EFFECTS OF EARLY-LIFE IMMUNE ACTIVATION ON MICROGLIA-MEDIATED SYNAPSE REMODELING AND HIPPOCAMPAL-DEPENDENT LEARNING IN THE JUVENILE RAT

by

Brittany F. Osborne

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Psychology

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TABLE OF CONTENTS

LIST OF TABLES ix
LIST OF FIGURES x
ABSTRACT xi

Chapter

1 INTRODUCTION 1

1.1 Microglia are the Immune Cells of the Brain 1
1.2 Microglia Shape Postnatal Neural Circuits 4
1.3 Early-life Programming of Microglia 9
1.4 Microglia in Neurodevelopmental Disorders 12
1.5 Conclusions, Hypotheses, and Predictions 14

2 METHODS 18

2.1 Experimental Subjects 18
2.2 Animal Treatments 18
2.3 Hippocampal-dependent Learning Task in Juveniles 19
2.4 Analysis of Brain Cytokine Expression 21
2.5 Immunohistochemistry 22
2.6 Image Acquisition and Three-dimensional Rendering of Microglia 24
2.7 Quantification of Microglia Engulfment of Synaptic Proteins 24
2.8 Acquisition of Neuronal Morphology and Spine Density 25
2.9 Quantification of Neuronal Morphology and Spine Density 25
2.10 Statistical Analysis 25

3 LPS ON POSTNATAL DAY 21 INDUCES DEFICITS IN THE EMERGENCE OF LEARNING IN JUVENILE MALES AND FEMALES 27

3.1 Introduction 27
3.2 Results 29
3.3 Discussion 32

4 LPS ON POSTNATAL DAY 21 PRODUCES ROBUST INFLAMMATION AND ALTERS EXPRESSION OF MICROGLIA-NEURON COMMUNICATION MOLECULES IN THE JUVENILE HIPPOCAMPUS 34

4.1 Introduction 34
4.2 Results 35
4.3 Discussion........ 42

5 LPS ON POSTNATAL DAY 21 ALTERS MICROGLIA-MEDIATED PHAGOCYTOSIS OF PRE- AND POST-SYNAPTIC PROTEINS IN THE JUVENILE HIPPOCAMPUS 46

5.1 Introduction 46
5.2 Results 47
5.3 Discussion........ 52

6 LPS ON POSTNATAL DAY 21 DECREASES BASAL DENDRITIC SPINE DENSITY IN CA3 PYRAMIDAL CELLS ON POSTNATAL DAY 24 IN THE JUVENILE HIPPOCAMPUS 54

6.1 Introduction 54
6.2 Results 55
6.3 Discussion........ 62

7 DISCUSSION........ 64

7.1 A Sensitive Period of Neural Circuit Remodeling May Explain Hippocampal Vulnerability to Inflammation during Juvenile Development 64
7.2 Complement C3 and Fractalkine Cx3cl1 Signaling May be Key Mechanisms Underlying Microglia-Mediated Phagocytosis of Synapses that Lead to Abnormal Neuronal Remodeling in CA1 and CA3 following Immune Activation........ 66
7.3 Broader Impact 69

REFERENCES 71

Appendix

A INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORMS 84
B PERMISSION FORMS........ 87

LIST OF TABLES

Table 2.1: Rat primers used for quantitative real-time PCR. 22

LIST OF FIGURES

Figure 3.1. Impact of neonatal infection on hippocampal-dependent learning and memory and pro-inflammatory gene expression in the hippocampus in juvenile male and female rats following a mild immune challenge of LPS (25µg/kg) 28

Figure 3.2. Learning in the Context Pre-exposure Facilitation Effect (CPFE) paradigm at P24 31

Figure 4.1. The inflammatory response in the hippocampus following treatment with LPS on P21 37

Figure 4.2. Characterization of changes in BDNF, Cx3cl1, and C3 in the hippocampus following treatment with LPS on P21 40

Figure 4.3. Characterization of changes in Cx3cr1, CD68, and CD47 in the hippocampus following treatment with LPS on P21 42

Figure 5.1. Juvenile males have a higher rate of microglia-mediated phagocytosis of PSD95 than females in CA1. 48

Figure 5.2. LPS on P21 significantly decreases microglia-mediated phagocytosis of PSD95 in CA3 of juvenile males. 50

Figure 5.3. Juvenile males have significantly higher rates of microglia-mediated phagocytosis of synaptic proteins compared to females in the DG. 51

