Early life stress effects on neuroimmune function in limbic brain regions and mood-related behavior in male and female Sprague-Dawley rats

Thesis

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Abstract

Adverse Childhood Experiences (ACEs) are widespread in the general population and appear to increase vulnerability to adult neuropsychiatric disorders. The biological mechanisms that confer this increased risk are poorly understood. Changes in neuroimmune cell number, phenotype, and activity are observed with stress, depression- and anxiety-like behavior in rodents, and mood disorders; these cells may also program adult behavior following early life stress. In this study, male and female Sprague-Dawley rats were subjected to early deprivation stress or control handling for 4hrs./day from PD2-20. Brains from rats killed at PD21 were processed for counting of mast cells and qPCR analysis of mRNA expression related to microglial phagocytosis, histamine synthesis, and blood-brain barrier proteins in limbic regions. Adult rats were tested for anxiety-like, stress coping, and risk assessment behavior. Stress increased granulated and total mast cell count in the hippocampus, as well as hippocampal expression of genes related to microglial phagocytosis. Stress decreased hippocampal histidine decarboxylase mRNA expression, amygdala cybb mRNA expression, exploratory behavior in the open field test (in males only), risk assessment behaviors, closed arm entries in the elevated plus maze, and time immobile in the forced swim test. When controlling for experimental condition, histidine decarboxylase mRNA expression and phagocytic gene expression were significantly negatively correlated. These experiments suggest potential neuroimmune mechanisms that program maladaptive adult behaviors following early life stress.

Dedication

For Margaret Fahey and Joseph Saulsbery, as always. I love you.

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Fields of Study

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Introduction

In humans, adverse childhood experiences (ACEs; including child abuse, neglect, and various forms of household dysfunction) show an exposure-response relationship to numerous poor health outcomes, including adult depression and suicide attempts (Chapman et al., 2004; Dube et al., 2001; Felitti et al., 1998). The relationship between ACEs and depression remains even when controlling for the presence of a mentally ill household member in childhood (Chapman et al., 2004). Among women, childhood physical and sexual abuse predict increased prevalence of anxiety disorders and major depressive disorder; in men, physical abuse is associated with an increased prevalence of anxiety disorders (MacMillan et al., 2001). Further, in depressed patients, a history of childhood maltreatment is associated with a more recurrent, persistent, and treatment-refractory disease course (Nanni, Uher, & Danese, 2012).

Vulnerability to early life stress may differ by gender and age at assessment: In the MacMillan et al. study cited above, childhood abuse predicted adult depression only in women (MacMillan et al., 2001); however, Zeanah et al. found that, in a sample of institutionalized children, girls experienced more symptom reduction than boys upon placement in foster care (Zeanah et al., 2009). Childhood abuse and neglect are also associated with reduced hippocampal volume (Bremner et al., 1997) and impaired working memory and emotional processing scores (compared with non-traumatized controls) (Gould et al., 2012). It is not fully understood how ACEs confer vulnerability to adolescent and adult disease, including psychological disorders, nor how gender and sex may mediate risk. Our lack of understanding has rendered effective

"treatments" for ACEs elusive.

Animal models can clarify the biological mechanisms that link toxic developmental stress to adolescent and adult depression- and anxiety-like behavior. Rodent models of childhood abuse and adversity include maternal separation stress (see for examples Andersen, Lyss, Dumont, & Teicher, 1999; Brenhouse & Andersen, 2011; Huot, Gonzalez, Ladd, Thrivikraman, & Plotsky, 2004; Suri et al., 2013), which approximates human experiences of childhood neglect and social isolation by separating rodent pups from their dam (and, depending on the protocol, possibly from each other) for several hours at a time. Although rat pups pass through a "stress hyporesponsive period" from postnatal day (PD) 2-14 (Sapolsky & Meaney, 1986), maternal separation is typically a robust enough stressor to activate the hypothalamic-pituitary-adrenal (HPA) axis (a neuroendocrine system that regulates the stress response) during this time (Schmidt et al., 2004). In adolescence and upon maturity, maternally stressed pups often present with abnormalities in behavior. For example, 3 hours per day of maternal separation stress from PD1-14 increases anxiety-like behavior in the open field test and impairs social interaction in adult Wistar rats (Rana, Pugh, Jackson, Clinton, & Kerman, 2015).

Immune pathways are a promising potential mediator of the link between early life stress and adult psychological disorders. In humans, various forms of early life adversity are associated with elevated interleukin-6 (IL-6) and C-reactive protein (CRP; an acute-phase reactant protein and an inflammatory marker that predicts cardiovascular disease risk (Miller, Chen, & Parker, 2011; Ridker, Buring, Cook, & Rifai, 2003)) later in life (Slavich & Irwin, 2014). Among people with high exposure to early life adversity, depression is associated with elevated CRP and IL-6—an association that is *not* observed in people without exposure to early life adversity (Miller &

Cole, 2012). Unmedicated depressed people have higher baseline tumor necrosis factor alpha (TNF-α) and IL-6 concentrations than healthy controls (Dowlati et al., 2010). Further, signs of mild neuroinflammation and circulating monocyte recruitment have been detected in the dorsal anterior cingulate cortex white matter of depressed suicides (Torres-platas, Cruceanu, Gang, Turecki, & Mechawar, 2014).

Several types of immune cells reside in the brain, including microglia. Microglia make up ~10% of total brain cells (Lawson, Perry, Dri, & Gordon, 1990) and respond to immune challenge (Wohleb et al., 2012), traumatic brain injury, and psychological stress by proliferating, altering their morphology, secreting proinflammatory mediators (such as tumor necrosis factor α, interleukin-1ß, and interleukin-6), and phagocytosing neurons or neuronal elements (Kettenmann, Kirchhoff, & Verkhratsky, 2013; Tian et al., 2017; Tynan et al., 2010; Wohleb, Terwilliger, Duman, & Duman, 2017). In the healthy brain, microglia participate in synaptic patterning (Weinhard et al., 2018), cell proliferation, cell survival, and myelination (Hagemeyer et al., 2017) (reviewed in Salter & Beggs, 2014). During development, microglia contribute to numerous processes critical for optimal brain function, including myelination, axon guidance, synaptic pruning (Paolicelli et al., 2011), hippocampal phagocytosis of neural progenitor cells (Nelson, Warden, & Lenz, 2017), and neurogenesis (Cunningham, Martinez-Cerdeno, & Noctor, 2013; Shigemoto-Mogami, Hoshikawa, Goldman, Sekino, & Sato, 2014) (reviewed in Mosser, Arnoux, & Audinat, 2017)**.** Developmental stress may disrupt or unbalance these processes, leading to suboptimal circuit organization and maturation and placing the organism at risk for cognitive and behavioral dysfunction.

Depression-like behavior has also been robustly associated with (neuro)inflammation,

including microglial activation, in rodents. Compared with handled controls, male Sprague-Dawley rats subjected to chronic restraint stress showed reduced sucrose preference (an indicator of anhedonia) along with increased density of microglia in numerous brain regions relevant to affective disorders, including the medial prefrontal cortex and nucleus accumbens (Tynan et al., 2010). In adult male mice, repeated social defeat stress (which is analogous to human bullying and abuse) is associated with monocyte trafficking into the brain, microglial activation, and anxiety-like behavior (reviewed in Reader et al., 2015; reviewed in Weber, Godbout, & Sheridan, 2017). Trafficked monocytes may contribute to neuroinflammation by secreting cytokines, which may activate brain-resident immune cells, leading to neuroinflammation and greater vulnerability to adult psychological disorders (Reader et al., 2015). Crosstalk between the central and peripheral immune systems may contribute to stress-related psychological disorders (Weber et al., 2017), and stress may promote this form of central-peripheral crosstalk via increased permeability of the blood-brain barrier. Adult stress alters the morphology and expression of BBB tight junction proteins that regulate BBB permeability, including claudin-5 and occludin (Menard et al., 2017; Sántha et al., 2016). Similarly, prenatal stress or postnatal MSS (compared with non-stress control) increases BBB permeability to Evans blue at PD20 and PD10, respectively (Gómez-González & Escobar, 2009). Although most research on neuroinflammatory responses to stress has focused on microglia, other stress-responsive immune cells reside in the brain and may compromise blood-brain barrier integrity.

Mast cells are innate immune cells that are usually associated with peripheral allergy and edema; they also reside in the brain, particularly in the hippocampal region (Joshi et al., 2019) where they increase BBB permeability via vasoactive products such as serotonin (Sharma,

Westman, Cervós Navarro, Dey, & Nyberg, 1995) and histamine under conditions that induce mast cell degranulation (Abbott, 2000; Esposito et al., 2001)**;** during development, mast cell histamine and serotonin could also program circuit development relevant to mood-related behavior. Mast cells also secrete other immune and neuromodulatory mediators such as proteases, prostaglandins, and cytokines; granulated mast cells may release low levels of these mediators in the absence of immune stimulation (Silver & Curley, 2013)**.** Stimulated, degranulated mast cells rapidly release their contents into the extracellular space (Silver & Curley, 2013). Further, mast cells communicate with microglia via histamine release. Histamine prompts microglia to upregulate pro-inflammatory receptors such as toll-like receptor (TLR) 4 and histamine 1 receptor (H_1 or H_1R) (Dong et al., 2017) and to release proinflammatory molecules, e.g. prostaglandins (Lenz et al., 2018), TNFα, and IL-6 (Dong et al., 2017)**.** Mast cells may mediate neuroinflammation by modulating the activity of microglia, and this relationship demands further study.

Mast cells are stress-responsive (Joshi et al., 2019): They express CRH receptors (Cao et al., 2014), and adult restraint stress induces mast cell degranulation and loss of BBB integrity (Esposito et al., 2001, 2002). Mast cells also participate in the regulation of anxiety-like behavior and cognition in mice: Compared with wildtype mice, adult mast cell-deficient mice display increased anxiety-like behavior (Nautiyal, Ribeiro, Pfaff, & Silver, 2008); these changes suggest a possible role of mast cells in the developmental programming of adult anxiety and mood. In humans, brain-resident mast cell numbers are highest during childhood and adolescence; their numbers fall in adulthood (Dropp et al., 1979). Therefore, there may be a sensitive period in early life for mast cell-mediated regulation of BBB permeability and brain function in response

to stress.

Here, I subjected male and female rat pups to early life stress to determine whether adult mood-related and risk assessment behaviors are associated with loss of BBB protein mRNA expression and mast cell degranulation. I hypothesized that early life stress would suppress BBB protein mRNA expression and increase passive coping behavior, anxiety-like behavior, risk assessment behaviors, and hippocampal mast cell degranulation. I also assessed the expression of genes related to microglial phagocytosis in the hippocampus, hypothalamus, and amygdala as a potential additional moderator of enduring, adverse effects of early life stress that is potentially downstream of mast cells. This study focuses on limbic brain regions because they are both stress-responsive and involved in mood disorders and mood-related behavior (Campbell & MacQueen, 2004; McEwen, 2001; Joëls et al., 2004; Roozendaal et al., 2009; Price & Drevets, 2010; Hill et al., 2015; Raineki et al., 2012); further, early life stress alters the development, structure, and function of these brain regions (Cohen et al., 2013; Bath et al., 2016; Johnson et al., 2018; Dahmen et al., 2018; Dannlowski et al., 2012; Veenema et al., 2006; Aisa et al., 2008).

Target gene	Forward sequence	Reverse sequence	Efficiency
ß-Actin	CTCTGAACCCTAAGGCCAA	GTACGACCAGAGGCATACA	99.2%
C1qa	AGGAGAATCCATACCAGAACC	AGGACACGATAGACAGACAAA	100.6%
Claudin-5	TGCAAAGTGTATGAGTCTGTG	TCAAGGTAACAAAGAGTGCC	100%
CD68	CCTCTCTGTATTGAACCCGA	TGTCCGTAAGGGAATGAGAG	97.1%
Cybb	CGAATTGTACGTGGACAGAC	CCACGATCCATTTCCAAGTC	99.8%
Histidine decarboxylase	TCTACCTCAGACATGCGAAC	AAGGACCGAATCACAAACCA	108%
Occludin	TATGATGAACAGCCCCCTAA	GACTCTTTCCGCATAGTCAG	104%
TJP1	TGAAAGAAGCGATTCAGCAG	GACAGACGATCATCATGCAA	96%
TJP2	AAGGCTGACTACTGAGCTAC	TATTCAACCGAACCACTCCA	92%
Tyrobp	CTGACTGTGGGAGGATTAAGT	CACAGAGGAACATTCGCATC	98.8%

Table 1. Quantitative polymerase chain reaction gene primer sequences and efficiency.

Methods

Animals

8 timed pregnant female Sprague-Dawley rats ordered from Harlan provided litters for this experiment. The gestating dams were single-housed with ¼" corn cob bedding and brown paper nesting material. The dams had *ad libitum* access to water and standard lab chow (Harlan #7912) and were housed in a temperature- and humidity-controlled room with a 12:12 standard light cycle (lights on at 06:00).

The day of birth was designated as PD0. On PD1, litters were culled to 10 ± 1 pups and were sex-balanced (except for one litter that was heavily skewed toward male pups). A random number generator (random.org) was used to assign half of the litters to the stress condition and half to control handling. All procedures were conducted in accordance with The Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and approved by The Ohio State University Institutional Animal Care and Use Committee.

Early maternal deprivation stress

Pups were stressed for 4hr/day from PD2-20. They were separated from the dam during the light cycle, when most maternal care occurs (Ader & Grota, 1970). Litters assigned to early deprivation stress were gathered in a tupperware container, transferred to a different room, and isolated from each other by placing them into individual plastic cups (Solo, Dart Container, Mason, MI) with a few shreds of home cage nesting material (as in Ganguly & Brenhouse,

2015). To avoid hypothermia, the cups were placed in an infant incubator (Petiatric, Wichita, KS) set to 35.6°C. During early deprivation, pups were left undisturbed, and the lights in the incubator room were left on to prevent circadian rhythm disruptions. Pups assigned to control handling were also gathered in a Tupperware container, then immediately returned to the home cage. All pups were weighed every other day.

Maternal observations

Maternal behavior was observed by trained observers for 10 minutes twice per day—once at baseline (before stress or control handling) and once upon pups' return to the nest. Pup-oriented behaviors were categorized as arched-back nursing, blanket nursing, passive nursing, nursing while licking, licking (of pups), and pup retrieval (De Guzman, Saulsbery, & Workman, 2018). Non-pup-oriented behaviors were categorized as nest building, self-grooming, tail chasing, and off-nest. Behaviors were scored in 5-second bins.

Perfusions and tissue collection

Half of the pups from each litter were randomly selected for tissue collection at PD21. Before perfusion, pups were overdosed with Euthasol (0.3mL, administered intraperitoneally). Depth of anesthesia was tested with toe pinch. Pups were perfused with 0.01M phosphate buffered saline (PBS) solution. One randomly selected hemisphere was post-fixed in 4% paraformaldehyde in PBS for 24 hours at 4ºC. Brains were then changed into 30% sucrose

solution and kept at 4ºC until they sank. Post-fixed brain hemispheres were then sectioned at 45µm thickness on a Leica cryostat in 10 series and stored in cryoprotectant at -20°C until staining. The hippocampi, amygdalae, and hypothalami from the other brain hemisphere were rapidly dissected and stored in cryotubes at -80°C until RNA extraction.

RNA extraction and cDNA synthesis

RNA was extracted from brain tissue samples with Qiazol and the Qiagen RNeasy Mini Kit (Hilden, Germany; #74106) according to the manufacturer's instructions. RNA quality and concentration were determined with absorbance spectroscopy. cDNA was then synthesized using the First Strand cDNA synthesis system (Origene).

