RESEARCH ARTICLE

The impact of early life immune challenge on behavior and microglia during postnatal development

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Sexual dichotomy exists in the development, presentation, and course of many neuropsychiatric disorders, including anxiety. Anxiety disorders are one of the earliest psychiatric illnesses to manifest and a role for immune system programming of the developing CNS has emerged in relation to anxiety. Adult rodents neonatally exposed to an immune challenge exhibit increased anxiety-related behaviors. The objective of this study was to determine the impact of postnatal immune challenge on behavior and microglia during the postnatal period and in adulthood. Mice were administered lipopolysaccharide (LPS; 0.05mg/kg, i.p.) or saline on postnatal days (P) three and five. Anxiety-related behavior was assessed during early development on P15 and P21, and re-assessed in adulthood at 10 and 12 weeks. Results reveal sex-specificity in the temporal emergence and phenotypic profile of behaviors displayed by LPS-treated mice. Male LPS-treated mice exhibited reduced exploratory in early development (P15 and P21) that persisted into adulthood. Female LPS-mice exhibited increased anxiety-like behaviors in the EPM in adulthood. These results demonstrate a role for interactions between sex and the immune system in shaping the developmental trajectory of exploratory and anxiety-like behaviors.

Keywords: lipopolysaccharide; immune-brain; neurodevelopment; sex differences; elevated plus maze; open field; light/dark test; neonatal; microglia

Abbreviations: LPS, lipopolysaccharide; P, Postnatal day; i.p., intraperitoneal; SAL, saline; OF, open field; EPM, elevated plus maze; PVN, paraventricular nucleus; DR, dorsal raphe; PBS, phosphate buffered saline; TX, Triton-X.

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Introduction

Early-life adversity is an established risk factor for the later development of psychiatric disorders. Both clinical and preclinical studies have demonstrated that early stressful life events can markedly alter adult stress-reactivity and predispose to anxiety[1]. Indeed, rodent models of early-life manipulation, such as maternal separation, result in a modification of adult anxiety-related behavior and associated neurobiological substrates with prolonged periods of maternal separation leading to enhanced fear and anxiety in adult mice[2] and dysregulation of the HPA axis[3, 4]. Many of the long-term behavioral and molecular consequences of early-life adversity exhibit a sexual dichotomy[2] demonstrating the importance of sex in influencing the development of adult

stress-related psychopathology. There are reported sex differences in the clinical onset, presentation, course, and/or treatment response of many psychiatric disorders with a proposed neurodevelopmental origin including depression and anxiety [5].

There has been a growing body of literature linking the immune system to stress-related behaviors [6]. Early immune challenge has emerged as a model of early-life adversity with a distinct focus on the impact of immune activation during CNS development on stress-related phenotypes [7]. Postnatal challenge with lipopolysaccharide (LPS), a cell wall constituent of gram-negative bacteria, during the first week of life (postnatal day (P)3 and 5) enhances the adult neuroendocrine and behavioral response to stress[8, 9] and increases basal or stress-induced anxiety-related behaviors [10]. Male and female rodents have different susceptibility to the acute[4] and long-term consequences of LPS challenge[3, 11] with a recent study demonstrating clear differences in anxiety-related behaviors of adult male and female LPS-challenged rats[12].

Whereas the majority of the early immune challenge literature has focused on alterations detected during adulthood, the possibility that anxiety-like behavior emerges earlier in development has not been fully explored. This has clinical relevance, given that anxiety disorders are often the first psychiatric illness to manifest [13]. In mice, the time window between the first and third postnatal week is essential to the establishment of anxiety. Conditional knockout of the serotonin (5-HT)-1A receptor[14] and transient inhibition of the 5-HT transporter[15] during this time modulates anxiety-related behaviors with differences in emotional-reactivity emerging as early as the third week of life[16, 17]. Here we examined the behavioral phenotype of LPS-treated male and female mice in early postnatal development and in adulthood.

Previous work in our laboratory has shown that serotonergic and CNS stress-related gene expression profiles are altered during the third and fourth post-natal week of life in mice following postnatal LPS challenge[17, 18]. In the present study, we examined microglia in stress-related brain regions during the same postnatal window. Microglia, the resident immune cell of the CNS, have a well-established role in the CNS response to injury and infection, however, recent work has focused attention on the importance of microglia to postnatal brain development [19,20]. In particular, microglial-synapse interactions have been demonstrated in the developing hippocampus[21,22], visual cortex[23], and the retinogeniculate system[24]. Microglial functions during postnatal development include synaptic pruning, synapse maturation, and structural remodeling [19]. Exposure to LPS during the first week of postnatal life has established long-term consequences on brain development and behaviour, and yet, the role of

microglia in this trajectory has not yet been considered.

