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Early life adversity drives sex-specific anhedonia and meningeal immune gene expression through mast cell activation

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Abstract

Exposure to early life adversity (ELA) in the form of physical and/or psychological abuse or neglect increases the risk of developing psychiatric and inflammatory disorders later in life. It has been hypothesized that exposure to ELA results in persistent, low grade inflammation that leads to increased disease susceptibility by amplifying the crosstalk between stress-processing brain networks and the immune system, but the mechanisms remain largely unexplored. The meninges, a layer of three overlapping membranes that surround the central nervous system (CNS)- dura mater, arachnoid, and piamater – possess unique features that allow them to play a key role in coordinating immune trafficking between the brain and the peripheral immune system. These include a network of lymphatic vessels that carry cerebrospinal fluid from the brain to the deep cervical lymph nodes, fenestrated blood vessels that allow the passage of molecules from blood to the CNS, and a rich population of resident mast cells, master regulators of the immune system. Using a mouse model of ELA consisting of neonatal maternal separation plus early weaning (NMSEW), we sought to explore the effects of ELA on sucrose preference behavior, dura mater expression of inflammatory markers and mast cell histology in adult male and female C57Bl/6 mice. We found that NMSEW alone does not affect sucrose preference behavior in males or females, but it increases the dura mater expression of the genes coding for mast cell protease CMA1 (*cma1*) and the inflammatory cytokine TNF alpha (*tnf alpha*) in females. When NMSEW is combined with an adult mild stress (that does not affect behavior or gene expression in NH animals) females show reduced sucrose preference and even greater increases in meningeal *cma1* levels. Interestingly, systemic administration of the mast cell stabilizer Ketotifen before exposure to adult stress prevents both, reduction in sucrose preference an increases in *cma1* expression in NMSEW females, but facilitates stress-induced sucrose anhedonia in NMSEW males and NH

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Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data
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females. Finally, histological analyses showed that, compared to males, females have increased baseline activation levels of mast cells located in the transverse sinus of the dura mater, where the meningeal lymphatics run along, and that, in males and females exposed to adult stress, NMSEW increases the number of mast cells in the interparietal region of the dura mater and the levels of mast cell activation in the sagittal sinus regions of the dura mater. Together, our results indicate that ELA induces long-term meningeal immune gene changes and heightened sensitivity to adult stress-induced behavioral and meningeal immune responses and that these effects could mediated via mast cells.

Keywords

Mast cells; Meninges; Early life adversity; Neonatal maternal separation; Sex differences; Inflammation; Depression

1. Introduction

Exposure to early life adversity (ELA), in the form of physical and/or psychological abuse or neglect, increases the risk of developing disorders at a multisystemic level later in life (Varese, 2012; Edwards et al., 2003; Goodwin and Stein, 2004; Jones, 2016): children with a history of ELA show higher rates of psychiatric diseases such as unipolar depression and substance abuse (Kessler et al., 1997; McLaughlin, 2012), but also increased prevalence of gastrointestinal disorders (Talley et al., 1994; Bradford, 2012), multiple sclerosis (Spitzer, 2012), migraines (Brennenstuhl and Fuller-Thomson, 2015), cardiovascular disease (Godoy, 2021), and metabolic syndrome (Rich-Edwards, 2010), among others. Since these disorders share a mechanistic origin involving inflammation (Levy, 2009; Lee and Giuliani, 2019; Martinez et al., 2018; Steven, 2019; Kaunzner, 2019; Monteiro and Azevedo, 2010), it has been proposed that ELA-driven changes in the immune system are the root underlying susceptibility to these diseases. Indeed, children (Slopen et al., 2013; Danese, 2011) and adults (Danese et al., 2007; Kiecolt-Glaser, 2011; Loucks, 2010) with a history of ELA display elevated levels of inflammatory markers. This, together with findings that ELA increases sensitivity of brain circuits involved in threat processing (Tottenham, 2011; van Harmelen, 2013; Kim, 2013), prompted the hypothesis ELA drives persistent systemic inflammatory states by amplifying the crosstalk between stress-processing brain networks and the immune system (Nusslock and Miller, 2016; Duffy et al., 2018).

A crucial interface between the brain and the peripheral immune system is the meninges, a layer of three overlapping membranes - dura mater, arachnoid, and piamater - that surround the central nervous system (CNS). Although originally thought to serve mostly as a physical protection, there is growing evidence the meninges play an active role in coordinating immune trafficking throughout the CNS (Coles et al., 2017) through a variety of mechanisms. First, vessels within the dura mater, unlike cerebral vessels, are fenestrated, and thus open to the passage of molecules present in the blood (Balin et al., 1986). Second, the meninges hold vascular channels connecting the skull bone marrow with the brain surface, allowing myeloid cell migration into the brain (Herisson, 2018). Third, the dura mater provides a network of lymphatic vessels that carry cerebrospinal fluid from the brain

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to the deep cervical lymph nodes (Louveau, 2015; Aspelund, 2015; Eide et al., 2018; Absinta, et al., 2017), which permits cerebrospinal-injected immune cells to traffic from the brain to the periphery. Finally, the meninges contain a rich population of resident immune cells, among which are mast cells (Rua and McGavern, 2018), master regulators of both innate and adaptive immune responses (González-de-Olano and Álvarez-Twose, 2018).

Mast cells are multifunctional innate immune cells characterized by their biphasic response to stimuli, which involves an early release of a wide range of preformed granule mediators (e.g. histamine, serotonin, proteases) followed by release of *de novo* synthesized mediators (e.g. cytokines). These mediators allow them to not only orchestrate the activity of innate and adaptive immune cells (González-de-Olano and Álvarez-Twose, 2018), but also modulate the activity of vascular and lymphatic endothelial cells (Kunder et al., 2011), nerves (Forsythe, 2019), and glia (Skaper et al., 2017; Dong, 2019). Meningeal mast cells, specifically, are key in maintaining blood brain barrier integrity (Sayed et al., 2010; Zhuang et al., 1996) and recruiting T-cells and neutrophils (Russi et al., 2018). Meningeal mast cells can also mediate the inflammatory responses associated with brain stroke (Sayed et al., 2010; Arac, 2014) and the activation of the trigeminal pain pathway associated with migraines (Levy et al., 2007). Importantly, mast cells can rapidly respond to stress (Ayyadurai, 2017; D'Costa, 2018; Theoharides, 1995), and ELA can persistently modify their activity and distribution (Pohl, 2017). Together, this suggests that meningeal mast cells are ideally positioned to play a key role in the brain-immune crosstalk driving persistent inflammation associated with ELA, but, to our knowledge, whether ELA has long-lasting effects on meningeal mast cell biology remains undetermined.