Quantitative polymerase chain reaction (qPCR)

qPCR was run in triplicate on 96-well plates with Bio-Rad Sso Advanced Universal Sybr Green Supermix in a Bio-Rad CFX-96 thermocycler. A sample from an unstressed male was used as an internal control, and reactions were normalized to actin. Gene primer sequences are listed in Table 1. Fold change was calculated using the Pfaffl method (Pfaffl, 2001).

Toluidine blue staining

To visualize mast cells, brains were stained with toluidine blue, which metachromatically

stains mast cell granules purple (Joshi et al., 2019). Brains were sliced into 10 series of 45µm sections on a Leica cryostat. The sections were stored at -20°C in cryoprotectant solution until they were mounted on charged slides (FisherBrand Superfrost Plus) and stained. One series of slides was stained with 0.5% toluidine blue in deionized water and 60% acidified ethanol (pH = 2.53) as follows: Slides were defrosted before staining, then washed in 60% EtOH for 2 minutes. The slides were then stained in the toluidine blue solution for 10 minutes, rinsed briefly in deionized water, and dehydrated in ascending graded alcohols (50% EtOH for 15 seconds, 70% EtOH for 45 seconds, 95% EtOH for 2 minutes, and 100% EtOH for 2 minutes). Slides were then allowed to dry for 30 seconds before clearing in xylene for 5 minutes x 2. Finally, slides were coverslipped with Permount and allowed to dry for at least two weeks before cell counting.

Mast cell counting

Mast cells were counted in the hippocampal-thalamic region of each rat at 20x magnification on a Zeiss Imager.M2 microscope with StereoInvestigator software (MBF Bioscience, Williston, VT). 5 hippocampal slices were selected for stereological analysis in each animal. A single investigator blinded to experimental condition performed all counts. Hypothalamic mast cells were not counted because no cells were detected at PD21 in 6 of the first 7 brains examined (in contrast to Joshi et al., 2019, who found small numbers of mast cells in the hypothalamus at PD11).

Mast cells were counted and categorized as either granulated or degranulated. Spherical mast cells with a uniform dark blue stain were categorized as granulated. Degranulated mast

cells were identified based on loss of rounded morphology, visible extruded granules, and loss of blue stain (Barbara et al., 2004; Esposito et al., 2001; Joshi et al., 2019). 5 brains (1 stressed female, 2 female controls, and 2 stressed males) were omitted due to processing error. One stressed female (final n = 8) was omitted from the percent degranulated mast cell analysis— no mast cells were detected in her hippocampus, rendering the percentage of degranulated mast cells meaningless for this animal.

Open field test

Rats selected for behavioral testing were weaned at PD21, housed with same-sex littermates, and raised to adulthood (~P75). Anxiety-like behaviors were then tested by allowing rats to explore an open field chamber for 10min under red light. The arena was thoroughly cleaned with 50% EtOH between each rat. All behavior tests were conducted in the afternoon (during the rats' light cycle) and were filmed with a video camera for future scoring. Open field test behaviors were scored automatically using EthoVision (Noldus).

Elevated plus maze test

1-2 days after the OFT, rats were further tested for exploratory and risk assessment behaviors in the elevated plus maze. The maze consists of two open arms and two walled arms; the maze is raised to increase its aversiveness and prevent rats from jumping onto the floor. Rats were placed in the center of the maze facing an open arm and were left to explore for 10 minutes

under red light. The maze was thoroughly cleaned with 50% EtOH between each rat. Open arm time, arm entries, and risk assessment behaviors (i.e., head dips, rearing, and stretch-attend postures) were scored by a single trained experimenter. The experimenter was blinded to animal sex and condition while scoring all behaviors except open arm time. An arm entry was scored when a rat placed all four paws in an arm. Closed arm entries and total arm entries were used as a measure of general locomotion (as recommended by Rodgers & Dalvi, 1997). Stretch-attend postures and stretch-approaches were combined into one category.

Forced swim test

1-2 days following EPM, stress coping behavior was assessed in the forced swim test (FST). The FST was conducted over two days— 15 minutes on day 1 and 5 minutes about 24 hours later. Tests were run under bright light in the afternoon and were recorded with a video camera. Two rats were tested at once. Rats were hidden from each other by a black divider. To begin the test, rats were gently placed into a plastic cylinder filled with about 30 cm. of tap water at a temperature of 24 ± 1 °C. At the end of the test period, rats were towel-dried and returned to their home cage. Water was changed between each rat.

Statistics

GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA) was used to conduct unpaired t-tests with Welch's correction, ordinary one-way ANOVAs, or two-way ANOVAs as

appropriate. Tukey's HSD test and Sidak's multiple comparisons test were used to explore multiple comparisons following significant and trending ANOVA results. R (version 3.5.1, "Feather Spray") and RStudio (version 1.1.456) were used to convert qPCR fold change values and risk assessment behavior frequencies into aggregate z-scores (similarly to z-scoring of anxiety-like and mood-related behaviors in Shepard et al., 2016; code included in appendix A), calculate Pearson's correlations, and build linear regression models. P-values at or under 0.05 were considered statistically significant.

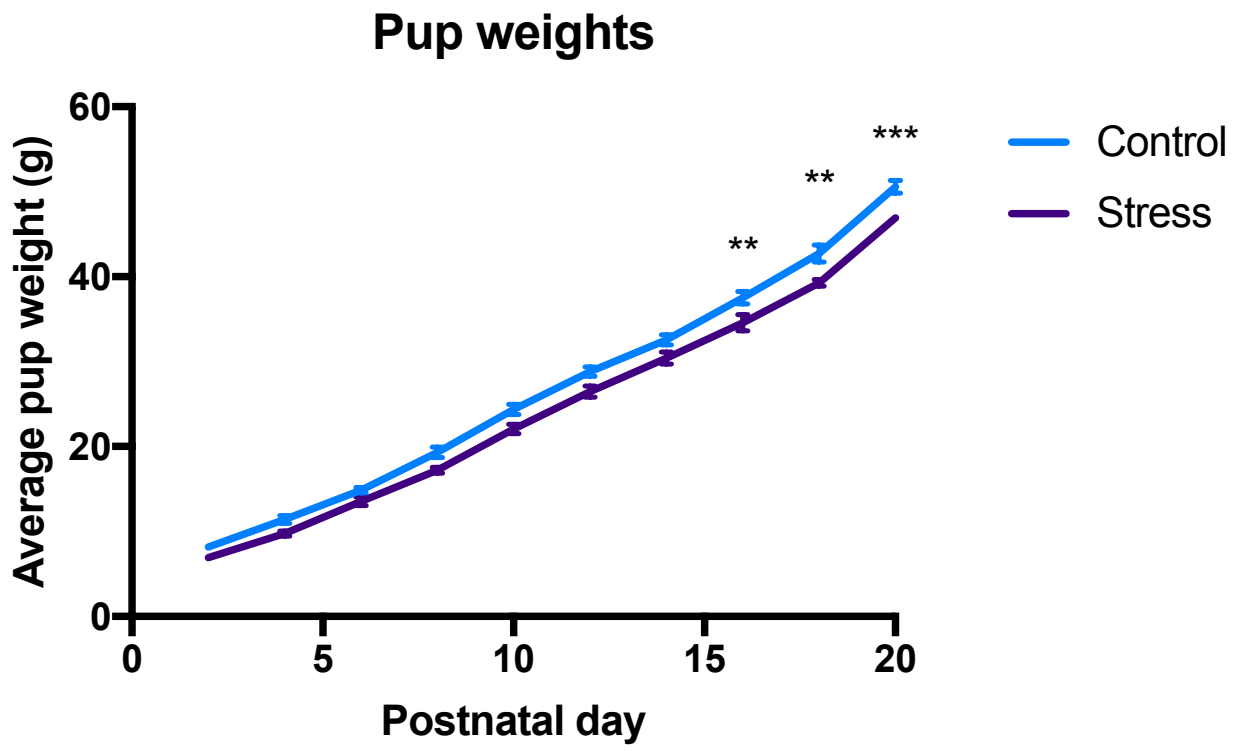

Figure 1. Average pup weights (by litter, n = 4/group, shown as mean ± SEM). On PD16, 18, and 20, early deprivation stress significantly decreased average pup weight compared with control handling.

*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

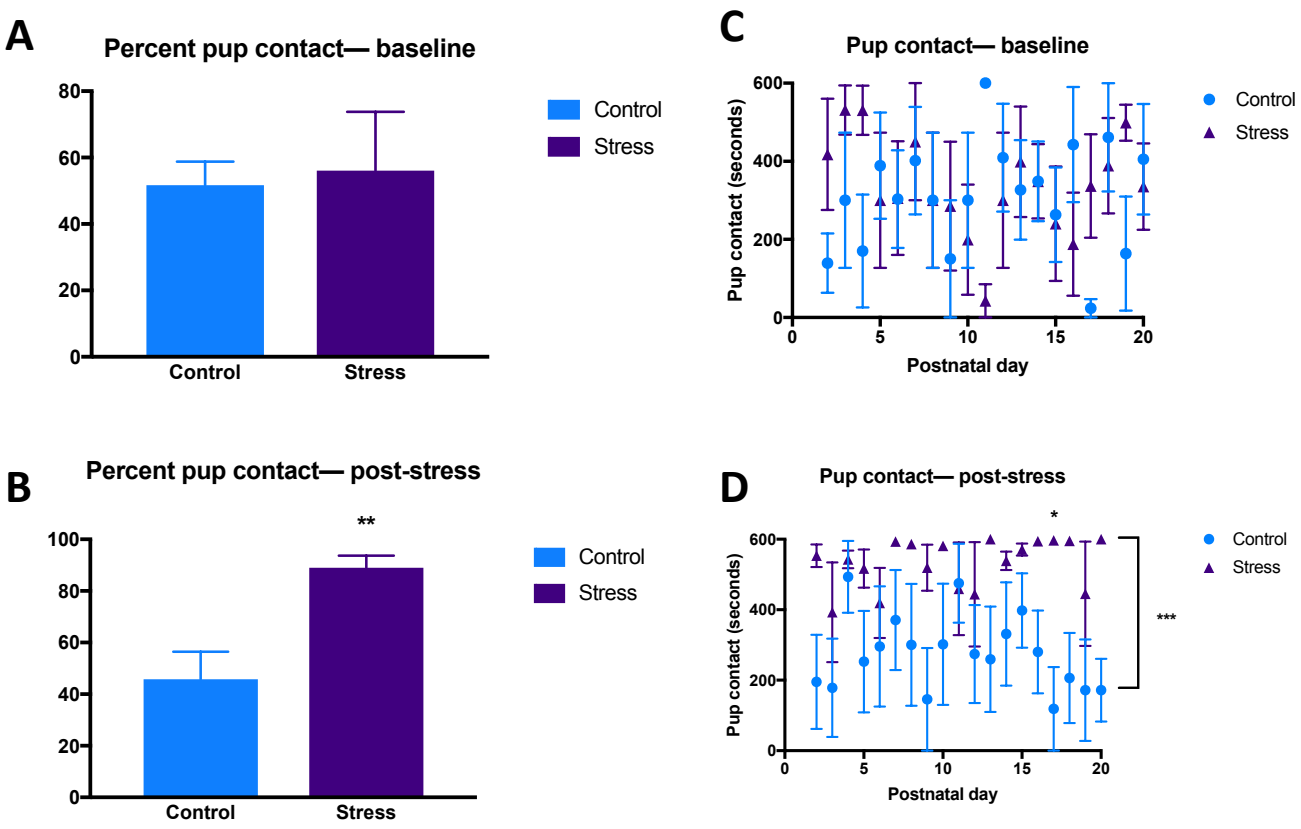

Figure 2. Percent pup contact (left column; n = 4, mean + SEM) and seconds of pup contact by day (right column; n = 4, mean ± SEM). Stress did not alter time spent in contact with pups at baseline (A; during the light cycle before a bout of early deprivation stress). Stress significantly increased pup contact after early deprivation stress ($p < 0.01$; B; compared with control handling and immediate return to the nest).

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

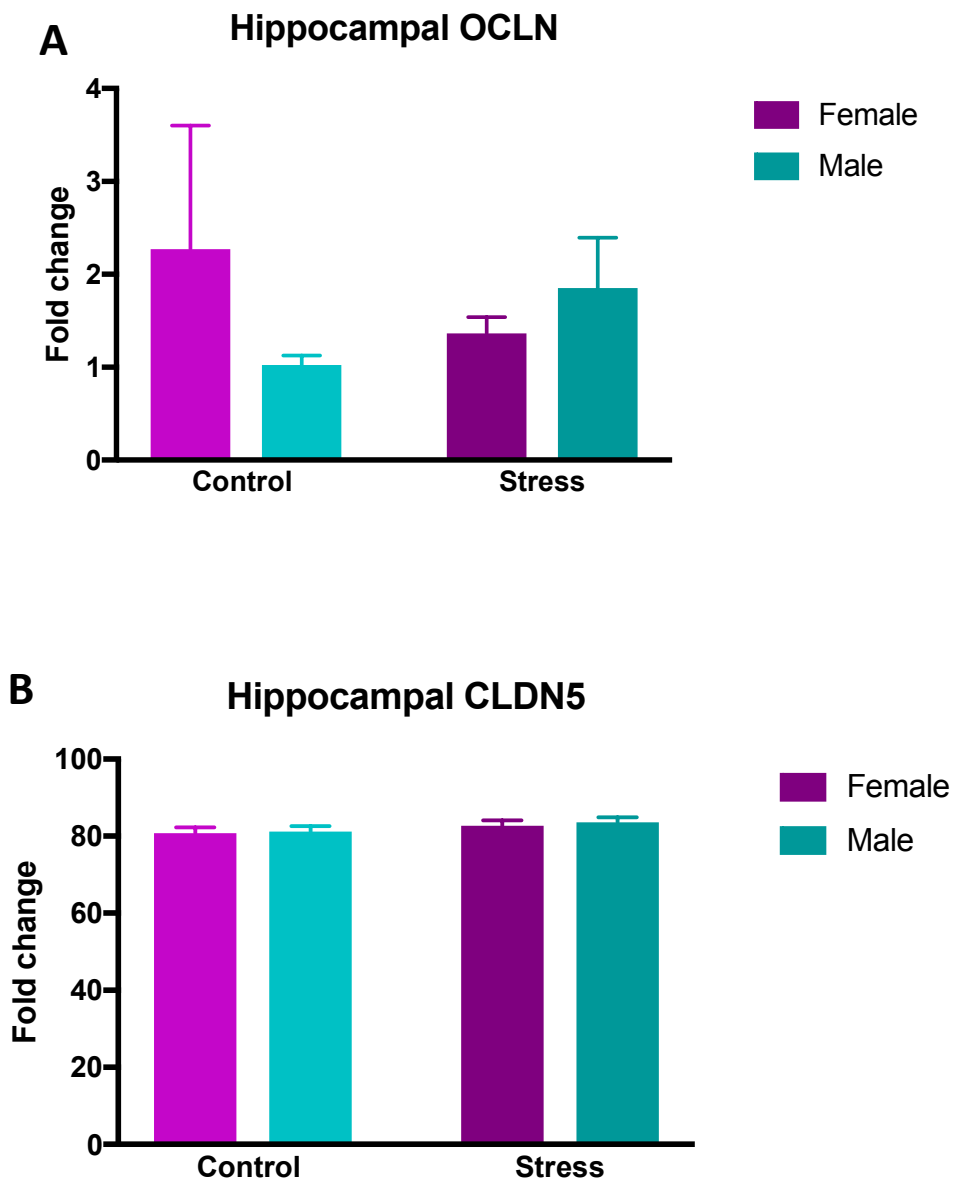

Figure 3. Fold change of hippocampal tight junction protein genes (n = 8-10, mean + SEM). Normalized to actin. Neither stress nor sex altered occludin (A) and claudin-5 (B) mRNA expression.