Materials and methods

Animals

CD-1 mice were bred in-house from mice (8-10 w) obtained from Charles River and the resulting male and female offspring were used in this experiment (n=11 litters). Postnatal day (P) 0 denotes day of birth. Litters were culled to a maximum of 12 on P1. Mice were weaned on P21 and housed by sex with 3-4 mice per cage. *All experimental procedures were approved by the Animal Research Ethics Board, McMaster University in accordance with the guidelines of the Canadian Council on Animal Care.*

Injections

On P3 and P5, mice were given an intraperitoneal (i.p.) injection of either 0.05 mg/kg lipopolysaccharide (E. coli LPS; Sigma, St. Louis, MO) in 50 µl/g or an equal volume of saline (SAL) between 07:00-9:00 h. Mice received LPS on both P3 and P5 or mice received SAL on both P3 and P5; entire litters received the same treatment. During injections dams were removed from the home cage and returned once all pups had received an injection, maternal separation did not exceed 10 min.

Behavioral Testing

Behavior was tested during postnatal development including open field testing at P15 and light/dark analysis at P21. At 10 weeks of age, mice were retested in the open field (OF) and at 12 weeks of age mice were tested in the elevated plus maze (EPM). Testing was performed during the animal's active cycle between 2000-0300h.

Open Field

P15 pups were separated from the mom and brought into a dimly lit testing room in their home cage. Pups were individually placed into Plexiglas chambers (Smart Cage Rack System; field dimensions: 9.5" wide x 18.0" long; Kinderscientific, Poway, CA) consisting of infrared beams that measured horizontal and vertical activity for 15 min. This system was interfaced to a PC computer running MotorMonitor software (Kinderscientific, Poway, CA). After all pups had undergone testing, they were returned to the mom as a group; maternal separation did not exceed 1 h. At 10 weeks of age, mice were allowed to habituate in a dark outer room before being placed into a brightly lit arena (Smart Cage Rack System). Distance travelled and time spent in the center versus periphery was recorded by MonitorMonitor software for 45 min.

Light/Dark

On P21, behavior was assessed in a 10 min light/dark test using the Smart Cage Rack system and behavior scored
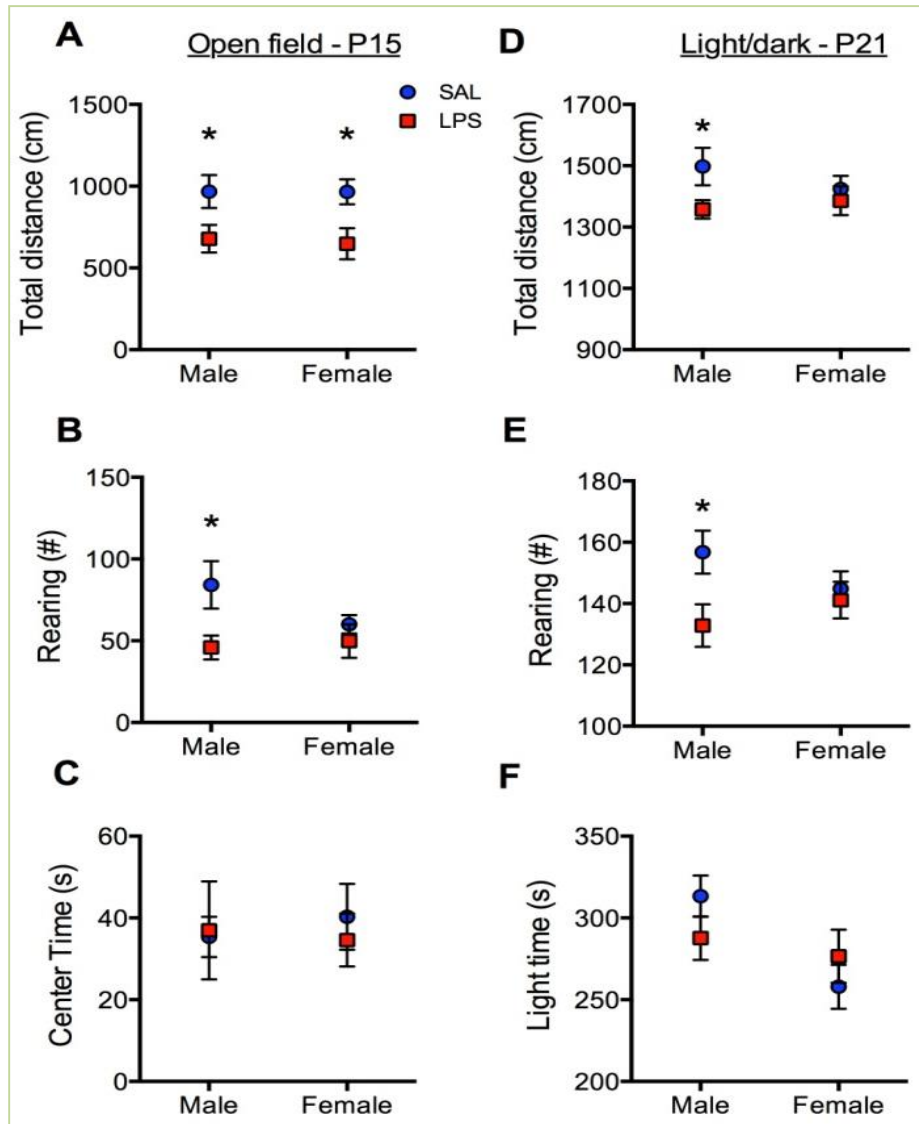
Figure 1. LPS challenge on postnatal day 3 (P3) and P5 resulted in changes in activity and exploratory behaviors in LPS-treated mice tested in the open field (OF) at P15 and in the light/dark box (L/D) at P21. Activity, measured by total distance travelled in the OF (A) was reduced in both male and female LPS-treated mice at P15. This difference persisted in male LPS-treated mice, as noted by the reduced total distance travelled in the L/D box at P21 (D). Exploratory behavior, measured by rearing, was reduced in male LPS-treated mice in both tests (OF - B; L/D - E), whereas female LPS-treated mice did not show this difference. Differences in anxiety-like behavior, measured by time spent in the aversive zone (center in OF - C, light side of L/D - F) were not present in LPS-treated mice compared to SAL-treated mice. Mean ± SEM is shown, *$p<0.05$, LPS compared to SAL.

