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Full-length Article

Neonatal lipopolysaccharide treatment alters hippocampal neuroinflammation, microglia morphology and anxiety-like behavior in rats selectively bred for an infantile trait

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ABSTRACT

Disruptions in homeostasis, such as the induction of inflammation, occurring during the neonatal period of development often produce changes in the brain, physiology, and behavior that persist through the life span. This study investigated the potential effects that an immune challenge delivered during neonatal development would have on anxiety behavior and stress reactivity later in life within a selectively-bred strain of rat. The rats have been bred for multiple generations to display either high or low anxiety-like phenotypic behavior. On postnatal day (P)3 and P5, male and female neonates were injected with saline or lipopolysaccharide (LPS). Brains were collected from a subset of neonates following injections. At P7, one male and one female per litter were tested for ultrasonic vocalizations (USVs). In adulthood, remaining litter mates were tested on the open field apparatus and the elevated zero maze (EZM) or on the EZM following 3 days of acute stress. Overall, we saw differences between the High and Low lines in neonatal anxiety-like behavior (USVs), neonatal peripheral immune response, adult anxiety-like behavior on the EZM, and adult anxiety-like behavior after stress induction, such that the High line rats display significantly more anxiety-like behavior than the Low line. Furthermore, we observed an effect of neonatal LPS during the neonatal peripheral immune response (e.g., increased inflammatory cytokine expression) and adult anxiety-like behavior on the EZM. We also observed an effect of sex within the anxiety-like behavior of LPS-treated adults exposed to stress paradigm. The combined results shed light on the relationships between neural development, early-life inflammation and anxiety throughout the lifespan.

1. Introduction

The immune system has a critical role in brain function in development, health, and sickness. For example, the primary immunocompetent cells of the brain, microglia, are increasingly implicated in the etiology of many neuropsychiatric disorders (Rico et al., 2010). Immune activation during neonatal development, a time of enormous maturation and increased vulnerability to environmental factors such as a bacterial infection (McGowan, 2015) has been shown empirically to affect both microglial function within the brain (Williamson et al., 2011), adult anxiety-like behavior within the rat model (Sominsky et al., 2012), disease susceptibility, reactivity to stress, and rates of neuropathologies (Bilbo and Schwarz, 2009; Karrow, 2006; Mouihate et al., 2010; Schwarz and Bilbo, 2012; Spencer et al., 2006; Williamson and Bilbo, 2013; Williamson et al., 2011).

Lipopolysaccharide (LPS) exposure during the neonatal period causes a rapid release of pro-inflammatory cytokines and chemokines in the periphery and within the brain, a dramatic increase in circulating corticosterone levels, an increase in TLR signaling molecule expression and other lasting physiological changes (Schwarz and Bilbo, 2012), peaking at about 2 h post-injection (Bilbo et al., 2005; Ortega et al., 2010; Schwarz and Bilbo, 2011; Xu and Ling, 1994). Furthermore, neonatal treatment with LPS leads to HPA axis hyperresponsiveness in adult behavioral paradigms and altered glucocorticoid responsiveness to stress (Granger et al., 1996; Hodgson et al., 2001; Shanks et al., 1995).

Anxiety disorders, depression, schizophrenia, posttraumatic stress disorder, and Rett Syndrome have all been linked to alterations in immune function (Abazyan et al., 2010; Ashwood et al., 2010, 2011; Careaga et al., 2010; Garay and McAllister, 2010; Muller and Ackenheil, 1998; Pace and Heim, 2011; Schwarz and

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Bilbo, 2012; Watanabe et al., 2010). Specifically, anxiety disorders rank among the most common neuropsychiatric disorders diagnosed within the United States, resulting from both familial inheritance and environmental factors (Hanamsagar and Bilbo, 2015; Weissman et al., 1996; Werner et al., 1999; Wickramaratne and Weissman, 2000) and causing clinically significant distress or impairment in social, occupational, or other important areas of functioning (American Psychiatric Association, 2013).

In a model of phenotypic anxiety in the rodent, we utilized two lines of selectively bred rats. Rodents emit ultrasonic vocalizations (USVs) to indicate positive and negative affect and to encourage or discourage prosocial behavior. The vocal response to isolation of rat pups consists of high-frequency ultrasonic vocalizations (USVs) within the range of 40–50 kHz (Allin and Banks, 1972; Brunelli, 2005b). Neonates isolated from their mothers emit USVs as immediate indicators of stress and defense, especially between the ages of postnatal days (P)3-18 (Brunelli, 2005b; Dichter et al., 1996; Hofer, 1996). Upon isolation, the stress response of the pup includes USVs as well as physiological changes within the HPA axis and noradrenergic activity (Brunelli, 2005b). Ultimately, the mother-infant interaction is a behavioral feedback loop that can be strongly modulated by pup USVs.

Selective breeding of rats based on their USVs upon isolation produced two distinct lines: pups with the highest rates of USV emission are the High line and pups with the lowest rates are the Low line (Brunelli, 2005a,b). The phenotypic differences are stable and extend beyond neonatal USVs to adult behavior on the elevated plus maze (EPM) (Dichter et al., 1996), and the Open Field test (Martinez et al., 2015). Based on this stable phenotypic difference between lines, we assessed the effects of neonatal immune challenge with LPS on affective changes in neonates (USVs) and adults (open field, elevated zero maze) as well as molecular changes in the peripheral and central immune and stress responses (e.g., interleukin-1β protein and mRNA expression, corticosterone levels, and microglial morphology) (see Fig. 1 for experimental timeline).

2. Materials & methods

2.1. Animals

Animals were N:NIH Norway rats derived from the 51st generation of High and Low Lines (Zimmerberg et al., 2005) raised in the animal facilities of Williams College. Rats were kept under standard conditions with a 12:12 light:dark cycle (lights on, 6AM) at 22℃ with 50% relative humidity. Rats had unlimited access to food and water. For breeding, females were mated with males from the same line. Pregnant females, determined by the presence of a vaginal plug, were separated from the male and individually housed in plastic cages (45 cm L × 25 cm W × 15 cm H). Dams were kept under the same standard housing conditions and had continuous access to food and water. The day of birth was denoted as P0. Offspring were weaned at 25 days of age, and pair-housed with a same-sex sibling within a plastic cage (45 cm L × 25 cm W × 15 cm H).

All housing and testing procedures were approved by the Williams College Institutional Animal Care and Use Committee.

2.2. Neonatal injections

At P3 and P5, chosen due to their significance in microglia development, the pups in both High and Low lines were separated from the dams and placed into a circular glass dish (20 cm D × 10 cm H) with bedding. All injections were done between 13:00 and 15:00. Their weights and sexes were recorded. Then they received a subcutaneous injection of either endotoxin-free saline (SAL) or *E. coli* – derived lipopolysaccharide (LPS) dissolved in saline (Fig. 1A). All pups in a given litter were given the same treatment and each litter was randomly assigned to a condition. Pups in the SAL group received 0.1 mL of saline, while pups in the LPS group received a dose of 50 μg/kg of LPS. To minimize heat loss, each litter was kept within a dish that rested on a heating pad during the separation from their dam. Following injections, pups were returned to the dams.

2.3. Experiment 1: maternal care assessment & USV testing

2.3.1. Maternal care assessment

Following P3 and P5 injections, pups were returned to the dams. As a measure of maternal care, we recorded the time (s) required for the dam to return all of her pups to the nest. Rather than continuing to disrupt maternal care by continuous observation, this procedure correlates the speed to create a nest with the quality of maternal care.

