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Sex differences in the effects of early life stress exposure on mast cells in the developing rat brain

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ABSTRACT

Early life stress leads to long lasting effects on behavior. Neuroimmune cells have been implicated as key mediators of experience-induced changes in brain and behavioral development, in that they are highly responsive to stress. Mast cells are one such type of neuroimmune cell, but little is known about their role in brain development or following early life stress. Here, we assessed the impact of three different early life stress exposure paradigms on mast cell dynamics in the developing brain of male and female rats, focusing on the hippocampus and hypothalamus, where most mast cells reside. We found that exposure to two weeks of chronic variable stress during gestation led to increased mast cell number and activation in the female offspring hypothalamus on the day of birth. Acute exposure to maternal separation stress on postnatal day (PN) 2 led to significant decreases in mast cells within the hypothalamus and hippocampus of females, but not males. In contrast, one week of exposure to brief daily maternal separation stress (e.g., handling), increased mast cell numbers in the female, but not male, hippocampus. We found significant sex differences in mast cell number and activation, including males having more mast cells than females in the hippocampus on the day of birth and males having significantly more degranulated mast cells on PN11. Thus, mast cells may be an unappreciated mediator of sex-specific brain development in response to early life perturbations.

1. Introduction

Brain development is driven by intrinsic cues that are modulated and shaped by environmental signals. Stress exposure during brain development is one of the most common of these early life experiences across species, and differences in the severity, duration, or timing of stress can lead to vastly different neurodevelopmental and behavioral outcomes (Bale and Epperson, 2015; Chen and Baram, 2016). Early life stress is a major risk factor for virtually every neuropsychiatric disorder, both neurodevelopmental disorders, such as autism and schizophrenia, and post-adolescent mood disorders, such as major depressive and anxiety disorders (Bale et al., 2010).

Stress exposure early in life can influence brain development via many physiological effectors, but recently the immune system has been shown to be highly responsive to stress and to mediate stress-induced changes in brain function and behavior (Ganguly and Brenhouse, 2015; Hodes et al., 2015). Innate immune cells colonize the embryonic brain and are highly responsive to environmental cues, including stressors (Bilbo et al., 2018; Nelson et al., 2018). Environmental perturbations may thus increase risk for adult psychopathology by modulating immune function in the brain (Hodes et al., 2015). Brain-resident macrophages, called microglia, regulate cell genesis, cell death, axon guidance and synaptic patterning during ontogeny (Lenz and Nelson, 2018). Stress-induced changes in microglial function during brain development may have lifelong implications for brain function and behavior given that microglia are such crucial regulators of normal brain development (Delpech et al., 2016). Yet microglia are not the only innate immune cells in the brain.

Another type of innate immune cell, the mast cell, has largely been implicated in anaphylaxis, peripheral allergic responses and itch (Metcalfe et al., 1997, 2009). Yet mast cells also populate the healthy rodent and human brain (Silver and Curley, 2013). Activated mast cells release serotonin, histamine, inflammatory cytokines, growth factors and proteases via a process called degranulation (Metcalfe et al., 1997; Silver and Curley, 2013). Mast cell-mediated conditions, such as asthma, allergy and mastocytosis are co-morbid with anxiety, depression and higher perceived stress (Georgin-Lavialle et al., 2016; Moura et al., 2012). Mast cell deficient mice have altered anxiety behavior and stress reactivity in adulthood (Nautiyal et al., 2008, 2012), yet the role mast cells play in brain development and the ontogeny of mood disorders remains unknown.

In the current experiments, we compared the effects of three early

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life stress exposure paradigms on mast cell colonization and degranulation in the developing brains of male and female rats. We found that two weeks of chronic variable stress during gestation led to increased mast cell number and activation in the hypothalamus of female, but not male, offspring when assessed on the day of birth. A single exposure to maternal separation stress on postnatal day (PN) 2 led to significant decreases in mast cell numbers within both the hypothalamus and hippocampus of females, but not males. In contrast, one week of exposure to brief daily maternal separation stress led to increased mast cell numbers in the female, but not male, hippocampus. We also found significant sex differences in mast cell number and activation across development, with males having more mast cells than females in the hippocampus on the day of birth and males having significantly more degranulated mast cells in both the hippocampus and hypothalamus on PN11. These studies suggest that mast cells may be more responsive to stress exposure during the early postnatal period than during the prenatal period.

2. Materials and methods

2.1. Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee at The Ohio State University. Studies were conducted with Sprague Dawley rats purchased while pregnant from Envigo (Indianapolis, IN) (postnatal acute stress experiment) or Taconic (Hudson, NY) (prenatal stress experiment) or bred in-house from animals originally from Envigo (postnatal repeated stress experiment). Animals were housed on a 12 h light/dark cycle (lights of at 0700) in standard ventilated cages in groups of 3, except when breeding, with food and water ad libitum. For in-house breeding, adult females were paired with males and separated when vaginal lavage was sperm-positive. Once sperm-positive (designated gestational day (GD) 0), pregnant females were isolated and allowed to deliver naturally. Cages were checked daily to determine the day of birth (designated postnatal day (PN) 0). For postnatal stress experiments, on the day of birth, pups from two or more litters born on the same day were grouped together, sexed, and then randomly assigned back to stress or control dams prior to stress exposure, to control for any differences in genetic background and maternal care. Entire litters were exposed to stress conditions or control treatment as detailed below, and offspring from the litters used across multiple studies, such that for the studies reported herein, only 1–3 pups per sex per litter were used to assess mast cell numbers.

