Differential Microglial Activation Following Immune Challenge in Peripubertal and Adult Outbred Mice

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DIFFERENTIAL MICROGLIAL ACTIVATION FOLLOWING IMMUNE CHALLENGE IN PERIPUBERTAL AND ADULT OUTBRED MICE

A Thesis Presented
by
DAVID J. PLACZEK

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

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Neuroscience and Behavior Program
DIFFERENTIAL MICROGLIAL ACTIVATION FOLLOWING IMMUNE CHALLENGE IN PERIPUBERTAL AND ADULT OUTBRED MICE

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ABSTRACT

DIFFERENTIAL MICROGLIAL ACTIVATION FOLLOWING IMMUNE CHALLENGE IN PUBERTAL AND ADULT OUTBRED MICE

MAY 2015

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Pubertal development is a sensitive period for the brain due to the high degree of changes an individual goes through. Stressors experienced during pubertal development can have long-lasting effects into adulthood. Female mice that receive peripubertal injections of the immune stressor, lipopolysaccharide (LPS), display altered responses to steroid hormones in adulthood. Altered behaviors include hormone-influenced sexual receptivity, cognition, anxiety-like behavior, and depression-like behavior. Microglia, the immune cells of the brain, may be involved in this mechanism due to their role in synaptic plasticity during developmental periods and their response to LPS. Female mice have more activated microglia in the brain during pubertal development than during adulthood, illustrating a hyperactive immune environment during this period. When injected with LPS, an inbred strain of female mice showed higher immunoreactivity of ionized calcium-binding adaptor (Iba1), a protein used as a marker for microglial morphology, during pubertal development than mice that received LPS injections as adults. The differential immune response between peripubertal and adult inbred mice occurred in the arcuate nucleus, the ventromedial hypothalamus, and the basolateral amygdala. The aim of this study was to test the hypothesis that differential microglial activation occurs between peripubertal and adult female outbred mice after an immune
challenge. We hypothesized that peripubertal mice injected with LPS would have higher Iba1 immunoreactivity (Iba1-ir) than adult mice injected with LPS. Female CD1 mice were injected with LPS or saline at either 6 weeks of age (during pubertal development) or at 11 weeks (during adulthood). Mice were euthanized 24 hours post injection, and tissue was immunostained for Iba1. Analysis of Iba1-ir in the arcuate nucleus, ventromedial hypothalamus, basolateral amygdala, posterodorsal medial amygdala, dentate gyrus, CA1, and CA3 using computer-assisted image analysis revealed main effects of treatment in several areas, demonstrating that LPS-treated animals had significantly more Iba1-ir than saline controls. However, no significant interactions between treatment and age were observed in any of the brain areas analyzed. This suggests a lack of differential microglial activation between peripubertal and adult CD1 mice injected with LPS. These results do not support our hypothesis and possible explanations will be further discussed below.
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CHAPTER 1
BACKGROUND AND SIGNIFICANCE

Pubertal development is a period of major reorganization of the body and brain, during which a reproductively immature juvenile transitions into one that is physically and chemically capable of sexual reproduction. Physical changes, including growth spurts and development of primary and secondary sexual characteristics (Marshall & Tanner, 1969; 1970) all accompany development of the brain. This brain reorganization brings forth adult-like behaviors of sexuality, aggression, and parenting.

The lasting cellular and molecular changes that the brain goes through render it highly vulnerable to environmental stress (Andersen, 2003). During pubertal development, girls may be exposed to stress from peers, such as bullying; exposure to these forms of adversity has been associated with depressive disorders, substance abuse problems, and eating disorders (Sontag, Graber, & Clemans, 2010). Other traumatic experiences and stressors including physical and sexual abuse during this period have been related to increased anxiety and depression as well as problems with attention and aggression, which persist into adulthood (Silverman, Reinherz, & Giaconia, 1996). More serious problems including suicidal thoughts and attempts, alcohol abuse, and other psychiatric disorders have also been observed in sexually-abused girls and women (Silverman, Reinherz, & Giaconia, 1996). These findings indicate the negative impact that peripubertal stress and trauma appear to have on mental health, persisting into adulthood.

Rodents can be used to study behavioral effects of stress occurring during pubertal development. Mice may be used due to their similarities with human pubertal
development in terms of timing. That is to say, the first outward sign of puberty onset in girls is breast-budding, culminating with menarche, taking, on average, between 2 and 3 years (Marshall & Tanner, 1969). Mice, as opposed to rats, have an extended peripubertal period beginning with vaginal opening and culminating with the onset of regular estrous cycles, which can take several weeks depending on environment (reviewed in Holder & Blaustein, 2014).