2.3.2. Ultrasonic vocalization (USV) testing

On P7, 1 male and 1 female pup were randomly selected from each litter (with 6 or more pups) and placed into a circular glass

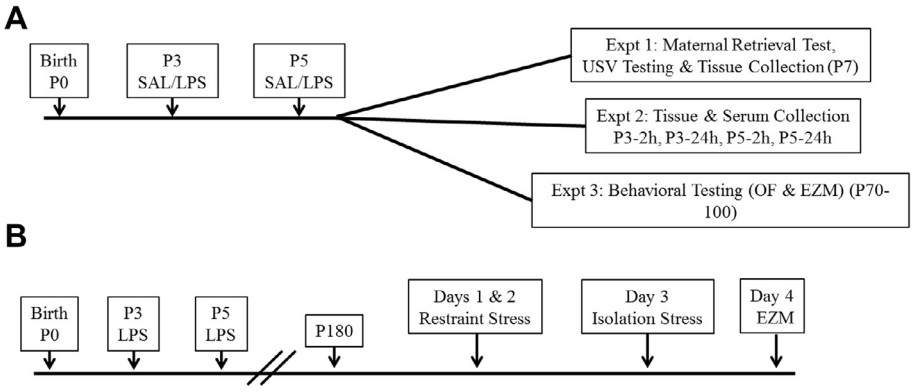

Fig. 1. Experimental timelines for all experiments. (*A*) Experiments 1, 2 and 3 all included rats that experienced neonatal treatment (SAL or LPS) at both P3 and P5. Experiment 1 measured maternal retrieval latencies and USVs in P7 pups. In experiment 2, after saline ($N = 80$) or LPS ($N = 79$) injections, one male and one female from each litter were euthanized at each time point post-injection ($N = 159$). In experiment 3, rats were aged to P70-P100 when adult anxiety-like behavioral assessments were conducted: open field test (OF) and elevated zero maze (EZM). (*B*) In experiment 4, all rats were treated as neonates with LPS at P3 and P5 and aged to P180 at which point they underwent three days of a stress paradigm ($N = 30$) or control handling ($N = 29$). On the fourth day, all rats were tested for anxiety-like behavior on the EZM and immediately euthanized for tissue harvest.

with bedding. The pups were transported into the habituation room and placed individually in a plastic container on a heating pad on a low setting, where they habituated for 10 min. After this brief maternal separation, pups were individually taken to the dark testing room and placed in an identical circular glass dish, this time with no bedding. This glass dish was located beneath an S-25 ultrasound bat detector (Ultra Sound Advice, London) set to detect signals at 50 ± 5 kHz. The ultrasonic vocalizations (USVs) for each pup were recorded for two minutes by listening through headphones attached to the detector and every individual vocalization was counted using LabChart to produce a total number of vocalizations for each pup ($N = 79$).

Pups were returned to small plastic containers on the heating pad until their brains could be collected. Following rapid decapitation, brains were post-fixed in 4% paraformaldehyde in 0.1 M PB for 48 h and cryoprotected in 30% sucrose for at least 48 h prior to tissue processing.

2.3.3. Tissue processing and immunohistochemistry

Using a freezing cryostat, the intact P7 brains were sliced into 30 μm thick coronal sections, taking 5 series of 50 sections through the anterior-posterior plane of the hippocampus. Sections were stored at 4 °C in 0.1% sodium azide solution until immunohistochemistry (IHC) was performed.

For IHC, several sections from each P7 brain were rinsed in 0.01 M phosphate buffered saline (PBS) and mounted on gel-coated slides. This series of sections was stained with ionized calcium binding adaptor molecule 1 (Iba1) to identify microglia. All the slides were first rinsed in 0.01 PBS. Next, the slides were incubated with cover slips at room temperature in 50% methanol plus 0.3% H_2O_2 for 30 min, followed by another rinsing by PBS. Slides were then incubated with cover slips in blocking buffer (5% normal goat serum and 0.3% Triton-X in PBS) for 1 h at room temperature. Next, the slides were incubated with cover slips overnight at room temperature in primary antibody (Iba1 antibody, 1:5000, rabbit polyclonal, Wako Pure Chemical Industries, Ltd.) in blocking buffer. The following day, slides were washed in PBS, incubated for 2 h at room temperature in a solution of biotinylated goat anti-rabbit secondary antibody (1:200, Vector Laboratories, Burlingame, CA, USA), in blocking buffer and then washed in PBS. The Avidin-Biotin Complex (ABC) method was used to bind a complex to the secondary antibody, 4 × 30 min. Slides were washed in 0.1 M phosphate buffer (PB). Lastly, slides were incubated with diaminobenzidine (DAB, Sigma-Aldrich, USA) for 25 min to produce a colorimetric stain. Each slide was dehydrated through ethanol washes, xylenes and finally coverslipped with Permount.

2.3.4. Densitometry

Stained slices were imaged at 10× magnification using a Nikon 4550L microscope and NIS Elements BR 3.0 software (Nikon Instruments). Due to the age of the brains, it proved to be very difficult in each slice to identify preferred areas of interest such as the hippocampus, so photos were not taken specific to a particular area of brain. Instead, for each brain 5 slices that did not have folds or tearing were photographed ($N = 29$ brains). Areas that were along a myelin tract or the edge of a slice were excluded. Densitometry analysis was performed in ImageJ64 to identify cells positive for Iba1. A signal pixel darkness value of more than three standard deviations darker than the threshold value was used to obtain the integrated area density in each photo. The average pixel darkness was multiplied by the area of the analyzed area to generate the integrated area density measurement, a procedure that has been previously used to estimate the amount of Iba1 staining (Williamson et al., 2012).

2.4. Experiment 2: neonatal central and peripheral immune response

2.4.1. Neonatal euthanasia & tissue harvest

As described above, each litter was randomly assigned to a treatment and received either endotoxin-free saline or LPS subcutaneous injections. At 4 separate time points post-injection (Fig. 1A), a male and a female were randomly selected from each litter and euthanized. Following rapid decapitation, the hippocampus was micro-dissected and flash frozen using isopentane cooled with dry ice. They were stored at −80 °C. At this time trunk blood from each pup was also gathered. The blood samples were centrifuged for 10 min at 16.1 × *g* and serum was separated and stored at −20 °C. Pups were euthanized at 2 h and 24 h after each injection and this process was repeated until an *n* of 6–8 was achieved within each experimental group ($N = 55$ rats) at all 4 time points post injection ($N = 159$ rats).

2.4.2. qPCR analysis

RNA was extracted from the hippocampal tissue of neonates using Trizol and bead-beating procedures (N = approximately 5 per group, maximum of 41 animals per time point). Approximately 200 mg of the tissue was suspended in 1 mL of cold Trizol in RNAase-free round bottom tubes with safe-lock caps (Eppendorf). A 15 mm stainless steel ball (Quiagen) was added to each tube and tubes were bead-beaten at maximum speed for 5 min. Next, 0.2 mL of chloroform was added, tubes were vigorously mixed and then centrifuged for 10 min at maximum speed at 4 °C. The supernatant was saved and 500 μL of isopropyl alcohol added. Tubes were incubated at −20 °C for 10 min followed by centrifuging for 10 min at maximum speed at 4 °C. The supernatant was discarded and the remaining pellet was cleaned with 150 μL of 70% EtOH and then centrifuged for 5 min at maximum speed. The supernatant was discarded, the RNA pellet was allowed to dry until translucent, dissolved in 20 μL of nuclease-free water, and stored at −80 °C.