Here, we assessed the effects of ELA and the combination of ELA plus exposure to an adult stressor on sucrose preference behavior as well as dura mater gene expression and mast cell histology in male and female C57Bl/6J mice. We used a second hit stressor based on previous findings showing that early life adversity increases sensitivity to future stress (Peña, 2019). Our gene expression analyses included mouse interleukin 6 (*il-6*) and mouse tumor necrosis factor alpha (*tnf alpha*), two major proinflammatory cytokines associated with ELA-related diseases (Baumeister et al., 2016; Kuhlman et al., 2020); mouse vascular endothelial growth factor C (*vegf-c*), critical for meningeal lymphangiogenesis (Antila, 2017) and recently shown to be actively involved in brain immunosurveillance (Song, 2020); as well as mouse chymotryptic serine proteinase (*cma1*), mouse histidine decarboxylase (*hdc*), and mouse tryptophan hydroxylase 1 (*tph1*), which reflect different pathways of mast cell activity (Koroleva, 2019; Nurkhametova, 2020; Galli, 2015). Our histological analyses included assessment of mast cell number, activation levels, and expression of pseudopodia in different subregions of the dura mater. Pseudopodia are cytoplasmic extensions used by mast cells to engulf pathogens (Rovere et al., 2009) and/or directly communicate with adjacent cells through transgranulation (Greenberg and Burnstock, 1983; Wilhelm et al., 2005), and thus modulation of pseudopodia is an important potential readout of changes in mast cell function. Finally, as a model of ELA, we used neonatal maternal separation plus early weaning (NMSEW), a widely used model shown to increase peripheral (Amini-Khoei, 2019; De Miguel et al., 2017; Avitsur et al., 2006; Wang, 2017) and central (Amini-Khoei, 2017; Ganguly et al., 2019; Viola, 2019) inflammatory markers as well as susceptibility to develop gastrointestinal (Estienne, 2010; Li, 2016), autoimmune (Stephan

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et al., 2002), cardiorespiratory (Kinkead and Gulemetova, 2010; Wigger, 2020) and mood related disorders (Amini-Khoei, 2019; Banqueri et al., 2017; Gracia-Rubio, 2016).

2. Materials and methods

2.1. Animals

All animal procedures were performed in accordance with the regulations of the Michigan State University animal care committee. Founding C57Bl/6J breeders were obtained from the Jackson Laboratory (Bar Harbor, Me). All animals were kept in cages enriched with Nestlet and Bed-r'Nest® at 20–23 °C under a 12 h light/dark cycle with *ad libitum* access to food and water and housed in same sex groups of 3–5 individuals or in adult male–female pairs for breeding purposes (male was removed from the cage before litters were born). For all experiments, newborn litters were randomly assigned to one of two conditions: neonatal maternal separation + early weaning stress (NMSEW) or normally handled (NH). Next, each litter was divided and and no more than one female and one male from each litter was randomly assigned to one of the the following experiments: no adult stress, adult stress, or adult stress + Ketotifen.

2.2. Neonatal maternal separation plus early weaning (NMSEW)

A diagram of the experimental timeline can be found in Fig. 1A. During postnatal days (PN) 1–17, pups in the NMSEW group were daily separated from their dams and placed in individual containers for 180 min, after which they were returned to their home cage with their dam and siblings. No additional heat source was provided for the pups during the separation time. Pups were left undisturbed between separation events. On day 17, NMSEW pups were weaned and housed in same sex sibling groups. HydroGel™ was provided for the first two days after weaning to ensure proper hydration. Pups in the NH groups were left undisturbed until postnatal day 28, when they were weaned. All animals were ear punched for individual identification on weaning day. After weaning, mice from NMSEW and NH groups were left undisturbed until they reached 8 weeks old (PN 56, adult).

2.3. Adult Stress

The adult stress consisted of a shortened version of subchronic variable stress (Brancato, 2017; Williams, 2019). In a separate set of studies (unpublished), we had previously found that this version does not affect sucrose preference behavior in control animals, and therefore was chosen to minimize ceiling effects based on evidence that early life adversity increases sensitivity to future stress (Peña, 2019). Starting at PN 56 adult animals were singly housed and exposed for 10 min to either tail suspension test, restraint stress, or foot shock stress (20 total 0.45 mA shocks at random) for three consecutive days (Fig. 1A).

2.4. Mast cell stabilization

20 mg/kg Ketotifen (Cayman Chemical, Catalog #20303, first dissolved in DMSO at 25 mg/ml and then diluted 4:100 with sterile PBS) or vehicle (4:100 DMSO in PBS) were administered intraperitoneally 30 min prior each episode of adult stress (Fig. 5A) or once a day for 3 consecutive days (Fig. 3A). Ketotifen fumarate is a histamine H1 receptor antagonist and mast cell stabilizer (prevents mast cell degranulation) (Baba, 2016; Finn and

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Walsh, 2013) that crosses the blood brain barrier (Tashiro, 2006). The chemical structure of Ketotifen fumarate is available in PubChem, PubChem CID: 5282408. 20 mg/kg dose was based on previously published studies showing successful mast cell stabilization in mice (Barnes et al., 1990; Lenz, 2018; Burks, 2019).

2.5. Sucrose preference

Mice were single housed and habituated to two bottles containing water for 24 h, after which one of the bottles was replaced with 1% sucrose solution. Mice were then allowed to drink from both bottles over 48 h, which placement was switched after the first 24 h to reduce effect of potential side bias. The content weight and volume of each bottle was manually recorded for 2 days. Mice were allowed to feed *ad libitum* during this time.

2.6. Tissue collection

For gene expression analyses, animals were euthanized via cervical dislocation on the last day of sucrose preference tests. The dura mater was freshly separated from the skull and immediately flash frozen using dry ice. For dura mater mast cell staining, animals were euthanized three days after the last stress episode, and the skull caps with attached dura mater were collected and immediately placed into 10% formalin solution.