Female C57Bl/6 mice (inbred strain) that are shipped from suppliers during pubertal development, around 6 weeks of age, demonstrate reduced sexual receptivity in adulthood following administration of estradiol and progesterone (Laroche, Gasbarro, Herman, & Blaustein, 2009b). Mice were ovariectomized prior to behavior testing to control for hormone influences on behavior. Shipping is a multi-factor stressor comprising a number of possible individual stressors. For example, during shipment, mice may be exposed to loud noises, predatory scents, food and water deprivation, and erratic changes in light exposure and temperature (Laroche, Gasbarro, Herman, & Blaustein, 2009b). In a different experiment to test which individual stressor could cause this robust effect in adulthood, female C57Bl/6 mice in the peripubertal period were exposed to several common stressors including restraint stress, food deprivation, a multiple stressor regimen including exposure to bright lights, and an immune stressor in varying doses. Only the immune stressor, lipopolysaccharide (LPS), a bacterial endotoxin obtained from Escherichia coli that activates the stress axis and immune system (Rivier, Chizzonite, & Vale, 1989; Spinedi, Suscun, Hadid, Daneva, & Gaillard, 1992), produced similar effects as shipping (Laroche, Gasbarro, Herman, & Blaustein, 2009a). This suggests the possibility of exposure to immune stressors during shipment. Mice that
were injected with a high dose of LPS (1.5 mg/kg body weight) at 6 weeks of age
demonstrated significantly lower sexual receptivity in three out of five behavioral trials
than mice that received saline at 6 weeks of age. Lower doses of LPS (less than 1.5
mg/kg body weight) produced effects compared to controls, but with more variation
(Laroche, Gasbarro, Herman, & Blaustein, 2009a). Mice that received LPS at 7 weeks of
age did not show long-term effects on hormone-induced sexual behavior. This indicates
an alteration of a hormone-dependent behavior after peripubertal LPS injection or
shipment (Laroche, Gasbarro, Herman, & Blaustein, 2009a). CD1 mice (outbred strain)
shipped or injected with LPS during the peripubertal period also exhibit altered sexual
receptivity, thus demonstrating a behavioral effect in both inbred and outbred mouse
strains (Ismail, Garas, & Blaustein, 2011).

CD1 mice shipped during pubertal development display lower levels of estrogen
receptor alpha (ER-α) (Ismail et al., 2011), which plays a large role in female sexual
behavior in rats (Ogawa et al., 1998). Areas of the brain analyzed included the
ventromedial nucleus of the hypothalamus (VMN), the arcuate nucleus (Arc), the
anteroventral periventricular nucleus (AVPV), and the medial preoptic area (MPOA). All
areas imaged except the AVPV showed lower numbers of ER-α cells after peripubertal
shipping compared to mice shipped at 4 weeks of age, before the sensitive period (Ismail
et al., 2011). Mice injected with LPS during pubertal development also displayed lower
levels of ER-α in the MPOA and the VMH (Ismail & Blaustein, unpublished
observations). This established an effect of stressors on the brain’s sensitivity to steroid
hormones, rather than stressors altering behavior through a different, non-hormone-
mediated, mechanism.
Sexual receptivity is only one of the hormone-dependent behaviors compromised after a peripubertal immune challenge. 17β-estradiol has an anti-depressive-like effect in rats (Okada, Hayashi, Kometani, Nakao, & Inukai, 1997) and in mice (Dalla, Antoniou, Papadopoulou-Daifoti, Balthazart, & Bakker, 2004). In both CD1 and C57Bl/6 mice, chronic estradiol treatment does not produce typical anti-depressant-like effects after peripubertal immune challenge (Ismail, Kumlin, & Blaustein, 2014). On the contrary, in the forced swim and tail suspension tests, mice injected with LPS during pubertal development exhibit longer durations of immobility, indicating increased, rather than decreased, depressive-like behavior in response to estradiol (Ismail et al., 2014).

Injections of progesterone and estradiol produce anxiolytic-like effects (Galeeva & Tuohimaa, 2001). Results from experiments using a marble-burying task, a light/dark box, and an elevated plus maze to assess anxiety-like behavior, demonstrate that following peripubertal LPS injection, estradiol and progesterone injections do not produce anxiolytic-like effects in either inbred or outbred strains of female mice (Olesen, Ismail, Merchasin, & Blaustein, 2011). Estradiol also enhances cognition, particularly on hippocampus-dependent tasks (Li et al., 2004). Female CD1 mice failed to respond to estradiol’s cognitively-enhancing effects after peripubertal immune challenge as measured by object recognition and placement, as well as social discrimination and recognition (Ismail & Blaustein, 2014). Therefore, an immune challenge during pubertal development directly affects the ability of ovarian hormones to modify certain behaviors including sexual receptivity, depressive-like, anxiety-like, and cognition. These behavioral modifications after peripubertal shipment and immune challenge raise the
question of an underlying mechanism or mechanisms that could account for the alteration.

Throughout the peripubertal period, the brain changes dramatically. White matter and cortical thickness both increase during pubertal development, as well as the size of subcortical regions including the amygdala and hippocampus (Capuron & Miller, 2011; Cooke, 2010; Goddings et al., 2014; Shen et al., 2010). On a smaller scale, cell body sizes in the posterodorsal nucleus of the medial amygdala (MePD) increase during pubertal development in Syrian hamsters (Cooke, 2010). Both the hippocampus and the amygdala play functional roles in social recognition in laboratory rodents (Broadbent, Squire, & Clark, 2004; Spiteri et al., 2010). The amygdala is also associated with anxiety (Yilmazer-Hanke, Roskoden, Zilles, & Schwegler, 2003) and depression (Dalla et al., 2004). Since these brain areas undergo drastic changes during pubertal development, they may be directly affected by a peripubertal immune challenge, ultimately changing the neural circuitry that underlies certain behaviors.