by MotorMonitor software. Each testing apparatus was equipped with a black Plexiglas chamber with lid (50% of total apparatus) that created a dark side with the other side of the apparatus open creating the light side; a small doorway allowed free exploration between the light and dark sides.

Elevated Plus Maze (EPM)

At 12 weeks of age, behavior was assessed using a 5 min EPM test (Kinderscientific). This device is a '+' shaped black Plexiglas maze, consisting of two opposing closed arms (flanked by 15cm opaque black Plexiglas walls), and two open arms (no walls). Mice were placed in the center of the plus maze and behavior scored using MotorMonitor software[25].

Tissue Collection

At P14, P21 and P28, brains were rapidly removed following decapitation, frozen in -60°C isopentane, and stored at -70°C until cryostat sectioning. At each time
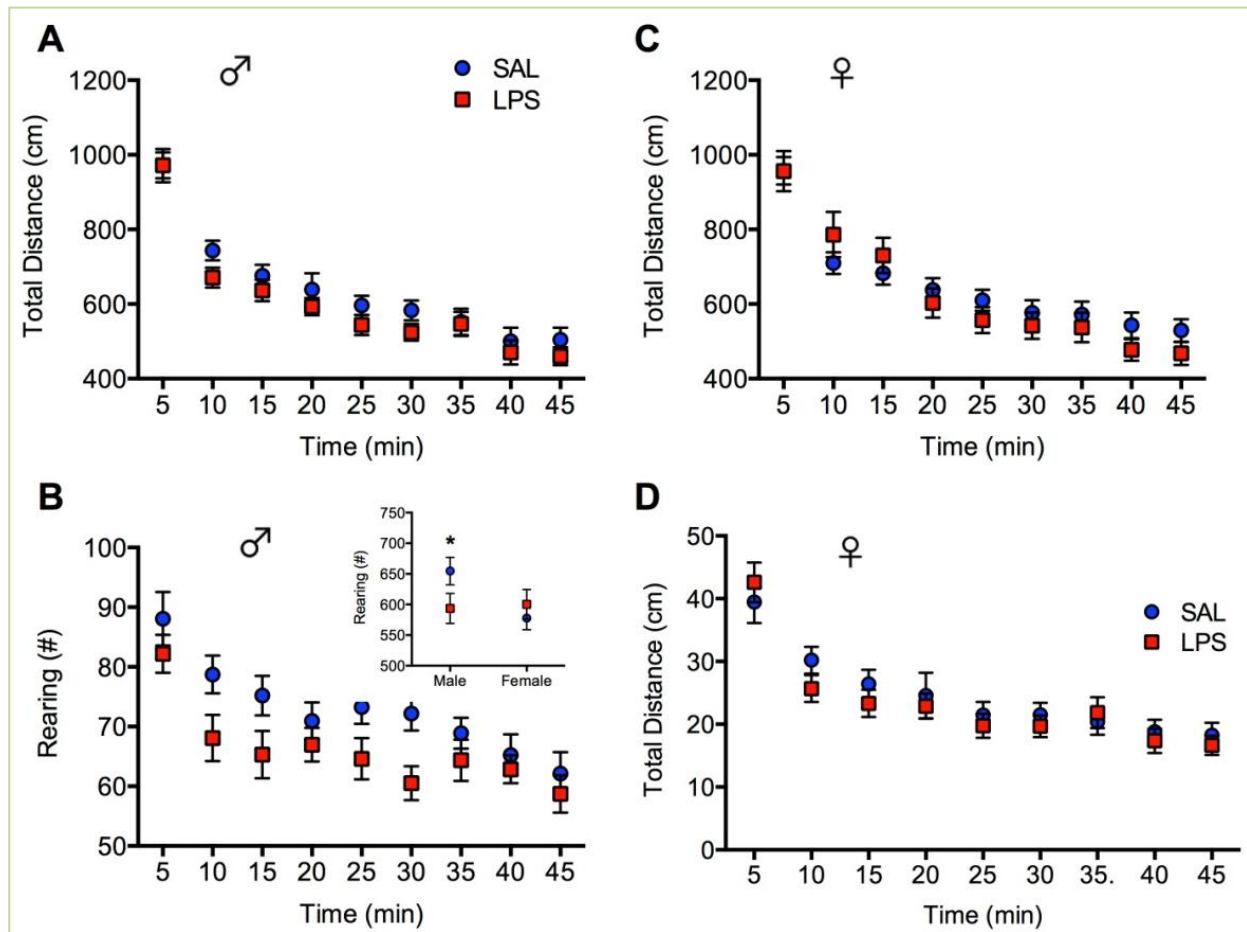
Figure 2. Open field behavior was retested in adulthood. No main effect of treatment was observed in distance travelled (A - male, C - female) or in rearing (B - male, D - female) when examined at 5 min intervals in the open field, however, LPS-treated male mice showed a significant reduction to total rearing over the 45 minute testing (B-inset). This was not observed in LPS-treated female mice. Mean ± SEM is shown at 5 min intervals over the 45 min testing period.