2.7. Gene expression analyses

Dura mater was homogenized using Trizol (Invitrogen), and total RNA was extracted using the RNAeasy MicroKit-Qiagen #74004. RNA integrity and concentration were assessed using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, California). Samples with RIN values equal to or above 8 were converted to cDNA using the Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (#4368814). The cDNA was used on the real-time RT (Edwards et al., 2003) Profiler PCR Array (QIAGEN, Cat. no. CLAM38697) in combination with RT (Edwards et al., 2003) SYBR®Green qPCR Mastermix (Cat. no. 330503). Each array plate contained one set of 96 wells with a panel of Qiagen designed primers for mouse IL-6, TNF alpha, Hdc, Tph1, VEGF-C, and CMA1. Positive PCR controls were included in each 96-well set on each plate. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as the assay reference gene. Each array contained samples from control and test groups.

2.8. Dura mater mast cell staining and quantification

After 24 h of postfixation with 10% formalin, dura mater was carefully separated from the skull, washed with DI water, and mounted on Superfrost plus microscope slides (Fisher Scientific). Once dry, slides were immersed for 1 h in a solution containing 0.5% Toloudine blue (t blue) in 0.5 N Hydrochloric acid, after which they were washed in DI water, dried, and coverslipped using Vectashield mounting medium. A Leica DM750 bright field Microscope with ICC50W Integrated Camera was used to take four 10x brightfield pictures per each dura mater region available (Fig. 4A,5A): parietal, interparietal, sagittal sinus, and transverse sinus. Finally, quantification was made by a blind experimenter using ImageJ. A 1000×780 μm box was used to limit counting areas:two random and non-overlapping counting areas were used per picture, 8 counting areas total per dura mater region. The

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boxes for parietal and interparietal regions were placed parallel to the sinuses, so that the sinus blood vessel and lumen were not included in the counting region. The total number of mast cells, number of active mast cells, number of inactive mast cells, and number of mast cells expressing pseudopodia were quantified per counting area. For detailed description of criteria used to classify mast cells please refer to Table 1. The level of activation was calculated as the number of active mast cells/number of inactive mast cells, and the relative expression of pseudopodia was calculated as number of mast cells expressing pseudopodia/ total number of mast cells per counting area. The data are presented as average numbers per area/mouse (total counts/number of counting areas). Since we could not find in the literature a detailed characterization of mast cells in different regions of the dura mater, our first assesment of dura mater mast cells was performed by pooling the samples from all NH groups (Fig. 4). After establishing general mast cell number, morphology, and distribution throughout dura mater regions, we proceeded to analyze the region-specific data separating by treatment groups (Fig. 5).

2.9. Statistical analyses

All figures and statistical analyses were done using Graphpad Prism 9 software. For all analyses, we used an alpha level criterion of 0.05 for statistical significance. Three-way ANOVAS were used to analyze the effects of sex, early life treatment, adult treatment, and their interaction, on the sucrose preference and gene expression data. Outliers identified by ROUT method (Q set to 1%) were excluded from analyses. Significant three way ANOVAS were followed by Fisher's LSD multiple comparisons test to detect differences between individual groups. Only significant comparisons relevant to our hypotheses are reported in the graphs, however all comparisons showing significance after Fisher LSD are provided in Supplemental Tables 1–4. One-way ANOVAs followed by Dunnett's multiple comparison tests were used to compare mast cells between dura mater regions, and two-way ANOVAs were used to analyze effects of sex, treatment, and the interaction on mast cells in specific subregions of the dura mater. Significant two-way ANOVAS were followed by Šídák's multiple comparison tests. For all analyses, effect sizes are expressed as Cohen's d and effect size correlation, r (Home, 0000).

3. Results

3.1. NMSEW plus adult stress reduces sucrose preference in females but not males, which is prevented by administration of Ketotifen

A three-way ANOVA of the sucrose preference behavior showed a significant sex*early life*adult treatment interaction ($F(2,175) = 4.78$, $p = 0.009$, Fig. 1B). While NMSEW alone did not affect adult behavior in the sucrose preference test in male or females, NMSEW + adult stress reduced sucrose preference in females compared with NH females (Fisher LSD female NH stress + veh vs. female NMSEW stress + veh $p = 0.02$, $d = 0.82, r = 0.38$). Ketotifen treatment prevented stress-induced reductions in sucrose preference in NMSEW females. In contrast, Ketotifen reduced sucrose preference in NH females exposed to stress (Fisher LSD female NH stress + veh vs. female NH stress + ket $p = 0.01$, $d = 0.92, r = 0.42$) and NMSEW males exposed to stress (Fisher LSD male NMSEW stress + veh vs. male NMSEW stress + ket $p = 0.007$, $d = 0.71, r = 0.33$).

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3.2. NMSEW induces long-term, sex-specific effects on immune-related gene expression in the meningeal dura mater

A three-way ANOVA showed a significant sex*early life*adult treatment interaction in the dura mater expression of the mast cell-specific protease *cma1* (F(2,72) = 3.9, p = 0.02, Fig. 2A). While there was no effect of NMSEW +/– adult stress in males, NMSEW alone in females increased meningeal *cma1* expression (Fisher LSD female NH no stress vs. female NMSEW no stress p = 0.01, $d = 0.97, r = 0.43$), which was further increased by the addition of an adult stressor (Fisher LSD female NMSEW no stress vs. female NMSEW stress + veh p < 0.0001, $d = 1.84, r = 0.68$, female NH stress + veh vs. female NMSEW stress + veh < 0.0001, $d = 3, r = 0.83$). Ketotifen treatment prevented the increased stress-induced *cma1* expression in NMSEW females (Fisher LSD no significant differences between female NMSEW stress + ket and any female NH groups; also female NMSEW stress + veh vs. female NMSEW stress ket p < 0.0001, $d = 3, r = 0.84$).