2.2. Experiment 1: prenatal stress

Pregnant Sprague Dawley rats were randomly assigned to the stressed or unstressed groups. From GD7-GD20, unstressed rats were handled daily while stressed rats were exposed to a chronic mild variable stress procedure that includes randomized daily exposure to two of the following battery of stressors, each exposure being 2–3 times total over the course of gestation: restraint, overcrowding, overnight food deprivation, housing with wet bedding, social novelty or foot shock. Restraint stress was performed using clear acrylic rat restrainers for either 45 min or 2 h under bright light illumination during the light portion of the light/dark cycle between 0900 and 1200. Overnight food deprivation and damp bedding exposure during the light phase were both 12 h in duration. Social novelty stress consisted of a novel male conspecific being added to the maternal dam's home cage for 30 min on three days of gestation, with a novel male each time. Overcrowding consisted of 12 h of 4 adult females co-habitating in a novel mouse cage. For foot shock, fear-conditioning boxes were used to administer 10 50-msec shocks over a 10 min period, with a 50–70 s inter-trial interval (tone CS onset- 1 ms, ISI 1 msec, US offset 2000 msec, total trial length 2050 msec). Following the completion of gestational stress or control handling, pregnant dams were allowed to rest undisturbed for the remaining 2 days of pregnancy and delivered litters naturally. On the day of birth, designated postnatal day (PN) 0, pups were removed from the home cage within 6 h of parturition and immediately sacrificed via transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde following lethal overdose with Fatal Plus (Vortech Pharmaceuticals, Dearborn MI). Only 1–2 animals per sex per litter were used for mast cell counts to minimize litter effects on observed endpoints. Other animals from the litters were used for other experiments not included in this report or euthanized via rapid decapitation.

2.3. Experiment 2: postnatal acute maternal separation stress

On PN2, male and female pups were either left undisturbed or removed from the maternal dam and placed in cups to isolate the animals from one another in a clean rat cage with bedding under a heat lamp and resting on a heating pad. Stressed litters remained away from the maternal dam for 4 h, and at the completion of the 4-h stress period, animals were immediately sacrificed via transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde following lethal overdose with Fatal Plus. For this experiment, 2–3 animals per sex per litter were used for mast cell counts to minimize litter effects on observed endpoints. Other animals from the litters were used for other experiments not included in this report.

2.4. Experiment 3: postnatal repeated brief handling stress

Pregnant Sprague Dawley rats were allowed to deliver their litters naturally in our animal facility. On PN0, within 6 h of detection in the nest, pups were sexed and returned to the dam. From PN4-PN11, pups underwent neonatal handling, a mild stressor that has previously been shown to have a buffering effect on later life stress reactivity (Fenoglio et al., 2005, 2006). Pups were removed as a litter from the maternal dam, and placed as a group in a clean rat cage with bedding under a heat lamp and resting on a heating pad. Pups were removed for 15-min and then returned to the maternal dam. Following the completion of handling stress on PN11, pups were returned to the maternal dams and removed one-by-one for sacrifice via transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde following lethal overdose of FatalPlus to minimize additional stress exposure on the final day of handling. Only 1–2 animals per sex per litter were used for mast cell counts to minimize litter effects on observed endpoints. Other animals from the litters were used for other experiments or endpoints not included in this report.

2.5. Tissue processing and histology

Following perfusion, brains were removed and placed in 4% paraformaldehyde overnight for postfixation, and cryoprotected in 30% sucrose in 0.1 M Phosphate Buffered Saline (PBS) until saturated. Brains were coronally sectioned into 2 alternate series on a Leica cryostat at a thickness of 45 μm. Sections were immediately mounted onto SuperFrost Plus slides (Fisher) and allowed to air dry. Once dry, one series underwent staining with acidic toluidine blue (Sigma; 0.2% in 60% ethanol; pH: 2.0; 10 min incubation with stain) to visualize mast cells. Toluidine blue metachromatically stains mast cell granules purple, and background brain tissue blue, thus allowing for visualization of mast cells within the brain, a procedure which we have recently used in the developing rat brain (Lenz et al., 2018). Following staining, sections were dehydrated with ascending alcohol, defatted with xylenes and coverslipped with Permount.

2.6. Cell counting

Up to 90% of brain-resident mast cells are located within or near the hippocampus (Fig. 1). Thus one major region of interest in our analysis