*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

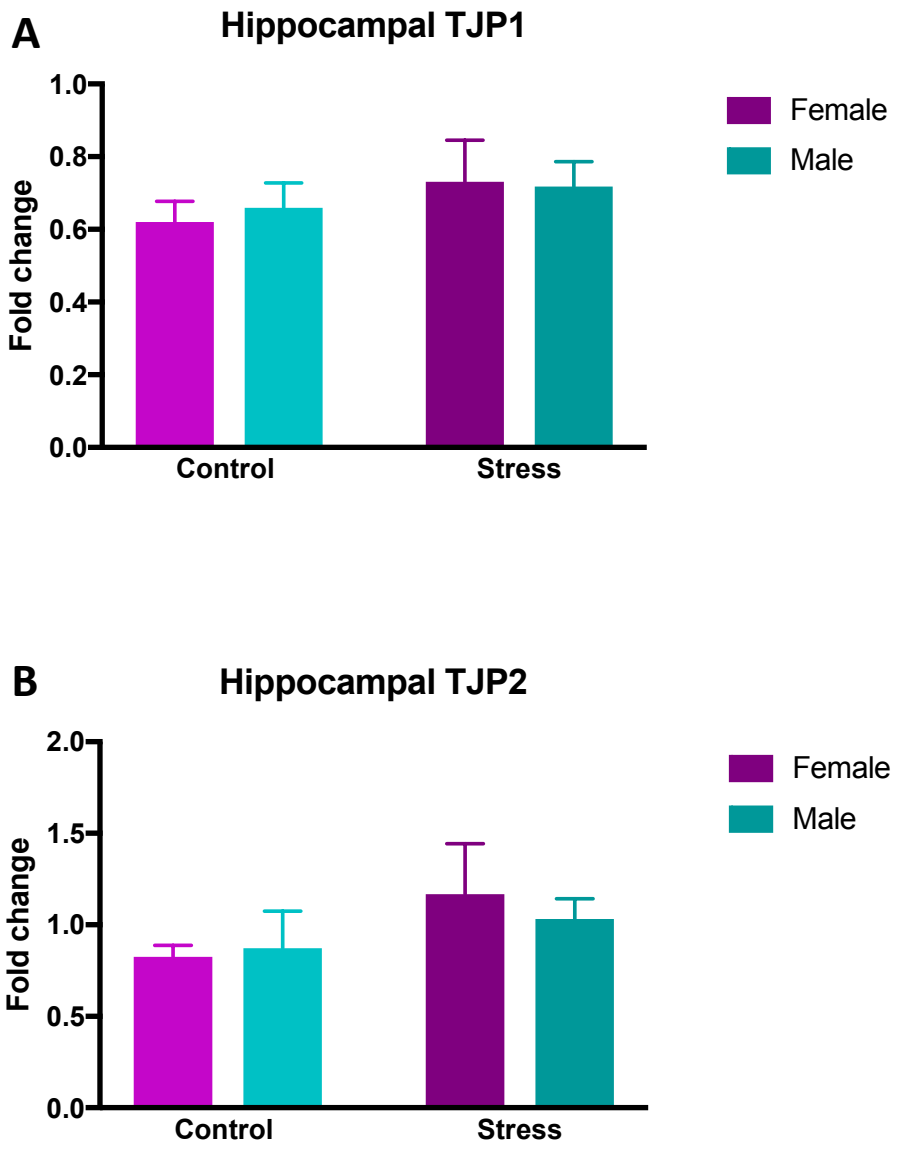

Figure 4. Fold change of hippocampal tight junction protein genes (n = 8-10, mean + SEM). Normalized to actin. Neither stress nor sex altered tight junction protein 1 (A) and tight junction protein 2 (B) mRNA expression.

*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

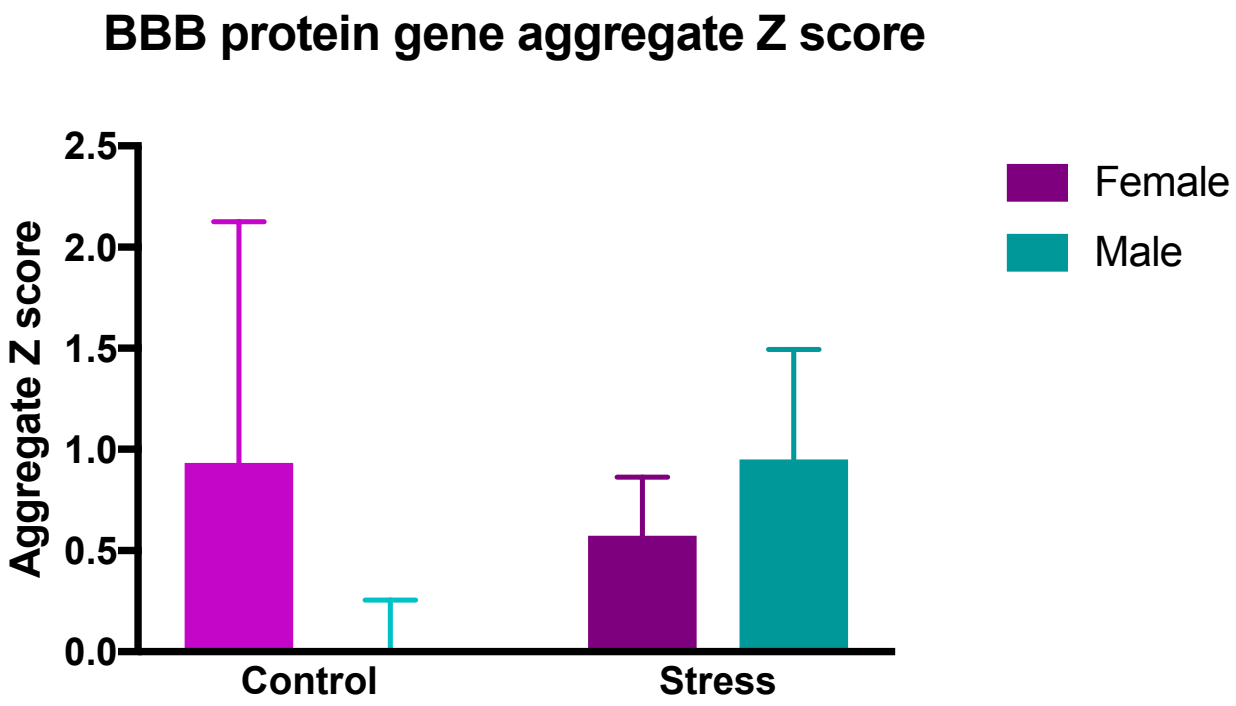

Figure 5. Aggregate z-scores of BBB protein mRNA fold changes in the PD21 hippocampus (mean + SEM). Control males were designated the reference group. Neither stress nor sex altered the pattern of BBB protein mRNA expression.

*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

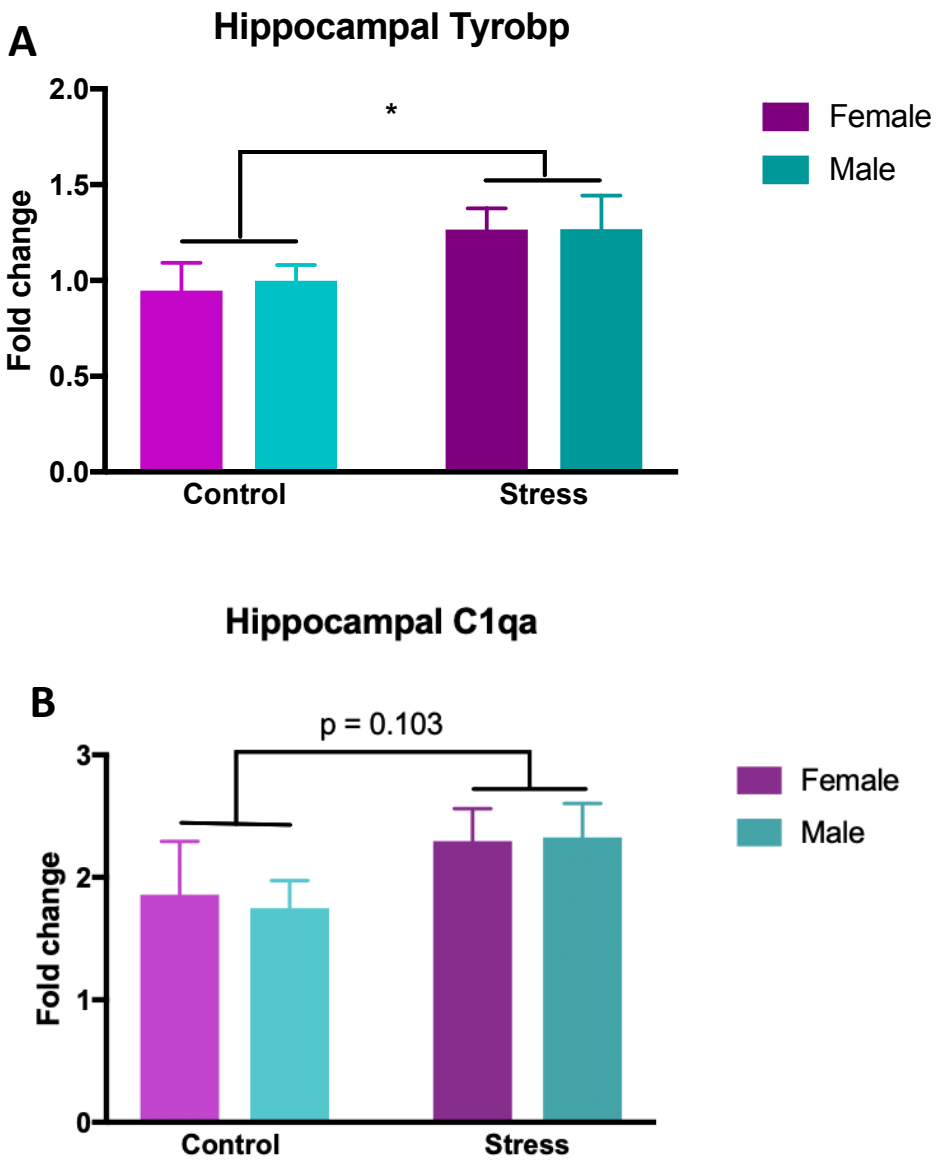

Figure 6. Fold change of hippocampal genes relevant for microglial phagocytosis (n = 8-10, mean + SEM). Normalized to actin. Stress significantly increased tyrobp mRNA expression (A; $p < 0.05$). Stress did not significantly alter C1qa mRNA expression (B).

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

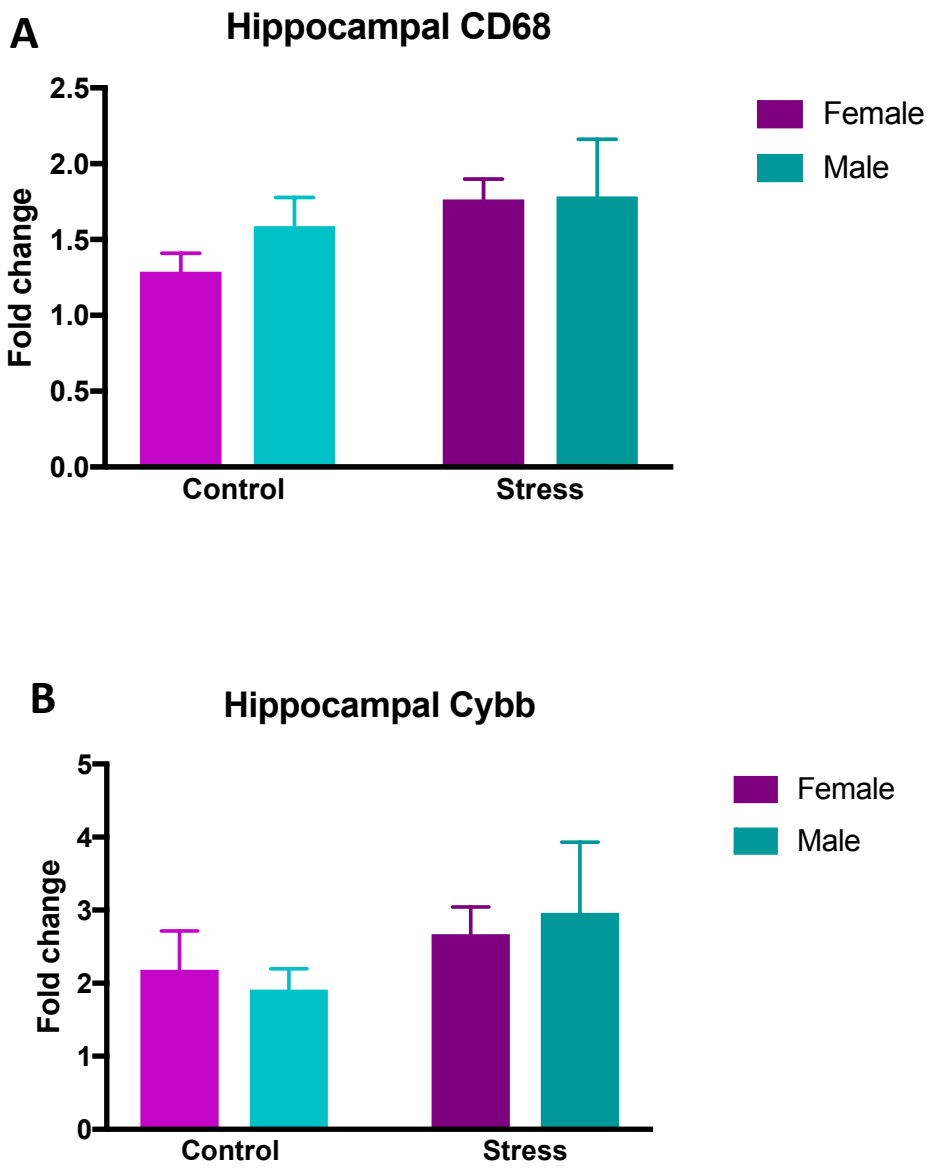

Figure 7. Fold change of hippocampal genes relevant for microglial phagocytosis (n = 8-10, mean + SEM). Normalized to actin. Neither stress nor sex significantly altered CD68 (A) or Cybb (B) mRNA expression.