point, pups from different litters were included (n=3/sex/treatment/time point). A series of 12 μm coronal sections were collected through the paraventricular nucleus (PVN Bregma -0.70 to -0.94 mm), and dorsal hippocampus (HIP Bregma -1.46 to -1.94 mm), and the dorsal raphe (DR; Bregma -4.48 to -4.60 mm), according to the stereotaxic atlas of Paxinos and Franklin[26]. For all regions collected, reference sections stained with Cresyl Violet were taken at regular intervals and compared to a mouse stereotaxic atlas for identification. Sections were mounted onto gelatin-coated glass slides, dried on a slide warmer (30°C) for 10 min, and stored at -35°C until processing.

Immunohistochemistry

Tissue sections were thawed and incubated in phosphate buffered saline (PBS) then treated with fresh 4% paraformaldehyde in PBS for 10 min. Following 3 five min washes with PBS, the tissue was incubated in 0.3% hydrogen peroxide in PBS for 10 minutes, to block endogenous peroxidase activity. Following 3 additional five min washes in PBS, the tissue was blocked in 10% Casein (Vector, Burlington, ON, Canada) solution in 1XPBS 0.1%Triton-X (TX) for 1 hour. The tissue was then incubated overnight at 4°C in primary antibody rat anti-mouse F4/80 (Abcam, Toronto, ON, Canada) at a 1:200 dilution. F4/80 is a marker of mouse macrophages, including mouse microglia. After 2 five min washes in PBS-TX, the tissue was incubated at room temperature for one h in biontinylated anti-rat secondary antibody (Vector, Burlington, ON, Canada) at a 1:500 dilution. Following 2 five min PBS washes, the tissue was incubated for 30 min with the avidin-biotin peroxidase complex (Vectastain ABC kit; Vector, Burlington, ON, Canada) prepared according to manufacturer instructions. Following 2 five min PBS washes, the tissue was incubated in diaminobenzidine contained in the DAB Peroxidase Substrate Kit (Vector, Burlington, ON, Canada) for 4 min to develop the peroxidase color. Tissue was then dehydrated using increasing concentrations of ethanol, cleared using xylene, and mounted.
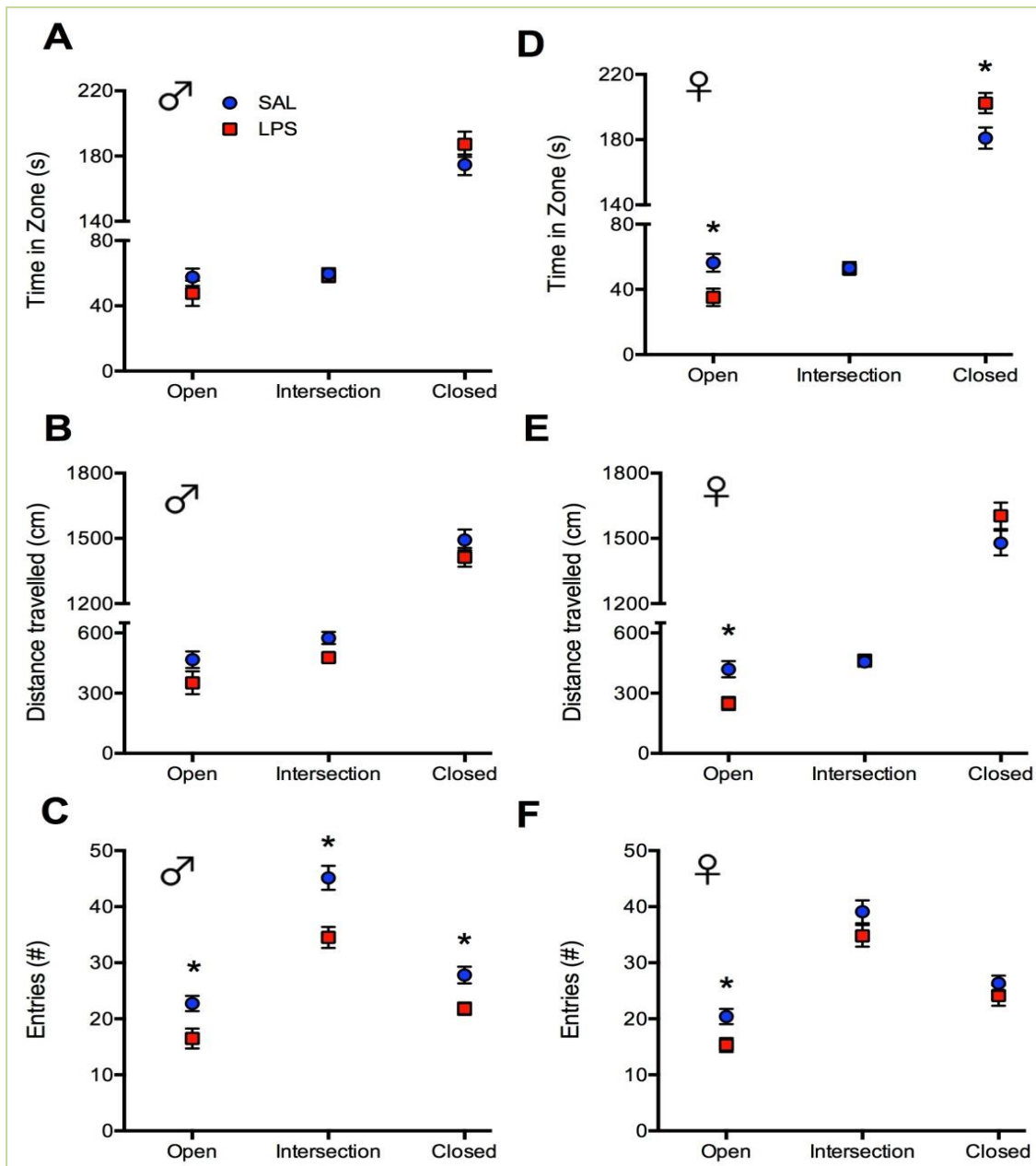
Figure 3. Postnatal LPS treatment resulted in changes in EPM behavior in adulthood. Male and female LPS-treated mice show different behavioral changes in the EPM.   Male LPS-treated mice showed no differences in time spent (A) or distance travelled (B) in EPM zones compared to male SAL-treated mice, however, male LPS-treated mice showed a significant reduction in # of zone entries into all zones of the EPM (C). Female LPS-treated mice showed increased anxiety-like behavior compared to female SAL-treated mice, including reduced open arm time (D), reduced distance travelled in the open arm (E), and reduced # of open arm entries. Mean ± SEM is shown, *$p < 0.05$, LPS compared to SAL.