Qualitative real-time PCR was analyzed as described in Williamson et al. (2016) and using SYBR green (Kapa Biosystems, Wilmington, MA). Threshold cycle (C_T: number of cycles to reach the threshold of detection) was determined for each reaction and relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (Williamson et al., 2016). The C_T of the target gene of interest (T) was normalized to that of the reference (housekeeping) gene (R), for both the test (A) and the calibrator samples (B): $\Delta C_{T(A)} = C_{T(T,A)} - C_{T(R,A)}$ and $\Delta C_{T(B)} = C_{T(T,B)} - C_{T(R,B)}$. We defined the calibrator sample as that with the lowest expression. Next, we normalized the ΔC_T of A to B: $\Delta\Delta C_T = \Delta C_{T(A)} - \Delta C_{T(B)}$. Lastly, we calculated the expression ratio: $2^{-\Delta\Delta CT}$ = normalized expression ratio. Gene expression was measured using primers against IL-1β and GAPDH that were designed originally by the Bilbo lab (Williamson et al., 2016). All primer sets were estimated as greater than 90% efficient, and within 5% of each other.

Primer sequence are as follows: IL-1β: F: GAAGTCAAGACCAA AGTGG, R: TGAAGTCAACTATGTCCCG; GAPDH: F: GTTTGT GATGGGTGTGAACC; R: TCTTCTGAGTGGCAGTGATG.

2.4.3. Trunk serum analysis

Interleukin-1β (IL-1β) was measured in serum using a commercially available ELISA kit (R & D Systems, Minneapolis, MN). The ELISA was run according to the manufacturer instructions with one exception. In the standard curve that is generated using each set of samples assayed, the highest concentration point (2000 pg/mL) was excluded in favor of including one point of lower concentration on the curve. Results are expressed as picograms per 1 mL of serum.

2.5. Experiment 3: adult behavioral assessment

All pups were housed with their dams until weaning at P25. After they were weaned, each rat was pair-housed with a same-sex sibling from its litter. Rats were housed under the same standard conditions with constant access to food and water and handled minimally through adolescence. Once the rats reached adulthood (P70-100), two males and two females from each litter were individually tested on the open field and the elevated zero maze (EZM), two behavioral paradigms designed to assess anxiety-like behavior in rats ($N = 32$ SAL, $N = 32$ LPS). On each behavioral task, males were tested first and females second to avoid female odor exposure to the males.

2.5.1. Open field testing

At approximately P70, each rat was individually removed from its home cage between 13:00 and 17:00 and transferred into a clear plastic transfer cage (45 cm L × 25 cm W × 15 cm H) with bedding and a ventilated plastic top. This was used to transport the rat into a separate testing room, maintained at the same temperature and humidity as the colony room. The lights were entirely turned off in the room with only a desk lamp aimed at the ground under the apparatus to provide a dim lighting for the experimenters. The test box consisted of a white open field (92.5 cm × 78 cm × 11.5 cm) marked in a 6 × 5 grid of squares. A dark emergence box (23.5 cm × 18.5 cm × 12.5 cm) was attached at one corner with a removable divider between the emergence box and the open field. The rat was placed into the emergence box and the divider was lifted so that the rat was able to emerge into the open field. Once the rat fully emerged from the emergence box (entire torso and all four paws), the divider was replaced so that the animal could not go back into the emergence box. If the rat failed to emerge from the box within 2 min, the rat was guided out into the open field and the divider was replaced. The rat was then allowed a 10-min trial within the open field. The entire trial was videotaped and scored for the total amount of time spent in the emergence box, total time spent in the outer rim of squares, and total time spent exploring the inner set of squares (middle 6 squares) within the open field. Methods adapted from Martinez et al. (2015). The rat was weighed and returned to its home cage. The behavioral testing apparatus was thoroughly cleaned between each rat with Odormute cleaner.

2.5.2. Elevated zero maze

At approximately P100, each rat was individually removed from its home cage between 13:00 and 17:00 and transferred into a clear plastic transfer cage (45 cm L × 25 cm W × 15 cm H) with bedding and a ventilated plastic top. This was used to transport the rat into a separate testing room, maintained at the same temperature and humidity as the colony room. The elevated zero maze (EZM) was in the center of the testing room. The EZM was a platform ring raised to 0.51 m off the ground. The ring of the maze was divided into two opposite "closed" sections resembling hallways and two opposite "open" sections resembling bridges. The room was illuminated by two spotlights that were aimed at the wall to provide fairly dim conditions for the rats. To begin a test, the rat was placed on the middle of an open arm of the EZM. Each animal remained on the EZM for five minutes during which its behavior was videotaped and scored for the total time spent in the closed sections of the EZM and the total time spent in the open sections of the EZM. The percent of time spent of time spent in the open arms is negatively correlated to anxiety level (Zimmerberg et al., 2005). The rat was moved back to the transfer cage, weighed, and returned to its home cage. The behavioral testing apparatus was thoroughly cleaned between each rat with Odormute cleaner.

2.5.3. Video scoring

All behavior in both EZM and OF tests was captured by a Black Box GS8000L Miniature Camera Video Recorder that was centered several feet above the testing apparatus. Videos were compressed, transferred to a Macintosh computer and scored using ODLog by a research assistant who was blind to the treatment group of the rat.

2.6. Experiment 4: stress induction in adulthood

All adult rats in Experiment 4 were treated with LPS as neonates (P3 & P5) to assess the effects of a "triple hit" on anxiety-like behavior (Fig. 1B). During adulthood (P180) each same-sex pair was randomly allocated to either the 3-day restraint and isolation stress ($N = 32$) or control condition ($N = 32$). Both the control and stress induction paradigms were adapted from Walker et al. (2009). On the fourth day the rats were tested for anxiety-like behavior on the elevated zero maze paradigm and immediately euthanized.

2.6.1. Stress induction paradigm and EZM

On the first day of stress induction, pairs that were assigned to the stress condition were separated and underwent individual restraint stress. Each rat was restrained in Plexiglas cylinder (6.5 cm inner diameter, 20 cm long, with mesh at the nasal end of the cylinder) for 30 min (09:00–13:00 h). Between animals, each cylinder was cleaned with Odormute and wiped dry. The rats in the control condition were gently handled for three minutes each (09:00–13:00 h). These procedures were repeated on the second day. On the third day, the animals assigned to the stress condition were isolated for 30 min. They were transferred individually into a clear plastic cage (45 cm L × 25 cm W × 15 cm H) with bedding, a ventilated metal top and a water bottle and placed in an isolated, closed, lit room for 30 min (09:00–12:00 h) before being returned to pair-housing. Bedding was changed between each animal. The rats assigned to the control condition received the same handling treatment as the previous 2 days. On the fourth day, all rats underwent behavioral testing on the EZM, as described previously. Behavior was captured by a Black Box GS8000L Miniature Camera Video Recorder and automatically scored by AnyMaze (Wood Dale, IL, USA) and the program coded for time spent within the closed arms and open arms of the EZM over the five-minute testing period.

2.7. Statistical analysis

Using SPSS, all of the statistical analyses for this study were three-way (2 × 2 × 2) ANOVAs comparing line (High and Low), treatment (Saline or LPS), and sex (Male and Female), excluding Experiment 4, which was a three-way ANOVA of line, sex, and stress treatment (Control or Stress). *F*-values for each analysis are reported in the results and an alpha level of $p < 0.05$ was used to determine statistically significant group differences. Where appropriate, data were further analyzed using a two-way ANOVA with Fishers LSD *post-hoc* tests. All data are presented as mean ± standard error of the mean.