Microglia, which are the immunoreactive cells in the brain, play an important role during pubertal development. Though microglia are essential for maintaining a healthy brain throughout life by monitoring the environment in a manner similar to macrophages, they also help with normal development by aiding in the refinement of synaptic connections (Paolicelli et al., 2011). Microglia maintain the brain’s health by scanning the environment and protecting against pathogens and other types of potentially harmful substances (Hanisch & Kettenmann, 2007). The fact that pubertal development is a time of brain reorganization via synaptic changes suggests an important role for microglia in this stage of an organism’s life.
LPS is widely used to induce an immune response in microglia, thus activating them. LPS is a component in the outer membrane of Gram negative bacteria and through the CD14 complex, binds to toll-like receptor 4 (TLR4) on microglia (Hou et al., 2014). Once activated, the ionized calcium-binding adaptor 1 (Iba1) protein is expressed in higher levels (Imai & Kohsaka, 2002). Iba1 is constitutively expressed in microglia, but higher levels of expression upon insult cause it to interact with Rac, a protein involved with actin cytoskeletal reorganization (Imai & Kohsaka, 2002). The actin cytoskeleton contributes to activation of microglia in that it aids in cell proliferation, migration, and other functions (Imai & Kohsaka, 2002). This makes Iba1 a good marker for analyzing microglia in that one can visualize morphological changes specific to microglial activity.

Microglia in a healthy (clear of neural insult) environment typically have what is known as “ramified” structure with long, thin, cellular processes (Hinwood et al., 2013). When responding to neural insult, microglia processes become thicker and shorter (Hinwood et al., 2013). Since microglia tend to increase in volume upon activation, Iba1 immunoreactivity (Iba1-ir) can be used to visualize differences between microglial activity in animals. In an experiment to test for morphological differences in microglia between peripubertal and adult mice, CD1 mice had more Type III microglia (short, thick processes indicating activation) at 6 weeks of age, during pubertal development, than at 10 weeks of age, at the beginning of adulthood (Holder and Blaustein, unpublished observations). Due to the increased number of Type III microglia during pubertal development, it is likely that microglia are highly active during this period. LPS would likely have a more detrimental effect in animals with baseline active microglia than in animals with primarily inactive microglia.
Activated microglia produce proinflammatory signal molecules (cytokines) such as tumor necrosis factor alpha (TNF-α). Cytokines can have pro-inflammatory or anti-inflammatory effects, and sometimes both (Hanisch & Kettenmann, 2007; reviewed in Holder & Blaustein, 2014). They are important for development, cell differentiation, synaptic plasticity, and response to injury (Bauer, Kerr, & Patterson, 2007). Cytokine levels are highly dynamic due to these functions. In times of chronic neural stress or inflammation, such as infectious disease, cancer, or arthritis, proinflammatory cytokines may inflict lasting damage on the brain. In humans, patients suffering from some of these ailments have higher levels of proinflammatory cytokines, which can contribute to major depression (Capuron & Miller, 2011). Since cytokines can have negative consequences, it is tempting to speculate that a stressor, such as LPS during pubertal development, could inflict long-lasting damage through a microglia-mediated immune response.

LPS injected in mice at 6 weeks of age leads to higher Iba1-ir than injection at 8 weeks in C57Bl/6 (inbred strain) mice in the basolateral amygdala (BLA), the VMH, and the Arc (Holder & Blaustein, unpublished observations). In an earlier study, the latter two brain areas had significantly lower of ER-α cells after peripubertal shipping (Ismail et al., 2011) and LPS injections (Ismail & Blaustein, unpublished observations). Hippocampal areas were also analyzed due to its increase in size during pubertal development and its high number of estrogen receptors (reviewed in Giedd et al., 2006). These areas included the dentate gyrus (DG), the CA1, and the CA3; however, no significant differences were observed between peripubertal and adult mice injected with LPS. The same behavioral deficits following a peripubertal immune challenge or shipment have been observed in
both inbred and outbred strains. Therefore, we aim to test whether or not the interaction of treatment and age after LPS injection extends to CD1 mice.

We hypothesize that female CD1 mice will show differential microglial activity patterns between ages following injection of LPS or saline during pubertal development or adulthood, such that peripubertal LPS-treated mice will have significantly higher Iba1-ir than adult LPS-treated mice. Observation of differential microglia behavior between peripubertal and adult CD1 and C57Bl/6 mice would suggest a common mechanism for altered behavioral responses to steroid hormones following immune challenge. Alternatively, failure to observe significant interactions between treatment and age would suggest that LPS does not interact with microglia differentially between peripubertal and adult CD1 mice.

