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Sex differences in prefrontal cortex microglia morphology: Impact of a two-hit model of adversity throughout development

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Abstract

Neuroimmune mechanisms play critical roles in brain development and can be impacted by early life adversity. Microglia are the resident immune cells in the brain, with both sex-specific and region-specific developmental profiles. Since early life adversity is associated with several neuropsychiatric disorders with developmental pathogenesis, here we investigated the degree to which maternal separation (MS) impacted microglia over development. Microglia are dynamic cells that alter their morphology in accordance with their functions and in response to stressors. While males and females reportedly display different microglial morphology in several brain regions over development and following immune and psychological challenges, little is known about such differences in the prefrontal cortex (PFC), which regulates several early life adversity-attributable disorders. Additionally, little is known about the potential for early life adversity to prime microglia for later immune challenges. In the current study, male and female rats were exposed to MS followed by lipopolysaccharide administration in juvenility or adolescence. The prelimbic and infralimbic PFC were then separately analyzed for microglial density and morphology. Typically developing males expressed smaller soma and less arborization than females in juvenility, but larger soma than females in adolescence. MS led to fewer microglia in the infralimbic PFC of adolescent males. Both MS and lipopolysaccharide administration affected morphological characteristics in juvenile males and females, with MS exposure leading to a greater increase in soma size following lipopolysaccharide. Interestingly, effects of MS and lipopolysaccharide were not observed in adolescence, while notable sex differences in PFC microglial morphology were apparent. Taken together, these findings provide insight into how PFC microglia may differentially respond to challenges over development in males and females.

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Introduction

Early life adversity is considered a risk factor for the development of several psychiatric conditions, including anxiety, depression and drug abuse [1, 71]. Alterations in brain structure and function, such as impaired hypothalamic-pituitary-adrenal axis stress response [81] and abnormal inflammatory state [74] are commonly reported in patients that experienced adversity early in life [54, 73]. Due to its late development when compared to other areas of the brain, the prefrontal cortex (PFC) is considered a sensitive region to the negative effects of postnatal adversity [3]. Previous studies have demonstrated that rodents exposed to maternal separation (MS) paradigm showed elevated PFC proinflammatory cytokine levels and decreased performance in a PFC-dependent cognitive task [20, 57]. However, these neuroimmune alterations that may link adversity early in life with the development of mental illnesses require further characterization.

In order to connect the altered neuroinflammatory state observed after early life adversity with development of later behavioral and cognitive impairments, microglia emerged as a possible mediating factor of the changes reported after stress exposure [14, 39]. Microglia are the resident macrophages of the brain, where they regulate neuronal function during an inflammatory challenge [50]. In response to a harmful stimulus, microglial cells undergo morphological and functional changes in order to phagocytose unwelcome agents and secrete inflammatory mediators to combat neural insults [25, 69]. These cells also regulate synaptic pruning, neurogenesis and synaptogenesis, especially during early stages of development [53, 63]. Although microglia are classically described as an immune regulator, microglia can also impact cognitive processes, such as learning and memory [4, 45]. When stimulated, these cells may release immunotransmitters, such as, tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interferon- γ that can directly modulate neuronal function and impact cognitive capabilities [33, 40, 79]. Regarding the effects of early life stressful events on microglia development, studies have not been conclusive. While some evidence indicates a long-term alteration in microglial function and cell count [15, 62], others report only a transient effect of early life adversity exposure [19].

It has been suggested that a single challenge may not be sufficient to promote long-term impairments on microglia activity, but it can indeed sensitize these cells to subsequent immune challenges, such as chronic stress, high-fat diet or inflammation [52]. This process, also known as microglial “priming” causes the cells to be over-activated when exposed to a second hit of stress, which may lead to exaggerated production of pro-inflammatory cytokines [13, 39]. These elevated levels of inflammatory biomarkers can result in cytotoxicity and cell damage, which may increase the susceptibility to neuropsychiatric conditions [5, 80]. In accordance with this two-hit model, recent evidence showed that MS followed by lipopolysaccharide (LPS) injection provoked depressive-like behavior, higher levels of pro-inflammatory cytokines and microglial abnormal function in the hippocampus and PFC of male rats [78]. However, males and females may respond differently to these environmental and immune challenges [6]. Studies have reported that males tend to show more pronounced neuroinflammatory responses after an immunological challenge when compared to females [23, 28]. Considering the close relationship of microglia to the immune

response and the development of psychiatric conditions later in life [42], it is essential to understand these sex-specific characteristics. Research to date has revealed that stress exposure during development impacts prefrontal cortex development [51], and later immune signaling [31] differently in males and females. Nevertheless, to the best of our knowledge little is known with respect to the sex-specific effects of early life adversity followed by a second hit on PFC microglia function and structure.