3. Results

3.1. Experiment 1 results

To ensure that differential maternal care was not a contributing factor to later-life anxiety-like behavior, we assessed nesting behavior and found no significant differences in maternal care at P3 or P5 in either line or treatment. Regardless of neonatal treatment or sex, there was a significant difference in USVs between

the selectively bred lines, such that the High line emitted more USVs than the Low line ($F(1,71) = 36.229$, $p < 0.005$; Fig. 2). This result replicated many previous findings (Brunelli, 2005a; Brunelli et al., 2006; Martinez et al., 2015; Zimmerberg and Germeyan, 2014).

Microglial activation within the P7 brain was assessed using densitometry on Iba1+ cells (Fig. 3). There was an interaction between neonatal treatment and sex ($F(1,21) = 6.48$, $p = 0.019$). *Post-hoc* pairwise comparisons revealed that females treated with LPS had an increased microglia density compared to the females treated with SAL ($p = 0.036$). Furthermore, SAL-treated males had increased microglia density compared to SAL-treated females ($p = 0.017$; Fig. 3C).

3.2. Experiment 2 results

We examined the effects of an LPS inflammatory challenge within the peripheral immune system and central immune system immediately (2 h) after the P3 and P5 challenges and after a longer period of time (24 h). We examined the peripheral immune response by assessing IL-1β protein levels in serum with an ELISA. At 2 h after the P3 injection, there was a main effect of neonatal treatment ($F(1,32) = 5.387$, $p = 0.027$), such that the LPS-treated rats had a significantly increased peripheral immune response compared to controls, regardless of line (Fig. 4A; $N = 50$ total, 4–6 per treatment). Twenty-four hours after the P5 injection, there was a main effect of line ($F(1,32) = 11.217$, $p = 0.002$), such that the Low line had lower expressed levels of IL-1β compared to the High line ($N = 45$ total, 4–5 per treatment) (Fig. 5). This novel finding shows that the High line pups have elevated peripheral inflammation and slower recovery post-injection following 2 immune challenges.

We also examined the central neuroimmune response between each line, treatment, and sex at two of the time points using qPCR techniques. At 2 h post-P3 injection, there was a main effect of LPS ($F(1,33) = 9.3$, $p = 0.004$) such that there was significantly higher IL-1β mRNA expression in the LPS-treated animals compared to the SAL-treated animals (Fig. 4B). There were no significant differences between groups after 24 h following the P3 injections or at either time point following the P5 injections (data not shown).

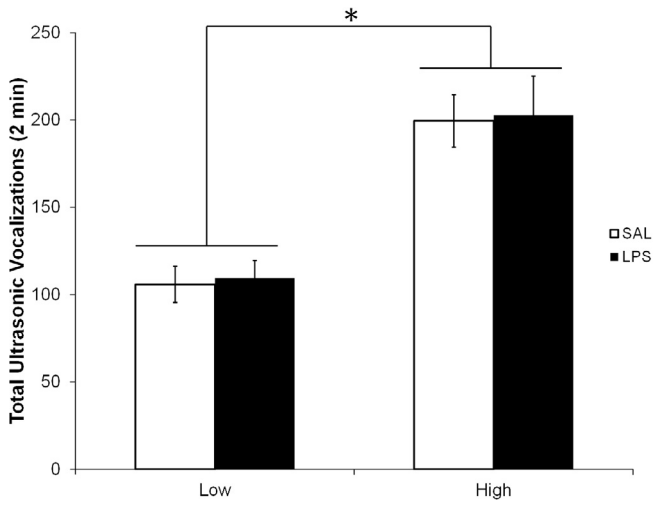

Fig. 2. High line rat neonates express significantly more ultrasonic vocalizations than Low line neonates at P7. There was a main effect of line in the amount of ultrasonic vocalizations emitted over the two minute period after maternal separation ($F(1,71) = 36.229$, $p = 0.001$) such that the High line emitted more USVs than the Low line, regardless of sex or neonatal treatment ($N = 20$ per group). (* indicates $p < 0.05$; error bars indicate SEM).

3.3. Experiment 3 results

Anxiety-like behavioral testing was conducted on Open Field (OF) and Elevated Zero Maze (EZM) apparatuses ($N = 64$ total, 8 rats per treatment). Two rats were excluded from the OF due to experimenter error, but included on the EZM. On the OF, there was a significant interaction between line and treatment in emergence latency ($F(1,54) = 5.532$, $p = 0.022$) (Fig. 6A). *Post hoc* comparisons revealed that, within the Low line, LPS-treated rats showed a significant increase in emergence latency compared to the SAL-treated rats ($p = 0.02$). Once inside the arena, all rats spent the majority of their exploration time at the edges (80–90% of the time; no significant differences between groups, $p > 0.05$). However, there was a significant interaction between line and treatment ($F(1,54) = 8.079$, $p = 0.006$), such that High line rats treated with LPS as neonates spent a significantly greater percentage of their time in the center 6 squares compared to their saline-treated counterparts, while both groups of Low line rats explored the center equally (Fig. 6B).

On the EZM, there was a main effect of line ($F(1,55) = 4.110$, $p = 0.047$) such that the High line rats spent more time in the closed arms than the Low line, indicating increased anxiety-like behavior (Fig. 7A). Furthermore, within the EZM there was a main effect of neonatal treatment ($F(1,55) = 10.061$, $p = 0.002$) such that the SAL-treated rats displayed more anxiety-like behavior than the LPS-treated rats by spending a greater percentage of their time in the closed arms (Fig. 7B). This SAL-treated behavioral result was unexpected and we sought to further investigate this interaction between line and treatment within the fourth experiment.

3.4. Experiment 4 results

We used the three-hit hypothesis set forth by Daskalakis et al. (2013), assessing both lines (High and Low) after neonatal immune challenge (LPS treatment) and adult stress status (No stress or Stress) ($N = 59$ total, 7–8 per treatment). In the EZM, there was a main effect of line ($F(1,52) = 28.812$, $p = 0.001$) such that the High line displayed more anxiety-like behavior than the Low line by spending more time in the closed arms of the EZM (Fig. 8A), similar to the EZM results in experiment 3. Within this experiment, there was also a main effect of sex ($F(1,52) = 19.959$, $p = 0.001$) such that males displayed more anxiety-like behavior than females, indicating that males were more sensitive to adult stress and handling than females (Fig. 8B). This sex difference was not observed within Experiment, indicating that the stress exposure and/or handling may drive the sex difference in anxiety-like behavior. Surprisingly, there was no effect of stress condition.

4. Discussion

The combined results reveal more about the relationships between neural development, early-life inflammation and anxiety throughout the lifespan. To our knowledge, no prior studies have investigated the neuroimmunological differences within the selectively bred High and Low lines. In general, the differences between the lines were robust, regardless of neonatal treatment, in that the High line showed increased anxiety-like behavior on affective tests and a delayed resolution of inflammation during the neonatal period. However, neonatal LPS reduced anxiety-like behavior on the EZM and increased microglial density in female neonates. We also observed sex differences following adult stress, such that males displayed more anxiety-like behavior than females on the EZM. Together, these findings further demonstrate the complicated relationship between inflammation, neonatal programming and anxiety.