A significant sex*early life interaction (three way ANOVA F(1,66) = 8.52, p = 0.005, Fig. 2D) was observed for meningeal *tnf alpha* (*tnf*) expression. Similar to *cma1* expression, NMSEW + /- adult stress influenced meningeal *tnf* expression in males. However, NMSEW alone significantly increased the *tnf expression* in females (Fisher LSD female NH no stress vs. female NMSEW no stress p < 0.0001, $d = 1.5, r = 0.6$). Surprisingly, the addition of an adult stressor reduced *tnf* expression to NH levels (Fisher LSD no significant differences between female NMSEW stress + veh or female NMSEW stress + ket and any female NH groups, also females NMSEW no stress vs. females NMSEW stress + veh p = 0.006, $d = 1.1, r = 0.48$, females NMSEW no stress vs. females NMSEW stress + ket p = 0.0003, $d = 1.4, r = 0.58$).

A three way ANOVA showed a significant sex by NMSEW interaction (F(1,69) = 5.99, p = 0.02, Fig. 2F). Similar to *cma1* and *tnf*, NMSEW +/– adult stress had no effect on *vegf-c* in males, but NMSEW + adult stress significantly increased *vegf-c* expression in females (Fisher LSD female NH stress + veh vs. female NMSEW stress + veh p = 0.042, $d = 1.9, r = 0.7$).

No significant differences were found in the meningeal dura mater expression of *hdc*, *il-6*, or *tph1* (Fig. 2 B,C,E). To rule out possible basal effects of Ketotifen, we ran an additional experiment comparing the effects of Ketotifen vs. vehicle on meningeal gene expression in non-stressed animals. We found no effects of Ketotifen on expression of *cma1*, *hdc*, *il-6*, *tnf alpha*, *tph1*, or *vegf-c* (Fig. 3 B-G).

3.3. NMSEW plus adult stress induces long-term alterations in dura mater mast cell number and morphology in a region-specific fashion

Given the effects of NMSEW and ketotifen on sucrose preference and gene expression, we further characterized meningeal dura mater mast cell populations bydura mater quantifying mast cell numbers and morphology using t-blue staining. Based on our findings that exposure to a “second hit” adult stress was needed to detect behavioral differences and exacerbation of meningeal *cma1* and *vegf-c* expression, we focused on NH and NMSEW groups following exposure to adult stress.

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Since the cellular compartment of the dura mater is not homogeneous (Coles et al., 2017), we first assessed whether there were overall differences in mast cell number, activation levels, and pseudopodia expression among the major regions of the cortical dura mater: interparietal, parietal, sagittal sinus, and transverse sinus. We found that the region of the dura mater had a significant effect on all the measures. A main effect of region on the average of mast cells per mm (Edwards et al., 2003) (one way ANOVA $F(3,198) = 185.5$, $p < 0.0001$, Fig. 4B) was found, with the interparietal dura mater showing significantly higher mast cell density compared with other regions (Dunnett's multiple comparisons test interparietal vs. parietal $p < 0.0001$, interparietal vs sagittal $p < 0.0001$, interparietal vs. transverse sinus $p < 0.0001$). Similarly, there was a significant effect of dura mater region on activation level (one way ANOVA $F(3,198) = 34.96$, $p < 0.0001$, Fig. 4C), with transverse sinus showing the highest ratio of active to inactive mast cells (Dunnett's multiple comparisons test transverse vs. parietal $p < 0.0001$, transverse vs interparietal $p < 0.0001$, transverse vs. sagittal sinus $p < 0.01$). Finally, the relative expression of pseudopodia was also different between dura mater regions (one way ANOVA $F(3,198) = 138.8$, $p < 0.0001$, Fig. 4D), with the interparietal dura mater showing the highest relative expression of pseudopodia (Dunnett's multiple comparisons test interparietal vs. parietal $p < 0.0001$, interparietal vs sagittal $p < 0.0001$, interparietal vs. transverse sinus $p < 0.0001$).

Because our results demonstrate heterogeneous characteristics of meningeal mast cells depending on the region of the dura mater, all subsequent comparisons between treatments were done within specific dura mater regions. In the interparietal region of the dura mater, early life treatment affected total mast cell number and relative pseudopodia expression. Compared to NH + adult stress animals, NMSEW + adult stress animals showed significantly higher mast cell number (two way ANOVA main effect of early life treatment $F(1,48) = 5.1$, $p = 0.03$, 95% CI of difference –14.97 to –0.863 Fig. 5D) and lower relative expression of pseudopodia (two way ANOVA main effect of early life treatment $F(1,48) = 8.2$, $p = 0.006$, 95% CI of difference 0.022 to 0.126, Fig. 5F). No differences were seen between groups in the activation levels. In the parietal dura mater, there were no differences between groups in the mast cell number or pseudopodia expression, but there was an interaction between NMSEW and sex in the activation levels (two way ANOVA interaction $F(1,47) = 7.9$, $p = 0.007$, 95% CI of difference –0.593 to –0.097, Fig. 5I): NH males showed increased proportion of activated mast cells levels compared with NH females ($p = 0.003$, $d = 1.3$, $r = 0.5$), and NMSEW reduced the activation level in males only (5p = 0.01, $d = 1$, $r = 0.45$). In the sagittal sinus region, NMSEW affected the relative expression of mast cells with pseudopodia, but, in contrast to the parietal region, NMSEW animals showed higher pseudopodia expression (two way ANOVA main effect of NMSEW $F(1,48) = 7.5$, $p = 0.008$, 95% CI of difference –0.041 to –0.006, Fig. 5N). There were no differences between groups in the total number or activation levels of mast cells in this region. Finally, in the transverse sinus region, while no effects of early life treatment were found in any of the measures, there was a main effect of sex on activation levels, with females showing higher activation levels than males (two way ANOVA main effect of sex $F(1,45) = 5.74$, $p = 0.02$, 95% CI of difference –2.196 to –0.252 Fig. 5Q).

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4. Discussion

In the present study we investigated the effects of early life adversity on sucrose preference behavior, dura mater expression of inflammatory markers and mast cell histology in adult male and female C57Bl/6J mice. We found that NMSEW alone did not affect sucrose preference behavior in males or females, but increased the meningeal dura mater expression of the genes encoding for mast cell chymas CMA1 (*cma1*) and the inflammatory cytokine TNF alpha (*tnf*) in females. When NMSEW is combined with an adult mild stress (that does not affect behavior or gene expression in NH animals) females show reduced sucrose preference and adult stress-induced increases in meningeal *cma1* levels, as well as increased meningeal expression of *vegf-c*, the molecule responsible for the development and maintenance of meningeal lymphatic vessels (Breslin, 2007). Systemic administration of the mast cell stabilizer Ketotifen prior to adult stress prevented the reduction in sucrose preference and the increases in *cma1* and *vegf-c* expression in NMSEW females, but facilitated stress-induced sucrose anhedonia in NMSEW males and NH females. Histological analyses showed that, compared with males, females have increased baseline activation levels of mast cells located in the transverse sinus of the dura mater, where the meningeal lymphatics run along (Aspelund, 2015), and that, in males and females exposed to adult stress, NMSEW increases the number of mast cells in the interparietal region of the dura mater and the levels of mast cell activation in the sagittal sinus regions of the dura mater. Together, our results show that ELA induces long-lasting sex-specific behavioral and meningeal immune sensitivity to later life stress, and implicates mast cells as important drivers of this response.