**Specific Aim**

**To test the hypothesis that differences occur in the microglial activation between peripubertal and adult female CD1 mice after LPS injection.** Observation of differential microglial activation patterns between peripubertal and adult mice would demonstrate differences in the brain’s response to immune stressors between times of rapid development and times of relative stability. This suggests that peripubertal mice are more sensitive to immune stressors, which aids in further understanding of the mechanism through which peripubertal immune stressors alter behavioral responses in adulthood.

**Methods**

**Animals**

Female CD1 mice were ordered from Charles River Laboratories in Kingston, NY. The peripubertal (6-week; n=16) group arrived at 3 weeks of age and the adult group (11-week; n=16) arrived at 10 weeks of age. These ages for shipment were not shown to
alter behavior in adulthood (Laroche et al., 2009). They were housed four to a cage with Carefresh bedding in temperature-controlled rooms (~72°F) with ad libitum access to food (Teklad 2014, phytoestrogen-reduced diet, Harlan Laboratories, Madison, WI) and water. The mice were on a reversed light/dark cycle, 14:10 hours respectively, with lights off at 1100 EST.

**Lipopolysachharide Injections**

LPS (Escherichia coli serotype O26:B6; no. L3755 Sigma Chemical Co., St. Louis, MO) was dissolved in sterile saline (1 mg/ml) and injected in the dose of 1.5 mg/kg *ip* (Laroche et al., 2009). After weighing the mice, two cages from each age group were randomly assigned to LPS injections, while the remaining cages received saline injections.

**Sickness Behavior Scoring**

Animal cages were letter-coded, so that scorers were blind to treatment conditions. Sickness behaviors were scored at 30 minutes, 4 hours, and 24 hours after injection. Sickness behaviors include lethargy, huddling, ptosis (eyelid-drooping), and piloerection (fur on back standing up) (Gandhi, Hayley, Gibb, Merali, & Anisman, 2007). Sickness scores were marked out of 4, with each behavior observed receiving a score of 1. Mice were weighed at 24 hours post injection to assess body weight loss.

**Tissue Processing**

Mice were injected with 100 mg/kg of sodium pentobarbital *ip* and decapitated. Brains were extracted and submersion-fixed in a 4% paraformaldehyde solution and stored at 4°C for 48 hours. After fixation, brains were cryoprotected in 30% sucrose in 0.05 M phosphate buffer and stored at 4°C for 72 hours. The brains were then frozen in powdered dry ice and placed in a freezer at -80°C. The brains were cut into a 1-in-4
series of 30 µm coronal sections on a cryostat at -20° C. The brain sections were stored in cryoprotectant at -20° C.

**Immunocytochemistry**

One series of brain sections from each animal was sorted for areas of interest and immunostained with an Iba1 antiserum (rabbit anti-Iba1, 1:10000; Wako Chemicals, Richmond, VA). Sections were rinsed in 0.5 M Tris-buffered saline (TBS) three times to wash out cryoprotectant. Sections were then rinsed three times in 0.05 M gel TBS (10% gelatin, 10% sodium azide, and 10% Triton-X). They were incubated in a blocking buffer for 1 hour to block nonspecific binding, composed of 10% bovine serum albumin (BSA), 20% normal goat serum (NGS), and 3.5% hydrogen peroxide. Tissue was then incubated in the primary antiserum for Iba1 at 4° C for 48 hours. The tissue was rinsed with three washes in gel TBS, and sections were incubated in secondary antiserum (1:800 of 2% NGS in gel TBS) for 90 minutes. Following two more washes each in gel TBS and TBS, the sections were incubated in an avidin-biotin horseradish peroxidase complex (1:800 avidin, 1:800 biotin; Vectastain ABC; Vector Laboratories) for 90 minutes.

Immunoreactivity was visualized with a nickel sulfate and 3,3’-diaminobenzidine tetrahydrochloride (DAB) solution (DAB Kit; Vector Laboratories) for 20 minutes. Tissue was mounted on gelatin-coated microscope slides, dehydrated in alcohol, cleared with xylene, and coverslipped.

**Image Analysis**

Slides were letter-coded prior to matching and analysis so that researchers were blind to treatment groups. Sections on the slides were anatomically matched on a Nikon compound light microscope using *The Mouse Brain Atlas in Stereotaxic Coordinates* (Franklin and Paxinos, 2004). Areas of interest include Arc, VMH, BLA, MePD, DG,
CA1, and CA3. The Arc, VMH, BLA, and MePD were matched to plate 43, while the CA1, CA3, and DG were matched to plate 44. Images were captured with a QImaging Micropublisher 5.0 RTV camera using the QCapture program. (Figure 1). Sections with the areas of interest were then analyzed using ImageJ for optical density and mean Iba1-ir per µm² using the default threshold function to quantify area and intensity of stain. Areas of analysis were drawn using a circle or square of predetermined-size on each image. All areas were imaged and analyzed bilaterally.

![Figure 1](image)

**Figure 1.** Tissue matching and analysis of images. Typical section for Arc image at 4X magnification with Arc border highlighted (A); Image used for analysis for Arc at 20X magnification with typical area of analysis drawn (B).