Figure 5.4. Microglia volumes for each region of interest in the juvenile hippocampus 24hrs following LPS treatment of P21 52

Figure 6.1. Neuronal morphology and dendritic spine density of pyramidal cells in the CA1 of juvenile males and females on P24 57

Figure 6.2. Neuronal morphology and dendritic spine density of pyramidal cells in the CA3 of juvenile males and females on P24. 60

Figure 6.3. Neuronal morphology and dendritic spine density of granule cells in the DG of juvenile males and females on P24. 61

ABSTRACT

Dysregulation of the immune system during childhood leads to an increased risk for a number of neurodevelopmental disorders including autism spectrum disorders (ASD), attention deficit hyperactivity disorder (ADHD), and schizophrenia. Microglia are the primary immune cells of the brain and are in constant communication with surrounding neurons. As such, activation of microglia can significantly influence the function of these neurons. Throughout development, microglia-neuron communication and interactions are necessary for the proper formation of neural circuits which support the emergence and long-term maintenance of cognitive function (Paolicelli et al., 2011; Bilbo, 2010). We found that immune activation caused by administration of the bacterial cell wall component, lipopolysaccharide (LPS; 100ug/ml/kg), specifically on postnatal day (P) 21 produces deficits in the emergence of hippocampal-dependent learning days later, on P24, in male and female rats. Next, we examined gene expression in the hippocampus at 2-, 4-, 8-, and 24-hr following immune activation with LPS and determined that there is a robust response of the inflammatory molecules IL-1β and IL-6. Additionally, there is a decrease in brain derived neurotrophic factor (BDNF) expression. We found decreases in fractalkine Cx3xl1 expression, a chemokine that is produced by neurons and directly communicates with microglia, in both males and females 24hrs after LPS administration. We also found that males, but not females, have a significant increase in the expression of complement C3 at 24hr post-LPS administration. Therefore, we next examined whether LPS administration on P21 alters microglia phagocytosis of synapses in the hippocampus and whether measures of neuronal morphology and spine density were subsequently altered on P24, when we observed learning deficits. We

found that LPS decreases microglia phagocytosis of the post-synaptic protein PSD95 in CA3 of males, and that this corresponds with an increase in spine density on basal dendrites in the CA3 of males on P24. Our data suggest that changes in microglia-neuron communication may underlie changes in hippocampal-dependent learning in the juvenile period of development. Changes in these unique microglial-neuronal interactions may be a key mechanism underlying the subsequent vulnerability to neurodevelopmental and learning disorders associated with early-life immune activation.

Chapter 1

INTRODUCTION

Early development represents a particularly unique time in the lifespan of an organism, during which time the brain is undergoing an enormous amount of growth, change and maturation. Development of the brain is marked by a number of important critical and sensitive periods, that is, periods during which heightened sensitivity to particular stimuli is maintained in order for the brain to develop in a certain manner. Thus, early brain development is a time during which external factors can shape the brain and its life-long function. While sensitive periods are a normal part of early brain development and necessary for healthy brain maturation, they also represent a time during which the brain can be particularly vulnerable to negative events or stimuli. Immune activation is one such stimulus that can result in profound deviations in normal brain development and later-life behavior. Microglia, the brain's resident immune cells, are a likely mechanism driving the life-long consequences of early-life immune activation.

1.1 Microglia are the Immune Cells of the Brain

Microglia were first described in depth in the 1932 landmark publication *Cytology and Cellular Pathology of the Nervous System* by Pio del Rio-Hortega. While others before him had observed microglia *in situ*, del Rio-Hortega was the first to truly characterize them by describing nine properties of these cells that are still valid by today's standards. These properties include, 1) Microglia enter the brain

during embryonic brain development; 2) These invading progenitor cells have an amoeboid morphology and are of a unique mesodermal origin relative to other neural progenitor cells; 3) They use vessels and white matter tracks as guiding structures for migration and enter all brain regions; 4) They transform into a branched, ramified morphological phenotype in the more mature brain; 5) In the mature brain, they are found almost evenly dispersed throughout the central nervous system and display little morphological variation; 6) Each cell seems to occupy a defined territory; 7) After a pathological event, these cells undergo a morphological transformation; 8) Transformed cells acquire an amoeboid morphology, similar to the one observed early in development; and 9) These cells have the capacity to migrate, proliferate, and phagocytose (Del Rio-Hortega, 1932). Despite this accurate definition and classification, a deeper understanding of these cells would not come until some 30 years later, when Georg Kreutzberg introduced his facial lesion model, which marked the beginning of the "modern era" of microglia research by identifying their traditional role as the immune cells of the brain. In their work published in (Blinzinger and Kreutzberg, 1968) described the role of microglia in the de-afferentation process of motor neurons following transection of facial nerves in rats. This model became extremely important as it allowed researchers to study the microglial response to injury with an intact blood-brain barrier, to distinguish between microglia and invading monocytes, as well as to decipher the role that microglia have in both de- and regeneration of the brain. Indeed, it is now well established that infection, trauma, or injury (i.e. any disturbance or loss of homeostasis in the brain) can result in a cascade of changes in microglia cells, including cell shape, gene expression, and function, a result that was termed "microglia activation" (Kettenmann et al., 2011; Davoust,