Sex differences have been well described regarding microglial density and morphology [9, 76, 77]. A recent study reported that only females showed reduced medial PFC microglial function after chronic stress in adulthood [10]. Investigating how a two-hit model can impact the different sex-specific characteristics of microglia over development can facilitate the investigation of the relationship between neuroimmune alterations and susceptibility to neuropsychiatric disorders. This study sought to evaluate microglial alterations in response to a two-hit model of MS and LPS administration in the PFC of male and female rats. Furthermore, we investigated the effects of LPS administration during juvenility and adolescence.

Material and Methods

Animals

This study was performed with male and female Sprague-Dawley rats. Pregnant females were obtained from Charles River Laboratories (Wilmington, MA) on day 15 of gestation. The day of birth was designated as postnatal day 0 (P0). At P2, litters were culled to 10 pups (5 males and 5 females), and randomly assigned to either MS or control (Con) group. P20 brains were collected after MS (4 hours after injection of either LPS or Veh). The remaining subjects were weaned on P21 and pair-housed with a same-sex littermate until brain tissue was collected (P40). Only one rat per litter was assigned to each experimental group to avoid potential litter effects. Rats were weighed briefly on P9, P11, P15, and P20. All animals were housed in wire-topped Plexiglas cages, under 12 h/12 h light–dark cycle (lights on 0700 to 1900, approximately 332 lux) with food (ProLab 5P00) and water available *ad libitum*. Animal facility was controlled for both temperature (22–23°C) and humidity. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH) with approval from the Institutional Animal Care and Use Committee at Northeastern University.

Maternal separation

MS protocol consisted of separating pups for 4 hours per day (0900 to 1300) between P2 and P20 (see Figure 1 for Experimental Timeline). From P2 to P14, pups were allocated in individual containers with bedding material from the home cage within a thermoneutral environment at a constant temperature of 36°C, maintained by a circulating water bath. From P15 to P20, when pups were able to regulate their own body temperatures, they were individually separated in small cages with home cage bedding. The dam and the pups were kept in different rooms to prevent any dam-pup ultrasonic communication. After MS period, pups were returned to their home cage and dam. Pups in the Con group were not disturbed after P2, except for routine weekly changes in cage bedding and weighing.

Lipopolysaccharide administration

LPS was used as a mild inflammatory challenge. At P20 and P40, MS and Con rats (n = 5–8) were randomly assigned to receive an intraperitoneal (i.p.) injection of LPS (0.1 mg/kg; L2630, Sigma, St. Louis, MO) or equal volume of saline vehicle (0.9% sodium chloride). Injections took place in the morning (0900; at the time of separation in MS groups) and brain tissue was collected 4 hours after injection.

Brain tissue collection

For immunohistochemical analyses, animals (ages P20 and P40) were deeply anesthetized with CO₂ and intracardially perfused with ice-cold 4% paraformaldehyde (PFA), made from 0.1 M phosphate buffered saline (PBS; pH 7.4). The brains were removed and stored for three days in 4% PFA at 4°C, then cryoprotected in 30% sucrose solution in 0.1 M phosphate buffer. Subsequently, P20 and P40 brains were sectioned with a freezing microtome at a thickness of 40 µm and stored free-floating in freezing solution at –20°C until staining was performed.

Immunohistochemistry

To stain for microglia and macrophages in the PFC, free-floating sections (P20 and P40) were blocked in 10% normal donkey serum (017-000-121, Jackson ImmunoResearch, West Grove, PA) and incubated for 1 hour at room temperature. Sections were then incubated overnight at 4°C in primary rabbit polyclonal anti-ionized calcium binding adaptor molecule (Iba1; 019–19741, 1:1000, Wako, Richmond, VA). On the second day, sections were incubated in Alexa Fluor 488 goat anti-rabbit secondary fluorescent antibody (1:500, A27034, Invitrogen, Eugene, OR) for 1 hour at room temperature. Sections were washed between steps in 0.1 M PBS containing Triton X-100 (Fisher Scientific). Lastly, sections were mounted on positively charged slides, dried overnight, and cover-slipped with ProLong Gold antifade mounting reagent (P36930, Invitrogen). All immunohistochemical batches included subjects from each experimental condition to obviate batch-specific variances in staining intensity.