**Statistical Analysis**

Sickness behavior was analyzed for main effects of treatment and time using a two-way, repeated measures analysis of variance (ANOVA). Bonferroni posttests were used to compare groups. Body weights were analyzed for main effects of treatment and time using a two-way ANOVA. A two-way ANOVA was used to analyze main effects of treatment and age on Iba1-ir and optical density for each brain. Bonferroni posttests were also used to compare treatment effects between ages. Graphpad Prism was used for all statistical tests and graph construction.
Results

Sickness Behavior and Body Weight Changes Following Saline or LPS Injections

A two-way, repeated measures ANOVA revealed a main effect of treatment ($F_{6, 56} = 29.29, p < .0001$), but not time ($F_{2, 56} = 3.08, p = .0537$) on sickness behavior. A significant interaction between treatment and time was observed ($F_{6, 56} = 29.29, p < .0001$). At 4 hours and 24 hours post injection, LPS-treated groups of both ages displayed significantly higher sickness behaviors than saline-treated animals ($p < .0001$). At 30 minutes post injection, there were significant differences between peripubertal saline and adult LPS ($p < .05$), peripubertal saline and peripubertal LPS ($p < .001$), adult LPS and pubertal LPS ($p < .001$), and between adult LPS and adult saline ($p < .001$). A two-way ANOVA for percent weight change revealed that LPS-treated animals lost significantly more weight than saline-treated animals over a 24-hour period post injection in both age groups ($F_{1, 28} = 163.87, p < .0001$). (Figure 1)

Figure 2. Sickness behavior and weight change. Sickness behavior scores 30 minutes, 4 hours, and 24 hours after LPS and saline injections (A). Percent change in body weight compared to baseline weights 24 hours after LPS and saline injections (B). * denotes $p < .05$, *** denotes $p < .001$. 

A

B
Analysis of Iba1 Immunoreactivity

In the Arc, there was a main effect of treatment ($F_{1, 23} = 20.00, p < .001$), but not age ($F_{1, 23} = 4.06, p = .0559$) on Iba1-ir. There was no significant interaction ($F_{1, 23} = 1.51, p = .2316$). Bonferroni posttests revealed that adult LPS-treated had significantly higher levels of Iba1-ir than saline-treated mice ($p < .001$). This effect was not observed in the peripubertal age group. There was a main effect of age ($F_{1, 23} = 5.51, p < .05$), but not treatment ($F_{1, 23} = 2.54, p = .1245$) on optical density. There was no significant interaction ($F_{1, 23} = 1.34, p = .2593$). (Figure 2)

![Figure 2](image)

**Figure 2.** Arcuate mean Iba1-ir per μm² and mean optical density. Immunoreactive Iba1 per μm² (Mean +/- SEM) in the Arc of female mice injected with LPS or saline and 6 or 11 weeks of age (A). The mean optical density of Iba1-ir (Mean +/- SEM) in the Arc (B). * denotes $p < .05$, *** denotes $p < .001$.

![Figure 3](image)

**Figure 3.** Arcuate mean Iba1-ir per μm² and mean optical density. Immunoreactive Iba1 per μm² (Mean +/- SEM) in the Arc of female mice injected with LPS or saline and 6 or 11 weeks of age (A). The mean optical density of Iba1-ir (Mean +/- SEM) in the Arc (B). * denotes $p < .05$, *** denotes $p < .001$. 

![Figure 4](image)
In the VMH, there was a main effect of treatment \((F_{1,23} = 39.63, p < .0001)\) on Iba1-ir, but not age \((F_{1,23} = 0.00, p = .9459)\). There was no significant interaction \((F_{1,23} = 0.21, p = .6546)\). Bonferroni posttests revealed that LPS-treated mice had significantly higher Iba1-ir than saline-treated mice in both the peripubertal group \((p < .01)\) and the adult group \((p < .001)\). There was a main effect of treatment \((F_{1,23} = 8.24, p < .01)\) but not age \((F_{1,23} = 1.40, p = .2489)\) on optical density. There was no significant interaction \((F_{1,23} = 0.01, p = .9060)\). (Figure 3).

**Figure 4.** VMH mean Iba1-ir per \(\mu m^2\) and mean optical density. Immunoreactive Iba1 per \(\mu m^2\) (Mean +/- SEM) in the VMH of female mice injected with LPS or saline and 6 or 11 weeks of age (A). The mean optical density of Iba1 stain (Mean +/- SEM) in the VMH (B). ** denotes \(p < .01\), *** denotes \(p < .001\).
In the BLA, there were no main effects of treatment \((F_{1, 26} = 2.71, p = .1116)\) or age \((F_{1, 26} = 1.46, p = .2374)\) on Iba1-ir. There was no significant interaction \((F_{1, 26} = 0.64, p = .4310)\). There were no main effects of treatment \((F_{1, 26} = 0.03, p = .8698)\) or age \((F_{1, 26} = 0.28, p = .6018)\) on optical density. There was no significant interaction \((F_{1, 26} = 0.16, p = .6938)\). (Figure 4)