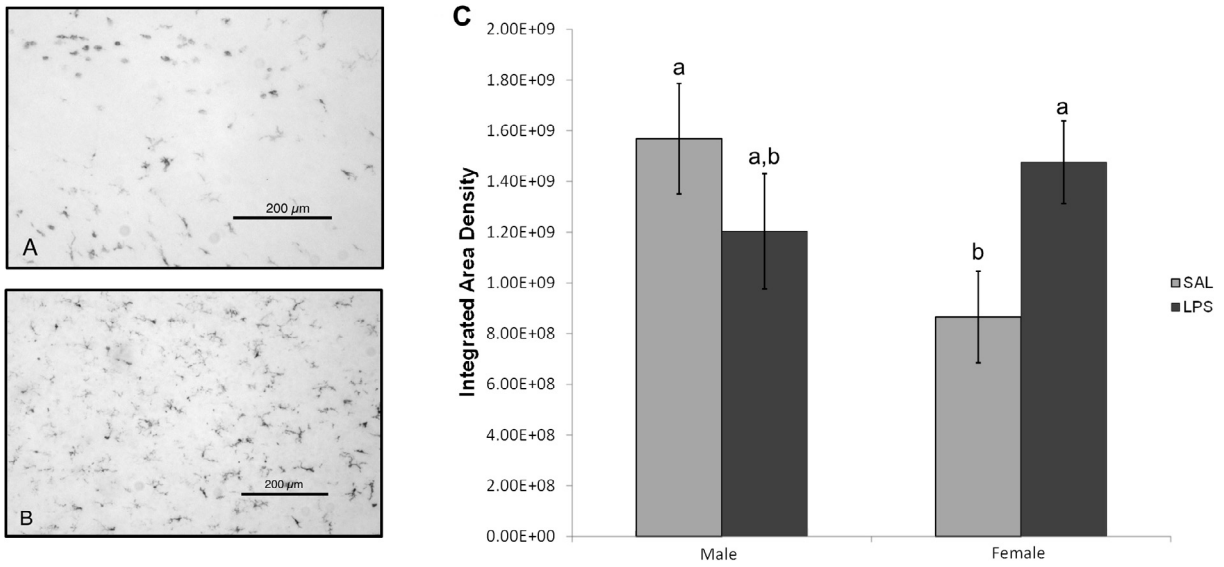

Fig. 3. Microglial density was affected by an interaction between sex and neonatal treatment. (*A&B*) Representative images of Iba1 + cells at 10× magnification from the P7 brains, demonstrating low and high expression in Iba1 + cells. (A) is a SAL-treated female and (B) is an LPS-treated male. (*C*) There was an interaction between treatment and sex ($F(1,21) = 6.48$, $p = 0.019$) such that LPS treatment increased microglial density in females compared to SAL-treated females, while neonatal treatment had no effect on microglial density in males ($N = 6–8$ per group). ([b]Significantly decreased from *a*, $p < 0.05$; error bars indicate SEM).

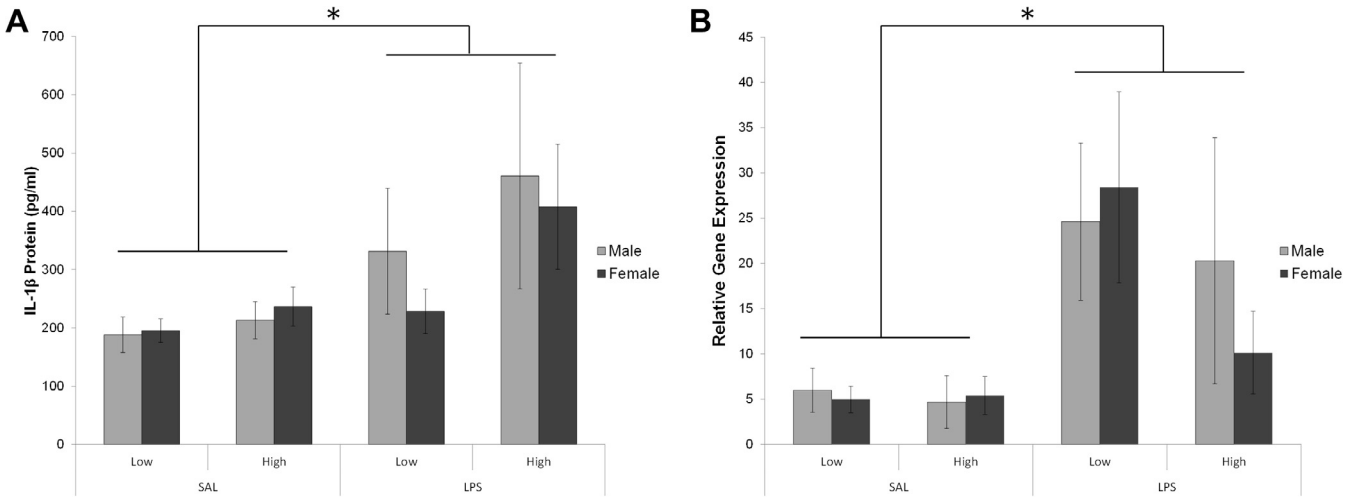

Fig. 4. Immune challenge (LPS) on P3 causes a significant increase in IL-1β protein in the periphery and a significant increase in IL-1β mRNA expression within the hippocampus at 2 h post-injection. (*A*) There was a main effect of neonatal treatment 2 h post-LPS injection on P3 ($F(1,32) = 5.387$, $p = 0.027$) such that the neonates treated with LPS had heightened IL-1β protein expression in serum compared to controls ($N = 4–6$ per group). The error bars depict standard error of the mean. (*B*) There was a main effect of neonatal treatment ($F(1,33) = 9.32$, $p = 0.004$) such that the neonates treated with LPS had increased IL-1β mRNA expression within the hippocampus compared to controls ($N = 4–6$ per group). (* indicates $p < 0.05$; error bars indicate SEM).

In Experiment 1, the High line rats vocalized more at P7 than the Low line, replicating many previous findings (Brunelli, 2005a; Brunelli et al., 2006; Martinez et al., 2015; Zimmerberg and Germeyan, 2014). However, the relative difference in USV vocalizations, despite its significance, is of a smaller magnitude compared to previous findings comparing the lines (Zimmerberg et al., 2005). This could be due to the effect of neonatal handling during the injection procedure because brief separation from the dam increases maternal care (Liu et al., 1997) and also because P7 is not the first time pups have been isolated from the dam. The lack of an LPS effect on neonatal USVs may be due to a) the robust nature of vocalizing behavior; b) the behavior's possible dissociation from inflammation or c) the effects of neonatal inflammation result in exclusively in a change of adult, not neonatal, anxiety-like behavior. At the cellular level, microglial activation within P7 brains was influenced by an interaction between neonatal treatment and sex such that the induction of inflammation increased the density of microglia in P7 LPS-treated females compared to the SAL-treated females. A higher Iba1+ densitometry can indicate a greater number of microglia or an increased number of primed microglia (Schulz, 2010). Generally we observed that males had higher Iba1+ density compared females, which has been observed previously such that between P4 and P30, males have more microglia that are also more reactive in specific regions of the brain compared to females (Schwarz and Bilbo, 2012). However, the induction of inflammation increased the density of female microglia to male levels. The lack of increase in microglia density in the LPS-treated males is likely due to the comparatively high baseline level of amoeboid microglia in males before the LPS challenge. Females, on the other hand, respond more strongly likely because