4.1. Early life adversity exerts sex-specific effects on behavioral sensitivity to adult stress

To assess whether exposure to early life adversity in the form of NMSEW affects emotional behaviors in adult male and female mice, we conducted the sucrose preference test, a classical paradigm to measure anhedonia-like behavior (Liu, 2018), a core symptom of depression. We found that, while NMSEW by itself did not affect sucrose preference in males or females, it increased the vulnerability to develop sucrose anhedonia after exposure to adult mild stress in females only. This is consistent with previous preclinical studies showing that females show increased sensitivity to develop depressive-like behaviors in response to early life adversity (Mourlon, 2010; Foley et al., 2014; Rincel, 2019; Goodwill, 2019), as well as epidemiological data showing that women are at increased risk for developing stress-associated psychiatric diseases (Kessler, 2003; Kendler and Gardner, 2014; Reynolds et al., 2015). Interestingly, pre-stress administration of the mast cell stabilizer Ketotifen exerted sex-and treatment-specific effects: while it prevented the reduction in sucrose preference in females exposed to NMSEW and adult stress, it induced sucrose anhedonia in NH + stress females and NMSEW + stress males. This suggests that mast cell activity could be affecting behavior in a context-specific fashion, which could be explained by the fact that mast cell activity, granule content and interaction with other cell types can greatly vary in response to different stimuli (Huber, 2019), although the functional relationship between mast cell activity and sucrose preference behavior remains unexplored.

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4.2. Early life adversity exerts sex-specific effects on immune-related molecules gene expression

To assess whether ELA has long-term effects on meningeal immunology, we performed gene expression analysis in dura mater samples of adult males and females. We found that, in females but not males, NMSEW increased the expression of the chymase protease *cma1*, indicating increased constitutive mast cell activity (Galli, 2015). Mast cell chymase proteases have been associated with multiple, context-specific physiological and pathological functions, including maintentance of blood brain barrier permeability (Pinke, 2020), remodeling and activity regulation of lymphatic and vascular vessels (Meyer, 2017; Jenne and Tschopp, 1991), as well as pro (Zhao, 2022) and anti-inflammatory (Piliponsky, 2012) effects, although the physiological significance of increased dura mater CMA1 is not yet understood. Interestingly, the addition of an adult mild stress resulted in further increases in the *cma1* expression in NMSEW females without affecting *cma1* in any other group, suggesting that, in addition to changing basal mast cell activity, NMSEW persistently increases mast cell sensitivity to stress in females. As reported previously in models of experimental autoimmune encephalomyelitis (Pinke, 2020), the administration of Ketotifen was sufficient to normalize *cma1* expression in both unstressed and stressed NMSEW females, suggesting that the dose and administration route chosen in our study exerted long-term stabilization of dura mater mast cell hyperactivity.

Our results also showed that NMSEW increased the expression of the *tnf alpha* in females but not males. This is in line with a wealth of research demonstrating that women in general exhibit stronger inflammatory responses (Klein and Flanagan, 2016; Casimir et al., 2013; Lasselin et al., 2018), and suggests that exposure to ELA results in a sex-specific, persistent meningeal proinflammatory state that may facilitate the development of women-biased diseases such as migraines (Levy, 2009; Nurkhametova, 2020) and depression (Troubat, 2021). Surprisingly, contrary to the effect of adult stress on *cma1*, the addition of an adult stressor normalized *tnf alpha* levels in NMSEW females, suggesting a mechanistic dissociation between factors modulating NMSEW induced *cma1* and *tnf alpha*. This could be explained by alternative yet nonmutually exclusive mechanisms: first, while both *cma1* and *tnf alpha* are produced by mast cells, their storage and release are regulated by separate molecular pathways (Moon et al., 2014). Second, unlike CMA1, which is exclusively expressed by mast cells, Tnf alpha is expressed by other immune cells found in dura mater, including T-cells (Allie, 2013) and macrophages (Baer, 1998), and therefore it is possible that adult stress is exerting cell-specific effects. Interestingly, previous studies have found that mast cell chymases can degrade Tnf alpha (Piliponsky, 2012), thus, the almost two-fold increase in *cma1* expression in NMSEW females exposed to adult stress could be directly downregulating tnf alpha through elevated CMA1 activity.

Finally, NMSEW plus adult stress also increased the expression of *vegf-c* in females only. VEGF-C is the key growth factor for lymphatic vessel formation and flow (Kuhlman et al., 2020; Schmahmann, 1998) and it is also actively involved in brain immunosurveillance (Antila, 2017), suggesting a female biased increased interaction between mast cells and meningeal lymphatic vessels, but further studies are needed to answer this question.

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4.3. Early life adversity exerts region-specific effects on dura mater mast cells in animals exposed to mild adult stress

We run an additional study assessing mast cell histological properties in NMSEW and NH animals exposed to an adult stressor, which was necessary to bring out behavioral influenced by NMSEW. We separated our analyses by dura mater subregions based on the knowledge that the dura mater has location-specific mechanical and biochemical properties (Walsh, 2018), innervation (Andres et al., 1987; Noseda et al., 2019), presence of blood and lymphatic vessels (Louveau, 2015; Aspelund, 2015), and cellular immunity (Rustenhoven, 2021), as well as our data showing that mast cell populations are heterogeneous across dura mater subregions.