**Figure 5.** BLA mean Iba1-ir per µm² and mean optical density. Immunoreactive Iba1 per µm² (Mean +/- SEM) in the BLA of female mice injected with LPS or saline and 6 or 11 weeks of age (A). The mean optical density of Iba1 stain (Mean +/- SEM) in the BLA (B).
In the MePD, there was a main effect of treatment ($F_{1, 20} = 14.55, p< .01$) but not age ($F_{1, 20} = 0.43, p = .5189$) on Iba1-ir. There was no significant interaction ($F_{1, 20} = 0.00, p = .9798$). Bonferroni posttests revealed that LPS-treated animals had significantly higher levels of Iba1-ir than saline treated animals in both peripubertal and adult groups ($p< .05$). There were no main effects of treatment ($F_{1, 20} = 0.71, p = .4082$) or age ($F_{1, 20} = 3.35, p = .0823$) on optical density. There was no significant interaction ($F_{1, 20} = 1.06, p = .3153$). (Figure 5)

Figure 6. MePD mean Iba1-ir per µm² and mean optical density. Immunoreactive Iba1 per µm² (Mean +/- SEM) in the MePD of female mice injected with LPS or saline and 6 or 11 weeks of age (A). The mean optical density of Iba1 stain (Mean +/- SEM) in the MePD (B). * denotes p< .05
In the DG, there was a main effect of treatment \((F_{1,27} = 4.50, p < .05)\), but not age \((F_{1,27} = 0.30, p = .587)\) on Iba1-ir. There was no significant interaction \((F_{1,27} = 0.51, p = .481)\). There were no main effects for treatment \((F_{1,27} = 0.21, p = .649)\) or age \((F_{1,27} = 0.06, p = .811)\) on optical density. There was no significant interaction \((F_{1,27} = 0.35, p = .557)\). (Figure 6)

**Figure 7.** DG mean Iba1-ir per µm^2 and mean optical density. Immunoreactive Iba1 per µm^2 (Mean +/- SEM) in the DG of female mice injected with LPS or saline and 6 or 11 weeks of age (A). The mean optical density of Iba1 stain (Mean +/- SEM) in the DG (B).
In the CA1, there was a main effect of treatment ($F_{1, 26} = 12.57, p < .01$) but not age ($F_{1, 26} = 1.44, p = .240$) on Iba1-ir. There was no significant treatment x age interaction ($F_{1, 26} = 0.04, p = .843$). Bonferroni posttests revealed that LPS-treated mice had significantly higher Iba1-ir than saline-treated animals in the peripubertal group ($p < .05$) but not in the adult group. There were no main effects of treatment ($F_{1, 26} = 0.09, p = .763$) or age ($F_{1, 26} = 1.13, p = .297$) on optical density. There was no significant interaction ($F_{1, 26} = 0.18, p = .671$). (Figure 7)

**Figure 8.** CA1 mean Iba1-ir per $\mu m^2$ and mean optical density. Immunoreactive Iba1 per $\mu m^2$ (Mean +/- SEM) in the CA1 of female mice injected with LPS or saline and 6 or 11 weeks of age (A). The mean optical density of Iba1 stain (Mean +/- SEM) in the CA1 (B). * denotes $p < .05$
In the CA3, there was a main effect of treatment ($F_{1, 25} = 8.65, p < .01$), but not age ($F_{1, 25} = 1.55, p = .225$) on Iba1-ir. There was no significant treatment x age interaction ($F_{1, 25} = 2.90, p = .101$). Bonferroni posttests revealed that LPS-treated mice had significantly higher levels of Iba1 immunoreactivity than saline-treated animals in the peripubertal group ($p < .01$) but not in the adult group. There were no main effects of treatment ($F_{1, 25} = 0.13, p = .719$) or age ($F_{1, 25} = 0.03, p = .861$) on optical density. There was no significant interaction ($F_{1, 25} = 0.01, p = .9057$). (Figure 8)

![Figure 9. CA3 mean Iba1-ir per µm² and mean optical density. Immunoreactive Iba1 per µm² (Mean +/- SEM) in the CA3 of female mice injected with LPS or saline and 6 or 11 weeks of age (A). The mean optical density of Iba1 stain (Mean +/- SEM) in the CA3 (B). ** denotes $p < .01$](image)

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Table 1. Animal representation in groups for Iba1-ir. Animals were removed from analysis if there were no sections representing the area, or if tissue was too damaged for image analysis.
CHAPTER 2

DISCUSSION

The objective of this experiment was to test the hypothesis that female outbred mice would have more Iba1 immunoreactivity (Iba1-ir), a measure of microglial activity, if injected with lipopolysaccharide during pubertal development rather than in adulthood. Based on previous data from a similar experiment using C57Bl/6 female mice (inbred), where mice showed significantly increased Iba1-ir in the Arc, VMH, and BLA if injected with LPS during pubertal development compared to adults (Holder & Blaustein, unpublished observations), we had predicted that microglial behavior patterns would be comparable in the CD1 mice (outbred). However, there were no significant age and treatment interactions on Iba1-ir in any brain areas. This does not support our hypothesis of differential microglial activation between peripubertal and adult CD1 mice. Further, it shows different patterns from what was observed in inbred mice (Holder & Blaustein, unpublished observations).