Visualization and Image Analysis

Stained tissue sections were visualized using a Zeiss Axioskop 2 Plus microscope and photographed using the AxioCam MRc microscope camera (Zeiss, Toronto, ON, Canada). Tissue sections containing the DR were imaged at 10X magnification, while tissue sections containing PVN or HIP were imaged at 20X magnification. Images were

taken in colour, and analyzed using AxioVision microscope software (Zeiss, Toronto, ON, Canada). Cells counts were obtained for each tissue section.

Statistics and Data Analysis

Postnatal behavioral data were analyzed using two-way ANOVA with treatment and sex as factors. Adult OF data were analyzed using repeated measures ANOVA with
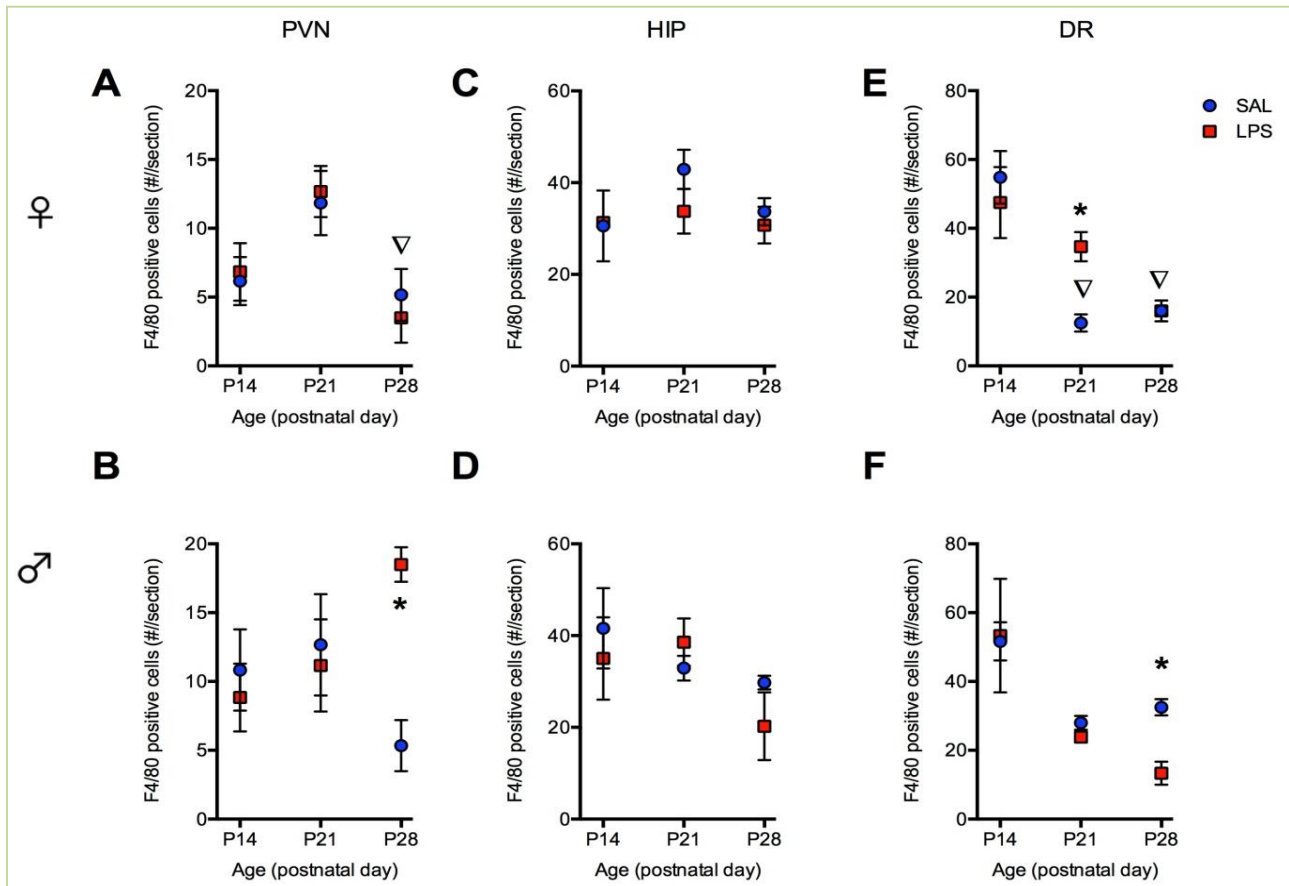
Figure 4. Postnatal LPS treatment resulted in treatment and sex-specific changes in microglial number in the paraventricular nucleus of the hypothalamus (PVN) and the dorsal raphe (DR). In the PVN, LPS-treated males showed an altered trajectory of development with an increase number of microglia in the PVN at P28 compared to SAL-treated males (B). No differences were observed between LPS- and SAL-treated females in the PVN, however, LPS-treated females had significantly less microglia than LPS-treated males (inverted triangles in panel A denote sex difference between panel A and B). No differences in microglia number were observed in female (C) and male (D) mice in the hippocampus (HIP). In the DR, LPS-treated female mice showed an increased number of microglia at P21 (E), whereas LPS-treated male mice showed a decreased number of microglia at P28 (F). In addition, SAL-treated female mice showed a significant reduction in the number of microglial cells at P21 and P28 in comparison to SAL-treated male mice (inverted triangles in panel E denote sex difference between panel E and F). Mean ± SEM is shown, *p<0.05, LPS compared to SAL.