Microscopy & Image Analysis

Images were acquired at 20x magnification (image size: 440µm × 330µm) on a Zeiss Axio Imager M2 microscope system. For each animal, twelve regions of interest per hemisphere, over three sections (between bregma 4.2 and 2.2 mm), were used to determine Iba1-labeled microglia quantification and morphological differences. All analyses were performed in the PFC prelimbic (PL) and infralimbic (IL) areas using ImageJ [64]. The total number of Iba1 positive cells per image was quantified using “Multi-Point” tool. To determine morphological alterations in microglia, soma size was measured using ImageJ “Freehand line” followed by “Analyze and Measure.” Images were then converted to binary and skeletonized to further identify microglial process complexity and ramification. The “Analyze Skeleton” ImageJ plugin was used to quantify the number of branches, junctions, and average branch length, for each identified cell [2]. Images were thresholded, where all foreground cells and respective processes were visible from the background, while adhering to an optimized cutoff range. Raw data for each image were organized in descending order

by branch number and all process fragments unconnected to a cell listed after the corresponding cell number for that image were removed. Measures were then summed and normalized to the number of cells per image. All microglia in all acquired images were included in analyses. All image acquisition and analyses were performed by an experimenter blind to experimental condition. Representative photomicrographs were cropped to better convey morphological differences between groups.

Statistical Analysis

Statistical analyses were performed with SPSS software v.25.0 (SPSS, Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). Group differences were analyzed using 3-way ANOVAs with a Rearing factor (Con and MS), Treatment factor (Veh and LPS), and Sex factor (Males and Females). Juveniles and Adolescents were analyzed separately. Relevant main effects or interactions (including effects of Rearing and/or Treatment) were followed up with 2-way ANOVAs within Sex (Rearing $\times$ Treatment). We also aimed to quantify the magnitude of observed effects. To do this, effect size was calculated in SPSS for interactions and main effects from 3- and 2-way ANOVAs. Values of effect size are presented as partial eta squared (η_p^2) and categorized as small (0.01), moderate (0.06), and large (0.14), as specified in [59]. To assess group differences, Sidak multiple comparison tests were performed when appropriate (when interactions or main effects of Rearing and/or Treatment were observed) within Treatment group.

Before analysis, data were assessed for outliers using Grubbs' Test. Normality of data distribution was analyzed for all variables, using the Shapiro-Wilk Test of Normality, and homogeneity of variances was tested via Levene's Test of Equality of Error Variances. All data are visually presented as group mean $\pm$ standard error of the mean (SEM) with individual subjects represented with dots, and statistical significance was considered as $p < 0.05$.

Results

Subjects exposed to neonatal MS (or Con rearing) were treated with an i.p. injection of LPS (or saline Veh) at P20 (juvility) or P40 (adolescence). Microglia cell count and measures of morphology were analyzed for Sex, Rearing, and Treatment differences.

Microglia cell count in the prelimbic prefrontal cortex

First, we quantified the number of Iba1+ microglia in the PL of juveniles and adolescents from acquired images of fluorescently stained microglia

Total microglia cell count did not vary by Sex, Rearing, or Treatment in the juvenile (Fig. 2A) or adolescent (Fig. 2B; See Table 1 for statistical analyses) PL.

Microglia morphology in the prelimbic prefrontal cortex

Measures of morphological alterations in the PL of males and females following neonatal MS and LPS injection were quantified and analyzed separately in juveniles and adolescents.

Juvenility.—Microglia morphology was first assessed by quantifying the circumference of each cell body averaged by cell count. While a 3-way ANOVA did not reveal a Sex $\times$ Rearing $\times$ Treatment interaction in juveniles ($F_{1,40} = 0.536$, $p = 0.468$, $\eta_p^2 = 0.013$), females overall displayed larger cell bodies (Main effect of Sex: $F_{1,40} = 4.533$, $p = 0.039$, $\eta_p^2 = 0.102$; Fig. 3A). Additionally, overall MS exposure was associated with increased soma size (Main effect of Rearing: $F_{1,40} = 8.119$, $p = 0.007$, $\eta_p^2 = 0.169$). Post-hoc 2-way ANOVAs (Rearing $\times$ Treatment) were run separately for males and females, revealing that the effect of Rearing was strong in females ($F_{1,18} = 7.009$, $p = 0.016$, $\eta_p^2 = 0.280$) and non-significant, but moderate, in males ($F_{1,22} = 2.999$, $p = 0.097$, $\eta_p^2 = 0.120$). A further Sidak's multiple comparison test showed that, within groups exposed to LPS, females exposed to MS had larger cell bodies compared to Con ($p = 0.0219$).

Morphology was also measured via the number of processes per cell. As illustrated in Fig. 3B, there was a strong main effect of Sex ($F_{1,40} = 30.849$, $p < 0.0001$, $\eta_p^2 = 0.435$), where females displayed more arborized microglia when compared to males. Interestingly, LPS increased branching in both males and females (Main effect of Treatment: $F_{1,40} = 5.009$, $p = 0.031$, $\eta_p^2 = 0.111$).

The number of branch junctions per microglial cell was also quantified in juveniles. While no 3-way interaction was revealed ($F_{1,40} = 1.209$, $p = 0.278$, $\eta_p^2 = 0.029$), a main effect of Sex was observed ($F_{1,40} = 34.393$, $p < 0.0001$, $\eta_p^2 = 0.462$), where females once again demonstrated increased ramification (Fig. 3C). Juveniles exposed to LPS also displayed more junctions than those treated with Veh (Main effect of Treatment: $F_{1,40} = 4.397$, $p = 0.042$, $\eta_p^2 = 0.099$).