In the interparietal region of the dura mater, NMSEW + adult stress increased the total mast cell number while decreasing the relative expression of pseudopodia, regardless of sex. This region of the dura mater is directly involved in the early life migration of cells from the external germinal layer into the internal granule layer in the cerebellum (Zhu et al., 2004; Zhu, 2002), a brain region involved in motor function but also emotional processes (Allen et al., 1997; Schmahmann, 2000; Schmahmann, 1998). The cerebellum has been shown to be affected by ELA: studies in humans showed that physical/psychological neglect during early life can affect cerebellar volume in healthy children (Bauer et al., 2009) as well as adults suffering from obsessive compulsive disorder (Brooks, 2016), and studies in rats showed that NMSEW is associated with reduced cerebellar metabolic activity (Gutiérrez-Menéndez et al., 2020). It is however unknown whether interparietal meninges have any role on ELA-induced changes in cerebellar function; future studies assessing this relationship could yield interesting findings.

In the parietal subregion, NMSEW + adult stress resulted in reduced activation levels in males, which was surprising since we had hypothesized that NMSEW would result in overall hyperactivation of mast cells. Currently we don’t know what mechanism is underlying this response, but it could be mediated by male biased, stress-induced local upregulation of mast cell inhibitors such as corticotropin-releasing factor receptor subtype 2 (D’Costa, 2018) or other inhibitory immunoglobulin-like receptors such as Allergin-1 and CD300a (Shibuya et al., 2015). Future studies are needed to identify subpopulation-specific mechanisms of mast cell regulation.

Finally, in the sagittal sinus region of the dura mater, NMSEW increased the relative expression of mast cell pseudopodia regardless of sex. This region is where CNS-derived antigens accumulate and are presented to patrolling T-cells (Rustenhoven, 2021) and where the meningeal lymphatic network, which provides an exclusive route for draining macromolecules and trafficking immune cells from the cerebrospinal fluid to the cervical lymph nodes (Louveau, 2015; Louveau, 2018; Hu, 2020), runs along (Louveau, 2015; Aspelund, 2015). There is substantial *in vivo* and *in vitro* evidence demonstrating that mast cells can modulate T-cells’ activity (Bulfone-Paus and Bahri, 2015; Nakae, 2005), and a recent study showed specifically that meningeal mast cells play a crucial role in T-cell recruitment and induction of pathogenesis in a model of experimental encephalomyelitis (Russi et al., 2016). Although the population of mast cells throughout the body is relatively small, their role in orchestrating the function of much larger immune cell populations

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is well established (Skaper et al., 2017; Bulfone-Paus and Bahri, 2015; Dahdah, 2014), indicating that the alterations we see in relatively small mast cell populations may still have behavioral and physiologically relevant effects. Therefore, it is possible that, through pseudopodia-facilitated release of cytokines, mast cells directly modulate meningeal T-cell recruitment, and an NMSEW increased expression of pseudopodia in the sagittal sinus could indicate an enhanced interaction between mast cells and T-cells, although the current findings are insufficient to establish whether the changes in the relative activation of pseudopodia expression are biologically relevant.

In sum, our behavioral and gene expression analyses showing that NMSEW increases sensitivity to develop sucrose anhedonia in response to a mild adult stressor as well as baseline and stress-induced dural *cma1* expression -both of which are prevented with administration of the mast cell stabilizer Ketotifen-together with our histological data showing that females have elevated baseline activity of mast cells located in the dural transverse sinus region, suggest that meningeal mast cells could be involved in sex-biased sensitivity to ELA. Although more data are needed to reach meaningful conclusions, a role for mast cells mediating sex-specific effects of ELA is further supported by previous studies showing that mast cells from female vs. male mice have an increased baseline mediator storage capacity and show exaggerated mediator release in response to adult psychological stress (Mackey, 2016; Mackey, 2020), and that early life stress increases mast cell sensitivity and mast cell-hyperactivation related diseases to a greater extent in females than males (Castagliuolo, 1996). Importantly, since this study used intraperitoneal administration of Ketotifen, it is also possible that mast cells residing in other tissues play a role: previous studies have found that psychological stress can acutely activate both peripheral (Spanos, 1997) as well as dural (Theoharides, 1995) mast cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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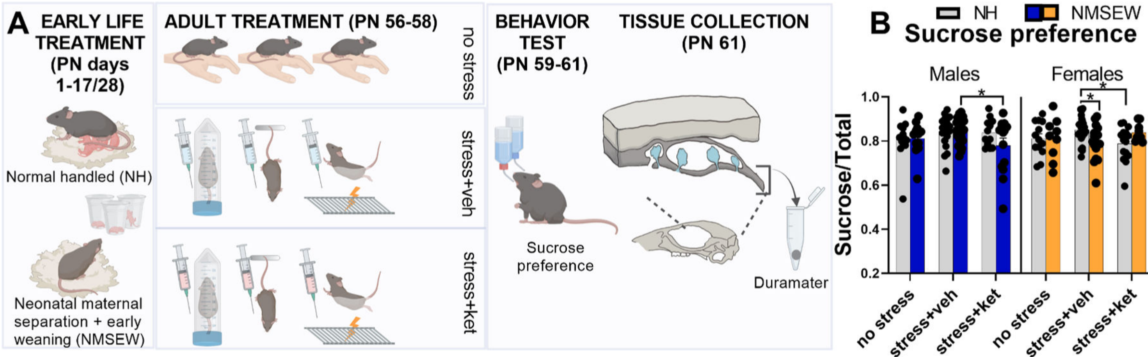

Fig. 1.

NMSEW plus adult stress reduces sucrose preference in females but not males, and administration of Ketotifen exerts sex and treatment specific effects. A. Experimental timeline. B. Three-way ANOVA assessing effects of sex, early life treatment, adult treatment and their interaction on sucrose preference behavior followed by Fisher LSD. *$p < 0.05$.