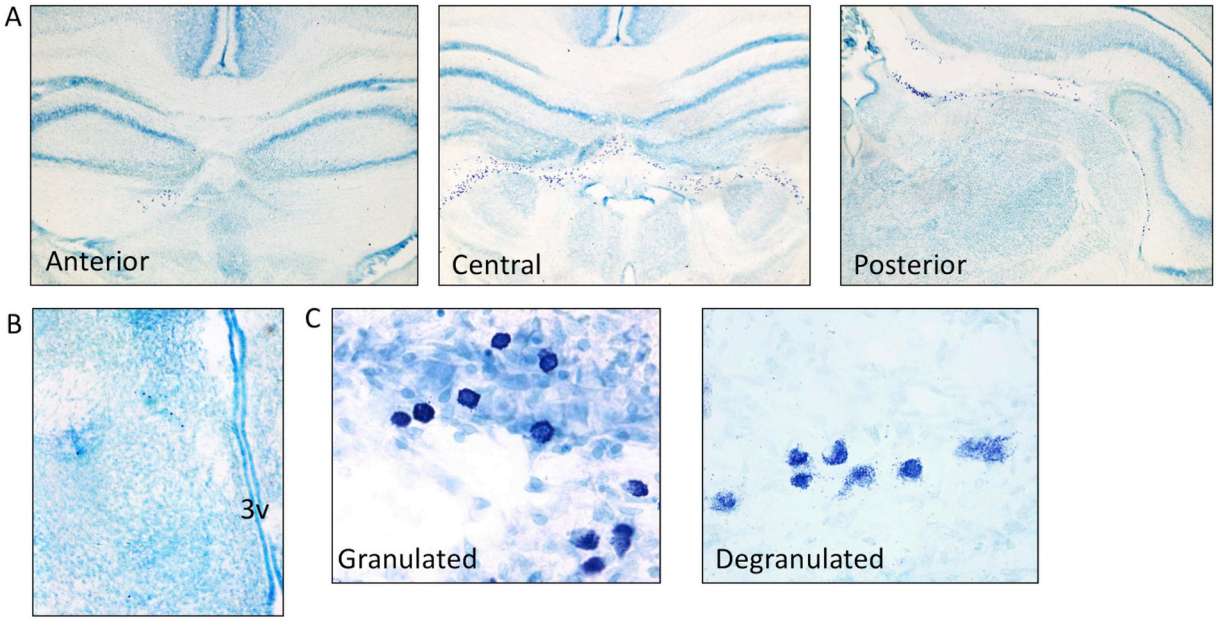

Fig. 1. Representative images of mast cells in the developing rat brain. Mast cells visualized by acidic toluidine blue staining in the rat brain on the day of birth (PN0). (A) A majority of mast cells locate to the fimbria and velum interpositum near the hippocampus and within the hippocampal formation itself. Mast cells are observed throughout the anterior-posterior extent of the hippocampus. (B) A much smaller number of mast cells can be seen in the hypothalamus. (C) Granulated mast cells are not actively releasing granules, which are packets filled with serotonin, histamine, and proteases. As such, granulated mast cells appear round and darkly staining (bottom center). Degranulated mast cells are in the active process of releasing granules, and are characterized by diffuse staining and a loss of a round morphology (bottom right). In the current experiments, mast cells were counted in both the hippocampus and hypothalamus and characterized as granulated or degranulated at the time of counting. 3v = third ventricle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was focused on this area (Fig. 1A). With the fimbria of the hippocampus and velum interpositum serving as the anterior border (Fig. 1A), mast cells were counted in every other section containing the hippocampus through the posterior extent of the ventral hippocampus. Cells were counted using stereological sampling and a computer-based stereology system (StereoInvestigator, MBF Biosciences, Inc., Willison, VT) coupled to a Zeiss AxioImagerM.2 microscope and a CCD camera. For each section, a contour around the region of interest (ROI) was traced at low magnification (2.5×), and counting frames of 200 × 200 μm were randomly placed across each ROI by the software program. Every mast cell within each counting frame was counted at 60× magnification and categorized as either granulated or degranulating. A mast cell was considered granulated if it had a uniformly darkly stained cell body with few to no visible individual granules and a round, intact membrane border, and was counted as degranulating if it had a light, granule appearance, an amorphous/non-spherical membrane border, and/or many extruded granules nearby the cell body (see Fig. 1 for examples). Stereological cell counts produce estimated total counts of mast cells across the hippocampus and nearby structures. Although a majority of brain-resident mast cells are in or around the hippocampus, there is also a smaller mast cell population in and near the hypothalamus (Fig. 1B), and we have previously shown in the nearby preoptic area that males have more mast cells than females (Lenz et al., 2018). Therefore, we also quantified mast cell number in the hypothalamus. Due to the small number of mast cells in this region, we did not use stereology to count these mast cells, but instead counted every mast cell in one of two series across the rostrocaudal extent of the hypothalamus.

2.7. Data analysis

Group sizes are reported for each condition and across experiments within the figure captions and summarized in Table 1. Total mast cell number and number and percent of degranulating mast cells were analyzed via two-way analyses of variance (ANOVAs), with sex and stress condition as main factors and alpha level set at 0.05. Where significant main or interaction effects were obtained via ANOVA, Tukey post-hoc tests were performed, and *p*-values adjusted for multiple comparisons. Data are presented as mean + standard error of the mean (SEM). For all results, we report exact F values, degrees of freedom, and exact *p* values, and for significant or trending (*p*s 0.05 > x > 0.08), we also report effect size values (η_p^2).

3. Results

3.1. Experiment 1: prenatal stress

For the hippocampus, two weeks of chronic variable stress during pregnancy did not induce alterations in mast cells in offspring brain on the day of birth (PN0), including the overall number of mast cells (Stress Treatment: $F(1,26) = 0.45$; $p = .51$), the number of granulated mast cells (Stress: $F(1,26) = 0.28$, $p = .60$), the number of degranulated mast cells (Stress: $F(1,26) = 0.10$; $p = .76$) or the percent of degranulated mast cells (Stress: $F(1,26) = 0.47$; $p = .50$), nor were there any stress by sex interaction effects (Fig. 2A). There was a trending sex difference in overall mast cell numbers on the day of birth, with males having more than females (Sex: $F(1,26) = 3.89$; $p = .059$, $\eta_p^2 = 0.13$; Fig. 2A), and a significant sex difference in the number of granulated mast cells, with males again having more than females (Sex: $F(1,26) = 9.72$, $p = .004$, $\eta_p^2 = 0.27$; Fig. 2A). There was no significant sex difference in the number of degranulated mast cells (Sex: $F(1,26) = 1.24$; $p = .28$), but a trend toward a significant sex difference in the percent of degranulated mast cells at PN0 (Sex: $F(1,26) = 3.77$, $p = .06$; $\eta_p^2 = 0.13$), with males having a lower percent degranulated than females.

For the hypothalamus (Fig. 2B), two weeks of chronic variable stress during pregnancy did not induce alterations in mast cells in offspring

Table 1
Summary of experimental details. Stressor column details the type of stress; "timing of stress" column details days and duration of each stress exposure; "Tissue harvest" column details the day animals were killed for brain harvesting and tissue analysis, "pups/sex/litter" column indicates the number of pups per sex from a given maternal dam used for each endpoint, and "group sizes" column summarizes the number of animals used for cell counts in each study for the hippocampus (HPC) and hypothalamus (HYPO). If no brain regions are specified, the N was the same for both brain regions.