Lastly, the average branch length per cell was assessed in juvenile animals. 3-way ANOVA revealed no Sex $\times$ Rearing $\times$ Treatment interaction ($F_{1,40} = 0.513$, $p = 0.478$, $\eta_p^2 = 0.013$; Fig. 3D). A main effect of Treatment, however, was observed ($F_{1,40} = 7.216$, $p = 0.010$, $\eta_p^2 = 0.153$), where LPS-treated juveniles demonstrated reduced length of processes. Post-hoc 2-way ANOVAs revealed a strong decrease in branch length in males (Main effect of Treatment: $F_{1,22} = 6.278$, $p = 0.020$, $\eta_p^2 = 0.222$), that was moderate, but not significant in females ($F_{1,20} = 1.890$, $p = 0.186$, $\eta_p^2 = 0.095$).

Adolescence.—When soma size was assessed in adolescent animals, only an effect of Sex was revealed via 3-way ANOVA ($F_{1,41} = 94.839$, $p < 0.0001$, $\eta_p^2 = 0.698$; Fig. 3E). In contrast to juveniles, however, females overall demonstrated smaller cell bodies than males. Also contrasting juveniles, no main effect of Treatment ($F_{1,41} = 0.869$, $p = 0.357$, $\eta_p^2 = 0.021$) or Rearing ($F_{1,41} = 0.078$, $p = 0.781$, $\eta_p^2 = 0.002$) was observed.

A non-significant, but moderate, Sex $\times$ Rearing $\times$ Treatment interaction was apparent ($F_{1,40} = 3.191$, $p = 0.081$, $\eta_p^2 = 0.072$) on the number of processes in the adolescent PL (Fig. 3F). Additionally, females displayed more processes than males (Main effect of Sex: $F_{1,41} = 51.638$, $p < 0.0001$, $\eta_p^2 = 0.557$).

Analysis of junction number also yielded a moderate 3-way interaction (Sex $\times$ Rearing $\times$ Treatment interaction: $F_{1,41} = 3.151$, $p = 0.083$, $\eta_p^2 = 0.071$; Fig. 3G). Adolescent females

also demonstrated more junctions per cell than males ($F_{1,41} = 50.472$, $p < 0.0001$, $\eta_p^2 = 0.552$).

While no 3-way interaction was present in adolescent animals ($F_{1,41} = 0.215$, $p = 0.645$, $\eta_p^2 = 0.005$), average branch length was decreased in females (Main effect of Sex: $F_{1,41} = 120.799$, $p < 0.0001$, $\eta_p^2 = 0.747$; Fig. 3H), unlike juveniles. As in contrast to juvenile animals exposed to LPS, however, Treatment did not alter branch length in adolescence ($F_{1,40} = 1.463$, $p = 0.233$, $\eta_p^2 = 0.034$).

Microglia cell count in the infralimbic prefrontal cortex

Next, cell count was assessed in the IL to determine varying effects of Sex, Rearing, and Treatment in different subregions of the PFC.

Juvenility.—There was no effect of Sex, Rearing, or Treatment on IL microglia cell count (Fig. 4A; See Table 2 for statistical analyses).

Adolescence.—A 3-way ANOVA revealed a moderate, but non-significant, effect of Rearing on microglia cell count in the adolescent IL ($F_{1,41} = 3.795$, $p = 0.058$, $\eta_p^2 = 0.085$; Fig. 4B). Follow-up 2-way ANOVAs in males and females demonstrated an interaction between Rearing and Treatment in males ($F_{1,20} = 5.224$, $p = 0.033$, $\eta_p^2 = 0.207$), but not females ($F_{1,21} < 0.000$, $p = 0.994$, $\eta_p^2 < 0.000$) that was driven by a moderate decrease in cell count in Veh animals exposed to MS, compared to Con ($p = 0.0606$).

Microglia morphology in the infralimbic prefrontal cortex

We then quantified morphological differences in the IL following MS and LPS exposure in juvenility and adolescence.

Juvenility.—When we assessed soma size differences in the IL of juvenile animals, no Sex $\times$ Rearing $\times$ Treatment interaction was apparent ($F_{1,40} = 0.119$, $p = 0.732$, $\eta_p^2 = 0.003$); however, females overall displayed larger cell bodies (Main effect of Sex: $F_{1,40} = 4.415$, $p = 0.042$, $\eta_p^2 = 0.099$; Fig. 5A). In addition, MS increased soma size (Main effect of Rearing: $F_{1,40} = 4.787$, $p = 0.035$, $\eta_p^2 = 0.107$) that, following post-hoc 2-way ANOVAs, was determined to be driven by females (Females: $F_{1,18} = 4.901$, $p = 0.040$, $\eta_p^2 = 0.214$; Males: $F_{1,22} = 0.903$, $p = 0.352$, $\eta_p^2 = 0.039$).