Vuaillat, Androdias, & Nataf, 2008; Block, Zecca, & Hong, 2007; Hanisch & Kettenmann, 2007; van Rossum & Hanisch, 2004; Kreutzberg, 1996). In this activated state, microglia have the capacity to become motile in order to move to the site of infection or injury, proliferate, phagocytose scattered debris and dying cells, and release and respond to a number of chemoattractants including pro-inflammatory and anti-inflammatory cytokines and chemokines. Notably, this response is very similar to that of other immune cells in the periphery. Thus, microglia activation is a tightly regulated and adaptive response directed at any disruption that threatens homeostasis in the brain.

Expanding upon this early work, two additional research findings came to light, yet until recent years, they received little attention within the expanding field of neuroimmunology. The first was that, contrary to original assumptions, various microglia populations residing throughout the brain *are not homogenous*. Van Weering and colleagues (2011) elegantly demonstrated this as they were investigating the chemokine Cxcl10 and its cognate receptor, Cxcr3, for their role in neuronal death and glial cell activation in the mouse hippocampus. They found that CA1 neurons and CA3 neurons were differentially vulnerable to NMDA-induced excitotoxicity. Importantly this difference was not dependent upon the neurons in these two brain regions, but rather it was dependent upon differences in the respective microglia cell populations in these two sub-regions of the hippocampus (van Weering et al., 2011). Others have similarly shown differences between microglia in grey matter versus white matter in their expression of the immunoregulator receptor Tim-3 (Anderson et al., 2007), differences in neurotrophin expression in microglia between and within regions (Elkabes et al., 1996), and differences in LPS- and INF-γ-induced expression

of TREM-2 in microglia between and within brain regions (Schmid et al., 2009; Schmid et al., 2002). These experiments point to both *constitutive* and *inducible* heterogeneity among the microglia populations throughout the brain. Since these seminal findings, it has been posited that microglia may have unique properties dependent upon their unique exposure to various neurotransmitters across various brain regions, their proximity to blood vessels, and the specific properties of the blood-brain barrier across different brain regions. These factors can influence the microenvironment and thus may provide "provincial" adaptations for the surrounding microglia populations (Abbott et al., 2010; Davoust et al., 2008), which could influence microglia function at many levels.

The second important finding was that "resting" microglia are actually quite active. The initial characterization of microglia as the immune cells of the brain used the term "resting" to denote a microglia cell that was not currently "activated" due to some immune stimulus or infection. While this distinction in function as being “resting” or “activated” was taken quite literally for many years, it is now recognized that "resting" microglia are hardly inactive. On the contrary, these cells are constantly surveying their environment, interacting with surrounding neurons and macroglia, which has led to the updated term “surveillance mode” rather than "resting". Microglia have an important role in constitutive brain function, and thus the shift to an "activated" state merely represents a shift in the type of activities these cells perform, rather than a change from “inactive” to "active" per se (Kettenmann et al., 2011).

1.2 Microglia Shape Postnatal Neural Circuits

Microglia are key regulators of synaptic activity, and continuous communication between microglia and surrounding neurons is a critical factor for the

development of neural circuits in the healthy brain. With the development of Cx3cr1-GFP transgenic mouse line, in which microglia are fluorescently labeled (Jung et al., 2000), and the use of *in vivo* two-photon laser scanning microscopy, it was possible to visualize surveying microglia in the intact brain of live animals for the first time just two decades ago. Using this approach, two groundbreaking studies provided the first insights into the dynamics of surveying microglia *in vivo* within the superficial layers of the somatosensory and motor cortices of adult animals (Davalos et al., 2005; Nimmerjahn et al., 2005). These studies revealed that microglia in "surveillance mode" are extremely dynamic, as they continuously extend and retract their processes, effectively changing their morphology on the time scale of minutes.

