about the effects of RSD on CCL2 and CXCL10 expression in the brain. Sawicki *et al.*[19] showed that six days of RSD increased CCL2 mRNA expression in the rostral cortex of male mice, but had no effect on CCL2 expression in the caudal cortex, hippocampus, or basal ganglia. More recently, McKim *et al.*[35] found that CCL2 mRNA expression increased in the brain following RSD; however, CCL2 was measured specifically in resident microglia. Thus, our observation that RSD does not affect CCL2 protein expression in the male brain is generally consistent with these reports. Importantly, we show that CCL2 expression in the female brain is also unaffected by RSD.

We did not detect any differences in brain CXCL10 levels in either male or female mice following RSD. In terms of comparison to other studies, there is relatively little information in the literature on the impact of RSD on CXCL10 expression in the brain; however, Tang *et al.*[53] recently reported that mRNA levels for this chemokine were upregulated in the brain of male mice following 10 days of RSD.

Similar to previous studies, we did not detect decreases in overall locomotor activity following RSD, suggesting that there was no induction of sickness behavior. Previous reports indicated that RSD leads to behavioral deficits, including anxiety- or depressive-like activity[25,35,53]. However, we did not observe these behavioral deficits in male mice following RSD. The basis for this difference is not clear. There was a marginal effect of treatment in the amount of time spent in the open arms by the males, but any effect was smaller than was the corresponding effect in females. It is possible that larger sample sizes may have revealed more substantial changes. In females, RSD actually increased exploratory activity. This finding is particularly interesting in the context of increased risk-taking behavior that is often observed in patients that have experienced psychosocial stress, including bullying[54-56]. Further investigation is of course required to definitively make this connection.

There are several possible explanations for the differences in the RSD-induced effects on proinflammatory cytokine/chemokine expression and behavior observed in our study, compared to the findings in previous reports. First, there are clear differences in the models of RSD, e.g., type of aggressor, duration and number of social defeat bouts, and presence/absence of continued visual and olfactory stimulation following the bout varied. Second, we assessed cytokine/chemokine expression at the protein level in whole brain homogenates, whereas others assessed mRNA in select brain regions and specific cell types. Lastly, the time point of tissue collection/behavioral assessment following RSD varied to some extent between these studies. For instance, we measured behavior and expression of inflammatory factors 12 h after the final bout of social defeat, whereas others typically measured at 14-24 h after the last bout of social defeat[19,25,45].

In our follow-up investigations, it will be important to assess inflammatory factors and behavior at additional time points both prior to and after the 12 h time point assessed in this study. One limitation of

our study was that we measured the expression of inflammatory factors in whole brain homogenates, which may explain the lack of treatment effect on cytokine/chemokine expression. Therefore, it will be important in future investigations to assess the expression of these inflammatory mediators in specific brain regions, such as the hippocampus and prefrontal cortex. Similarly, we expect to measure mRNA expression of cytokines/chemokines in microglia and astrocytes following RSD. Lastly, it will be important to include additional behavioral tests, and assess additional behaviors, such as depressive-like behavior.

In conclusion, to our knowledge, this is the first report on the effects of female-female social defeat on proinflammatory cytokine/chemokine expression in liver and brain, and on exploratory behavior. This report provides interesting and novel findings about the differential effects of acute and chronic social defeat on proinflammatory cytokines/chemokines and about the extent to which these effects are tissue- and sex-dependent. There is considerable evidence that liver-brain crosstalk contributes to mood disorders, and thus, the effects of RSD on inflammatory factors in the liver and brain may ultimately yield critical insights into the detrimental effects of psychosocial stress, particularly on anxiety and mood disorders.

DECLARATIONS

Authors' contributions

Concept, experimental design, literature review, statistical analysis, manuscript preparation: Davis RL
Performed experiments, data acquisition and analysis, and manuscript editing: McCracken K, Buck DJ
Experimental design, statistical analysis, manuscript editing: Curtis JT

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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