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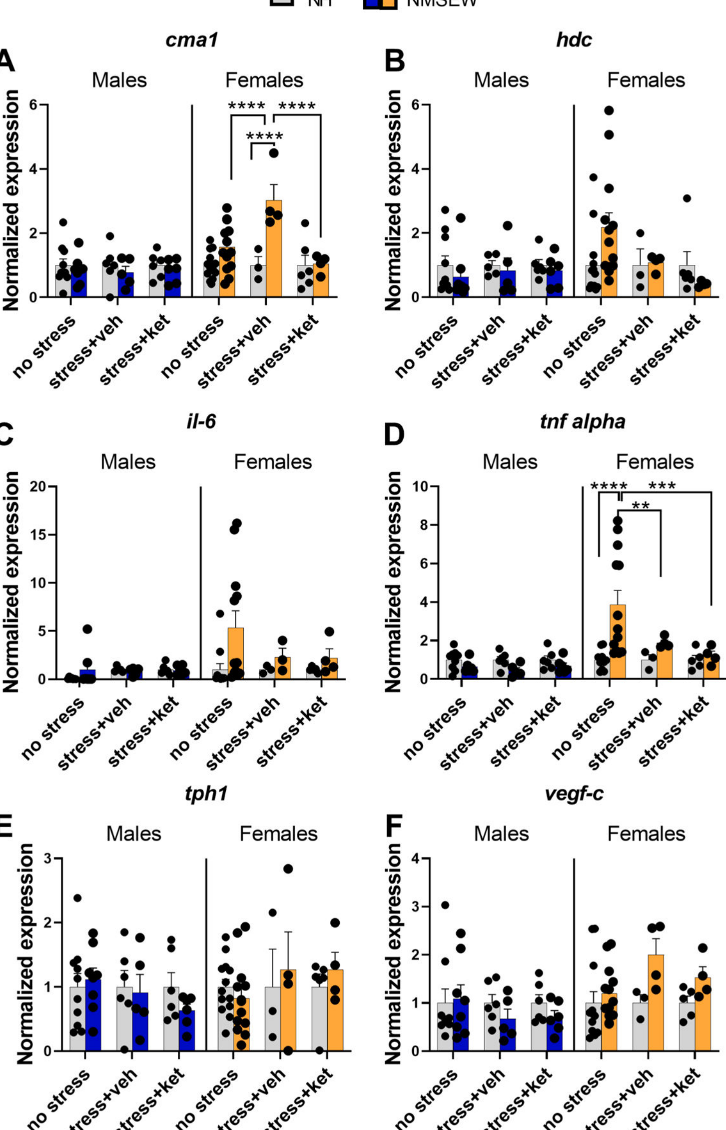

Fig. 2.

NMSEW exerts sex-specific effects on dura mater gene expressionThree-way ANOVA assessing effects of sex, early life treatment, adult treatment and their interaction on the dura mater gene expression of A. mast cell-specific protease *cma1*, B. the rate limiting enzyme for histamine synthesis *hdc*, C. the inflammatory cytokines *il-6* and D. *tnf alpha*, E. the tryptophan catalizing enzyme *tph1*, and F. the mouse endothelial growth factor *vegf-c* followed by Fisher LSD. *p < 0.05, **p < 0.01 ***p=<0.001, ****p < 0.0001.

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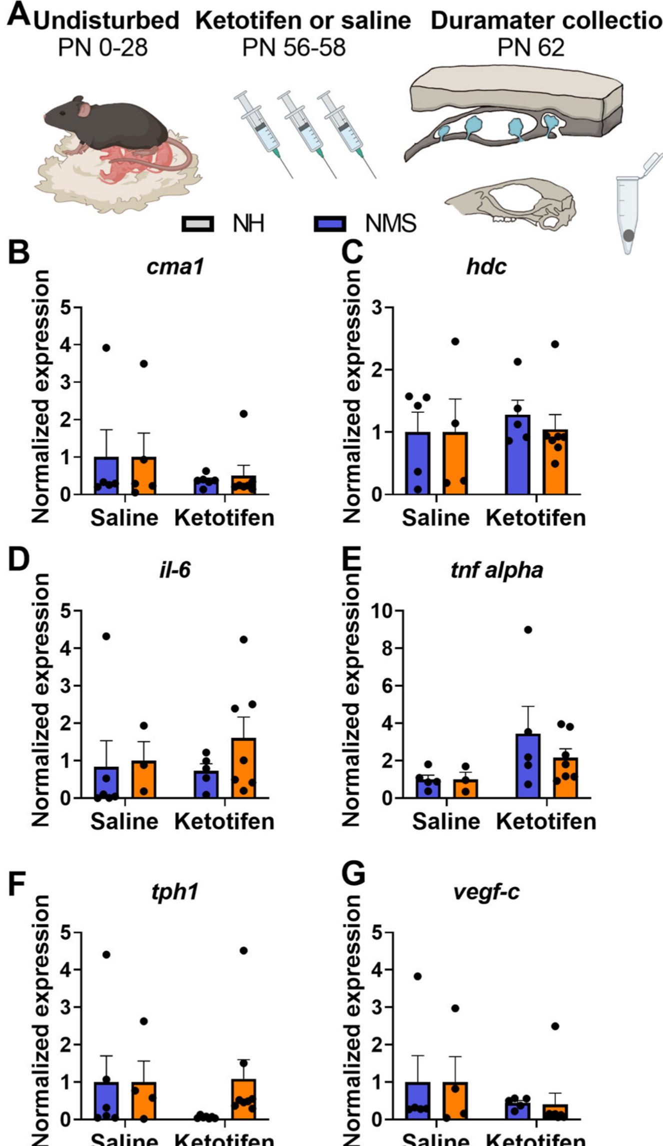

Fig. 3.

Administration of Ketotifen does not affect dura mater gene expression in NH animals. A. Experimental timeline. B-G. No effects of Ketotifen are seen on expression of *cma1*, *hdc*,*il-6*,*tnf alpha*, *tph1*, or *vegf-c* in NH males or females.

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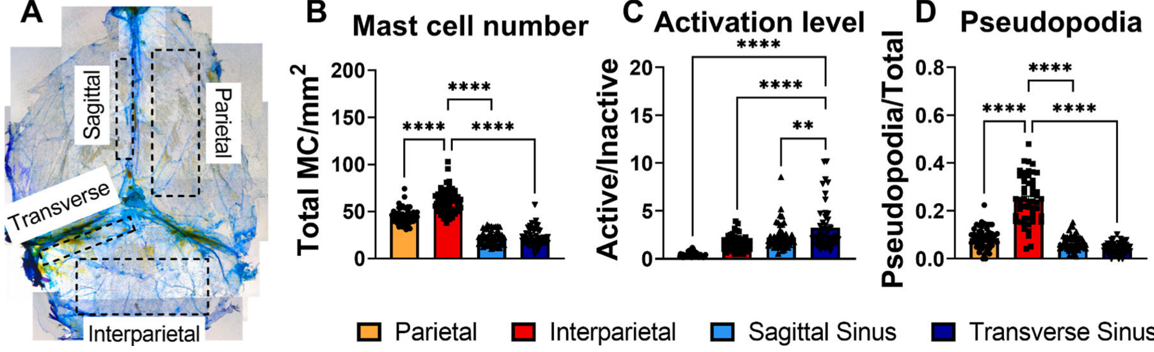

Fig. 4.