During early brain development an exuberant number of synaptic connections are formed that are later refined via the important process of synaptic pruning. Synaptic pruning is an activity-dependent developmental process during which a number of synapses are tagged and eliminated while others are retained and subsequently strengthened. It is likely that spontaneous neuronal activity plays an instructional role in synaptic pruning; however, the precise cellular and molecular mechanisms underlying this developmental process are not well understood. One mechanism that is known to have at least a partial role in this type of pruning is astrocytes. Astrocytes release a number of signaling molecules (Bosworth & Allen, 2017), primarily with the goal of regulating synaptogenesis (Christopherson et al., 2005) and synapse maturation (Risher et al., 2014). Astrocytes are also involved in synaptic pruning during early development (López-Murcia, Terni, & Llobet, 2015),

however, interestingly, one of the main ways in which they are involved is indirectly by tagging synapses for phagocytic elimination by microglia. Emerging evidence continues to demonstrate that microglia, along with the receptors and molecules they express, are likely candidates participating in the process of synaptic pruning. To determine whether microglia engulf synapses undergoing synaptic pruning, Schafer and colleagues (2012) examined retinal ganglion cells (RGC) of $\mathit{Cx3cr1}^{\mathrm{GFP/+}}$ mice in the developing visual system on postnatal day 5 (P5), when synaptic pruning in this brain region is at its peak. In these mice, microglia express enhanced green florescent protein (EGFP) under the control of the fractalkine receptor, Cx3cr1, which is exclusively expressed on microglia in the CNS. Using this technique, they were able to determine that microglia engulf RGC presynaptic elements in the dorsal lateral geniculate nucleus (dLGN), allowing them to conclude that phagocytosis is one mechanism by which microglia induce synaptic pruning during early brain development. When Schaefer and colleagues examined microglia on P9, when synaptic pruning in the visual system is nearly complete, they found that engulfment of RGC elements was significantly reduced compared to P5, even though microglia maintained their ability to phagocytose as indicated by their expression of CD68 and cell morphology (Schafer et al., 2012).

Thus, what mechanisms underlie microglia-mediated engulfment or pruning of RGC synapses? In the peripheral immune system, classical complement proteins promote the identification and clearance of invading pathogens as well as cellular debris by attracting macrophages that express the complement receptor-3 (CR3) to the

site of infection. In the CNS, complement-3 (C3), which is the ligand for CR3, is expressed on immature synapses and this expression was necessary for subsequent engulfment of synaptic elements during the process of synaptic pruning (Stevens et al., 2007). In the CNS, microglia also express CR3 suggesting that C3 binding to CR3 on microglia may be one mechanism by which microglia are directed to engulf immature or weak synapses. Consistent with this hypothesis, on P5, synapses in the dLGN have increased CR3 expression (Schafer et al., 2012) and immunohistochemistry showed enrichment of C3 at synapses (Stevens et al., 2007). Subsequently both of these molecules were downregulated in the dLGN by P9. Furthermore, injection of TTX or forskolin in one eye and vehicle injection into the other eye resulted in microglia engulfing significantly *more* synaptic inputs from the less active eye into the dLGN (i.e. from the TTX-treated eye) than from the vehicle-treated eye. Lastly, microglia from CR3 or C3 knockout (KO) mice have significantly reduced phagocytic capacity compared to wild-type (WT) controls in the P5 dLGN and significantly increased retinogeniculate synaptic density as adults (Schafer et al., 2012).Though CR3 is expressed on other phagocytic cells including peripheral monocytes and macrophages that can infiltrate the CNS, the authors confirmed that these peripheral immune cells were not present in the dLGN at P5. Together, these data demonstrate that microglia engulf synapses during a critical period of synaptic development. CR3 is expressed on microglia and its ligand, C3, are an important mechanism underlying this important process of synaptic pruning.