Group	Stressor	Timing of stress	Tissue harvest	Pups/sex/litter	Group sizes
Prenatal stress	Chronic variable stress (maternal dam): restraint, overcrowding, overnight food deprivation, wet bedding, social novelty, foot shock	2 stressors/day; GD7–20	PN0	1–2	M control: HPC: 8, HYPO: 7 M stress: HPC: 7, HYPO: 8 F control: HPC: 8, HYPO: 5 F stress: HPC: 7, HYPO: 5
Acute maternal separation	Pups separated from mother; isolated in cups on heating pad	4 h; Once on PN2	PN2	2–3	M control: HPC: 6, HYPO:5 M stress: HPC: 6, HYPO: 7 F control: HPC: 6, HYPO: 5 F stress: 7
Repeated brief maternal separation	Pups separated from mother; kept together on heating pad	15 min/day; PN4–11	PN11	1–2	M control: 9 M stress: 9 F control: HPC: 8, HYPO: 10 F stress: 9

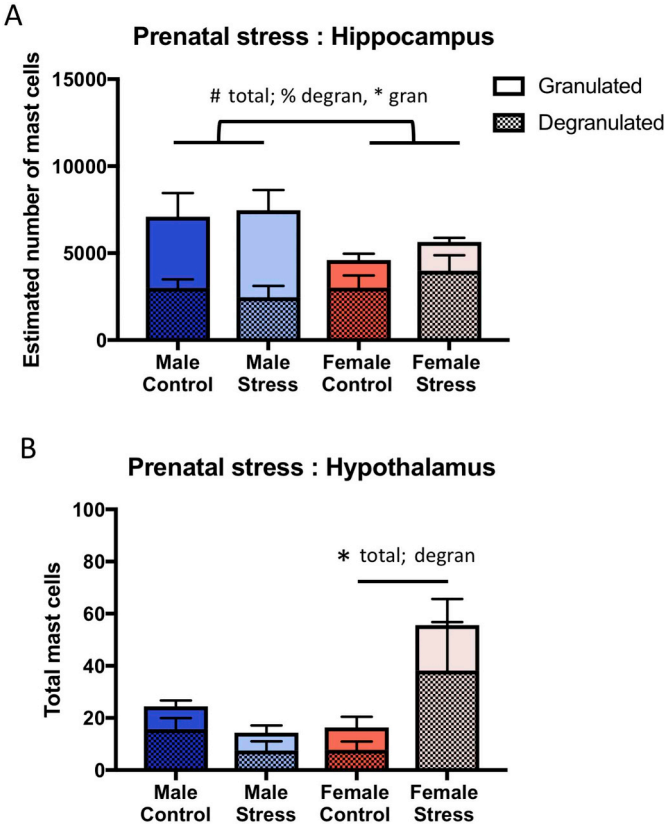

Fig. 2. Effects of prenatal chronic variable stress on mast cells in offspring brain. Two weeks of chronic variable stress during pregnancy led to region-specific changes in mast cell number in offspring brain. (A) In and around the hippocampus, there was no observed stress effects on mast cell number, but males had marginally more total mast cells percent degranulated mast cells and significantly more granulated mast cells on the day of birth (PN0) than females. (B) In the hypothalamus, stress led to a significant increase in total (full bars) and degranulated (crosshatched bars) mast cell numbers in females, but not males. Group sizes: (A): Male control: 8, Male stress: 7, Female control: 8, Female stress: 7. (B): Male control: 7, Male stress: 8, Female control: 5, Female stress: 5. Data analyzed via 2-way ANOVA for each brain region/mast cell type (Sex × Stress Condition as factors). * indicates $p > .05$, # indicates $p > .08$. Exact F and p values presented in text.

brain on the day of birth (PN0), including the overall number of mast cells (Stress Treatment: $F(1,21) = 1.45$, $p = .18$), the number of granulated mast cells (Stress: $F(1,21) = 0.51$; $p = .48$), the number of degranulated mast cells (Stress: $F(1,24) = 1.80$; $p = .19$) or the percent of degranulated mast cells (Stress: $F(1,24) = 0.18$; $p = .68$) (Fig. 2B). There were no significant sex differences in any parameters. There were significant stress by sex interaction effects for overall mast cell numbers ($F(1,21) = 4.13$; $p = .05$; $\eta_p^2 = 0.16$) and degranulated mast cell numbers ($F(1,21) = 5.46$, $p = .029$; $\eta_p^2 = 0.21$), such that there were no significant sex differences in mast cell numbers in controls, but following stress, females, but not males, showed significant elevations in numbers of degranulated and total degranulated mast cells.

3.2. Experiment 2: postnatal acute stress

Four hours of acute maternal separation stress had no significant effect on total mast cell number in and around the hippocampus on PN2 (Stress Treatment: $F(1,21) = 2.03$; $p = .17$; Fig. 3A). There was also no main effect for sex or interaction effect on total mast cell numbers (Sex: $F(1,21) = 0.01$; $p = .99$; Interaction $F(1,21) = 1.83$, $p = .19$). There were no effects of stress or sex on the number of granulated mast cells