The sickness behavior scores demonstrate the effectiveness of the LPS injections. Both LPS groups (peripubertal and adult) showed significantly higher sickness behavior scores at 4 and 24 hours than saline-treated animals. The peripubertal saline group that showed high sickness scores at 30 minutes post injection has not been observed in previous experiments in this laboratory. In previous experiments, mice were injected within one hour of the onset of the dark phase (Laroche, Gasbarro, Herman, & Blaustein, 2009a; Olesen et al., 2011). Here, the mice were injected starting at 0900 and injections were finished by 0930. Sickness scoring began at 1000, exactly 30 minutes after finishing injections. Since the lights turned off at 1100 and the mice were likely still sleeping at the
onset of scoring, this possibly caused the scorers to observe huddling and lethargy, two behaviors that may be misinterpreted for sleeping. The behavior scores for this group decreased by 4 hours post injection, so it is unlikely that the saline was contaminated with LPS. Further, there was no main effect of time on sickness behavior, as observed in past experiments. This is likely due to the inconsistencies of observed behaviors, and the data would more resemble sickness scores observed previously, had injections been timed closer to the onset of the light cycle. LPS groups lost significantly more body weight by 24 hours post injection than saline-treated groups. This is consistent with LPS effects.

Animals in several groups needed to be removed from image analysis. In the VMH and MePD, there were no more than half of the animals in the peripubertal LPS group represented in the image data. (Table 1). Tissue from these animals was either damaged or cut improperly to yield sections not representative of brain areas of interest. Using tissue from these animals would have produced unreliable data.

There were main effects of treatment on Iba1-ir in the VMH and MePD in both age groups, showing that LPS-treated mice had significantly higher Iba1-ir than saline-treated mice. There were no differences between peripubertal and adult mice, showing an absence of differential activation between ages, which does not support our hypothesis. However, in the CA1 and CA3, only mice injected with LPS during pubertal development had significantly higher Iba1-ir than mice injected with saline during pubertal development. This effect was not observed in adults. In the Arc, only adult mice treated with LPS had significantly higher Iba1-ir than saline-treated mice. In the DG and BLA, there were no main effects of treatment on Iba1-ir in either age group. Possible reasons for this include improper analysis of microglial activity using ImageJ. The
program analyzes immunostain intensity on the image using a threshold function. Since activated microglia have short, thicker projections while inactivated microglia have longer projections (Hinwood et al., 2013), these projections from cell bodies in different coronal planes were often present on images in saline-treated animals. ImageJ factored these projections from other planes into the analysis, though they aren’t accurate measurements of immunostain present on the section that was imaged. (Figure 9)

Figure 10. Side-by-side treatment comparison of BLA images. Image of BLA in animal treated with saline during pubertal development (A). Image of BLA in animal treated with LPS during pubertal development (B).

In the Arc, there was a main effect of age on mean optical density. There was a main effect of treatment on mean optical density in the VMH. The lack of significant effects in other brain areas can be attributed to the way in which images were analyzed. As previously described, ImageJ analyzes all stain on the slide, including stain from projections on other planes. Mean optical density measures the average darkness of pixels above threshold. Here, images were given thresholds manually rather than using the automatic threshold. The automatic threshold function detected stain that was out of focus, likely from projections originating from a different coronal plane. By thresholding manually, a better measure was obtained of what we considered to be stain on the slide. However, this method limited variation in terms of mean optical density, thus preventing
observation of differences. The reason the Iba1-ir was present in such high levels is likely due to the omission of an additional TBS wash after DAB incubation. This wash would have likely removed excess DAB that still had the potential to bind. This has been done previously and was likely the reason for the degree of background stain present on many images from animals treated with saline.

These data are not consistent with data collected previously in this laboratory using C57Bl/6 mice. In the previous experiment, mice treated with LPS during pubertal development had significantly higher levels of Iba1-ir in the Arc, VMH, and BLA than mice treated with LPS as adults (Holder & Blaustein, unpublished observations). This effect was not observed in any brain areas in this experiment. Iba1-ir from the CA1 and CA3 here is closer towards the effect predicted, but this was not observed in the previous experiment. Rather, CA1 and CA3 Iba1-ir from the previous experiment more resembled MePD and VMH data here.

Though it is unlikely, differences between the mechanism by which LPS activates microglia could be present between inbred and outbred mouse strains. Behavioral differences have been documented between several inbred strains (reviewed in Crawley et al., 1997). Therefore, it is possible that the finer molecular organization in this immune response may differ between CD1 and C57Bl/6 mice. However, inconsistencies between experiments are likely to have influenced differences in data. Ideally, both strains would have been studied under the same conditions. Researchers, housing conditions, as well as methods of tissue processing and image analysis would have been consistent. The high variability of Iba1-ir between LPS-treated animals could have also been limited if the mice were monitored for pubertal timing. Since the typical onset of pubertal development
occurs at post-natal day 25 and 30 for C57Bl/6 and CD1 mice respectively (Ismail & Blaustein, unpublished observations), the two strains were likely at different developmental trajectories at the time of injection (6 weeks). Using this time for injection and shipment has led to positive data in the past for both strains. However, a possible shift in the sensitive period may be a contributing factor to the absence of replicated behavior data in recent years and monitoring each mouse for pubertal timing would give a more accurate and consistent indication of when to inject.