*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

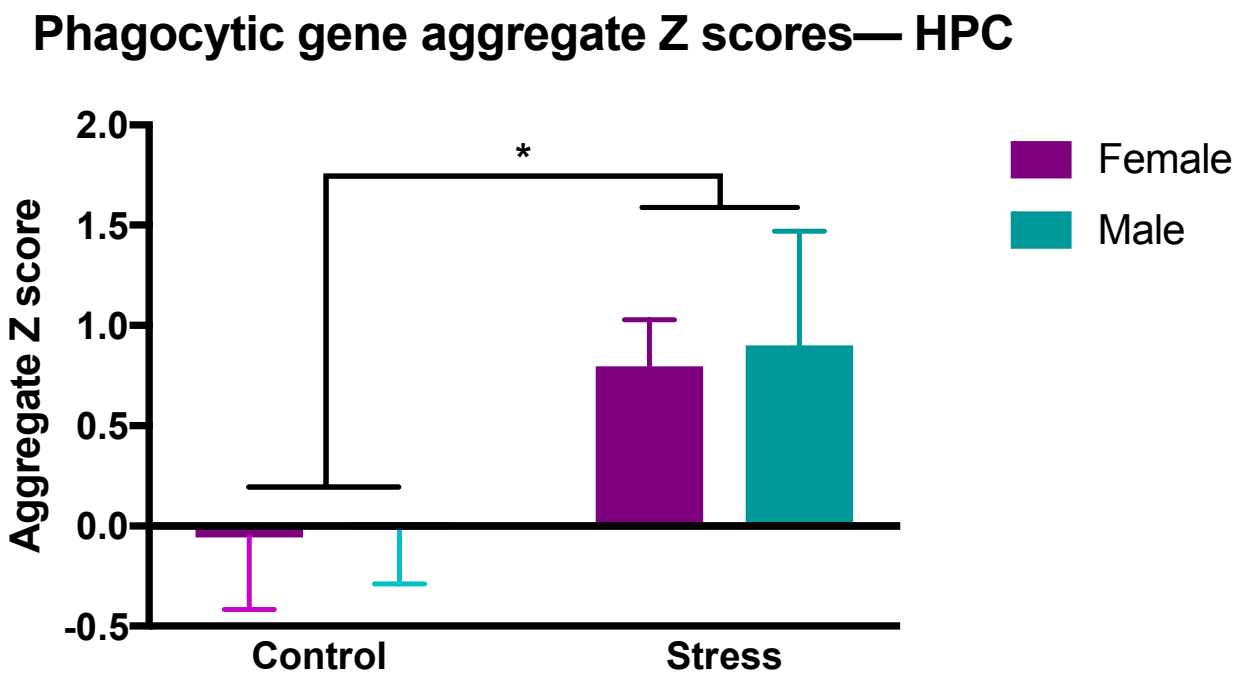

Figure 8. Aggregate z-scores of genes related to microglial phagocytosis in the PD21 hippocampus (mean + SEM). Control males were designated the reference group. Stress significantly increased the expression of phagocytic genes (mRNA) irrespective of sex ($p < 0.05$).

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

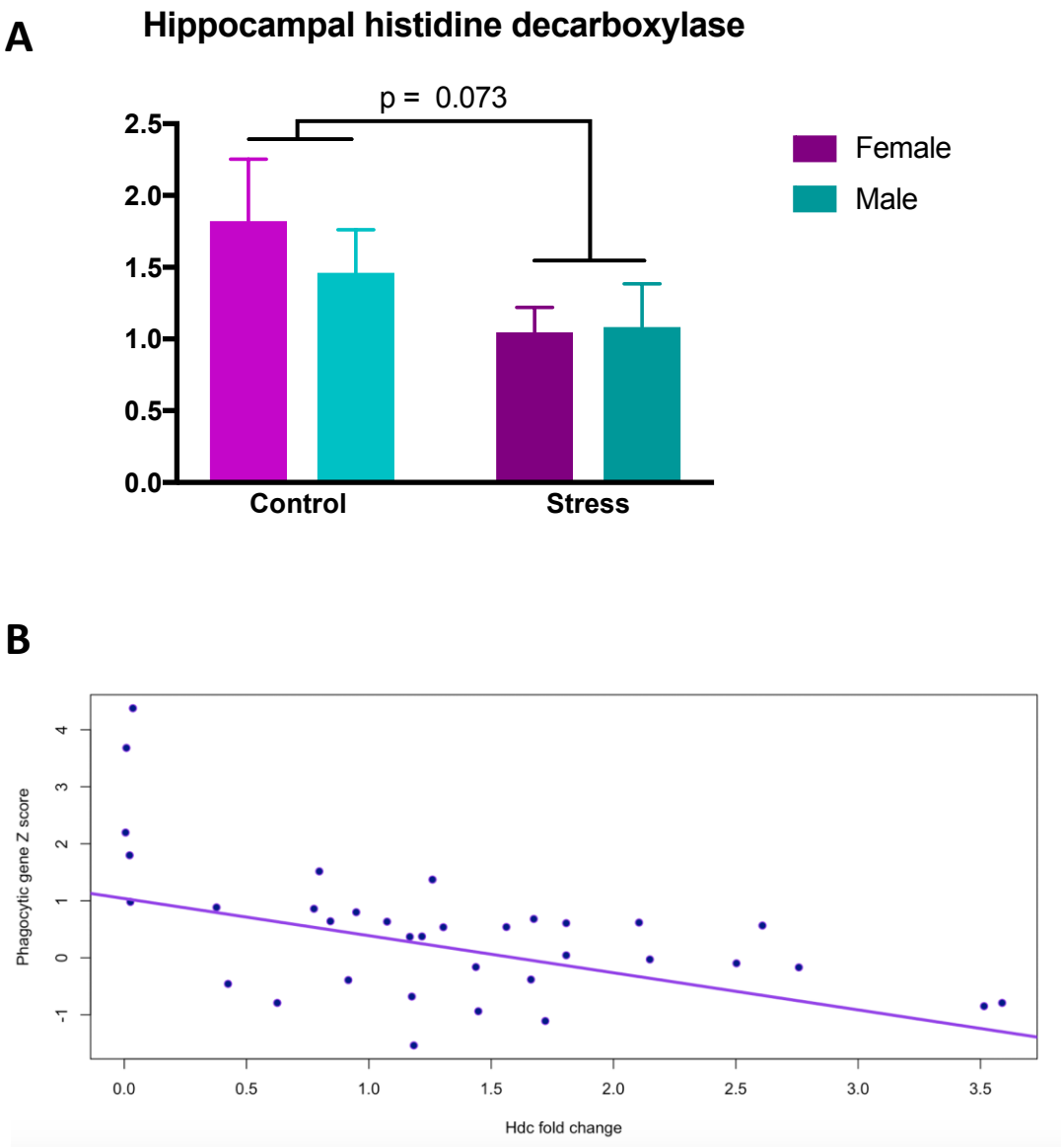

Figure 9. Fold change of histidine decarboxylase (Hdc) in the PD21 hippocampus (mean + SEM). Normalized to actin. Stress led to a trending decrease in histidine decarboxylase mRNA expression (A). In the bottom panel (B), Hdc fold changes are shown plotted against phagocytic gene aggregate z-scores with a least squares regression line.

*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

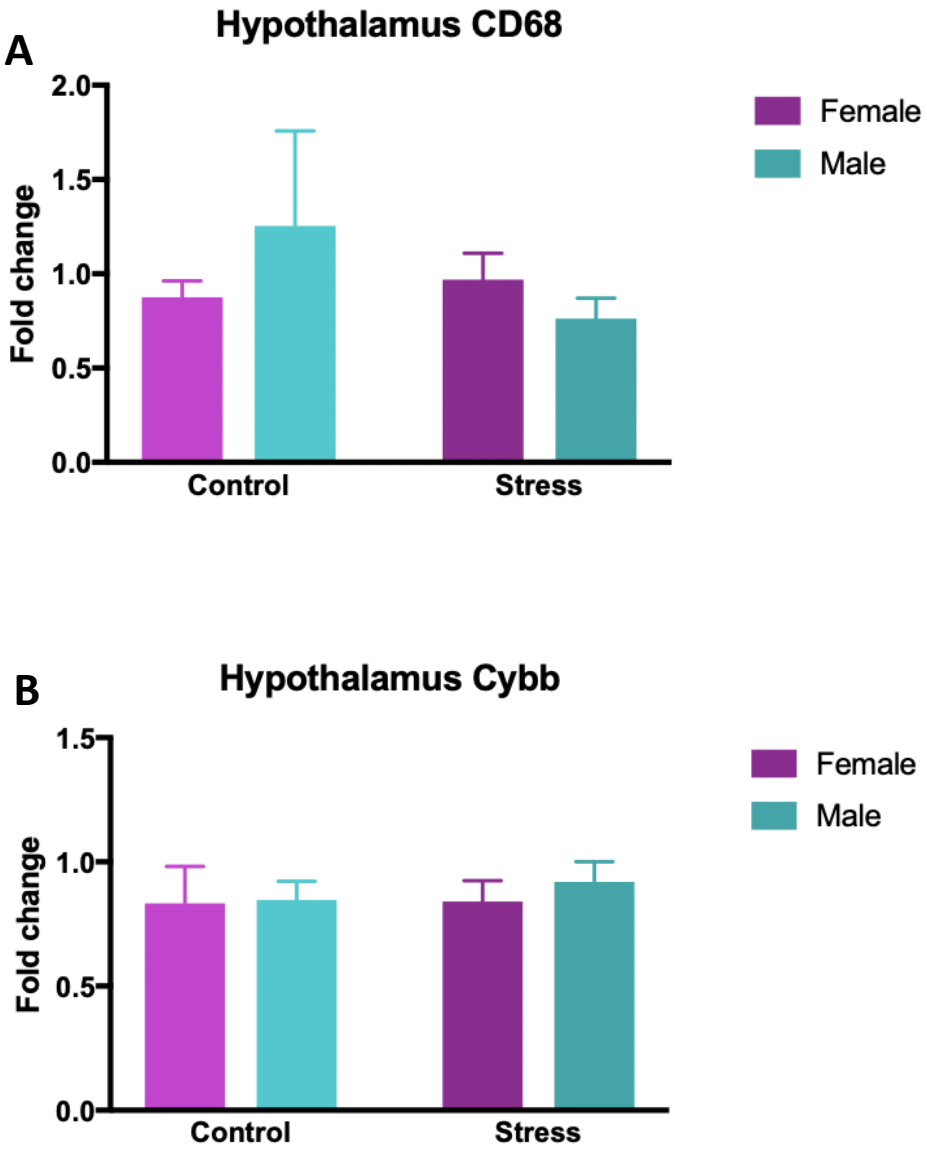

Figure 10. Fold change of hypothalamic genes relevant for microglial phagocytosis (n = 7-10, mean + SEM). Normalized to actin. Neither stress nor sex significantly altered CD68 (A) or Cybb (B) mRNA expression.

*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

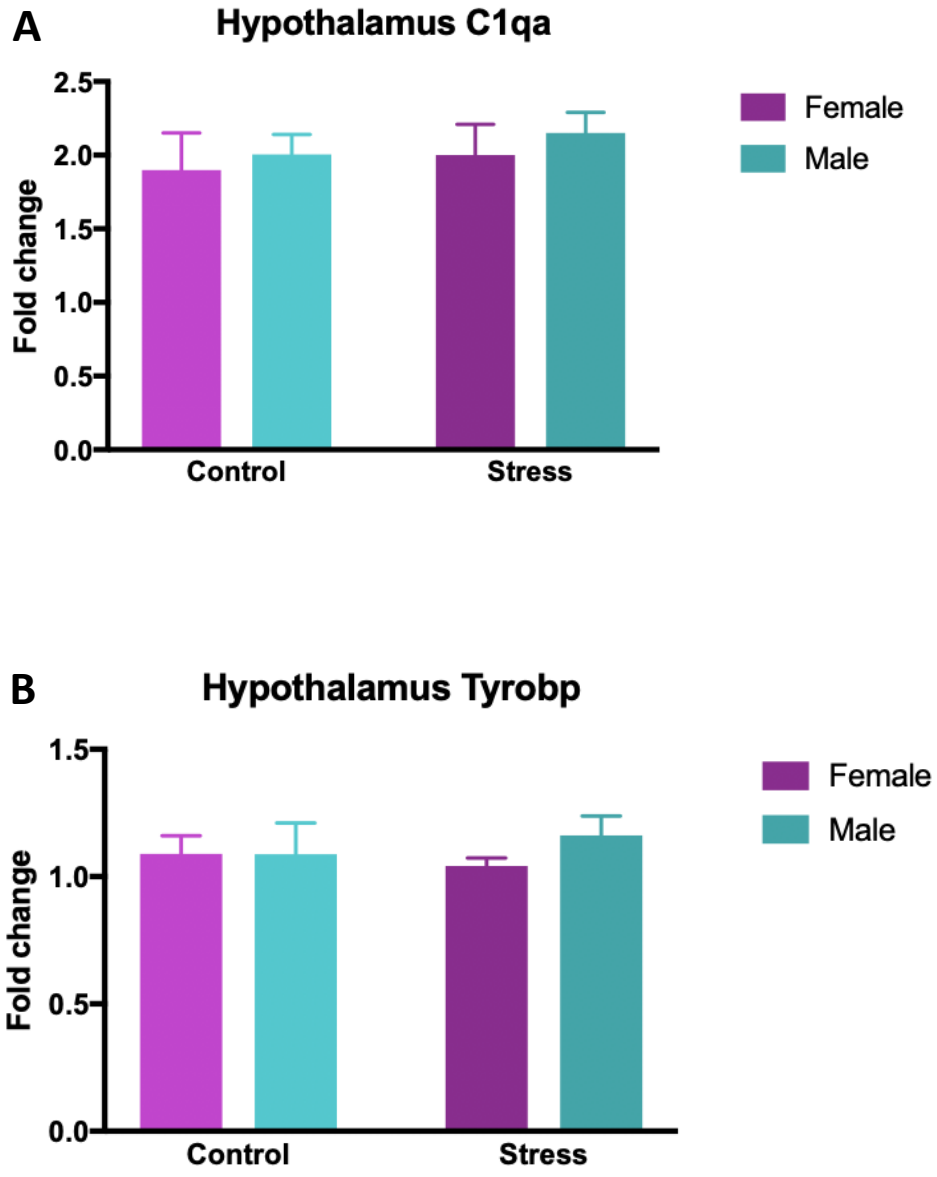

Figure 11. Fold change of hypothalamic genes relevant for microglial phagocytosis (n = 7-10, mean + SEM). Normalized to actin. Neither stress nor sex significantly altered C1qa (A) or Tyrobp (B) mRNA expression.

*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

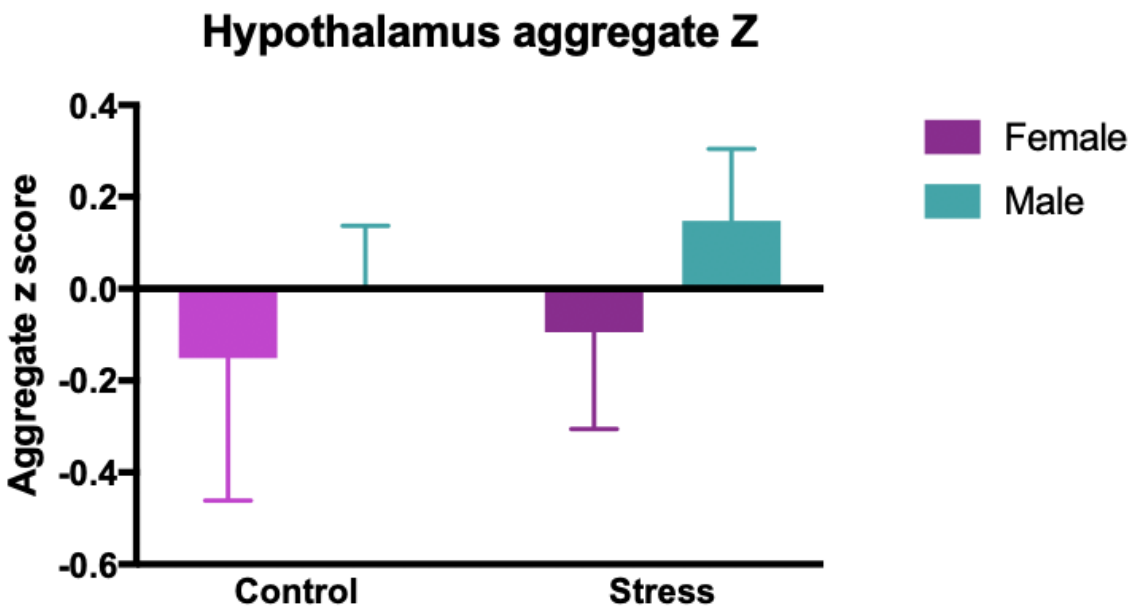

Figure 12. Aggregate z-scores of genes related to microglial phagocytosis in the PD21 hypothalamus (mean + SEM). Control males were designated the reference group. Neither stress nor sex altered the expression of phagocytic genes (mRNA).