3-way ANOVA revealed no Sex $\times$ Rearing $\times$ Treatment interaction ($F_{1,40} = 1.555$, $p = 0.220$, $\eta_p^2 = 0.037$) on the number of processes per cell (Fig. 5B). There was a main effect of Sex, however, ($F_{1,40} = 14.207$, $p = 0.001$, $\eta_p^2 = 0.262$), where females demonstrated longer processes than males.

The number of junctions per cell in the IL was also higher in females than males (Main effect of Sex: $F_{1,40} = 15.926$, $p < 0.0001$, $\eta_p^2 = 0.285$; Fig. 5C).

While no 3-way interaction (Sex $\times$ Rearing $\times$ Treatment interaction: $F_{1,40} = 0.621$, $p = 0.435$, $\eta_p^2 = 0.015$) was apparent in the juvenile IL on average branch length per cell, LPS significantly decreased process length (Main effect of Treatment: $F_{1,40} = 4.684$, $p = 0.036$,

$\eta_p^2 = 0.105$; Fig. 5D). Post-hoc 2-way Rearing $\times$ Treatment analyses in males and females revealed that the effect of Treatment was specific to males ($F_{1,22} = 4.765$, $p = 0.040$, $\eta_p^2 = 0.178$), not females ($F_{1,18} = 0.560$, $p = 0.464$, $\eta_p^2 = 0.030$).

Adolescence.—Analysis of soma size in the IL of adolescents revealed a strong main effect of Sex ($F_{1,41} = 61.215$, $p < 0.0001$, $\eta_p^2 = 0.599$; Fig. 5E), where females overall displayed smaller cell bodies. Unlike juveniles, however, there was no effect of Rearing ($F_{1,41} = 0.496$, $p = 0.485$, $\eta_p^2 = 0.012$).

Similar to juveniles, adolescent females demonstrated more processes per cell (Main effect of Sex: $F_{1,41} = 54.143$, $p < 0.0001$, $\eta_p^2 = 0.569$; Fig. 5F).

While there was no Sex $\times$ Rearing $\times$ Treatment interaction on the number of branch junctions per cell in the adolescent IL ($F_{1,41} = 0.455$, $p = 0.504$, $\eta_p^2 = 0.011$), there was a main effect of Sex ($F_{1,41} = 53.784$, $p < 0.0001$, $\eta_p^2 = 0.567$; Fig. 5G). Specifically, females were more arborized than males, displaying a higher number of junctions overall than males.

When average branch length was assessed, 3-way ANOVA revealed no Sex $\times$ Rearing $\times$ Treatment interaction ($F_{1,41} = 0.566$, $p = 0.456$, $\eta_p^2 = 0.014$), while, in contrast to juveniles, females demonstrated shorter processes than males (Main effect of Sex: $F_{1,41} = 96.703$, $p < 0.0001$, $\eta_p^2 = 0.702$; Fig. 5H). Also, unlike results in juveniles, Rearing did not affect length of processes ($F_{1,41} = 1.561$, $p = 0.219$, $\eta_p^2 = 0.037$).

Discussion

We assessed the impact of two challenges throughout development on microglial cell count and morphology in the PFC of males and females. Quantified measures of morphological characteristics included soma size, arborization (number of branches and junctions), and process length. Our findings suggest basal sex and age differences in arborization and soma size, as well as sex-dependent effects of early life adversity (see Figure 6 for summary).

Previous evidence in rodents suggests that the rate of colonization and maturation of microglia throughout the brain is dependent on sex [43, 66]. To our knowledge, however, the current work is the first to report morphological differences in PFC microglia in males and females throughout development. While we did not observe basal sex differences in microglia cell count, the present study suggests that female microglia are more branched than males in both juvenility and adolescence, while showing shorter processes than males in adolescence. Moreover, we found that female microglia have larger cell bodies in juvenility and smaller cell bodies in adolescence, as compared to males. Schwarz et al. [66] also found that the morphological characteristics of microglia shift differentially in males and females between neonatal and early adolescent stages. These developmental sex disparities in basal morphology may mediate functional neuroimmune sex disparities. For example, juvenile peripheral cytokine levels (specifically, IL-1 β , interleukin-6 (IL-6), and interleukin-10) have been seen to predict later-life cognitive dysfunction in females, but not males [32]. Indeed, it has been postulated that women are more at risk of developing autoimmune and neuropsychiatric disorders because of a stronger immune response [65].

Microglia, therefore, may play different roles in the male and female brain, implicating them in sex-dependent effects of adversity.