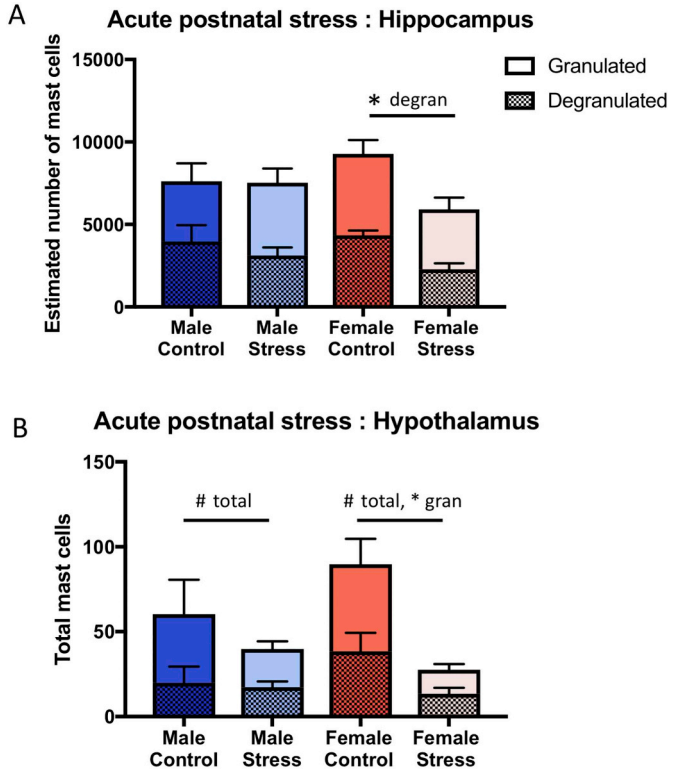

Fig. 3. Effects of acute maternal separation stress on mast cells in male and female rat brain. (A) A single exposure to 4 h of maternal separation stress on PN2 led to significant decreases in degranuated mast cell numbers in the hippocampus of female neonates, but not males. (B) In the hypothalamus, stress led to a trending decrease in total mast cell numbers in males and females, and a significant decreased in granuated cells in females. Group sizes: (A): Male control: 6, Male stress: 6, Female control: 6, Female stress: 7. (B): Male control: 5, Male stress: 7, Female control: 5, Female stress: 7. Data analyzed via 2-way ANOVA for each brain region/mast cell type (Sex × Stress Condition as factors). * indicates $p > .05$; # indicates $p > .08$. Exact F and p values presented in text.

(Stress: F(1,21) = 0.09; $p = .76$; Sex: F(1,21) = 0.08; $p = .78$; Interaction F(1,21) = 1.40; $p = .25$). However, there was a main effect of stress on the number of degranulated mast cells, leading to a significant decrease in degranulated cells F(1,21) = 6.31, p = .02; $\eta_p^2 = 0.23$), with females showing a significant reduction in pairwise post-hoc analyses. No main effect of sex or stress by sex interaction (Sex: F(1,21) = 0.16; $p = .69$; Interaction F(1,21) = 1.10, $p = .31$) were observed for degranulated cells. Neither stress nor sex had a significant effect on the percentage of degranulated mast cells at PN2 (F(1,21) = 1.50; $p = .23$; Sex: F(1,21) = 0.18; $p = .67$; Interaction: F(1,21) = 0.007; $p = .93$).

In the hypothalamus (Fig. 3B), there were no significant main effects of stress on total mast cell number, though there was a trend toward a stress effect (F(1,20) = 3.65, $p = .07$; $\eta_p^2 = 0.15$), with stressed animals showing almost half the number of mast cells as controls. There was a significant effect of stress on the number of granulated cells (F(1,20) = 4.35, $p = .05$; $\eta_p^2 = 0.18$), such that stressed animals had less than half of the number of granulated mast cells seen in controls, and post-hoc tests showed a significant difference in the female groups. There was no significant effect of stress on the number (F(1,20) = 2.03; $p = .17$) or percentage (F(1,21) = 2.23; $p = .15$) of degranulated mast cells. With regard to sex, there were no sex differences in total mast cells (F(1,21) = 0.124; $p = .73$), granulated (F(1,20) = 0.003; $p = .96$) or number (F(1,21) = 0.98; $p = .33$) or percentage (F(1,21) = 2.82; $p = .11$) of degranulated mast cells, nor any sex × stress interaction effects in the hypothalamus at PN2.

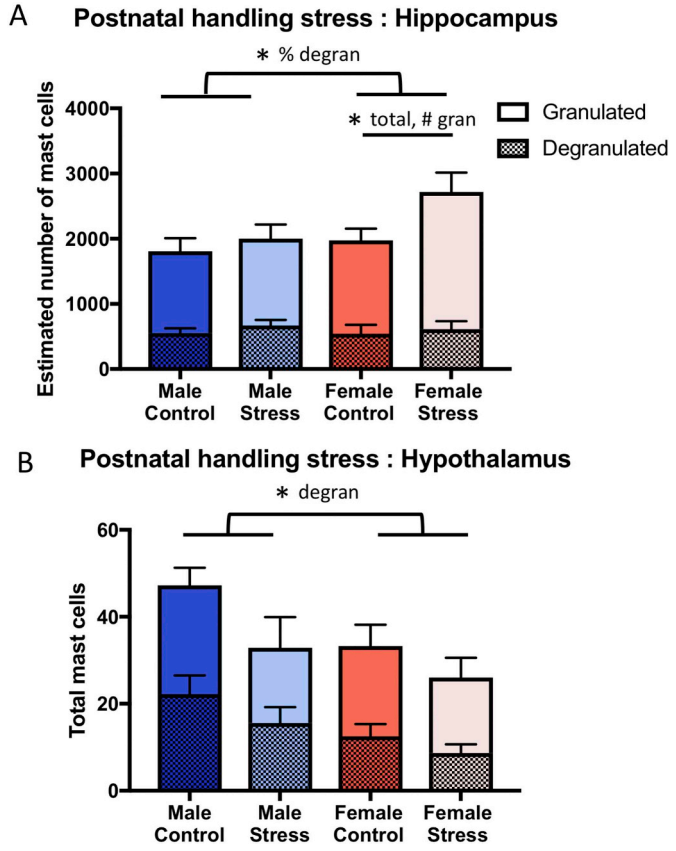

Fig. 4. Effects of daily repeated brief handling stress on mast cells in male and female rat brain. Exposure to one week of brief handling stress (15 min/day from PN4-11) led to (A) increased total mast cell number and a trend toward increased granulated mast cell number in the hippocampus of females, but not males. Males had a significantly higher percentage of degranulated mast cells than females irrespective of condition. (B) In the hypothalamus, we found no significant effects of stress, but found that males had higher numbers of degranulated mast cells than females, irrespective of stress condition. Group sizes: (A): Male control: 9, Male stress: 9, Female control: 10; Female stress: 8. (B): Male control: 9, Male stress: 9, Female control: 10; Female stress: 9. Data analyzed via 2-way ANOVA (Sex × Stress Condition as factors) for each brain region/mast cell type. * indicates $p > .05$, # indicates $p > .08$. Exact F and p values presented in text.