During the second and third weeks of a rodent's postnatal development, synaptic maturation across various brain regions begins via the process of experience-dependent pruning of synapses. This type of synaptic pruning is distinct in several ways from the early postnatal synaptic pruning discussed above (for a review see Katz & Shatz, 1996), and represents a unique period of neural development necessary for the construction of *mature* neural circuits through the refinement of synapses. Paolicelli and colleagues (2011) provided the first direct evidence that microglia engulf synapses during this period of synaptic maturation. Using the same *Cx3cr1*$^{GFP/+}$ mouse line described in Schafer and colleagues (2012), they found that PSD95+ and SNAP25+ puncta were co-localized with GFP+ microglia. Moreover, 3D reconstruction confirmed that PSD95+ and SNAP25+ puncta were completely surrounded by the GFP-labeled microglia cytoplasm in CA1 *stratum radiatum* in the hippocampus at P15, indicating that microglia are phagocytosing these puncta at this time. Cx3cr1 is expressed exclusively on microglia in the CNS and is important for the migratory ability of these cells. The Cx3cr1 ligand, Cx3cl1, is expressed by neurons and is hypothesized to be a mediator of microglia-neuron communication. Knockout of Cx3cr1 on microglia resulted in an increased density of PSD95 puncta as well as an increased density of dendritic spines on CA1 pyramidal neurons compared to WT controls specifically during the second and third postnatal weeks suggesting a deficit in synaptic pruning in KO mice. Furthermore, Cx3cr1 KO mice had a decrease in the frequency of sEPSCs in CA1 pyramidal cells at P13-P16, indicating that in the

absence of appropriate microglia-mediated synaptic pruning, the synaptic connections within the CA1 remain relatively immature (Paolicelli et al., 2011).

Expanding on these findings, Zhang and colleagues (2012) examined whether the immature connectivity observed at P13-P16 in Cx3cr1 KO mice persisted into adulthood. As predicted, the researchers found that Cx3cr1 KO mice had synaptic connectivity that remained relatively immature, even into adulthood, though the number of synapses eventually reached adult levels at a later date. Conversely, WT controls displayed molecular and electrophysiological characteristics indicative of mature synapses, suggesting that experience-dependent synaptic pruning by microglia within the first few postnatal weeks represents a *critical window* for normal circuit maturation (Boulle et al., 2016; Zhang et al., 2012).

Taken together, these data highlight four important points: 1) the first few weeks of postnatal life represent an important period for the formation and maturation of neural circuits, 2) microglia have a pivotal role in sculpting neural circuits, 3) C3 and Cx3cl1/Cx3cr1 have an important role in supporting the microglia-neuron communication necessary to establish mature neural circuits, and 4) perturbations in microglia function during the first few weeks of postnatal development can have lasting, negative consequences on the brain and behavior.

1.3 Early-life Programming of Microglia

Early-life programming is the idea that events that occur during perinatal development have the potential to alter the course of normal development, leading to

significant and often permanent changes in physiological, behavioral, and cognitive outcomes later in life. This idea has been studied in many contexts including exposure to early-life stress (Agorastos et al., 2019), maternal separation or neglect (Blaze & Roth, 2017), exposure to alcohol or other drugs of abuse (Klintsova et al., 2012; Murawski et al., 2012), and changes in diet that affect life-long metabolism (Bolton & Bilbo, 2014); however, less is known about early-life programming of the immune system and the potential enduring consequences this may have on neurocognitive outcomes.

During early brain development, microglia migrate into the brain from the periphery where they release cytokines and chemokines important for brain development (Ginhoux & Prinz, 2015; Nikodemova et al., 2015; Lenz, et al., 2013; Schafer & Stevens, 2013; Schwarz et al., 2012). During embryonic development and the first few postnatal week in rats, microglia in many brain regions exhibit an amoeboid morphology, which is followed by a gradual change in their morphology to a more heterogeneous population of morphologies (e.g., stout microglia and/or microglia with thick processes) around postnatal day (P) 4. By the end of the third postnatal week, the most predominate microglial morphology is one with long, thin processes (i.e. "ramified") (Schafer & Stevens, 2013; Schwarz et al., 2012), and this phenotype is maintained into adulthood. Developmental programming of the immune system in the brain is thought to occur mainly through a mechanism described as glial priming. That is, exposure to a robust immune challenge during the first few weeks of life, when microglia are still developing, is thought to cause microglia to remain in a

chronically sensitized or primed state (Bland et al., 2010). Notably, priming does not result in microglia that constitutively over-produce inflammatory molecules, rather "primed" microglia produce an exaggerated response to *subsequent* immune challenges later in life.