A vast literature points to microglia as mediators of neuroimmune response to adversity [8, 27]; however, the majority of this research focuses on males [65]. Microglia express glucocorticoid receptors that help regulate their response to stress [70]. Importantly, Sierra and colleagues also found that whole-brain microglia in ovariectomized females expressed higher levels of glucocorticoid receptors than males, suggesting a non-gonadal potential sex difference in adversity-induced immune response. Indeed, analysis of the functional neuroimmune response has shown sex differences in peripheral and PFC cytokine levels following MS [20, 32] and LPS [67], where males are generally more likely to express elevated levels of IL-1 β and TNF- α after stress. The current work found that microglia cell count was not affected by LPS in the PFC, which corroborates previous findings [41]. MS, however, decreased the number of microglia in the IL of adolescent males. An MS-induced decrease in cell count was unexpected, given past literature suggesting that behavioral stress in adulthood increases PFC microglia density [35, 75]. It is important to consider that microglia are in constant baseline motility [24]. This allows microglia to effectively explore the environment in order to clear cellular debris and remodel extracellular matrix [30, 50]. Past evidence has shown that early life stress exposure increased microglia motility in the adult mice brain [72], which may indicate that these cells could have been recruited to support other brain regions that were affected by our stress model.

Microglia undergo extensive morphological restructuring in response to stress that may underlie central nervous system dysfunction [49, 55]. Evidence suggests that MS increases morphological correlates of microglia abnormal activation in the male hippocampus, resulting in shorter, thicker processes and larger soma [61]. Similarly, we report that MS increased soma size in the PFC; however, process length and arborization was unaffected by rearing. In contrast with our findings in response to MS, it was revealed that both male and female microglia become more arborized after LPS injection in juvenility. Past work shows that unchallenged, ramified microglia prune newborn cells in the hippocampus [69], suggesting an important role for non-amoeboid microglia in neuronal interactions and plasticity. Indeed, microglia can be considered “partners” in neuronal processes, such as synaptic transmission [12]. Therefore, stress-induced morphological changes could potentially signify altered neuron-microglia interaction and subsequent neuronal activity. Moreover, increased branching after immune challenge was only seen in the PL. Increased branching following LPS occurring in the PL but not the IL can be viewed in the context of previous work indicating the distinct functional contributions of the PL and IL regions to corticolimbic connectivity and social behavior. Microglial hyper-ramification in the PFC is associated with alterations in neuronal activity [35], and PL connectivity with limbic regions such as the basolateral amygdala has been related to anxiogenic effects [21], while IL connectivity has been shown to promote anxiolytic effects [44]. Relatedly, abnormal function of the PL but not the IL has recently been shown to reduce social behaviors in mice [37]. Therefore, increased microglial branching in the PL could incite or indicate a change in neuronal activity within the PL that might explain reduced social investigation following LPS administration in juveniles [22].

Interestingly, juvenile males experienced microglial process shortening in both the PL and IL, suggesting a sex-specific effect of LPS on process complexity. These findings do not coincide with the traditional concept of morphological alterations, where microglia typically acquire an amoeboid morphology following a stressor [11, 47]. While LPS shortened microglia processes – a hallmark of the transition to an amoeboid shape – these processes also increased in complexity, which is more common in a hyper-ramified state [13]. Hyper-ramification, however, is generally used to describe a morphological state including both increased arborization and process length [34, 36]. Research investigating the sex-dependent effect of adversity in the PFC found that both acute and chronic restraint stress increased microglia ramification in females, but not males [10]. We have, however, previously demonstrated non-traditional morphological alterations in microglia [29]. Specifically, MS paired with food restriction decreased arborization in females and increased process length in males, with food restriction alone increasing process length in females. Similar to the current work, increased arborization and process length did not occur together and, at times, seem to oppose each other. This could indicate a transitional state between ramified and activated morphological states. Importantly, there is evidence that microglial phagocytosis and cytokine secretion can occur without concurrent morphological changes [48, 56, 60]. The findings reported here provide further evidence that the relationship between environmental insults, microglia morphological states, and functional activation is context-dependent. Care must, therefore, be taken when generalizing stress-induced morphological alterations to the diverse roles of microglia.