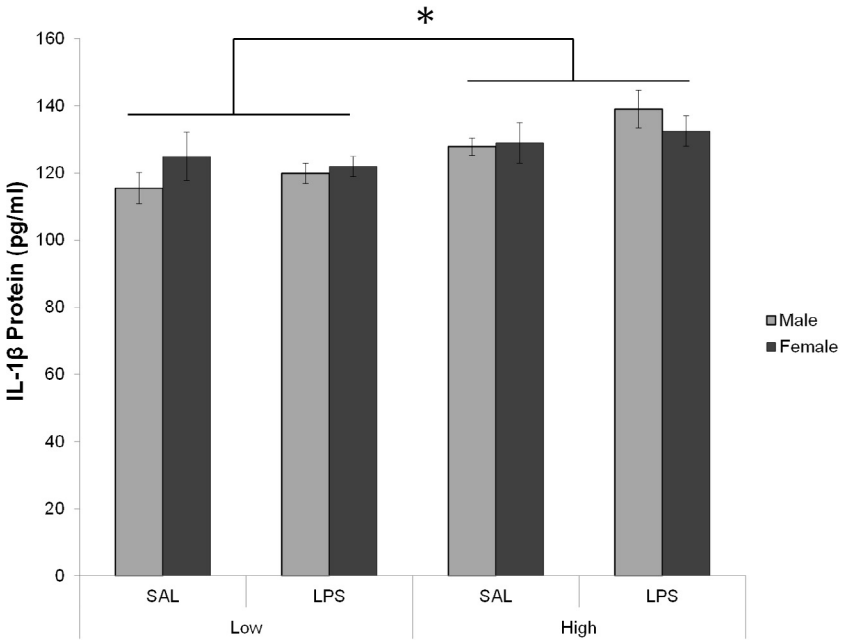

Fig. 5. High line rats have increased serum IL-1β compared to Low line rats 24 h after neonatal injections. There was a main effect of line on serum IL-1β expression ($F(1,32) = 5.387$, $p = 0.027$) such that the High line neonates had increased IL-1β expression compared to the Low line neonates (N = 4–6 per group). (* indicates $p < 0.05$; error bars indicate SEM).

they have fewer microglia than males on P3 and P5, and LPS challenge alters existing microglia morphology and may induce the microglia to replicate. The changes in microglial morphology are very important because they can induce long-term functional alterations in immune function, metabolism, cognition, and neuroendocrine processes that have life-long implications for the organisms (Bilbo and Schwarz, 2009; Sominsky et al., 2012).

In Experiment 2, we observed increased serum IL-1β within LPS-treated pups 2 h after the P3 injection, which replicates many previous findings using LPS to induce peripheral inflammation (Hodgson et al., 2001; Schwarz and Bilbo, 2011; Shanks et al., 1995). LPS-treated neonates also had increased hippocampal mRNA expression of IL-1β compared to the SAL-treated neonates. Furthermore, the results from Experiment 2 demonstrate a novel physiological difference between the High and Low lines following neonatal inflammation. At 24 h following the P5 injection, the High line had significantly higher levels of peripheral IL-1β expression, indicating that the High line rats had an elevated peripheral inflammatory response and slower recovery from inflammation. This prolonged increase in serum IL-1β was not mirrored in the hippocampus, such that all groups showed no differences in IL-1β mRNA expressed at that time point. The peripheral immune response is not always proportionally reflected in the CNS due to the distinct populations of IL-1β-producing cells in these regions and their relative frequency (i.e., peripheral leukocytes are a vast majority of serum while microglia are a small population within the CNS) as well as the signaling mechanisms between the periphery and CNS that modulate cytokine signals (for review, see Dantzer et al., 2008). The dissociation between the two responses indicates normal resolution in the brain with slower resolution in the periphery.

In Experiment 3, we investigated the consequences of early life inflammation in adult anxiety-like behavior. In the OF paradigm, latency to emerge into the open field was modulated by an interaction between line and treatment. Within the Low line, LPS significantly increased emergence latency compared to SAL, but there was no difference due to treatment within the High line. This difference could indicate that the Low line is more vulnerable to the effects of neonatal inflammation or that the High line rats exhibit a ceiling effect. Our study also assessed a much shorter time limit for emergence than a previous study (2 min vs. 15 min, respectively) (Martinez et al., 2015). High line rats, on the other hand, were affected by neonatal treatment in their exploration behavior within the OF, such that neonatal LPS treatment increased exploration behavior.

Anxiety-like behavior appeared slightly differently on the EZM compared to the OF. Within the same cohort of rats, there were main effects of both line and neonatal treatment. The High line rats demonstrated more anxiety-like behavior than the Low line, staying in the closed arms for more time, replicating previous studies (Brunelli et al., 2006; Zimmerberg et al., 2005; Zimmerberg and Germeyan, 2014) and recapitulating the line-dependent difference in neonatal anxiety-like behavior seen in Experiment 1. This EZM finding, however, did not correspond to the increased exploration of the center in the OF. Furthermore, the SAL-treated rats demonstrated more anxiety-like behavior than the LPS-treated rats, regardless of line or sex. Based upon this finding alone, it appears that LPS seems to have an anxiolytic effect on behavior, in contrast with studies that have all demonstrated that the neonatal administration of LPS is associated with anxiety-like behavior in adulthood (Bilbo and Schwarz, 2009; Bland et al., 2010; Hofer, 1996; Schwarz and Bilbo, 2012; Sominsky et al., 2012; Walker et al., 2009, 2004). These unexpected results may have two explanations: first, many researchers use the Sprague Dawley strain, while our lines were derived from the N:NIH strain and selectively bred for many generations. Due to differences in physiology and behavior seen in different strains of laboratory rats, the effects of neonatal inflammation may not be identical or consistent. Secondly, the EZM was the second behavioral apparatus all of the animals were tested on in adulthood, following the OF. Because of previous handling and testing, the EZM might not have elicited the anxiety-like behavior commonly seen on the apparatus.

Given the extensive relationship between the immune system, the HPA axis and neuropsychiatric disorders, we examined the effects of acute stress on anxiety-like behavior in combination with the effects of neonatal immune challenge in Experiment 4 (Granger