*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

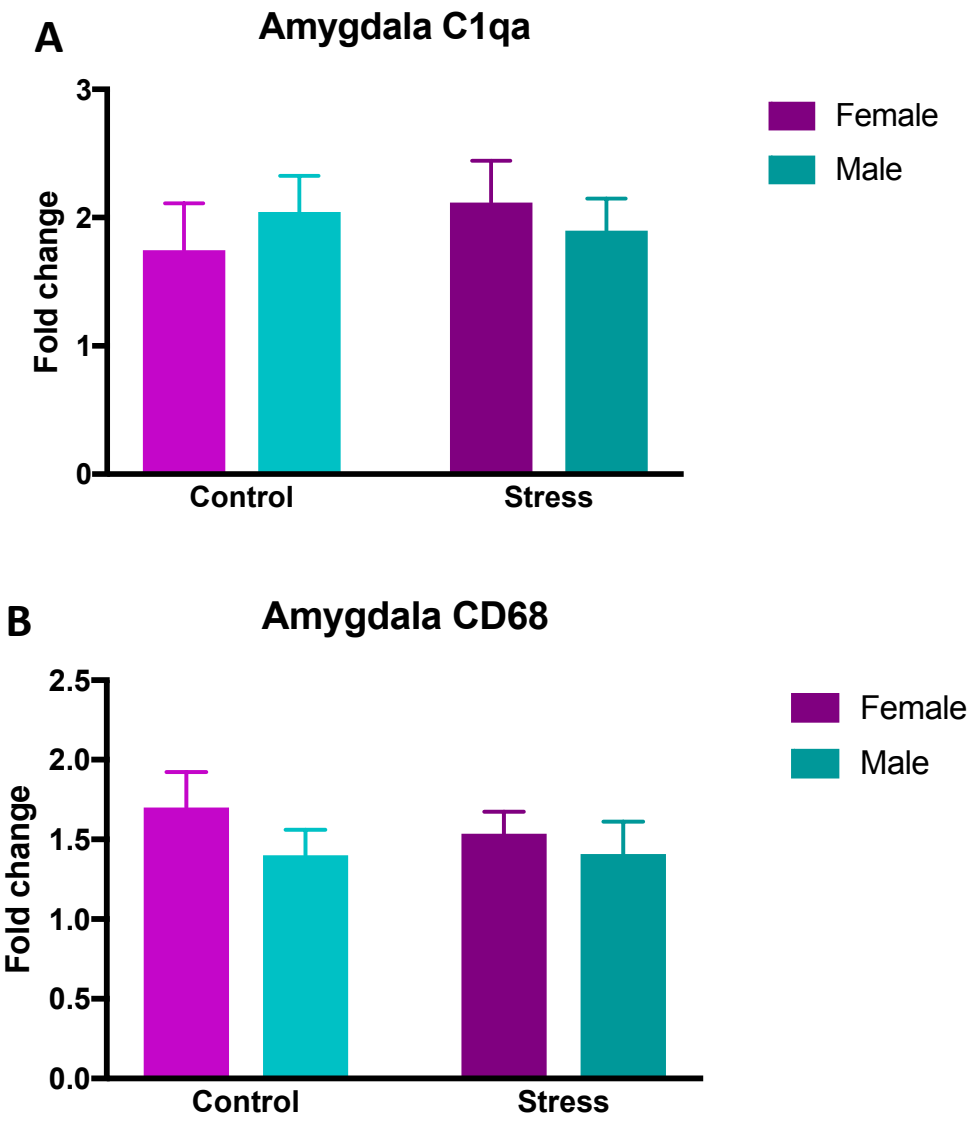

Figure 13. Fold change of amygdala genes relevant for microglial phagocytosis (n = 5-10, mean + SEM). Normalized to actin. Neither stress nor sex significantly altered C1qa (A) or CD68 (B) mRNA expression.

*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

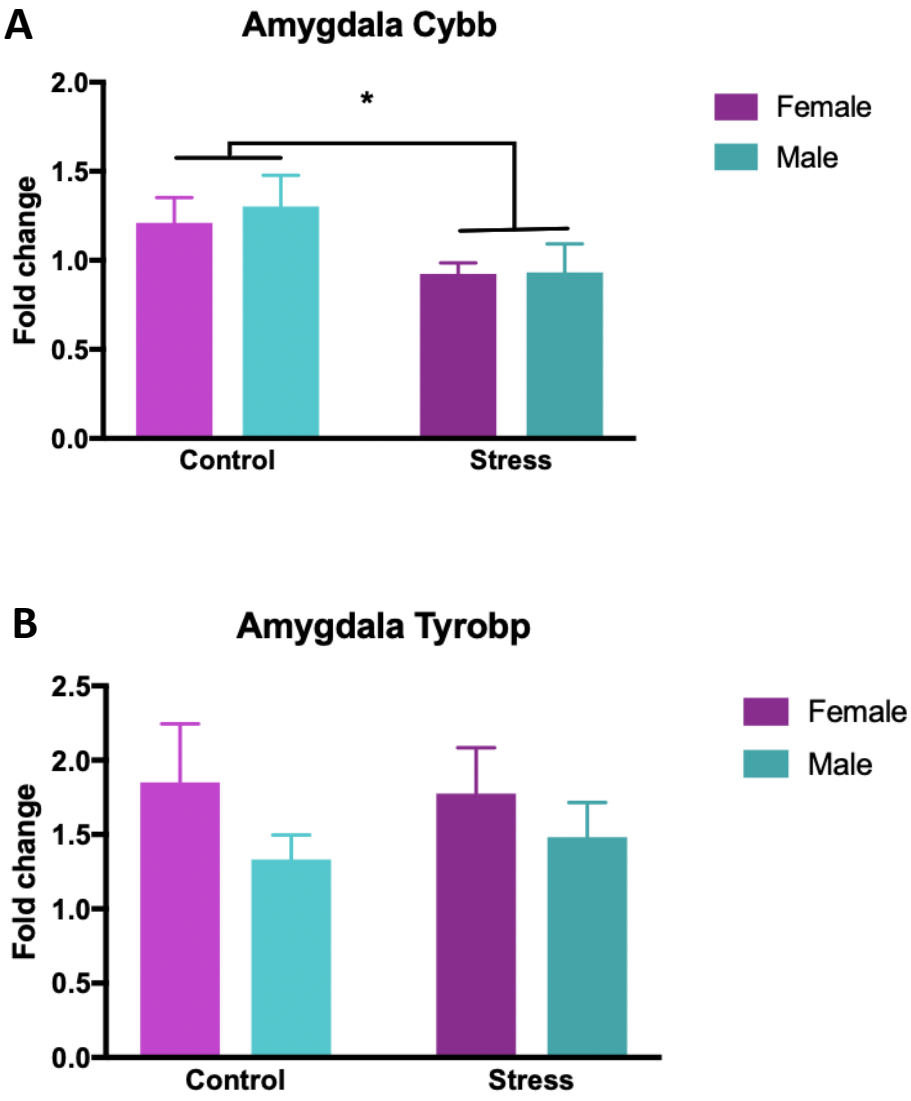

Figure 14. Fold change of amygdala genes relevant for microglial phagocytosis (n = 5-10, mean + SEM). Normalized to actin. Stress decreased Cybb mRNA expression (A; $p < 0.05$). Neither stress nor sex significantly altered Tyrobp mRNA expression (B).

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

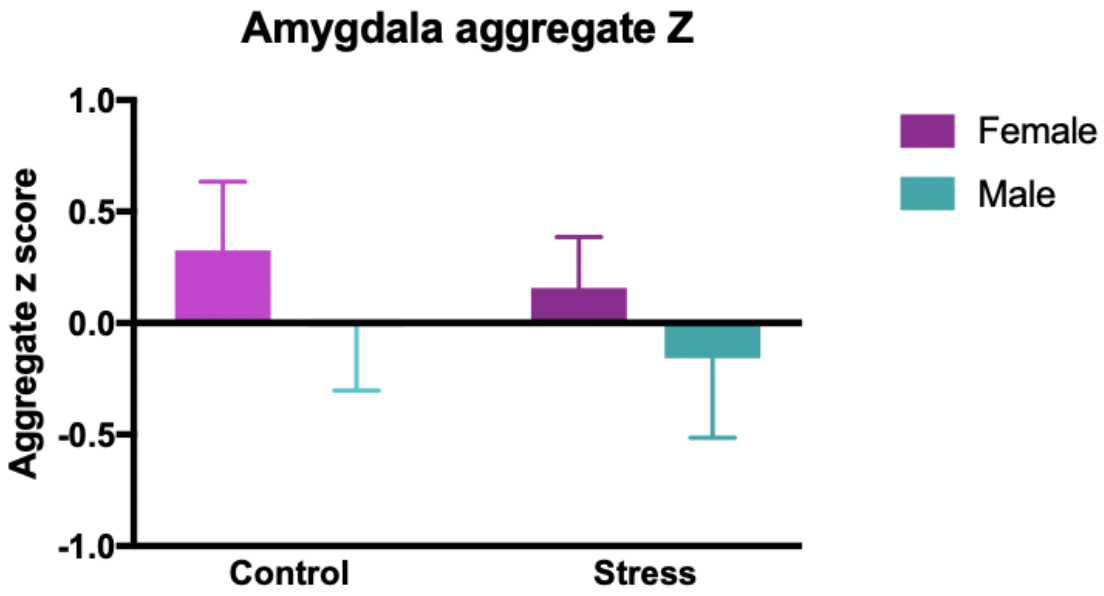

Figure 15. Aggregate z-scores of genes related to microglial phagocytosis in the PD21 amygdala (mean + SEM). Control males were designated the reference group. Neither stress nor sex altered the expression of phagocytic genes (mRNA).

*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

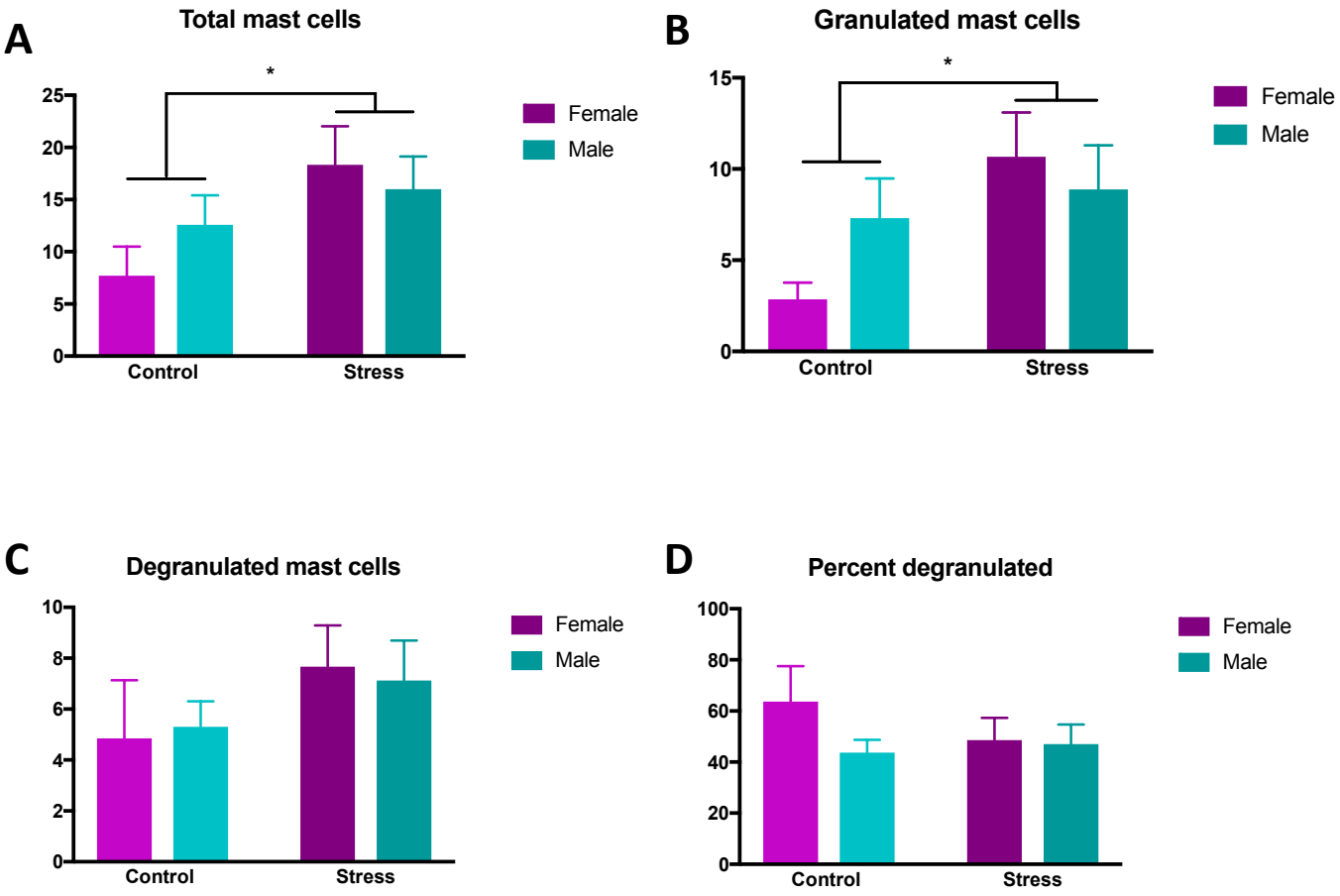

Figure 16. Number of total mast cells, granulated mast cells, and degranulated mast cells and percent degranulated mast cells in 5 sections of the PD21 hippocampus (n = 7-10, mean + SEM). Stress increased total mast cell count (A) and granulated mast cell count (B). Neither stress nor sex altered the number (C) or percent of degranulated mast cells (D).