Indeed, early-life exposure to infection, injury, or immune activation can have significant, lasting consequences for cognition and behavior in adulthood. For example, neonatal exposure to bacterial or viral mimetics (e.g. lipopolysaccharide [LPS] or Poly I:C) can produce permanent alterations in behavior and cognitive functions such as altered social interactions and memory (Hanisch & Kettenmann, 2007), increased anxiety (Ransohoff & Perry, 2009), and schizophrenia-like behaviors (Laskaris et al., 2016) across a number of species later in life. Bilbo and colleagues (2006) found that neonatal infection with live *Escherichia coli* (*E.coli*) during the neonatal period produces learning impairment in male rats, but only when learning is paired with a second, mild immune challenge with a low dose of LPS in adulthood. Furthermore, early-life immune activation results in exaggerated expression of the pro-inflammatory cytokine interleukin-1β (IL-1β) in the hippocampus in response to the LPS challenge administered at the time of learning (Bilbo et al., 2006). When IL-1β synthesis was prevented via a caspase-1 inhibitor, learning deficits were prevented (Bilbo et al., 2005) indicating that this inflammatory molecule released from "primed" microglia has a critical role in the cognitive deficits observed in these rats. These data suggest that the immune system, in particular microglia, is permanently altered or programmed by *early-life* immune activation or

inflammation, and that the immune system and the molecules it produces (e.g. cytokines) are key mediators of the cognitive and learning impairments observed in adulthood. Additionally, primed microglia cells express higher levels of the surface antigens CD11b and MHCII in the hippocampus which is sustained into adulthood (Frank et al., 2006). Williamson and colleagues (2011) confirmed these characteristics of primed microglia with the use of flow cytometry showing that cells from neonatally infected rats were larger and expressed more CD11b on a per cell basis, while the overall number of cells was not different from controls (Bland et al., 2010). Together, these data demonstrate that early-life immune activation can program microglia cell function, permanently altering their reactivity to subsequent immune challenges, which can ultimately impair cognitive function in adulthood.

1.4 Microglia in Neurodevelopmental Disorders

Epidemiological data indicate a strong correlation between early-life immune activation from infection or injury and later diagnosis with a number of neurodevelopmental such as autism and schizophrenia (Laskaris et al., 2016; Yirmiya et al., 2015; O'Connor et al., 2014; Leckman, 2014; Frick et al., 2013; Maezawa et al., 2011). Moreover, evidence suggests that chronic immune dysregulation in adults, such as that caused by early-life programming, is associated with cognitive disorders including depression, schizophrenia, dementia, and Alzheimer's disease later in life. Important to the work presented here, there is an emerging literature to suggest that changes in the brain that may underlie the pathology of these disorders actually begin early in development (Leckman, 2014; O'Connor et al., 2014; Frick et al., 2013;

Maezawa et al., 2011). For example, a number of neurodevelopmental disorders including schizophrenia, autism spectrum disorder (ASD), and Rett syndrome have all been linked to early-life immune activation and subsequent dysregulation of immune function in early childhood (Maezawa et al., 2011). For example, individuals with ASD and Rett syndrome have altered peripheral cytokine levels, low immunoglobulin levels, and altered T cell activation (Maezawa et al., 2011).

Evidence of altered neuroimmune function in the CNS is emerging that suggests a key role for microglia in these disorders. In fact, neuroinflammation, which is primarily associated with microglia, has been found in patients with psychiatric disorders (Najjar et al., 2013) and an increase in microglial cell number in cortical areas and visual cortex in patients with ASD has been reported (Tetreault et al., 2012). Postmortem studies of patients with schizophrenia have found microglia activation and increased numbers of microglia cells (Radewicz et al., 2000; Bayer et al., 1999) and, using PET imaging, van Berckel and colleagues (2008) found microglial activation within the first 5 years of symptom onset. Similarly, in patients with Major Depressive Disorder (MDD), increased microglia activation as well as macrophage recruitment was found in postmortem dorsal anterior cingulate cortex (Torres-Platas et al., 2014). Neuroinflammation is also observed in the majority of these patients suggesting that increased release of pro-inflammatory cytokines may be involved in the cognitive and behavioral changes observed in these neurodevelopmental or psychiatric disorders, however, it is not clear to what extent this inflammation is either

detrimental or beneficial; nor is it clear whether microglia activation is a primary cause or consequence of these disorders. To this point, a meta-analysis of cytokine profiles in individuals with MDD found no differences between expression levels of IL-10 compared to non-depressed subjects suggesting that normal levels of anti-inflammatory cytokines are not predictive of mood disorder rates (Dowlati et al., 2010), thus a simple understanding of pro- versus anti-inflammatory, or activated versus not activated is not, on its own, predictive of the risk for these disorders. Furthermore, increased microglia *activity* (e.g. pruning) is correlated with the intensity of psychotic symptoms in prodromal patients who are at a high risk of psychosis, but have not yet converted to schizophrenia (Bloomfield et al., 2016), indicating that microglia-neuron interactions, not just microglia "activation" (i.e. inflammatory response), could be important for determining the onset and severity of schizophrenia. Together with the recent evidence from animal models indicating the importance of microglia-neuron communication in the proper developmental of neural circuits and the implications this has for long-term cognitive and behavioral outcomes, it appears that there is still much to be learned about the role that microglia have in the development of neurodevelopmental and psychiatric disorders.