A shift in the sensitive period could be the period moving forward or back in time, or it could be a lengthening or shortening. Three different stressors were found to decrease mRNA of kisspeptin in the MPOA and Arc of female rats, likely as a result of elevated corticosterone and corticotrophin-releasing factor (CRF) levels (Kinsey-Jones et al., 2009). Kisspeptin is a puberty onset and fertility-related protein. If environmental stressors can disrupt pubertal timing, certain factors may have shifted the timing of puberty onset in recent years. Nearby construction has been documented to raise plasma corticosterone roughly twofold in rats, even if researchers could not hear noise or detect vibrations (Raff, Bruder, Cullinan, Ziegler, & Cohen, 2011). The presence of a male researcher can also be a source of stress for laboratory rodents (Sorge et al., 2014), possibly impacting puberty onset through a glucocorticoid-mediated mechanism. Examination into the possible sensitive period shift could define a new time point to begin assessing pubertal timing, and thus injection timing, in future experiments.

Estradiol is highly variable in concentration throughout pubertal development and adulthood. During vaginal opening, blood plasma levels of estradiol are relatively low compared to levels at the onset of vaginal estrus (Safranski, Lamberson, & Keisler,
Estradiol has been widely documented as a neuroprotectant. In LPS-treated brain tissue of rats, estradiol blocked upregulation of the proinflammatory cytokine, tumor necrosis factor alpha (TNF-α) (Liu et al., 2005). In contrast, removal of ovaries in mice prior to LPS injection attenuated increases of TNF-α and Interleukin-12 (IL-12) (Soucy, Boivin, Labrie, & Rivest, 2005a). Though the ovaries secrete many other chemicals, estradiol replacement rescued the ability of LPS to produce a proper immune response, illustrating estradiol’s evident facilitation in microglial activation following LPS. It is unlikely that between rats and mice, steroid hormones act in an opposite manner in terms of this mechanism. Possible causes for differences in data could include estradiol acting in a dose-dependent manner, where one concentration is beneficial, while another is damaging. It is also difficult to compare findings between two studies when one was in vitro while the other was in vivo. Though the nature of estradiol’s impact on microglial activity is difficult to conclude, the fact remains that it plays a role in microglia-mediated immune responses.

Estrogen receptors are found in multiple areas of the brain and are involved in reproductive behaviors. As mentioned, female CD1 mice expressed lower numbers of ER-α cells in the Arc, MPOA, and VMH after peripubertal shipment compared to mice shipped prior to the sensitive period (Ismail et al., 2011). This effect was also observed in the MPOA and VMH after peripubertal LPS injections in female mice (Ismail & Blaustein, unpublished observations). Estrogen receptor presence on microglia can be illustrated by its modulation of the immune response to LPS (Soucy, Boivin, Labrie, & Rivest, 2005b). Therefore, there is a direct connection of estrogens, their receptors, and microglial activation.
Since estradiol is involved with microglial activation, steroid hormones and their receptors may be involved in the general function of the central nervous system’s immune responses. The methyltransferase, Set and MYND Domain-Containing Protein 2 (SMYD2), decreases levels of certain proinflammatory cytokines, including Interleukin-6 (IL-6) and TNF-α during times of inflammation in macrophages (Xu et al., 2015).

SMYD2 also downregulates ER-α by methylating the gene (Jiang et al., 2014). The same protein plays a role in cardiac development of mice (Diehl et al., 2010), and is expressed in both the heart and the brain (Brown, Sims, Gottlieb, & Tucker, 2006).

SMYD2 could be involved in the mechanism for downregulation of estrogen receptors after a peripubertal stressor, specifically through a microglia-mediated immune response. Perhaps during microglial activation, SMYD2 interacts with proinflammatory cytokines and estrogen receptors in concert. Though it is variable between strains, mice have higher Iba1-ir during pubertal development. This is consistent with activated microglia, which produce proinflammatory cytokines. This may lead to higher levels of SMYD2, possibly leading to lower levels of ER-α at this age. It would be interesting to contrast SMYD2 expression in tissue samples from peripubertal and adult mice in order to test for possible differences between ages and treatments. From there, mice such as the C3H/HeJ strain (Jackson Laboratory), with a toll-like receptor 4 mutation rendering them resistant to certain endotoxins like LPS, could be analyzed for sex behavior and estrogen receptor expression after peripubertal and adult LPS injection. That would identify a causal relationship between LPS, microglia, and altered sensitivity to steroid hormones in adulthood. A similar experiment could be carried out with SMYD2 knockouts to attribute that protein as a step in the mechanism.
This area of research is relevant both to neuroinflammation and to the debilitating effects that stress and trauma have on the brain and behavior. Identifying a mechanism between pubertal development, immune stressors, and altered behavior could give insight as to how the brain responds to environmental stressors during sensitive periods of development.
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