*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

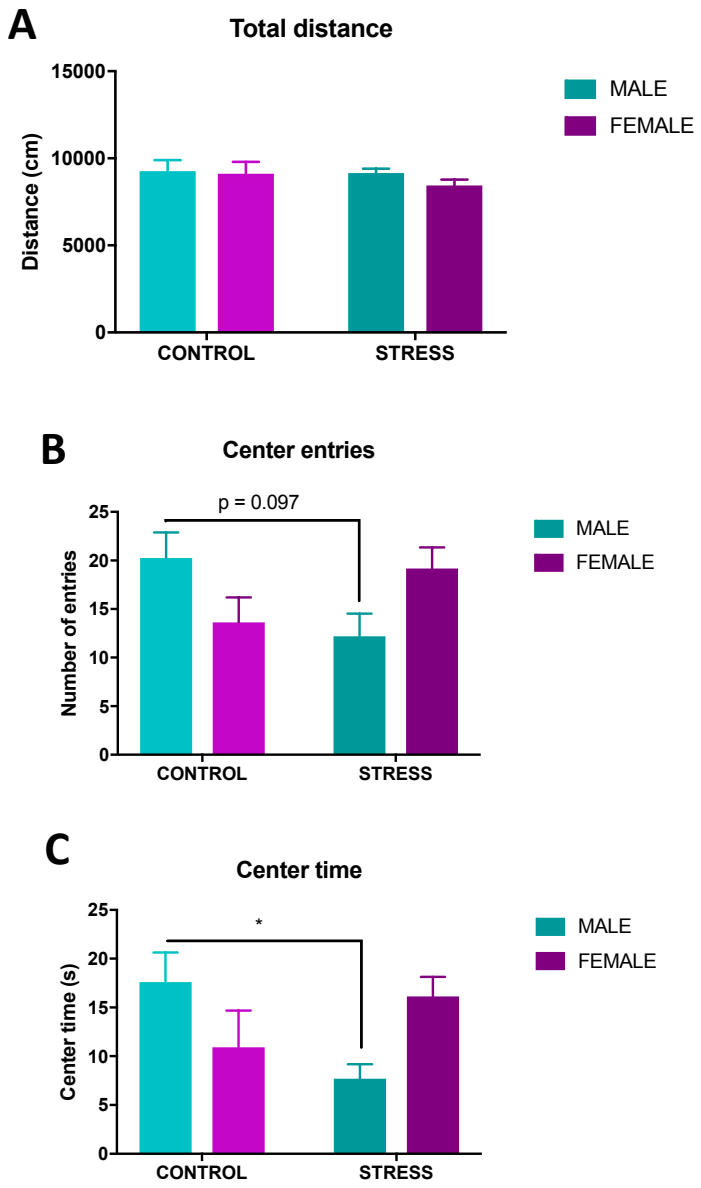

Figure 17. Anxiety-like behavior in the open field test (n = 8-12, mean + SEM). Neither stress nor sex altered total distance traveled in the open field (A). In male rats, stress decreased center entries at the 0.1 level of significance (B; $p = 0.097$). Also in male rats, stress (compared with control handling) decreased time spent in the center of the open field (C; $p < 0.05$).

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

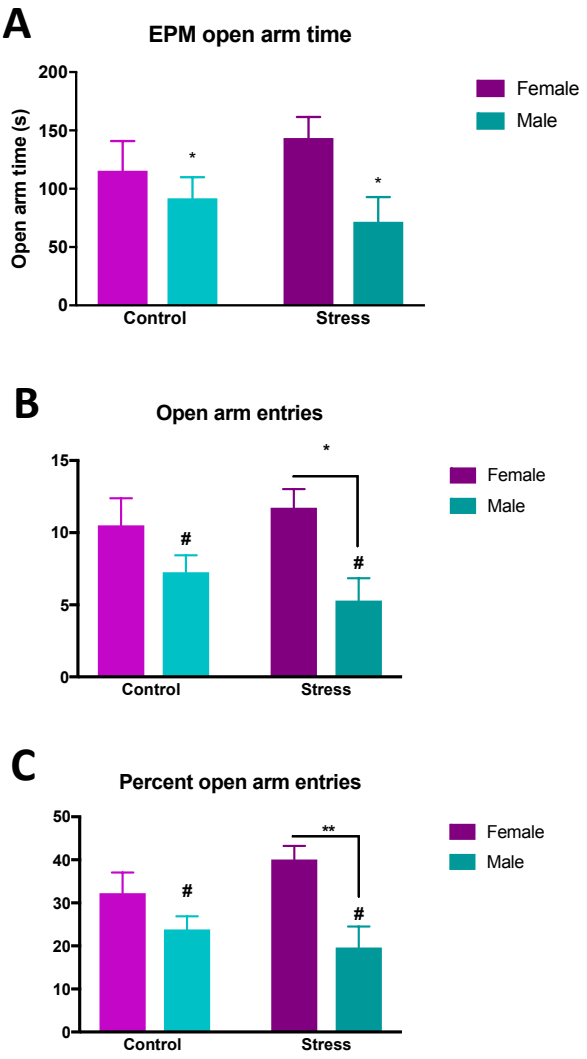

Figure 18. Anxiety-like behavior in the elevated plus maze (n = 8-12, mean + SEM). Stress did not alter open arm time in the EPM; however, male rats spent less time in the open arms of the maze than female rats did (A; $p < 0.05$). Male rats also made fewer entries into the open arms of the maze and had a lower percent of open arm entries than females did; specifically, stressed males entered the open arms fewer times (B) and had a lower proportion of total arm entries (C) than stressed females did ($p < 0.05$).

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

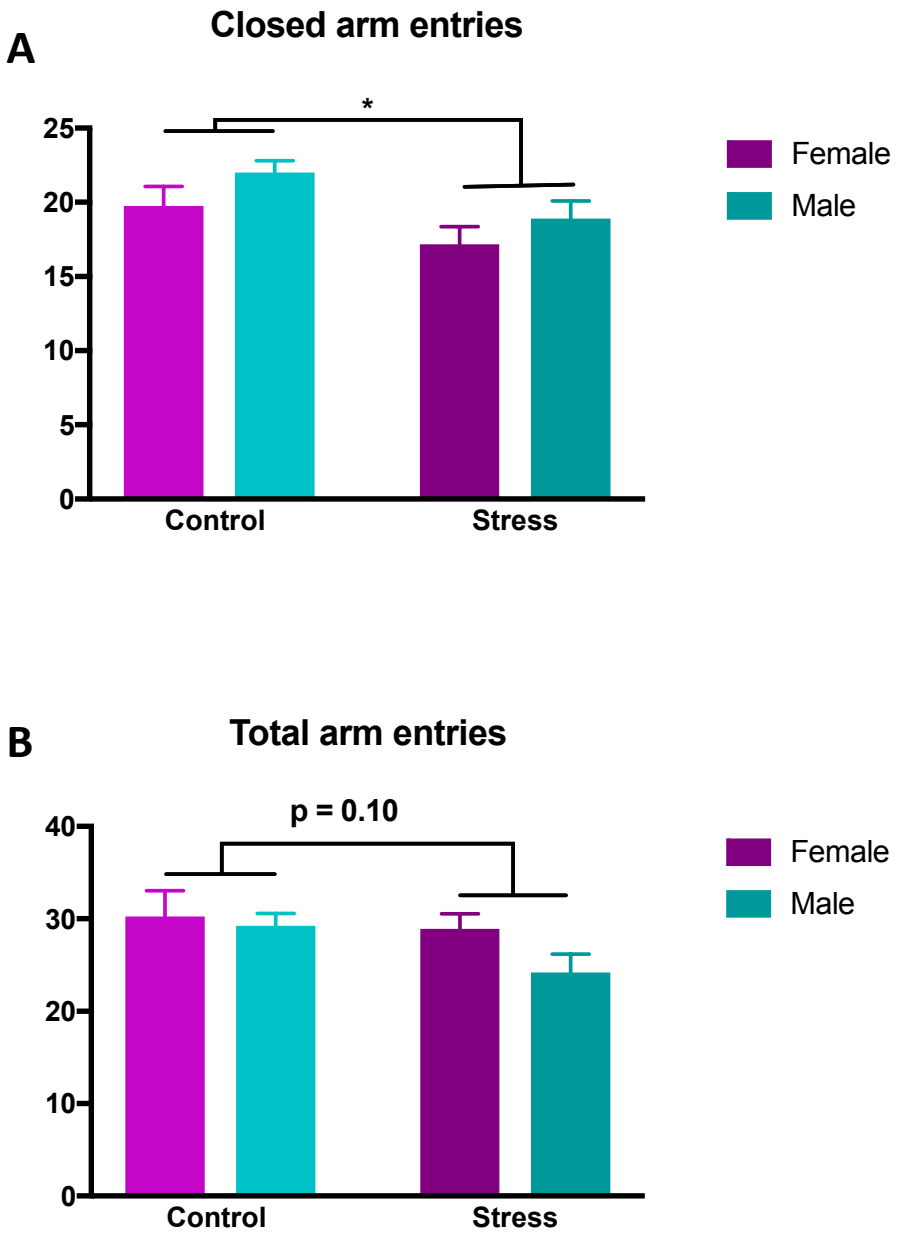

Figure 19. Locomotion in the elevated plus maze (n = 8-12, mean + SEM). Stress decreased closed arm entries (A; $p < 0.05$). Stress also led to a trending decrease in total arm entries (B; $p = 0.10$).

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

Figure 20. Risk assessment behaviors in the elevated plus maze (n = 8-12, mean + SEM). Males head-dipped significantly less than females (A; $p < 0.05$); stress did not alter head dip frequency. Stress decreased the frequency of rears in both sexes (B; $p < 0.0001$). Neither sex nor stress altered number of stretch-attend postures; however, the interaction effect p-value showed a trend towards significance (C; $p = 0.067$).

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

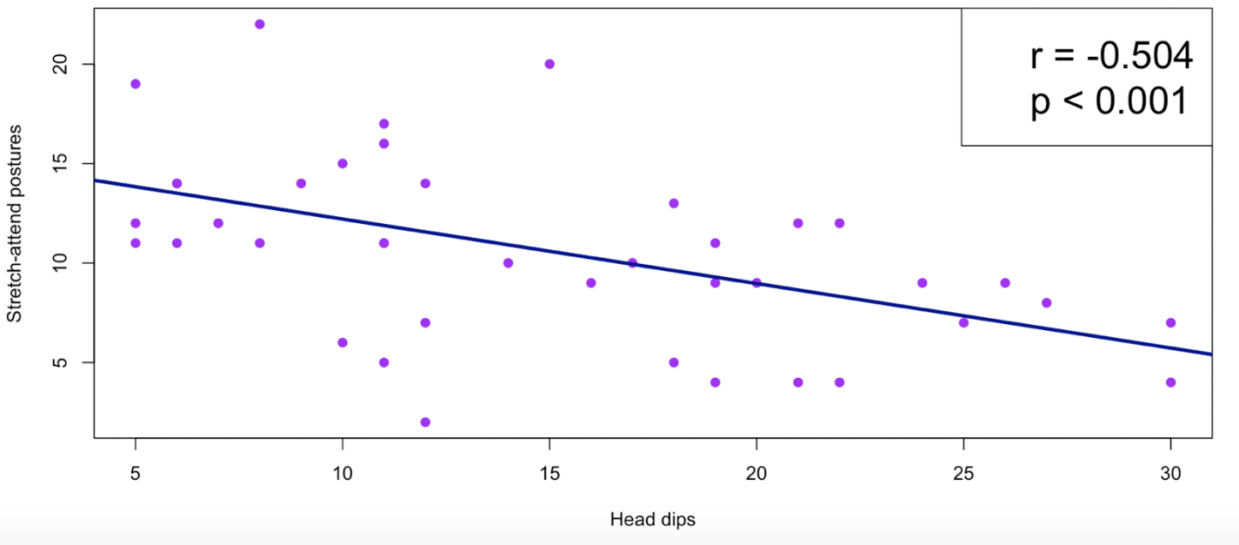

Figure 21. Scatterplot of head dips vs. stretch-attend postures. These risk assessment behaviors were significantly negatively correlated ($r = -0.504$, $p < 0.001$), such that rats who head-dipped relatively more adopted stretch-attend postures relatively less and vice versa.

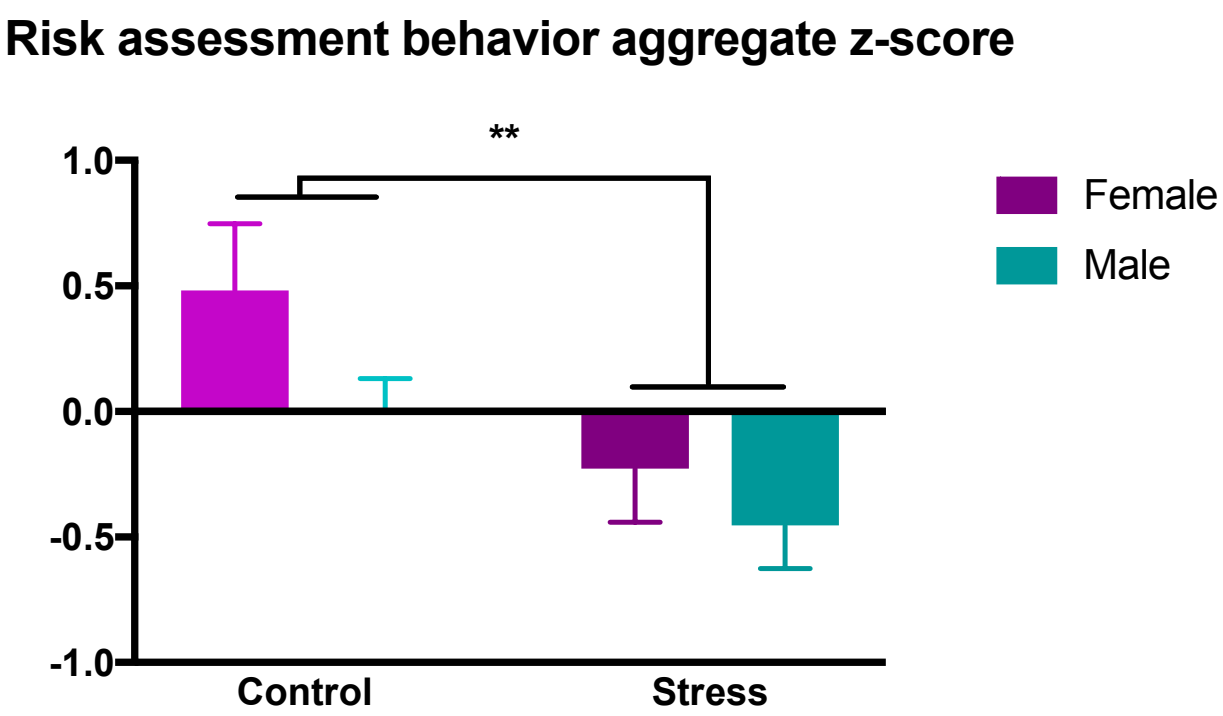

Figure 22. Aggregate z-scores of risk assessment behaviors (head dips, rears, and stretch-attend postures) in the elevated plus maze (n = 8-12, mean + SEM). Control males were designated the reference group. Stress significantly decreased risk assessment behaviors in the EPM ($p < 0.01$).