Past literature shows that a secondary immune challenge can potentiate microglial response following an initial hit [16, 18]. While we did not observe an interaction between Sex, Rearing, and Treatment, we report that females exposed to both neonatal MS and juvenile LPS had larger cell bodies than females exposed only to LPS. This increase in soma size was not apparent after MS alone and was not observed in males. We, therefore, cannot confidently report an additive effect of MS and LPS exposure; however, our findings may suggest a sex-specific priming effect of MS on microglia morphology in juvenility. Increased soma size was also apparent in microglia following chronic unpredictable stress with enhanced expression of the danger-associated molecular pattern, High-mobility group box 1 [26]. This suggests that chronic stress induces the potentiation of the neuroimmune response that occurs in conjunction with altered soma size. While some evidence suggests that functional sensitization to a secondary stressor is more common in males than females [38, 58], female microglia have also been found to experience abnormal function following multiple hits of stress [23]. Specifically, females expressed similarly elevated IL-1 β , TNF- α , and IL-6 levels after inescapable tail shock and LPS. This suggests microglia may play an important role in the brain's response to adversity in both sexes, however as recent work has supported [68], sex-specific effects of stress exposure on inflammatory mechanisms may drive differential neuronal outcomes in males and females.

Certain limitations should be considered when interpreting the current work. First, rats in this study were injected with small dose of LPS (0.1 mg/kg), which may affect the results. Our goal was to induce mild systemic inflammation; therefore, we avoided higher doses that have been shown to increase mortality rates in treated animals [11]. While studies report neuroimmune response using much higher doses [58], others have used the same [17] – and

even smaller [23] – doses to potentiate behavioral and immune response to following an initial stressor. Second, the length of time between LPS injection and brain removal also varies throughout the literature. In the present work, we harvested brains four hours after LPS treatment, which may explain smaller effects and lack of sensitization in our data. Evidence suggests, however, that altered neuroimmune state can be observed two [46], three [23], four [58], and 24 hours [41] after treatment. Last, the functional significance of morphological changes must be addressed. There is limited understanding of whether microglia morphology can be used as a predictor of function [7]. While we did not assess functional consequences of multiple hits of adversity, our findings indicate a continuing need to further investigate the link between functional and morphological activation states.

The findings presented here provide leading evidence for sex- and age-dependent effects of adversity on microglia reactivity. We report basal sex differences in microglia morphology and non-traditional morphological responses to adversity that was specific to juvenility, which may reflect developmental transitory states. Importantly, there were sex differences in microglial response to MS and LPS that may underlie gender differences in adversity-induced neuropsychiatric disorders. Taken together, these findings illustrate the complex nature of microglial morphology, demonstrating the dynamic nature of adversity-induced morphological alterations in both sexes.

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Abbreviations:

Iba1	Ionized calcium binding adaptor molecule
IL	Infralimbic prefrontal cortex
IL-1β	Interleukin-1 β
IL-6	Interleukin-6
LPS	Lipopolysaccharide
MS	Maternal separation
P	Postnatal day
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PL	Prelimbic prefrontal cortex
TNF-α	Tumor necrosis factor- α

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Highlights

- Female PFC microglia are more arborized than males in juvenility and adolescence
- MS by itself increased microglial soma size in the PL and IL of juvenile males and females
- LPS administration increased arborization in the PL of juvenile males and females
- LPS increased soma size to a higher degree in females exposed to MS

Figure 1.

Experimental timeline. (A) Male and female Sprague-Dawley rat pups were exposed to maternal separation (MS) or control (Con) rearing daily from postnatal day (P) 2 to 20. At P20 or P40, lipopolysaccharide (LPS) or saline vehicle (Veh) was administered via intraperitoneal injection. Four hours after injection, brains were harvested and stored until immunofluorescent staining. (B) Example diagram of prelimbic (PL) and infralimbic (IL) prefrontal cortex (PFC). Images were acquired and quantified between bregma 4.2 and 2.2 mm. (C) Full-size, uncropped representative image of Iba1+ staining (image size: 440µm × 330µm; scale bar = 50µm), as well as a representation of the skeleton analysis procedure (scale bar = 10µm. Raw images were thresholded and binarized, followed by skeletonization. The “Analyze Skeleton” ImageJ plugin was then used to quantify morphological measures in skeletonized images.

Figure 2.

Microglia cell count in the prelimbic (PL) prefrontal cortex (PFC) following maternal separation (MS) or control (Con) rearing and lipopolysaccharide (LPS) or vehicle (Veh) in juvenility and adolescence. There were no effects of Sex, Rearing, or Treatment on cell count in juveniles (A) or adolescents (B). (C) Representative photomicrographs of Iba1+ microglia. $n = 5-8$ animals/group; scale bar = 100 μm .

Figure 3.

Microglia morphology in the prelimbic (PL) prefrontal cortex (PFC) following maternal separation (MS) or control (Con) rearing and lipopolysaccharide (LPS) or vehicle (Veh) injection in juvenility and adolescence. (A) Juvenile females overall had larger soma sizes than males and MS increased perimeter of cell bodies. Specifically, microglia cell bodies in females exposed to both MS and LPS were larger compared to Con females treated with LPS. Both Sex and Treatment affected the number of processes (B) and junctions (C) per cell, where females and animals treated with LPS were more arborized than males and Veh animals, respectively. (D) Males, but not females, treated with LPS had shorter processes than (Veh) juveniles. (E) In adolescence, females had smaller somas than males, but were unaffected by MS or LPS. Females also had more processes (F) and junctions (G) per cell than males. (H) Neither Rearing nor Treatment affected average branch length, but females overall had shorter processes than males. Lines outside of graphs signify a main effect of Sex, Rearing, or Treatment. *: $p < 0.05$; ****: $p < 0.0001$; $n = 5-8$ animals/group.