1.5 Conclusions, Hypotheses, and Predictions

Microglia have a more significant role in the development and programming of the brain and behavior than was previously appreciated. Furthermore, researchers are finally starting to elucidate the precise mechanisms underlying their important role in

early brain development via an understanding of not only their role as the immune cells of the brain, but also their active role in normal developmental processes that establish mature neural circuits. Exposure to a number of early-life events that activate the immune system can alter the function of microglia cells, which may underlie the *onset* and *maintenance* of abnormal developmental trajectories, thereby increasing the risk for neurodevelopmental or psychiatric disorders that have their origins in early development. However, little research has investigated the effects of immune activation during ***juvenile brain development***, when the brain is undergoing a tremendous amount of experience-dependent maturation, and when functional neural circuits are established, necessary for the emergence of cognitive and social behaviors. Thus, the goal of these experiments was to determine how immune activation alters microglia function in the hippocampus, including changes in microglia-mediated synaptic sculpting of hippocampal neural circuits, and how these changes may lead to deficits in hippocampal-dependent learning in juvenile rats.

The experiments presented here test the central hypothesis that: ***Immune activation during a "sensitive" period of neural circuit remodeling in the hippocampus will disrupt microglia-mediated synaptic remodeling of neural circuits and produce deficits in the emergence of cognitive function.***

To test this central hypothesis, the following individual hypotheses and their predictions were addressed:

Experiment 1.1 & 1.2: We hypothesize that postnatal day 21 is a "sensitive" developmental time period in the hippocampus which is particularly vulnerable to

immune activation, and that exposure to immune activation will result in deficits in the emergence of hippocampal-dependent learning.

Prediction 1: Immune activation on P21 will produce learning deficits in the hippocampal-dependent learning task the Context Pre-exposure Facilitation Effect (CPFE) paradigm when tested on the day the ability to learn the CPFE emerges, P24.

Prediction 2: Immune activation on P24, an age at which rats can already learn the CPFE, will *not* result in learning deficits.

Experiment 2.1a: We hypothesize that immune activation on P21 will result in significant increases in inflammatory gene expression and alter the expression of microglia-neuron signaling molecules in the hippocampus.

Prediction 1: Immune activation will increase pro-inflammatory gene expression, but these changes will be resolved within 24 hours after the immune challenge.

Prediction 2: Immune activation will alter the expression of molecules that are critical to microglia-neuron communication in the hippocampus.

Experiment 2.1b: We hypothesize that immune activation on P21 will disrupt microglia-mediated phagocytosis of pre- and post-synaptic proteins in the hippocampus.

Prediction: Immune activation will result in a significant decrease in the microglia-mediated phagocytosis of pre- and post-synaptic proteins in the hippocampus.

Experiment 2.2: We hypothesize that immune activation on P21 will produce changes in neuronal morphology and dendritic spine density in the hippocampus on P24.

Prediction: Immune activation on P21 will lead to increases in dendritic complexity and increases in the number of dendritic spines on hippocampal neurons at P24.

POTENTIAL IMPACT of this Research: These experiments will further our understanding of developmental periods that are sensitive to immune activation, and the factors responsible for the subsequent emergence of neurodevelopmental and cognitive disorders. Specifically, these experiments will advance our understanding of the molecular and cellular mechanisms of immune dysregulation and they will identify unique molecular signatures across critical time points throughout this developmental window. Identifying these mechanisms will allow future research to target these molecules and their associated neural circuits to inform future therapies aimed at helping those with neurodevelopmental disorders.

Chapter 2

METHODS

2.1 Experimental Subjects

Adult male and female Sprague Dawley rats were ordered from Envigo Laboratories in Indianapolis, Indiana. Rats were housed in same sex pairs in clear, polyethylene cages (45cm x 20.5cm x 24cm) and allowed one week of acclimation to the facility prior to breeding. The colony room was maintained at 22°C on a 12:12 hr light:dark cycle (lights on at 7:00 a.m.) and all rats had *ad-libitum* access to food and water. For breeding, male and female pairs were housed together for five days and the presence of sperm plugs was checked daily to determine the date of conception, designated as embryonic day one (E1). Pregnant females were housed individually two days prior to the calculated date of birth. Litter sizes and male to female ratios were not adjusted at the time of birth; however, no more than 1-2 pups from a given litter were represented in each experimental group to minimize possible litter effects. All rats were weaned on P21. Sentinel rats were housed in the colony room and periodically examined for the presence of common rodent diseases. All tests came back negative. All experiments were approved by the University of Delaware Institutional Animal Care and Use Committee.