3.3. Experiment 3: postnatal handling stress

Following 15 min of daily maternal separation stress from PN4–11, stressed animals showed a significant increase in mast cell numbers in and around the hippocampus (F(1,31) = 4.21, $p = .048$; $\eta_p^2 = 0.12$; Fig. 4A), with post-hoc tests showing female stress animals had a significantly higher number of mast cells than control females ($p = .03$). There was no main effect of sex at this age nor a stress by sex interaction (Sex: F(1,31) = 0.38; $p = .54$; Interaction F(1,31) = 1.70, $p = .20$). There was a trend toward an effect stress on the number of granulated mast cells, with stress increasing granulated mast cell numbers (Stress: F(1, 31) = 3.12, $p = .08$; $\eta_p^2 = 0.09$), but no effect of sex or sex by stress interaction (Sex: F(1, 31) = 1.85; $p = .18$ Interaction: F(1, 31) = 2.03; $p = .16$). There were no significant effects of stress or sex on the number of degranulated mast cells (Stress: F(1, 31) = 2.28, $p = .14$; Sex: F(1,31) =1.99, $p = .17$, Interaction: F(1,31) = 0.10; $p = .75$). There was no effect of stress condition nor a stress by sex interaction on the percent of degranulated mast cells at PN11 (Stress Treatment: F(1,31) = 0.20; $p = .66$; Interaction: F(1,31) = 0.03; $p = .88$). However, at this age, males had a significantly greater percentage of degranulated mast cells than females, irrespective of stress condition (Sex: F(1,31) = 8.11, $p = .008$; $\eta_p^2 = 0.21$).

In the hypothalamus (Fig. 4B), there were no significant effects of stress condition or stress × sex interactions, however there was a significant sex difference in the number of degranulated mast cells at PN11 ($F(1, 33) = 6.34$, $p = .017$; $\eta_p^2 = 0.16$), with males having more degranulated mast cells than females. There was no sex difference in total mast cell counts ($F(1,33) = 1.79$, $p = .19$), granulated mast cell counts ($F(1,33) = 0.16$) or the percentage of degranulated mast cells ($F(1,33) = 2.99$, $p = .09$).

4. Discussion

In the current studies, we compared the effects of three early life stress paradigms on the number and activation profile of brain-resident mast cells in male and female rat pups in and around the hippocampus as well as in the hypothalamus. We found that two weeks of prenatal stress induced no major changes in the number or granulation state of mast cells in offspring's brains on the day of birth in either the hippocampus or hypothalamus. However, on the day of birth, we found that males had more mast cells and more granulated mast cells in their hippocampi than females, irrespective of stress condition. A single acute maternal separation stress exposure on postnatal day 2 caused a significant reduction in degranulated mast cells in the hippocampus. In contrast, in the control hypothalamus, females at PN2 had significantly more mast cells, and stress rapidly decreased those numbers to male levels. Exposure to a week of mild handling stress from postnatal days 4–11 caused an increase in total mast cells in the hippocampus by PN11. Overall, these data show that mast cell dynamics in the brain are developmentally regulated, region specific, display sex differences during development, and are responsive to early life stressors.

4.1. Effects of prenatal stress

Surprisingly, two weeks of exposure of the maternal dam to chronic variable stress during pregnancy did not alter the number or activation profile of hippocampal mast cells in offspring assessed on the day of birth. In the hypothalamus, in contrast, prenatal stress led to increases in mast cell number and degranulation in female, but not male, offspring on the day of birth. Pregnant dams experienced stress from gestational days 7–20, and parturition typically occurred on GD 21 or 22, thus a 24–48 h latency between the last bout of stress and brain tissue collection from newborn pups usually occurred. This delay between the last stress exposure and mast cell assessment may account for the lack of observed effects; perhaps assessing mast cells in the brains of fetuses on the same day as prenatal stress exposure would yield significant stress effects on mast cell dynamics. It is also possible that the stress of parturition led to a ceiling effect, such that all groups showed similar elevations in mast cell activation that masked any effects of prenatal stress. It may be that the mild chronic stress protocol was not sufficiently severe enough to alter fetal development. Indeed, this protocol was developed to induce affective changes and maternal care deficits in new mothers while not altering gross measures of offspring development, such as birth weight or litter size (unpublished observations). A more severe prenatal stressor may be necessary to induce mast cell changes in offspring. Finally, it is possible that developing fetuses are protected from stress effects on mast cells by the placenta or significantly suppressed maternal immune system during pregnancy. Currently underway experiments are assessing the extent to which prenatal stress alters mast cell numbers and broader inflammatory tone in the placenta and fetal brain.

4.2. Effects of acute postnatal stress

A single exposure to 4 h of maternal separation stress on postnatal day 2 led to a significant main effect of stress on the number of mast cells in both the hippocampus and hypothalamus, and post-hoc comparisons showed that the main effect of stress was largely driven by significant differences between female groups. Surprisingly to us, however, there was a *decrease* in the number of total mast cells relative to undisturbed controls. These results are suggestive that a single acute stress does impact mast cells, however future work will be necessary to ascertain the full nature of these effects. It is possible that the timing of tissue collection relative to the onset of separation is responsible for our observed effects. Mast cells are thought to be the first responders following a variety of immunogenic insults, often undergoing degranulation within seconds to minutes of an insult (Zhang et al., 2016). Thus, if maternal stress caused mast cell numbers to increase rapidly following initial separation from the maternal dam, for example 15 or 30 min following separation, we would have missed such an increase. It is also a possibility that acute stress could lead to the recruitment of mast cells out of the brain into the meninges or peripheral tissues. Mast cells are known to release vasodilators and to promote blood brain barrier permeability (Esposito et al., 2001; Wernersson and Pejler, 2014), thus stress-induced mast cell activation could allow mast cells to subsequently migrate out of the brain, potentially to aid in peripheral immune responses. It is also possible that stress could have led mast cells to be recruited to other brain regions not assessed in the current experiment.