Mast cells are not homogeneous across dura mater regions. A: diagram showing regions of dura mater analyzed:: parietal, interparietal, Sagittal sinus and Transverse sinus. B: Total mast cell (MC) number per mm^2 significantly differs by dura mater region, with interparietal region showing the highest mast cell density. C: Activation level of mast cells differs by dura mater region, with transverse region showing the highest activation. D: Relative expression of pseudopodia varies by dura mater region, with interparietal region showing the highest expression. One way ANOVA followed by Dunnett's multiple comparisons test, **$p < 0.01$, ****$p < 0.0001$. n = 49–52 per group.

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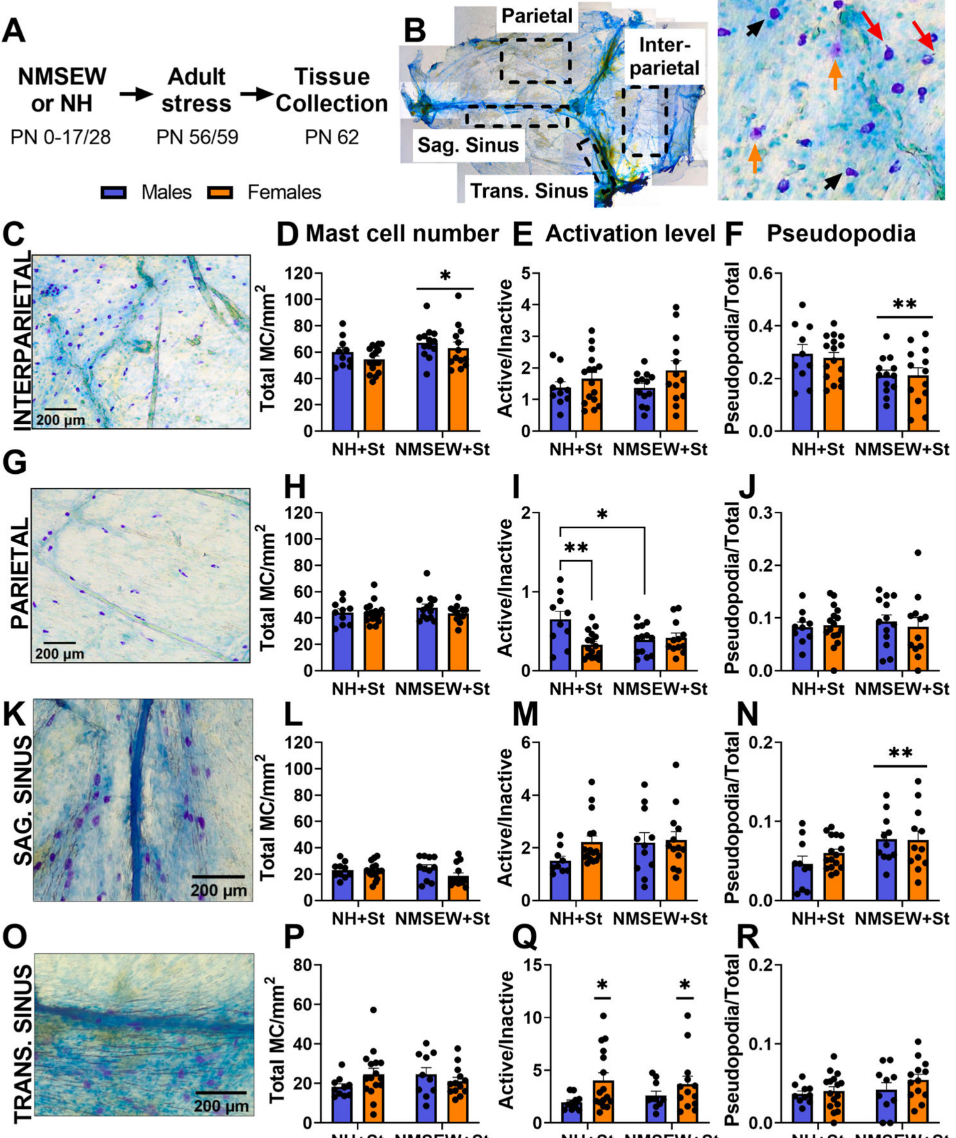

Fig. 5.

NMSEW plus adult stress induces histological changes in dura mater mast cells. A. Experimental timeline. B. Photomicrograph showing subregions of dura mater and examples of mast cell features quantified: active mast cell (orange arrows), inactive mast cell (black arrows), and presence of pseudopodia (red arrows). C, G, K, O. Photomicrograph showing examples of mast cells in interparietal, parietal, sagittal sinus, and transverse sinus regions, respectively. D, F. Compared to NH plus adult stress (NH + St), NMSEW plus adult stress (NMSEW + St) increases total mast cell number (two way ANOVA main effect of early life treatment, *p = 0.03) and reduces relative pseudopodia expression (two way ANOVA main effect of early life treatment, *p = 0.01) in the interparietal dura mater.I. In the parietal region, NH males showed increased activation levels compared to NH females (Šídak's multiple comparisons test *p = 0.003), and NMSEW reduced the activation level in males only (Šídák's multiple comparisons test *p = 0.01) N. NMSEW increases relative

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pseudopodia expression in the sagittal sinus region (two way ANOVA main effect of early life treatment, *p = 0.01). Q. Females show increased activation levels of mast cells located in the transverse sinus region (two way ANOVA main effect of sex, *p = 0.02).

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Table 1

Classification of duramater mast cells

Type	Description	Examples
Inactive (red arrows)	Mast cell is densely packed, most of the cytoplasm is dark purple, and the cell surface can be clearly delimited	
Active (green arrows)	Mast cell is not densely packed, the cytoplasm purple stain is scattered, and the cell surface is blurry	
Pseudopodia (yellow arrows)	One or more cytoplasmic extensions arise from the surface of the cell.	