treatment and time as factors. EPM data were analyzed using two-way ANOVA with treatment and EPM zone as factors. Bonferroni multiple comparisons tests were used for post-hoc comparisons. All values are expressed as mean ± SEM. Microglial data were analyzed using repeated measures univariate analysis with sex and treatment as factors. Independent t-tests were used for pairwise comparisons. All values are expressed as mean ±SEM.

Results

Impact of LPS on behavior in the preweaning/ weaning period

Open field behavior was assessed at P15. LPS administration reduced the total distance travelled in the OF by both male and female pups (Fig.1A; F(1,96)=10.4, p=0.0012). Male LPS pups also showed reduced rearing in

the OF at P15 (F(1,97)=5.47, p=0.017), however no differences were observed in rearing when LPS female pups were compared to SAL female pups (Fig. 1B). In addition, no difference in time spent in the center of the OF was observed in LPS pups compared to SAL pups (Fig. 1C). These data reveal locomotor/activity deficits in LPS-treated male and female pups at P15, and a male-specific exploratory deficit following LPS treatment.

Light/dark behavior was tested on P21 (day of weaning). A main effect of treatment (F(1,93)=4.13, p=0.047) on total distance travelled was found in the L/D test at P21. In particular, male LPS-treated pups showed reduced total distance travelled compared to male SAL-treated pups (Fig. 1D). In addition, in male LPS-treated pups, the rearing deficits observed at P15 in the OF persisted in the L/D test at P21 (Fig. 1E). In contrast, there were no differences in

total distance travelled or rearing in female LPS-treated pups compared to female SAL-treated pups in the L/D test. No treatment differences were observed in time spent in the light chamber for either male or female pups in the L/D test at P21 (Fig. 1F).