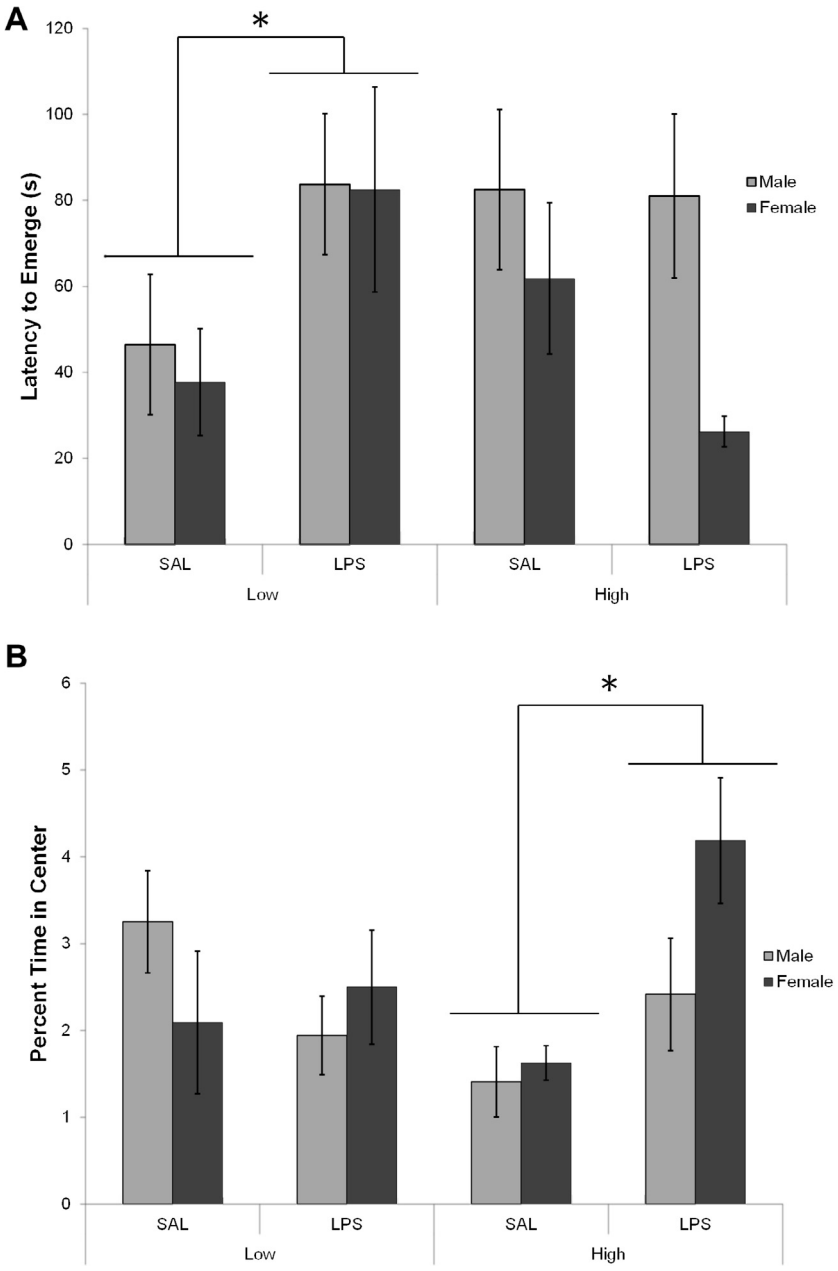

Fig. 6. Neonatal treatment with LPS increases anxiety-like behavior in the Low line and decreases anxiety-like behavior in the High line on the Open Field Test. (*A*) There was a significant interaction between line and treatment ($F(1,55) = 5.532$, $p = 0.022$) in the expression of anxiety-like behavior, such that LPS increased latency to emerge into the open field in Low line rats but not the High line. (*B*) There was also a significant interaction between line and treatment ($F(1,54) = 8.079$, $p = 0.006$) in exploration of the open field, such that LPS-treated High line rats explored the center of the field significantly more than SAL-treated High line rats, while both groups of Low line rats explored the center equally (N = 15–17 per group). (* indicates $p < 0.05$; error bars indicate SEM).

et al., 1996; Hodgson et al., 2001; Shanks et al., 1995). When tested on the EZM after a three-day stress paradigm or control handling, the High line demonstrated more anxiety-like behavior than the Low line, similar to the results in Experiment 3. Furthermore, within Experiment 4 there was a main effect of sex such that the males demonstrated more anxiety-like behavior than the females. Increased male sensitivity to stress after neonatal inflammation has been shown previously across multiple studies (Walker et al., 2012, 2009). In the Hide Box/Open Field test used (similar to the OF paradigm used in this study), only males showed significant anxiety-like behavior compared to females after adult stress exposure. Furthermore, on the EPM, males treated neonatally with LPS and exposed to adult stress displayed a significantly increased amount of anxiety-like behavior compared to the other treatment groups (Walker et al., 2009). However, to expand on the findings from Walker et al. (2009), future studies could incorporate SAL-treated rats into the stress paradigm and subsequent testing.

The most intriguing and unexpected result from Experiment 4 is the lack of a stress effect. Because the chronic stress paradigm was our experimental manipulation that was intended to serve as the third challenge for these rats, on top of phenotypic vulnerability and neonatal immune activation, we expected it to result in an increase of anxiety-like behavior for the stressed animals. The lack of effect of our experimental manipulation could imply two possibilities. First, it could be that despite being adapted from the methods of Walker et al. (2009), our stress paradigm was too innocuous

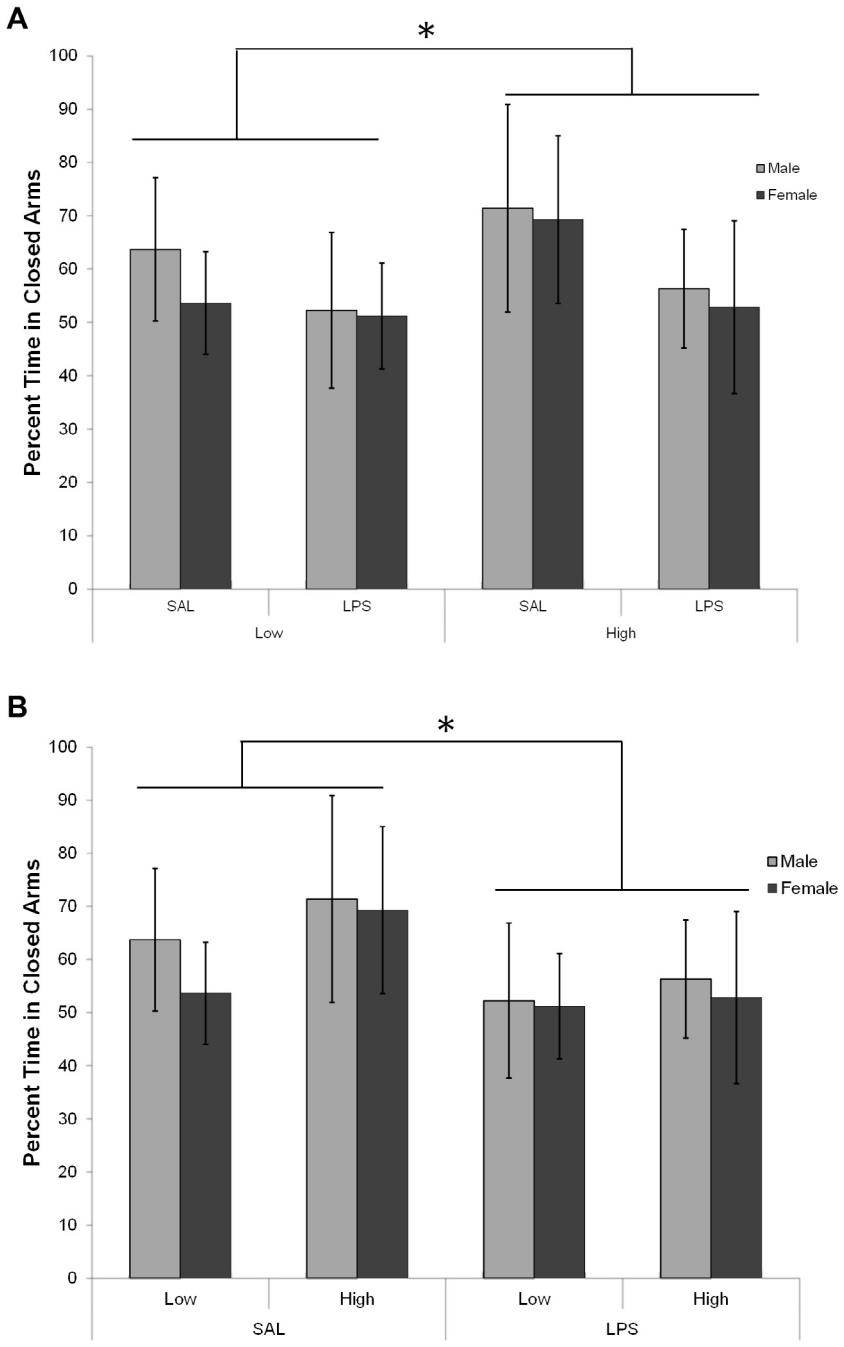

Fig. 7. Both selective breeding and neonatal treatment alter anxiety-like behavior on the Elevated Zero Maze. (*A*) There is a main effect of line ($F(1,55) = 4.110$, $p = 0.047$) such that the Low line spend less time in the closed arms on the EZM and display less anxiety-like behavior than the High line ($N = 7–8$ per group). (*B*) There was also a main effect of neonatal treatment ($F(1,55) = 10.061$, $p = 0.002$) such that the saline-treated rats display more anxiety-like behavior than the LPS-treated rats, spending more time in the closed arms of the EZM ($N = 7–8$ per group). (* indicates $p < 0.05$; error bars indicate SEM).