4.3. Effects of chronic postnatal handling stress

We found that one week of daily brief maternal separation stress (e.g., handling) led to region-specific changes in mast cell number and degranulation in the hippocampus and hypothalamus. In the hippocampus, we found that stress led to increased total mast cell number, an effect that was primarily driven by increased mast cells in females. Interestingly, within the hypothalamus of the same animals, mast cell number showed no significant effect of stress treatment. Stress did not lead to significant changes in degranulated, or actively signaling, mast cells. This is interesting especially in the hippocampus, where stress increased mast cell number, because it suggests that repeated brief stress may be altering the recruitment of mast cells into that region. Handling stress is known to have a stress-buffering effect on offspring behavior in later life, possibly by leading to increased maternal care following separation (Fenoglio et al., 2006). In addition, mast cell deficient animals show elevated anxiety and stress responsivity (Nautiyal et al., 2012) (see below for further discussion), thus it may be that elevations in mast cell number in response to handling could be one means through which handling is protective against future stress-responsivity and anxiety-like behavior in females.

4.4. Contrasting effects of stressor mode, timing and duration

The three early life stress paradigms used in the current experiments induced differential effects on mast cell number and activation state in the immature rat brain. A single acute maternal separation stress on postnatal day 2 had a significant effect on total mast cell numbers in both the hippocampus and hypothalamus of females, but not males. But contrary to our prediction, as discussed above, acute stress led to a decrease, not an increase, in mast cell number. In contrast, one week of brief, daily postnatal maternal separation stress (e.g., handling) led to a significant increase in mast cell number within the offspring brain, particularly in females (see Fig. 4). These results together suggest that the postnatal window is a sensitive period for the effects of early life stress on mast cell number and activation within the developing brain, and that a single versus repeated exposures to stress lead to opposite effects on mast cell dynamics, at least in terms of their number. Future studies will be necessary to determine whether a single acute stressor always leads to decreased mast cell number in the brain, or whether this is dependent upon the developmental timing of stress exposure. It would also be important to determine whether a longer repeated daily stress during the neonatal period, for example 3–4 h daily maternal separation stress, produces greater increases in mast cell number in the

brain, or instead if it leads to decreased mast cell number as seen with the acute maternal separation stressor used in the current study.

Brief daily maternal separation stress, or handling, is thought to be a form of stress inoculation (Fenoglio et al., 2006), in that it often leads to increased resilience to stress and mood dysregulation later in life. The stress buffering effects of early life handling is thought to be mediated by a 'rebound' in maternal care following the brief separation (Fenoglio et al., 2006), not by alterations in stress hormones. Thus, the mechanisms through which handling or prolonged maternal separation stress influence mast cells may be different. In the current experiment, we did not assess maternal behavior or HPA axis response to the stress paradigms, thus future studies will be necessary to tease out whether maternal care, elevated stress hormones, or a combination of these and other factors are responsible for stress effects on mast cell properties.

Previous work using mast cell deficient mice has shown that without mast cells, animals display reduced brain levels of serotonin and increased anxiety-like behavior (Nautiyal et al., 2008, 2012). This data suggests that mast cells normally play an adaptive, potentially stress-buffering role in the brain. Perhaps following repeated handling, increased mast cell numbers contribute to the resilience phenotype of these animals. Indeed, previous work on early life handling shows lifelong changes in stress reactivity, HPA negative feedback, cognition, and markers of neurodegeneration (Meaney et al., 1988; Lesuis et al., 2016). Some of the protective effects of early life handling against drug reinstatement behavior are mediated via anti-inflammatory function within microglia (Schwarz et al., 2011) thus handling-induced changes in mast cell function could contribute to this early life programming of lifelong behavior and physiology.

4.5. Sex differences

On the day of birth, we found that males have more mast cells and more granulated mast cells than females in the hippocampus, whereas males and females had similar numbers of mast cells in the hypothalamus. On PN2 and PN11, the sex difference in mast cells in the hippocampus was not detected in the hippocampus or hypothalamus. This timing suggests that male-typical hormone exposure during development may be responsible for this sex difference. The male androgen surge in rats begins prenatally, around embryonic day 18, and then there is a second surge of androgens on the day of birth, following which androgen levels decrease in males on the day of birth (Konkle and McCarthy, 2011; reviewed in Lenz et al., 2012). Thus, if high levels of androgens or estrogens aromatized from androgens in the brain are responsible for programming this sex difference, it makes sense that sex differences would be detected during this androgen surge. Indeed, we have previously shown in the preoptic area that males have significantly more mast cells than females during the critical period for sexual differentiation (Lenz et al., 2018). Approximately 50% of mast cells express estrogen receptor alpha, and treating newborn females with estradiol leads to masculinization of mast cell number in the preoptic area within days (Lenz et al., 2018). The current results suggest that this sex difference is generalizable to other brain regions, such as the hippocampus. Although we did not observe a significant baseline sex difference in mast cell number in the hypothalamus on PN0, this may be the result of both the very low numbers of mast cells observed in this region as well as the lack of statistical power in our time course × sex design, because the shape of the data in the hypothalamus is once again in the direction of males having more mast cells than females at PN0.

How might sex steroid hormones lead to sex differences in mast cell number in the brain? Our previous work has shown that estradiol treatment leads to increased mast cell number within the preoptic area, and these effects cannot be attributed to mast cell proliferation (Lenz et al., 2018). Therefore it is more likely that estrogens lead to mast cell recruitment into particular brain regions. Previous work in doves has shown that mast cell recruitment into the brain occurs during reproduction, are responsive to gonadal steroids, and subsequently that mast cells release the sex hormone, gonadotropin releasing hormone (GNRH) (Wilhelm et al., 2000; Zhuang et al., 1997). Similarly, work in rats has demonstrated that mast cells are dynamic within the female brain across the estrus cycle (Kovács and Larson, 2006). Thus it is possible that mast cells are recruited into the brain by hormones, and in turn may further contribute to sex-specific brain function via mediating release of hormones.