Figure 4.

Microglia cell count in the infralimbic (IL) prefrontal cortex (PFC) following maternal separation (MS) or control (Con) rearing and lipopolysaccharide (LPS) or vehicle (Veh) injection in juvenility and adolescence. (A) In juvenility, cell count was unaffected by Sex, Rearing, and Treatment. (B) Rearing had a moderate effect on adolescent cell count, where microglia in males experienced a moderate decrease in number after MS. (C) Representative photomicrographs of Iba1+ microglia. $n = 5-7$ animals/group; scale bar = 100 μm .

Figure 5.

Microglia morphology in the infralimbic (IL) prefrontal cortex (PFC) following maternal separation (MS) or control (Con) rearing and lipopolysaccharide (LPS) or vehicle (Veh) injection in juvenility and adolescence. (A) Both Sex and Rearing affected soma size in juvenile, where females and animals exposed to MS displayed larger cell bodies. While there were no effects of Rearing or Treatment, Sex affected arborization in juvenility, where females had more processes per cell (B) and junctions per cell (C) than males. (D) There was a main effect of Treatment on average branch length, driven by male juveniles treated with LPS that had shorter processes than Veh. Neither MS nor LPS exposure changed the number of processes (F) or junctions (G) per cell, females overall displayed more arborized branches than males. (H) Sex, but not Rearing or Treatment, affected the length of processes, where females had shorter branches per cell than males. Lines outside of graphs signify a main effect of Sex, Rearing, or Treatment. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.0001$; $n = 5-7$ animals/group.

Figure 6.

Cartoon representation of main observations. In juvenility, females overall had larger cell bodies and more processes and junctions than males. In adolescence, males instead showed larger cells bodies; however, females had more highly arborized branches that were shorter than males. When exposed to LPS in juvenility, microglia became more arborized in both males and females (in the prelimbic, but not infralimbic cortex), while males showed shorter processes following LPS administration. LPS alone did not alter morphology when administered in adolescence. Neonatal MS exposure alone resulted in increased soma size in juvenility and juvenile females exposed to both MS and LPS had larger cell bodies than females exposed to LPS alone. (Note that microglial morphology is represented with exaggerated features to provide clarity of differences).

Table 1.

Statistical analyses for microglia cell count in the juvenile and adolescent PL.

Comparison	Juvenility			Adolescence		
	<i>F</i>	<i>p</i>	η_p^2	<i>F</i>	<i>p</i>	η_p^2
Effect of Rearing	$F_{1,40} = 1.059$	0.310	0.026	$F_{1,41} = 1.381$	0.247	0.033
Effect of Treatment	$F_{1,40} = 2.305$	0.137	0.054	$F_{1,41} = 1.627$	0.209	0.038
Effect of Sex	$F_{1,40} = 1.638$	0.208	0.039	$F_{1,41} = 0.715$	0.403	0.017
Rearing $\times$ Treatment Interaction	$F_{1,40} = 1.590$	0.215	0.038	$F_{1,41} = 1.950$	0.170	0.045
Rearing $\times$ Sex Interaction	$F_{1,40} = 0.189$	0.666	0.005	$F_{1,41} = 0.099$	0.754	0.002
Treatment $\times$ Sex Interaction	$F_{1,40} = 0.014$	0.905	< 0.0001	$F_{1,41} = 0.871$	0.365	0.021
Rearing $\times$ Treatment $\times$ Sex Interaction	$F_{1,40} = 0.427$	0.517	0.011	$F_{1,41} = 2.040$	0.161	0.047

Table 2.

Statistical analyses for microglia cell count in the juvenile IL.

Comparison	Juvenility		
	<i>F</i>	<i>p</i>	η_p^2
Effect of Rearing	$F_{1,40} = 1.640$	0.208	0.039
Effect of Treatment	$F_{1,400} = 2.589$	0.115	0.061
Effect of Sex	$F_{1,40} = 1.278$	0.265	0.031
Rearing $\times$ Treatment Interaction	$F_{1,40} = 2.252$	0.141	0.053
Rearing $\times$ Sex Interaction	$F_{1,40} = 0.565$	0.457	0.014
Treatment $\times$ Sex Interaction	$F_{1,40} = 0.242$	0.626	0.006
Rearing $\times$ Treatment $\times$ Sex Interaction	$F_{1,40} = 0.048$	0.828	0.001