Impact of LPS on behavior in the adulthood

OF behavior was retested at 10 weeks of age for 45 min. Total distance travelled and rearing data are presented in Fig. 4. No long-term treatment-related differences were observed in total distance travelled in the OF for male (Fig. 2A) and female (Fig. 2C) mice. A main effect of treatment on rearing in the OF was observed for male mice $(F(1,441)=19.2, p<0.0001)$. While post hoc tests did not reveal specific time point differences between LPS- and SAL-treated mice, a significant reduction in total rearing was observed in male LPS-treated mice compared to male SAL-treated mice (Fig. 2B inset), but no difference was observed in female mice (Fig. 2B inset).

EPM behavior was tested at 12 weeks of age. Female LPS-treated mice showed increased anxiety-like behavior in the EPM (Fig. 3). There was a main effect of EPM zone in female mice $(F(2,150)=481.8, p<0.0001)$ and an interaction effect $(F(2,150)=8.1, p=0.0004)$. A significant decrease in time spent in the open arms and a significant increase in time spent in the closed arms was observed in female LPS-treated mice compared to female SAL-treated mice (Fig. 3D), whereas no differences were observed in the time spent in the intersection (Fig. 3D). Distance travelled in the open arm was also reduced in female LPS-treated mice compared to female SAL-treated mice (Fig. 3E; main effect of zone $F(2,150)=461.2, p<0.0001$; interaction $F(2,150)=5.8, p=0.0038$). The number of open arm entries was also reduced in female LPS-treated mice (Fig. 3F; main effect of zone $F(2,150)=62.4, p<0.0001$; main effect of treatment $F(1,150)=7.3, p=0.0076$) revealing across these outcome measures a long-term impact of early life exposure to LPS on anxiety-like behavior.

Male LPS-treated mice showed reduced exploratory behavior in the EPM (Fig.3). There were no differences in time spent in EPM zones in male LPS-treated mice compared to male SAL-treated mice (Fig. 3A). There was a main effect of treatment on distance travelled in EPM zones in male LPS-treated mice $(F(1,147)=7.99, p=0.0053)$. While posthoc analysis did not reveal a significant difference in distance travelled in the different EPM zones (Fig. 3B), there was a significant difference in total distance travelled in the EPM in male LPS-treated mice $(2242.12\pm52.7))$ compared to male SAL-treated mice (2534.8 ± 63.5). No difference in total distance travelled in the EPM was observed in female mice (LPS - 2313.9 ± 72.6; SAL - 2354.2 ± 71.1; main effect of treatment $F(1,99)=6.35$, $p=0.013$). In addition, there was a main effect of treatment on EPM zone entries in male mice $(F(1,147)=31.6,$

$p<0.0001)$ with male LPS-treated mice showing significantly less entries into all EPM zones compared to male SAL-treated mice (Fig. 3C).

Impact of LPS on microglia cell number

Microglial cell number was determined in the PVN, HIP, and DR (Fig. 4) at P14, P21, and P28. We have previously reported changes in serotonergic and stress-related gene expression during the postnatal window in these brain regions in LPS-treated mice[17, 18]. Microglia cell numbers in the PVN were low in general in our tissue sections, with univariate analyses showing a significant effect of sex $(F(1,35)=5.97, p=0.022)$, a significant interaction between sex and age $(F(2, 35)=3.74, p=0.039)$ and a significant interaction between sex, treatment, and age $(F(2, 35)=4.36, p=0.024)$. In female mice, there was an increase in microglia cell number at P21 that returned to P14 levels at P28. There was no difference detected between LPS-treated and SAL-treated female mice in PVN microglial number (Fig.4A). In male SAL-treated mice, microglia cell number showed a similar trajectory to female SAL-treated mice (Fig. 4 A and B). In contrast, male LPS-treated mice showed an increase in microglia cell number compared to male SAL-treatment mice at P28 revealing an altered trajectory for microglia development in male mice in the PVN. Accompanying this treatment effect in male mice was a significant difference between male and female LPS-treated mice at P28 (Fig. 4 A and B). In the HIP, microglia cell number was more abundant that in the PVN but remained stable over this postnatal window (Fig. 4 C and D). No differences in microglia cell number were detected in the HIP related to age, sex, or treatment. In the DR, microglia cell number was highest at P14 and reduced further at P21 and was lowest at P28. Our ANOVA analysis revealed a significant effect of age on microglia number $(F(1,35)=27.5, p<0.0001)$. Independent t-test was used for additional pairwise comparisons. In female mice, LPS-treated mice showed a delayed trajectory compared to SAL-treated mice with increase microglia cell number at P21 in the LPS-treated group (Fig. 4E). Interestingly, microglia cell number differed between male and female SAL-treated mice with increased microglia cell number in male SAL-treated mice at P21 and P28 compared to female SAL-treated mice. A further difference was detected, as LPS-treated male mice showed reduced microglia cell number at P28 compared to SAL-treated male mice. Overall, these data show microglia numbers during postnatal development were affected by postnatal age, sex, treatment, and brain region.