Later in development, on PN11, males have higher number or percentage of degranulated mast cells than females in both the hypothalamus and hippocampus. While male-typical hormone levels derived from the testes have decreased by PN11, it still remains possible that brain-derived androgens or estrogens are higher in the male than female brain at this age, as central levels of steroid hormones often do not track with peripheral levels, especially within the hippocampus (Konkle and McCarthy, 2011). Alternatively, factors beyond sex steroid hormones could contribute to sex differences in activated mast cells at this age, including sex differences in stress reactivity or maternal care received, given that these factors also influence brain development (Bale and Epperson, 2015; Lenz and Sengelaub, 2006; Mueller and Bale, 2008; Murgatroyd and Spengler, 2011).

4.6. Mechanisms of mast cell effects on brain development

Mast cells are fascinating to consider in the context of brain development and brain physiology, because they release mediators that are known to be critically important for brain function, most notably the neurotransmitters, serotonin and histamine (Silver and Curley, 2013). In addition, mast cells release cytokines and prostaglandins when activated, which have also been implicated as neuromodulators that can impact brain development (Amateau and McCarthy, 2004; Huang et al., 2003; Silver and Curley, 2013; Wu et al., 2017; Zhang et al., 2016). Mast cells are constitutively active cells, engaging in a steady process of degranulation, whereby they release these neuroactive mediators into the surrounding neural microenvironment. The main contents of mast cell granules are histamine, serotonin, and proteases, whereas other mast cell mediators are released in a degranulation-independent manner (Silver and Curley, 2013). Thus, differences in mast cell number across sexes, brain regions, or by age in the absence of stress may be a source of variability in brain physiology that has not been accounted for or explored. A recent study has shown significant sex differences in the transcriptome of peripheral mast cells (Mackey et al., 2016), thus such differences in brain mast cells are likely as well.

Studies using the mast cell deficient mouse have shown that the loss of mast cells leads to significant decreases in hippocampal neurogenesis, increased anxiety, an elevated stress response, and impaired learning and memory (Nautiyal et al., 2008, 2012). These studies illustrate the important role for mast cells in regulating hippocampal function, however the lifelong nature of the mast cell deficiency makes it impossible to determine the extent to which mast cells are programming brain development versus supporting ongoing brain function and plasticity in adulthood. Future studies will be necessary to tease out these developmental versus adult effects, but given that early life stress also impacts later life anxiety and cognition, it is possible that mast cells in the developing brain are contributing to early life stress's programming effects on later life behavior, including cognition and mood.

Immunocompetent cells, both mast cells, as well as microglia and astrocytes, are present in the developing brain and shape many normal processes of development, including cell genesis, apoptosis, cell migration, synaptic patterning, and myelination (Reemst et al., 2016; Lenz and Nelson, 2018). We have recently shown that mast cells in the rat brain are essential to the development of the preoptic area, namely promoting sex-typical synaptic patterning that is necessary for adult male sexual behavior (Lenz, Pickett, et al., 2018; Lenz et al., 2019). In this recent work, we found that mast cell crosstalk with microglia is necessary for this sex-typical synaptic patterning process to occur.

Therefore mast cells may likewise be contributing to both neonatal cell genesis and synaptic patterning of the hippocampus and hypothalamus, both at baseline and in response to stress, and possibly via mediating effects on microglia. In addition, given that mast cells release neurotransmitters, such as serotonin and histamine, as well as growth factors and inflammatory cytokines (Silver and Curley, 2013), they could be shaping both neuronal and/or astroglial function in the developing brain. Future work will be necessary to address these mechanistic questions as well as delve into the nature of mast cell crosstalk with other cell types in the brain.

Although mast cells undergo constitutive degranulation, physiological and environmental factors can increase mast cell degranulation, and lead to degranulation-independent release of inflammatory mediators, such as cytokines. Most relevant to the current studies is past work suggesting that mast cells express receptors for the stress hormone, corticotropin releasing hormone (CRH). CRH can stimulate mast cells to degranulate (Cao et al., 2005, 2006). Conversely, mast cells themselves have been shown to release CRH (Kempuraj et al., 2004), which could amplify the brain's response to stress. Additionally, acute restraint stress in adulthood has been shown to activate mast cells in the skin (Donelan et al., 2006; Lytinas et al., 2003) and lead to the release of proinflammatory mediators, such as interleukin 6 (Huang et al., 2003). Acute stress in adulthood can also lead to increased blood brain barrier permeability in rats (Esposito et al., 2001) and this effect appears to be mast cell dependent in that the inhibitor of mast cell degranulation, sodium cromolyn, prevents this increase. It is unknown if early life stress can lead to similar mast cell signaling, or downstream changes in blood brain barrier permeability, but this is an important future question to address.

4.7. Conclusions

Overall, the current studies show that brain-resident mast cells respond dynamically to early life stress exposure, and that the effects of stress are dependent upon the timing of stress, the duration of stress, and the severity of stress. Moreover, mast cells in different parts of the brain are not equally affected by stress. In general, mast cell numbers and activation following stress were more dramatic in females than in males. This may mean that sex differences in the early life programming of behavior by stress may be mediated by mast cells more in females than in males. Having this knowledge about the timing of peak mast cell number in the brain may have broader implications for understanding why neuroimmune cells and their signaling impact different aspects of brain development depending upon the timing of immune perturbations.

Author contributions

AJ planned experiments, performed stress procedures, processed tissue, counted cells, and performed data analysis. CEP and MD performed experiments, counted cells, performed data analysis. CD counted cells, performed data analysis, and made figs. AH and BL performed prenatal stress experimental manipulations. KML planned experiments, counted cells, performed data analysis, made figures, and wrote the manuscript.

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