to generate a difference in anxiety-like behavior, because the manipulation was not stressful enough or was not variable enough to elicit a strong HPA axis reaction. The restraint in their paradigm (2009) was a wire mesh that completely immobilized the rats, while our tube restraint allowed the rats some mobility, which may have been less stressful. Variable, long-term stress has been proposed to elicit a greater HPA axis activation compared to repeated stress paradigms (Dhabar, 2014). Because the stress paradigm was intense and identical for the first two days, followed by a lower stress experience on the third day, and all stress paradigms and behavioral testing occurred at approximately the same time of day, it is possible that the stress paradigm itself was not stressful enough for the animals. While stress induction performed during the light cycle can alter cytokine expression both centrally and peripherally (Dunn et al., 1972; Fonken et al., 2015, 2016), isolation stress on the third day may not have enhanced cytokines enough to alter subsequent anxiety-like behavior. Previous work has also shown that chronic stress induced during the light cycle increased depressive-like behavior, but not anxiety-like behavior, while stress during the dark cycle increased anxiety-like behavior in males (Huynh et al., 2011). Restraint stress alters affective behaviors on circadian and sex-related bases, indicating that our stress during the light cycle might not have altered EZM behavior at all in any of our groups. Alternatively, the lack of significant difference

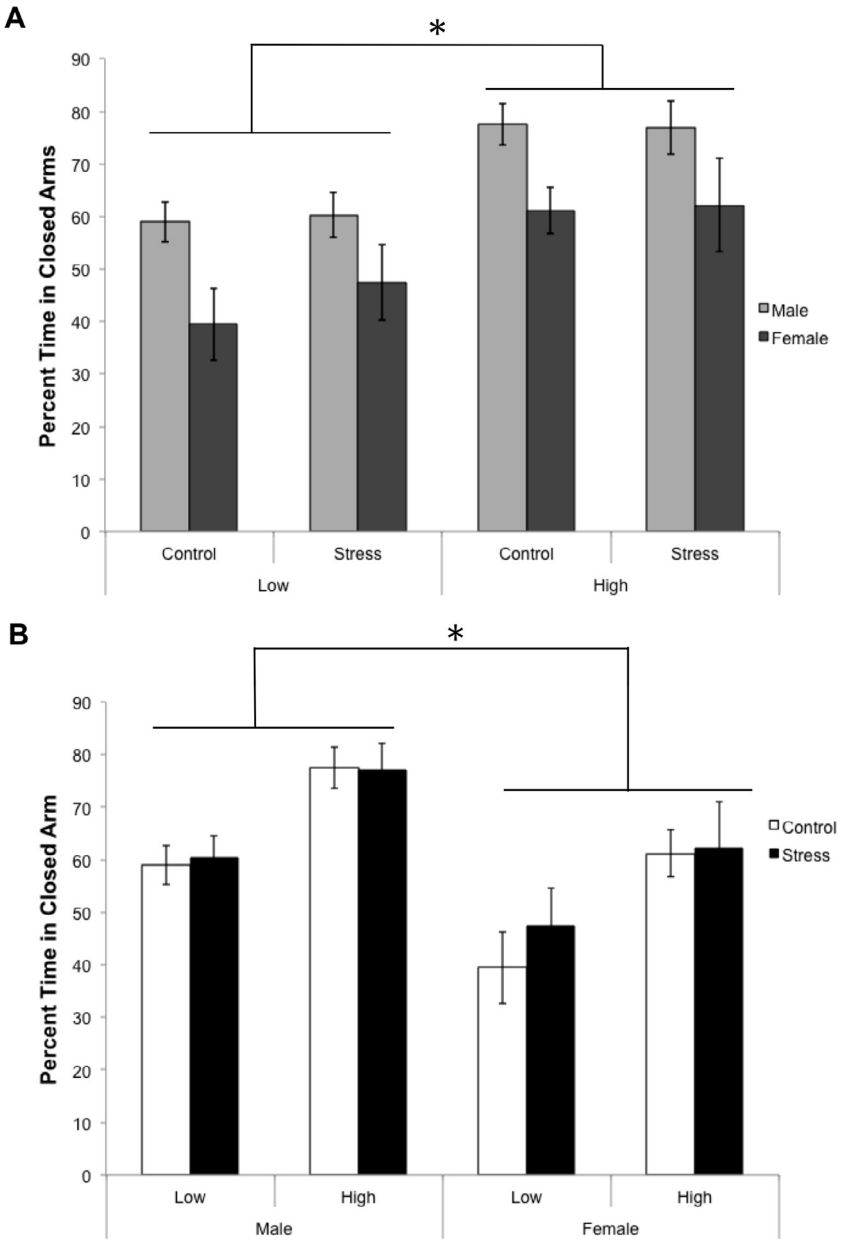

Fig. 8. Both selective breeding and sex alter anxiety-like behavior on the Elevated Zero Maze following a three day stress paradigm. (A) There was a main effect of line ($F(1,52) = 28.812$, $p = 0.001$) such that the High line displayed more anxiety-like behavior compared to the Low line rats, recapitulating previous results (N = 7–8 per group). (B) There was also a main effect of sex ($F(1,52) = 19.959$, $p = 0.001$), such that males spent more time in the closed arms, displaying more anxiety-like behavior than females (N = 7–8 per group). (* indicates $p < 0.05$; error bars indicate SEM).

between both treatment groups could also imply that the handling control procedure was stressful enough that the control rats also endured stress to induce a change in anxiety-like behavior. The rats in this experiment had not been handled for significant amounts of time since P25 with the exception of brief cage transfers by the animal care staff.

To our knowledge this was the first study that utilized rat lines selected for high and low anxiety-related phenotypes and assessed the effects of neonatal inflammation during the neonatal period and into adulthood. In sum, we saw complex effects of neonatal inflammation between lines in terms of neonatal anxiety-like behavior, peripheral neonatal immune response, adult anxiety-like behavior on the EZM, and adult anxiety-like behavior after stress induction. Furthermore, we also observed an effect of neonatal treatment in peripheral immune response 2 h post injection on P3 and during adult anxiety-like behavior on the EZM. We also observed an effect of sex within the anxiety-like behavior of LPS-treated adults exposed to stress paradigm. A sex difference was also observed via an interaction with treatment in the microglial morphology within the brains of P7s after being induced with neonatal inflammation.

While it is difficult to make conclusions regarding the etiology of human neuropsychiatric disorders directly from these results, inconsistencies within the results demonstrate significant gaps in the field. Many neuropsychiatric disorders have all been linked to immune dysfunction, but an added layer of complexity when looking at the human model is the possibly genetic or epigenetic inheritance or alterations experienced by a given individual. For this reason, the selectively bred populations serve as an important component of this study by representing rodent models of the

two possible extreme ends of the human spectrum of anxiety behavior. However, we do not know if the differences between these lines are due to alterations in the same subset of genes or two separate genetic profiles in each phenotype, preventing a mechanistic explanation for these findings. Many factors, both genetic and otherwise, may contribute to the cumulative resilience or vulnerability to future disruptions in homeostasis in life such as the induction of stress. Anxiety disorders are one of the most common neuropsychiatric disorders diagnosed within this country and the impairment and distress they cause affects many aspects of normal human function. Taken together, the results of this study suggest that there is a complex interaction between phenotype, neonatal inflammation, sex, and stress in the etiology of anxiety disorders that must be further explored.

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