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

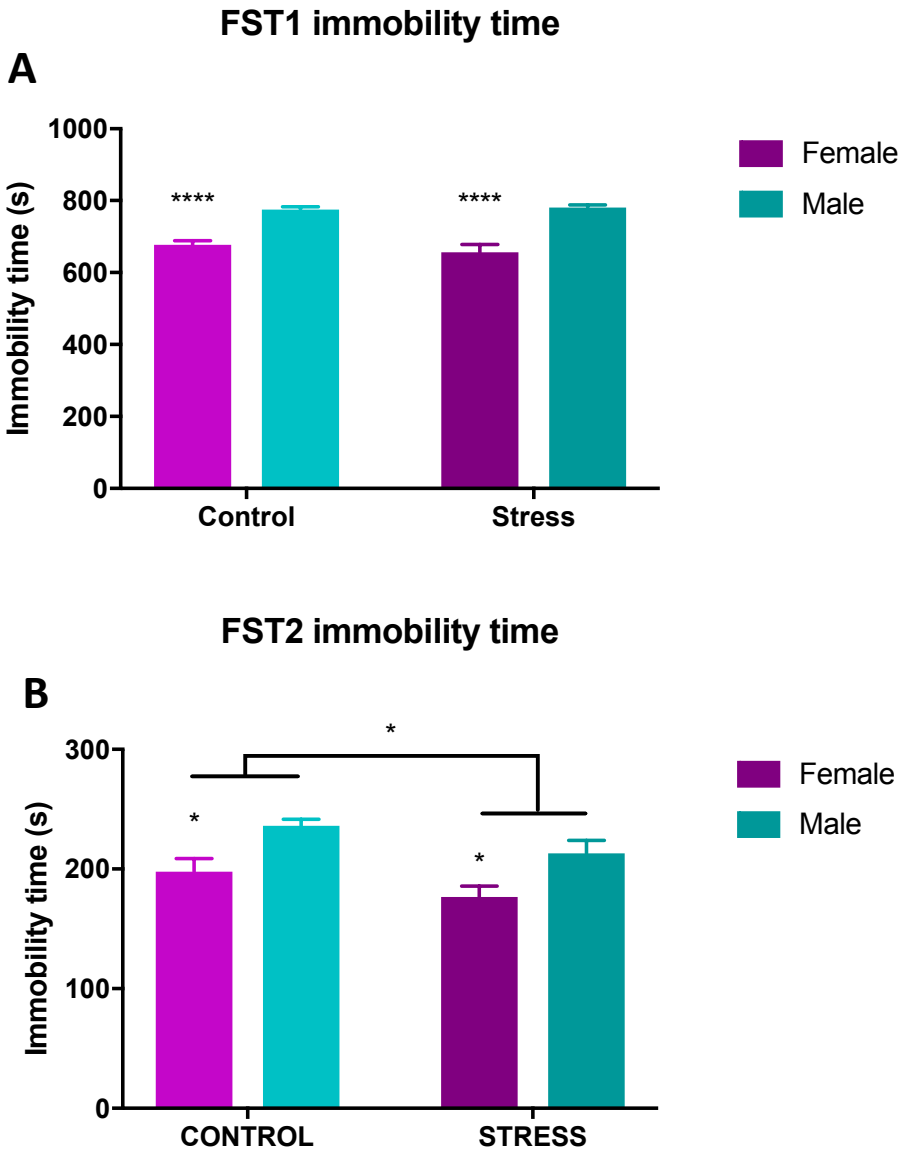

Figure 23. Stress coping behavior in the forced swim test (n = 8-12, mean + SEM). Compared with control handling, stress did not alter coping behavior on either the first or second day of the FST. Female rats spent less time immobile than male rats did on both day 1 (A; $p < 0.0001$) and day 2 (B; $p < 0.05$) of the test.

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

Results

Pup weights

Early deprivation stress significantly decreased average pup weight on postnatal days 16, 18, and 20 (respectively, $t_{(60)} = 3.498, p < 0.01$; $t_{(60)} = 4.072, p < 0.01$; $t_{(60)} = 4.329, p < 0.001$, Sidak's multiple comparisons test, Figure 1). Stress also decreased average litter weight on PD 10 and 12 at the 0.1 alpha level ($t_{(60)} = 2.825, p = 0.062$; $t_{(60)} = 2.76, p = 0.074$; Figure 1).

Maternal behavior

Following early deprivation stress or control handling with immediate return to the nest, dams of stressed litters spent significantly more time in contact with their pups ($F_{(1,6)} = 54.85, p < 0.001$; Figure 2b). However, stress did not alter baseline pup contact ($F_{(1,6)} = 0.2125, p = 0.661$; Figure 2a).

BBB tight junction protein gene expression

Early deprivation stress did not significantly alter hippocampal mRNA expression for the blood-brain barrier tight junction protein genes occludin, claudin-5, tight junction protein (TJP1), and TJP2 (respectively, $F_{(1,32)} = 0.004, p = 0.953$; $F_{(1,32)} = 2.294, p = 0.140$; $F_{(1,32)} = 1.081, p = 0.306$; $F_{(1,32)} = 1.872, p = 0.181$, Figure 3). No sex differences were observed in

the expression of any of these genes (occludin: $F_{(1,32)} = 0.323, p = 0.574$; claudin-5: $F_{(1,32)} = 0.206, p = 0.653$; TJP1: $F_{(1,32)} = 0.025, p = 0.875$; TJP2: $F_{(1,32)} = 0.057, p = 0.813$). For each gene, the interaction of sex and stress also failed to reach statistical significance (occludin: $F_{(1,32)} = 1.667, p = 0.206$; claudin-5: $F_{(1,32)} = 0.029, p = 0.866$; TJP1: $F_{(1,32)} = 0.102, p = 0.752$; TJP2: $F_{(1,32)} = 0.247, p = 0.622$).

Further, when fold change values for this qPCR panel were converted to z-scores (see Appendix A) and aggregated, neither stress nor sex altered the pattern of BBB protein mRNA expression (Figure 3.3; stress: $F_{(1,32)} = 0.214, p = 0.647$). No sex difference was observed in the pattern of expression for these genes ($F_{(1,32)} = 0.191, p = 0.665$).

Phagocytic gene expression

Stress increased hippocampal mRNA expression of tyrobp ($F_{(1,32)} = 4.692, p < 0.05$, Figure 4.1a). Stress did not significantly alter hippocampal mRNA expression of C1q, CD68, or Cybb ($F_{(1,32)} = 2.826, p = 0.103$; $F_{(1,31)} = 1.806, p = 0.189$; $F_{(1,32)} = 1.458, p = 0.236$, respectively, Figure 4). No sex differences were observed in the expression of any of these genes (Tyrobp: $F_{(1,32)} = 0.039, p = 0.845$; C1q: $F_{(1,32)} = 0.017, p = 0.896$; CD68: $F_{(1,31)} = 0.411, p = 0.526$; Cybb: $F_{(1,32)} = 0.001, p = 0.982$). When fold change values for this qPCR panel were converted to Z-scores (see Appendix A) and aggregated, stress significantly increased mRNA expression for genes related to microglial phagocytosis (Figure 4.3; $F_{(1,31)} = 4.604, p < 0.05$). No sex difference was observed in the pattern of expression for these genes ($F_{(1,31)} =$

$0.039, p = 0.844$).

No stress or sex differences were observed in hypothalamic CD68 (Figure 6.1a; stress: $F_{(1,31)} = 0.609, p = 0.441$; sex: $F_{(1,31)} = 0.113, p = 0.739$), Cybb (Figure 6.1b; stress: $F_{(1,31)} = 0.168, p = 0.684$; sex: $F_{(1,31)} = 0.234, p = 0.632$), tyrobp (Figure 6.2b; stress: $F_{(1,31)} = 0.026, p = 0.873$; sex: $F_{(1,31)} = 0.566, p = 0.457$), or C1qa (Figure 6.2a; stress: $F_{(1,31)} = 0.436, p = 0.514$; sex: $F_{(1,31)} = 0.456, p = 0.505$) mRNA expression. Further, no sex or stress differences were observed in the aggregate z-score of hypothalamic phagocytic gene expression (Figure 6.3; stress: $F_{(1,31)} = 0.246, p = 0.627$; sex: $F_{(1,31)} = 0.906, p = 0.349$).

Stress decreased Cybb mRNA expression in the amygdala (Figure 7.2a; $F_{(1,27)} = 4.915, p = 0.035$). Sex did not significantly affect Cybb mRNA expression in the amygdala, nor was there a significant interaction effect of sex and stress (sex: $F_{(1,27)} = 0.113, p = 0.740$; interaction: $F_{(1,27)} = 0.081, p = 0.778$). Neither stress nor sex altered the mRNA expression of C1qa (Figure 7.1a; stress: $F_{(1,27)} = 0.131, p = 0.720$; sex: $F_{(1,27)} = 0.016, p = 0.900$), CD68 (Figure 7.1b; stress: $F_{(1,27)} = 0.168, p = 0.686$; sex: $F_{(1,27)} = 1.245, p = 0.274$), or tyrobp (Figure 7.2b; stress: $F_{(1,27)} = 0.018, p = 0.900$; sex: $F_{(1,27)} = 2.02, p = 0.167$) in the amygdala. Neither stress nor sex altered the aggregate z-score of phagocytic gene expression in the amygdala (Figure 7.3; stress: $F_{(1,27)} = 0.247, p = 0.623$; sex: $F_{(1,27)} = 0.952, p = 0.338$).

Histidine decarboxylase mRNA expression

Stress induced a trending decrease of hippocampal histidine decarboxylase (Hdc) mRNA

(Figure 5a; $F_{(1,31)} = 3.452, p = 0.073$). No sex differences were observed in Hdc mRNA expression ($F_{(1,31)} = 0.272, p = 0.606$), and the effects of stress did not depend on sex ($F_{(1,31)} = 0.414, p = 0.525$).

Linear regression models

Using least-squares linear regression, I explored a potential relationship of hippocampal histamine synthesis to hippocampal microglia phagocytic mRNA expression (aggregate z-score) while controlling for experimental condition (i.e., stress vs. control handling). The best-fitting least-squares regression model of this relationship is $\hat{Y}_{phagoZ} = 1.040 - 0.651X_{Hdc} + 0.506X_{condition}$. This model predicts phagocytic mRNA expression better than would be expected by chance ($F_{(2,32)=} 8.466,\ R^2 = 0.346,\ p < 0.01$), and only Hdc fold change significantly predicted phagocytic gene aggregate z-score ($t_{(32)} = -3.251, p < 0.01$). Experimental condition did not significantly predict phagocytic gene aggregate z-score when Hdc mRNA expression was accounted for ($t_{(32)} = 1.376, p = 0.178$).

Mast cell counts

Stress increased total mast cell count (Figure 8a; $F_{(1,30)} = 4.764, p = 0.037$); however, no multiple comparison was significant. Total mast cell count did not differ by sex ($F_{(1,30)} = 0.158, p = 0.694$). The interaction of sex and stress was not significant ($F_{(1,30)} = 1.263, p =$

0.270). Stress also increased granulated mast cell count (Figure 8b; $F_{(1,30)} = 4.537, p < 0.05$); granulated mast cell count did not differ by sex ($F_{(1,30)} = 0.362, p = 0.552$), and the interaction between sex and stress was not significant ($F_{(1,30)} = 2.002, p = 0.167$). No multiple comparisons were significant, but the difference in granulated mast cell count between control females and stressed females approached significance ($q_{(30)} = 3.442, p = 0.092$). Neither stress nor sex altered the percent of degranulated mast cells (relative to total mast cells) in 5 sections of the PD21 hippocampus (Figure 8d; stress: $F_{(1,29)} = 0.458, p = 0.504$; sex: $F_{(1,29)} = 1.54, p = 0.225$). Neither stress nor sex altered degranulated mast cell count (Figure 8c; stress: $F_{(1,30)} = 2.109, p = 0.157$; sex: $F_{(1,30)} = 0.001, p = 0.976$).

Open field test

Stress did not alter total distance travelled in the open field (Figure 9a; $F_{(1,37)} = 0.582, p = 0.451$). Sex and stress interacted to determine center entries in the open field (Figure 9b; $F_{(1,37)} = 7.501, p < 0.01$); however, neither sex nor stress alone altered center entries. Stressed male rats entered the center of the open field less than control males at the 0.1 level of significance ($q_{(37)} = 3.382, p = 0.097$). Similarly, neither sex nor stress altered time spent in the center of the open field, although the interaction of sex and stress was statistically significant (Figure 9c; $F_{(1,37)} = 8.099, p < 0.01$). Stressed males spent significantly less time in the center of the field than control males did ($q_{(37)} = 3.892, p < 0.05$).

Elevated plus maze test

Stress did not alter open arm time in the EPM (Figure 10.1a; $F_{(1,37)} = 0.038, p = 0.846$, Figure 7). Male rats spent significantly less time in the open arms of the maze than females did (Figure 10.1a; $F_{(1,37)} = 5.374, p = 0.026$). The interaction of sex and stress was not statistically significant ($F_{(1,37)} = 1.383, p = 0.247$).

Female rats made more open arm entries than males (Figure 10.1b; $F_{(1,37)} = 11.09, p < 0.01$); in particular, stressed females made more open arm entries than stressed males ($q_{(37)} = 4.524, p < 0.05$). Stress did not alter open arm entries in the EPM ($F_{(1,37)} = 0.062, p = 0.805$). Females also had a higher percentage of open arm entries (relative to total arm entries) than males did (Figure 10.1c; $F_{(1,37)} = 13.55, p < 0.001$); stress did not alter percent open arm entries ($F_{(1,37)} = 0.214, p = 0.646$). Stressed rats made fewer closed arm entries than control rats (Figure 10.2a; $F_{(1,37)} = 6.444, p < 0.05$). At the $\alpha = 0.10$ level of statistical significance, males made more closed arm entries than females did ($F_{(1,37)} = 3.158, p = 0.084$). Sex and stress did not interact to alter closed arm entries ($F_{(1,37)} = 0.057, p = 0.813$). At the $\alpha = 0.10$ level of statistical significance, stress decreases total arm entries (Figure 10.2b; $F_{(1,37)} = 2.854, p = 0.10$). Sex does not alter total arm entries ($F_{(1,37)} = 2.278, p = 0.14$), nor did sex and stress interact to alter total arm entries ($F_{(1,37)} = 0.961, p = 0.333$).

Males head-dipped significantly less than females did (Figure 10.3a; $F_{(1,37)} = 6.913, p < 0.05$), with no effect of stress ($F_{(1,37)} = 0.0008, p = 0.978$). Stress significantly decreased the number of rears (Figure 10.3b; $F_{(1,37)} = 21.88, p < 0.0001$), with no effect of sex ($F_{(1,37)} =$

$1.103, p = 0.301$). Stressed females reared significantly less than control females ($q_{(37)} = 4.663, p < 0.05$), and stressed males reared significantly less than control males ($q_{(37)} = 4.702, p < 0.05$). Although neither stress nor sex altered the number of stretch-attend behaviors (Figure 10.3c; respectively, $F_{(1,37)} = 0.836, p = 0.366$ and $F_{(1,37)} = 0.906, p = 0.347$), the interaction of stress and sex was significant at the $\alpha = 0.10$ level ($F_{(1,37)} = 3.575, p = 0.067$). When head dips, rears, and stretch-attend behavior frequencies were converted to z-scores and averaged to derive aggregate risk assessment behavior z-scores (Figure 10.5), stress significantly decreased the expression of risk assessment behaviors ($F_{(1,37)} = 9.125, p < 0.01$), and males expressed fewer risk assessment behaviors than females at the $\alpha = 0.10$ level of significance ($F_{(1,37)} = 3.386, p = 0.074$). Sex and stress did not interact to alter the expression of risk assessment behavior ($F_{(1,37)} = 0.441, p = 0.511$). Head dips and stretch-attend postures were significantly negatively correlated (Figure 10.4; r = -0.504, p < 0.001).

Forced swim test

Stress did not alter immobility time on day 1 of the FST (Figure 11a; $F_{(1,37)} = 0.260, p = 0.613$); however, male rats spent significantly more time immobile than female rats did ($F_{(1,37)} = 65.41, p < 0.0001$). Stress decreased immobility time on day 2 of the FST (Figure 11b; $F_{(1,37)} = 5.943, p < 0.05$). Males spent more time immobile than females did on day 2 of the test (Figure 11b; $F_{(1,37)} = 17.18, p < 0.001$); the interaction between sex and stress was statistically insignificant, however ($F_{(1,37)} = 0.009, p = 0.924$).

Discussion

In this study, early deprivation stress altered neuroimmune function in the hippocampus and amygdala, as well as adult locomotor and stress coping behavior. Specifically, stress increased the hippocampal expression of genes relevant for microglial phagocytosis, suppressed hippocampal histidine decarboxylase expression (suggesting a potential dampening of histamine synthesis), suppressed Cybb mRNA expression in the amygdala, and increased hippocampal mast cell counts. Stress also reduced activity in the elevated plus maze while increasing it in the forced swim test, suppressed open field exploratory behavior in males only, and decreased risk assessment behaviors (particularly rearing) in the EPM. Interestingly, except in the open field test, there were no sex differences in the effects of stress. These findings further characterize the behavioral phenotype that results from early deprivation stress in rats; these data also suggest potential mechanisms responsible for early life stress effects on adult behavior.