Discussion

The current study determined the impact of early immune challenge on the behavioral profile of mice in the immediate postnatal period and in adulthood. These results

provide important evidence to demonstrate a sexual dichotomy in the emergence and presentation of immune-related changes in behavior and support a growing body of literature linking the immune system to stress-related behaviors. Early immune activation was associated with a sex-specific temporal and phenotypic emergence of anxiety-related behavior - a result that extends previous reports of sex differences in the acute and long-term effects of neonatal LPS exposure [3, 4, 11]. Specifically, behavioral characterization through development revealed differential timing for the onset of behavioral effects in male and female LPS-treated mice. Male LPS-treated mice showed exploratory deficits that emerged early in life and persisted into adulthood. Female LPS-treated mice showed increased anxiety-like behavior that had an adult-onset. This differential timing is interesting in light of the increased incidence of psychiatric diagnoses in women after puberty[27] and an increased incidence of psychiatric illness in boys prior to puberty[28]. Furthermore, the distinct behavioral profile displayed between female and male LPS-mice recapitulates the different clinical features that men and women present with across a range of anxiety disorders in terms of incidence, age of onset, and clinical presentation[5, 29].

Sexual dimorphic changes in behavior following early immune challenge have been reported. In mice, postnatal immune challenge on P4 and P5 disrupted avoidance learning in adulthood in male LPS-treated mice but not in female mice[30]. In addition, female LPS-treated mice showed altered motor responses to a second adult immune challenge compared to SAL-treated mice.[30] Our data showed that LPS challenge specifically influenced exploratory behavior in male mice, which is similar to previous work in rats [11]. In that report, neonatal LPS challenge in rats resulted in reduced distance travelled, reduced horizontal movements, and reduced vertical movements in the OF in response to a second adult LPS challenge, an effect that was not observed in females[11]. Recently, prenatal exposure to immune challenge was shown to influence the development of social play behavior, specifically in male and not female mice, an effect that was accompanied by central changes in vasopressin mRNA expression in the medial amygdala of male mice[31]. These data demonstrate that the domains of behavior that are altered following immune challenge is different in male andfemale rodents and therefore it is important to include both sexes in studies of animal behavior. Further, more studies are needed to identify sexually dimorphic molecular changes that underlying sex-specific alterations in behavior.

The mechanism by which peripheral immune activation may disrupt developing stress-related behavior may involve direct immune-CNS signaling. LPS is known to act on brainstem neuromodulatory systems, leading to activation of serotonin neurons in distinct sub-regions of the dorsal raphe[32, 33]. Indeed, previous work in our lab has shown that early LPS challenge disrupts CNS serotonin signaling specifically during the third week of development in mice[17] - a time window crucial for the development of anxiety neurocircuitry[16, 17]. In addition to this, immune activation may indirectly affect the developing brain via HPA activation and the increase in glucocorticoids that occur in response to LPS administration. The possibility of early life immune challenge reprogramming the peripheral system to influence adult immune response has also been suggested[34].

Glial-neuronal signaling may also be a key mechanism that influences long-term changes following early immune challenge as postnatal exposure to *E.coli* infections has been shown to lead to immediate and long-term changes in microglia[35]. Certainly, microglia are emerging as a key regulator of CNS development and in particular, evidence is building that sex differences in microglia during early postnatal life may contribute to long-term sex differences in brain function.[36-38] Microglia numbers are increased in the early postnatal period, compared to adolescence and adulthood, with peak numbers occurring in the second week of postnatal life with a gradual reduction in number in the following weeks[39]. The morphology of microglia in the first postnatal week in rodents is consistent with activated ameboid microglia found in the adult CNS[24, 38, 39]. Most interestingly, differences in both morphology and number have been reported in postnatal rat pups with increased microglial numbers in male pups by P4 compared to female pups[38]. The brain's inflammatory response to central infection also develops in the first week of life as the microglial and leukocyte infiltration response to central administration of LPS is greatest at P7 in rat pups compared to P0, and the kinetics of the response are distinct from that observed in the adult rat[39]. Notably, we observed sex differences in microglial number in the dorsal raphe between SAL-injected male and female mice and treatment effects in male mice in response to LPS in both the PVN and DR. These findings are consistent with the observation that there are increased numbers of microglia in male mice during the first week of postnatal life and increased immune sensitivity in male mice in response to immune challenge during this postnatal window[38, 40]. Previous work has shown that in the absence of immune activation, immune genes are upregulated during postnatal development in several brain regions, and sex differences in levels of gene expression have been reported[38]. Our work using the early immune challenge model in mice, showed changes in gene expression in response to early life immune challenge during the third week of postnatal life prior to the treatment effects we see in microglial number, suggesting that changes in microglial number and activation state may be an important long-term change in the CNS and contribute to the long-term sex differences in behavior we and others

have observed.

Overall these results add to the growing body of literature that demonstrates that immune-brain crosstalk and CNS immune signaling involving microglia are critical mediators of CNS development and brain function. In addition, these data support a role for immune signaling in the development of sex differences in CNS circuitry and behaviour. Systematic studies are needed across different species and strains to pull together the related studies and to better understand the developmental mechanisms involved.

Conflict of interest

The author(s) declare that they have no Conflicting interests.

Acknowledgements

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