In particular, chronic early deprivation stress increased total and granulated mast cell counts in the PD21 hippocampus. This finding is comparable to that of Joshi et al. (2019), which demonstrated increased granulated and total mast cell count in the PD11 hippocampus following postnatal handling stress, although this change occurred only in females. This discrepancy may be due to the relative severity of early deprivation stress when compared with postnatal handling— rats in my study were stressed two weeks longer and 3.75 more hours/day than postnatally handled rats. Male mast cell counts may respond only to severe stressors. Different timepoints may also account for the discrepancy— PD11 rats differ from PD21 rats in a number

of ways, including the dynamics of brain-resident mast cells and stress response (Joshi et al., 2019; Sapolsky & Meaney, 1986).

Because hippocampal mast cell counts drop precipitously during the first two weeks of life (Joshi et al., 2019), increased mast cell counts in stressed animals suggest that the hippocampus may retain mast cells longer under stress, potentially extending the sensitive period for mast cell modulation of hippocampal circuitry. Alternatively, early deprivation stress may promote mast cell proliferation or migration to the hippocampus, raising the total number of mast cells beyond that observed in control animals.

Further, hippocampal histidine decarboxylase mRNA expression significantly predicts microglial phagocytic gene expression in the hippocampus even when controlling for experimental condition (i.e., stress vs. control handling)— higher histidine decarboxylase expression is associated with lower microglial phagocytic gene expression. This finding suggests that hippocampal histamine synthesis (either by mast cells or by microglia (Katoh et al., 2001; Moon et al., 2014) is linked to microglial phagocytosis, although causal direction cannot be determined from these data. Mast cells may suppress microglial phagocytosis via histamine synthesis and release; this hypothesis requires further exploration in more detailed, mechanistic studies, however. Interestingly, although sex differences are observed in genes relevant to microglial phagocytosis in the PD2 hippocampus (Nelson, Warden, & Lenz, 2017), those sex differences are no longer apparent at PD21.

Microglia also express histidine decarboxylase and may participate in paracrine and autocrine histamine signaling (Iida et al., 2015). When microglia are already activated (e.g., by

stress), histamine may exert anti-inflammatory actions via the H_4 receptor (Ferreira et al., 2012); histamine may also suppress phagocytosis via the H_3 receptor (Iida et al., 2015). It is also possible that early deprivation stress increases the expression of a pro-inflammatory histamine receptor (H_1) in hippocampal microglia, rendering these cells more sensitive to the pro-inflammatory actions of relatively low concentrations of histamine. Future studies could address this possibility via immunofluorescent labelling for histamine receptors in Iba1-immunoreactive cells.

The findings of this study do not support a role for the compromised blood-brain barrier in the maladaptive behavioral sequellae of early life stress. If mast cells contribute to the programming of adult mood-related and coping behavior in the context of early deprivation stress, they aren't likely mediating these changes via the BBB (in contrast to adult social defeat stress, which produces neurovascular pathology in mice (Menard et al., 2017)). This finding also contrasts with studies that show loss of BBB integrity with maternal separation and adult restraint stress (Esposito et al., 2001, 2002; Gómez-González & Escobar, 2009). The work of Esposito et al. used an acute stress manipulation and measured BBB permeability immediately post-stress, whereas I used a chronic stress manipulation and collected brain tissue the day following the final bout of stress. Esposito et al. and Gómez-González & Escobar also used a direct measure of BBB permeability (although Evans blue assays tend to overestimate BBB permeability, see Saunders, Dziegielewska, Møllgård, & Habgood, 2015) rather than indirectly assessing BBB function via gene expression. It is possible that stress compromises BBB integrity without altering gene expression for BBB proteins. Alternatively, BBB function may have been unaffected by stress in this study because stress did not alter mast cell degranulation here.

Further, early deprivation stress had enduring effects on adult behavior, particularly risk assessment behaviors in the elevated plus maze. Stress decreased risk assessment behaviors irrespective of sex. This finding can be better understood in light of the sizable literature on revictimization in humans. Child abuse and neglect are associated with sexual and physical revictimization in adulthood, e.g. in dating relationships (Fiorillo, Papa, & Follette, 2013; Messman-Moore & Long, 2000; Widom, Czaja, & Dutton, 2008); deficits in self-protection and risk assessment have been proposed as mediators of revictimization (Messman-Moore & Brown, 2006). Early deprivation stress is a potentially useful model to explore neurodevelopmental and neuroimmune correlates of impaired risk assessment, and to determine how adequate risk assessment behavior can be preserved in the face of early life stress.

The use of a single stress manipulation limits this study. Early life stressors are diverse—they occur in many distinct forms, in many combinations, at various ages of onset and with inconstant chronicity. The conclusions of this study are not necessarily generalizable to all forms of early life stress. Given that early deprivation stress approximates child neglect, additional studies could be conducted using limited bedding and nesting (a manipulation analogous to child *abuse*).

Further, because tissue analysis and behavioral testing were performed in separate cohorts, I can draw only vague associations between gene expression and stereological endpoints and altered adult behavior. Future research could firm up these associations by, for example, administering H_3 or H_4 receptor antagonists to control handling rats and comparing their adult behavior to stressed rats.

Such an experiment could also clarify the relationship between hippocampal histamine synthesis and microglial phagocytosis. I did not count or categorize microglia in this study, so stressed rats may simply have more hippocampal microglia than control handling rats; however, it is more likely that microglia in the stressed hippocampus are functioning differently from control microglia— when considered singly, only one phagocytic gene was upregulated; if stressed rats simply had more hippocampal microglia, I would expect significant upregulation of all phagocytic genes assessed. A future study could address the question of microglial number and identify any stress-related changes in the types of cells or cell elements that microglia preferentially phagocytose. To determine whether microglia or mast cells (or both) are responsible for the stress-induced Hdc expression changes observed here, qPCR for Hdc should be performed in isolated mast cells and microglia from the hippocampi of stressed and control animals. Actual histamine content could also be quantified *in vivo* in the hippocampus via microdialysis or *in vitro* in cultured mast cells and microglia.

Further studies are needed to determine whether the hippocampi of stressed rats actually contain more phagocytic microglia than the hippocampi of control rats; if microglial phagocytosis *is* upregulated in stressed rats, further studies should also identify which cell types phagocytic microglia target following early deprivation stress. In the healthy brain, phagocytic microglia regulate the number of neural progenitor cells in the developing hippocampus (Nelson et al., 2017), as well as neuroblasts in the adult hippocampus (Sierra et al., 2010) and newborn astrocytes in the developing amygdala (vanRyzin et al., 2019); phagocytic microglia also contribute to synaptic patterning and circuit organization (Schafer et al., 2012)**.** Microglia may upregulate phagocytic activity to adapt hippocampal circuitry to a stressful environment. Further,

microglial phagocytosis of apoptotic cells often prevents inflammation (Sierra et al., 2013); therefore, upregulation of phagocytosis following early deprivation stress could represent a strategy to lessen stress-related damage to the hippocampus. Alternatively, if microglia are over-pruning neural progenitors and newborn neurons following stress, phagocytosis could mediate the relationship between early life stress and adult cognitive deficits, including memory impairment (Bremner et al., 1995; Navalta et al., 2006; Pechtel & Pizzagalli, 2011).

Finally, future studies should consider additional timepoints (e.g., adolescence) and brain regions. For example, altered risk assessment behaviors may be associated with neuroimmune alterations in the bed nucleus of the stria terminalis, a brain region responsible for assessing safety contexts (reviewed in Lebow & Chen, 2016). Future studies should also assess hippocampal-dependent cognition with tasks such as the Morris water maze and novel object recognition (as in Suri et al., 2013).

In conclusion, early deprivation stress is associated with alterations in microglial function, mast cell dynamics, and histamine synthesis at PD21, as well as changes in anxiety-like, stress coping, and risk assessment behavior in adulthood. Stress did not alter gene expression relevant to BBB function. Surprisingly, sex differences were observed only in central tendency in the open field test. These results suggest that early deprivation stress may promote mast cell retention in the juvenile hippocampus, extending the sensitive period for mast cell programming of stress-sensitive hippocampal circuitry. Early deprivation stress also suppresses hippocampal histidine decarboxylase mRNA expression during development; reduced expression of this gene suggests a possible suppression of hippocampal histamine synthesis, which may disinhibit microglial phagocytic activity, which may, in turn, organize local circuitry that

contributes to the expression of the adult behavioral phenotype observed here. These data suggest several potentially fruitful future directions for the study of early life stress effects on adult mood-related, coping, and risk assessment behavior.

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Appendix A: R code written to obtain aggregate Z scores for qPCR panels

```
Phago = read.table(file = '/Users/angelaleemarlowe/Dropbox/Ohio State University/Lenz
    Lab/Adventures in MSS (Thesis Project)/HPC_MSS Phagocytosis.txt', header = T)

head(Phago)

ref_group_A <- subset(Phago, Sex=="Male" & Condition=='Control')

ref_mean_A <- mean(ref_group_A$C1qa)

ref_mean_A

ref_sd_A <- sd(ref_group_A$C1qa)

ref_sd_A

ref_mean_B <- mean(ref_group_A$CD68)

ref_mean_B

ref_sd_B <- sd(ref_group_A$CD68)

ref_sd_B

ref_mean_C <- mean(ref_group_A$Cybb)

ref_mean_C

ref_sd_C <- sd(ref_group_A$Cybb)

ref_sd_C

ref_mean_D <- mean(ref_group_A$Tyrobp)

ref_mean_D

ref_sd_D <- sd(ref_group_A$Tyrobp)

ref_sd_D

install.packages("dplyr")

library("dplyr")

Phago <- mutate(Phago,

        z_C1qa = (Phago$C1qa-          ref_mean_A)/ref_sd_A,
```

```
        z_CD68 = (Phago$CD68-ref_mean_B)/ref_sd_B,
        z_Cybb = (Phago$Cybb-ref_mean_C)/ref_sd_C,
        z_Tyrobp = (Phago$Tyrobp-ref_mean_D)/ref_sd_D)
head(Phago)
Phago <- mutate(Phago,
          z_score = (z_C1qa + z_CD68 + z_Cybb + z_Tyrobp)/4)
head(Phago)
write.table(Phago, file="/Users/angelaleemarlowe/Dropbox/Ohio State University/Lenz
    Lab/Adventures in MSS (Thesis Project)/HPC_MSS Phagocytosis Z.txt")
```

Author note: The above code was written to obtain aggregate z-scores for phagocytic gene expression in the hippocampus. Nearly identical code was written to obtain aggregate z-scores for phagocytic gene expression in the hypothalamus and amygdala. For brevity, I have included only one set of code.

```
BBB = read.table(file = '/Users/angelaleemarlowe/Dropbox/Ohio State University/Lenz
    Lab/Adventures in MSS (Thesis Project)/HPC_MSS.txt', header = T)
head(BBB)
ref_group_B <- subset(BBB, Sex=="Male" & Condition=='Control')
ref_mean_E <- mean(ref_group_B$CLDN5)
ref_mean_E
ref_sd_E <- sd(ref_group_B$CLDN5)
ref_sd_E
ref_mean_F <- mean(ref_group_B$OCLN)
ref_mean_F
ref_sd_F <- sd(ref_group_B$OCLN)
```

```
ref_sd_F

ref_mean_G <- mean(ref_group_B$TJP1)

ref_mean_G

ref_sd_G <- sd(ref_group_B$TJP1)

ref_sd_G

ref_mean_H <- mean(ref_group_B$TJP2)

ref_mean_H

ref_sd_H <- sd(ref_group_B$TJP2)

ref_sd_H

install.packages("dplyr")

library("dplyr")

BBB <- mutate(BBB,

        z_CLDN5 = (BBB$CLDN5-ref_mean_E)/ref_sd_E,

        z_OCLN = (BBB$OCLN-ref_mean_F)/ref_sd_F,

        z_TJP1 = (BBB$TJP1-ref_mean_G)/ref_sd_G,

        z_TJP2 = (BBB$TJP2-ref_mean_H)/ref_sd_H)

head(BBB)

BBB <- mutate(BBB,

        z_score = (z_CLDN5 + z_OCLN + z_TJP1 + z_TJP2)/4)

head(BBB)

write.table(BBB, file="/Users/angelaleemarlowe/Dropbox/Ohio State University/Lenz
    Lab/Adventures in MSS (Thesis Project)/HPC_MSS BBB Z.txt")
```

Appendix B: R code for risk assessment behavior analysis

```
install.packages("openxlsx")

library("openxlsx")

RISK = read.xlsx(xlsxFile = '/Users/angelaleemarlowe/Dropbox/Ohio State University/Lenz
    Lab/Adventures in MSS (Thesis Project)/EPM ARM ENTRIES & RISK
    ASSESSMENT.xlsx', sheet = 1, startRow = 1, colNames = TRUE, rowNames = FALSE,
    detectDates = FALSE, skipEmptyRows = TRUE, skipEmptyCols = TRUE, rows = NULL,
    cols = NULL, check.names = FALSE)

head(RISK)

cor.test(RISK$Head.dips, RISK$Stretch.attend)

plot(RISK$Head.dips, RISK$Stretch.attend, pch = 21, col = 'purple', bg= 'purple', xlab = "Head
    dips", ylab = "Stretch-attend postures")

abline(lm(RISK$Stretch.attend~RISK$Head.dips), col="dark blue", lwd = 3) #statmethods.net

legend("topright",

    legend = c('r = -0.504', 'p < 0.001'), cex = 2)

ref_group_A <- subset(RISK, Sex=="MALE" & Condition=='CONTROL')

ref_mean_A <- mean(ref_group_A$Head.dips)

ref_sd_A <- sd(ref_group_A$Head.dips)

ref_mean_B <- mean(ref_group_A$Rears)

ref_sd_B <- sd(ref_group_A$Rears)

ref_mean_C <- mean(ref_group_A$Stretch.attend)

ref_sd_C <- sd(ref_group_A$Stretch.attend)


install.packages("dplyr")

library("dplyr")

RISK <- mutate(RISK,
```

```
        z_Head.dips = (RISK$Head.dips-ref_mean_A)/ref_sd_A,
        z_Rears = (RISK$Rears-ref_mean_B)/ref_sd_B,
        z_Stretch.Attend = (RISK$Stretch.attend-ref_mean_C)/ref_sd_C)
head(RISK)
RISK <- mutate(RISK,
        z_score = (z_Head.dips + z_Rears + z_Stretch.Attend)/3)
head(RISK)
write.table(RISK, file="/Users/angelaleemarlowe/Dropbox/Ohio State University/Lenz
    Lab/Adventures in MSS (Thesis Project)/MSS_EPM Risk Z.txt")
```

Appendix C: R code for least squares linear regression models

```
MCMG = read.table(file = '/Users/angelaleemarlowe/Dropbox/Ohio State University/Lenz Lab/Adventures in MSS (Thesis Project)/HPC_MSS Phagocytosis Z.txt', header = T)

head(MCMG)

plot(MCMG$Hdc, MCMG$z_score, pch = 21, col = 'purple', bg= 'dark blue', xlab = "Hdc fold change", ylab = "Phagocytic gene Z score")

abline(lm(MCMG$z_score~MCMG$Hdc + MCMG$Condition), col="purple", lwd = 3)

summary(lm(MCMG$z_score~MCMG$Hdc + MCMG$Condition))

summary(lm(MCMG$z_score~MCMG$GranMC + MCMG$Condition))

plot(MCMG$GranMC, MCMG$z_score, pch = 21, col = 'blue', bg= 'blue', xlab = "Granulated mast cell number", ylab = "Phagocytic gene Z score")

abline(lm(MCMG$z_score~MCMG$GranMC), col="violet", lwd = 3)
```