2.2 Animal Treatments

Lipopolysaccharide (LPS) Injections. All experiments used LPS derived from *E.coli* 0111:B4 obtained from Sigma Aldrich (Cat. No. L2630). Sterile, pyrogen-free

DPBS was used to dilute the stock concentration of LPS (2,500μg/mL) to a final concentration of 100μg/mL for injections. On P21, rats in all experiments received an intraperitoneal (i.p.) injection of either 1mL/kg of 100μg/mL of LPS or the equivalent volume of DPBS as a control. Immediately following injections rats were weaned and left undisturbed until the start of additional experimental procedures.

2.3 Hippocampal-dependent Learning Task in Juveniles

Context Pre-Exposure Facilitation Effect (CPFE) Paradigm

Procedure. Hippocampal-dependent learning was assessed using a modified version of contextual fear conditioning called the Context Pre-exposure Facilitation (CPFE) paradigm (Burman et al., 2009; Rudy et al., 2004; Fanselow, 1990). All rats were numbered (i.e. blind to treatment) throughout the duration of the experiment. Rats began this task on P24, which is the earliest age at which robust and reliable learning of the CPFE task emerges (Jablonski et al., 2012). CPFE is a 3-day task where on P24 (day 1) rats were placed in a context and allowed to freely explore and learn about the context for 5 minutes. Twenty-four hours later on P25 (day 2), rats were put back in the same context, received an immediate shock (2, 2-second, 0.7mA [direct current] shocks spaced 1 second apart), and then immediately removed from the context. Twenty-four hours following the immediate shock, on P26 (day 3), rats were placed back into the context for 5 minutes and learning was measured by assessing the amount of time spent freezing.

Immediate Shock Deficit. To control for hippocampal-dependent learning, the Immediate Shock Deficit (ISD) paradigm was performed on a separate group of

juvenile male and female rats. Rats in this task do not receive the day 1 pre-exposure to the context on P24, but do receive the immediate shock on day 2 and test on day 3 as described above. This control task was used to confirm that freezing on test day was a result of a hippocampal-dependent, learned-association between the context on day 1 and the shock on day 2, rather than a more generalized form of fear learning to the shock alone (Rudy et al., 2004; Fanselow, 1990).

Apparatus. Two identical chambers were obtained from TSE Systems (2560606 Series; Germany) and connected to the TSE Systems software program (TSE Multi Conditioning System – Fear Conditioning). Each chamber was white with two doors in the front of the chamber that locked closed and housed the conditioning boxes. Each chamber was fitted with sound attenuating foam, interior lights and a TSE Systems camera (Type No. 256060-VID-KIT/CAM) that was mounted at the top of the chamber directly above each conditioning box. All four walls of each conditioning box (44cm x 37cm x 44 cm) were made of black Plexiglas. Two walls had vertical, white stripes covering them (front and back walls of the boxes) constructed from painter's tape, the left wall had a large white outline of a triangle constructed from painters tape, and the right wall had a large white outline of a square, also constructed from painters tape. The shock was delivered through a removable floor of stainless steel rods that were 0.5cm in diameter, spaced 1cm apart, and wired to a TSE Systems generator (PROCESS CONTROL MultiConditioningSystem 256060 Series). The chambers were cleaned with Quatricide and then water before each rat was placed inside. Videos from the test session on day 3 were exported and an observer, blind to

the treatment conditions, scored the freezing behavior for each rat. Freezing behavior was defined as the complete absence of movement with the exception of that required for breathing. A frequency score was generated by assessing every 5 seconds whether the rat was freezing or not throughout the entire 5-minute testing session (60, 5-second intervals).

2.4 Analysis of Brain Cytokine Expression

Two, four, eight, and twenty-four hours following saline or LPS injections on P21, rats were euthanized by administration of an overdose of Euthasol® (ANADA 200-071) via i.p. injection. Once anesthetized, rats were perfused via cardiac puncture with ice-cold, 0.9% saline solution to remove blood and peripheral immune cells from the brain. Following perfusion, the dorsal hippocampus was collected and immediately flash frozen on dry ice. Samples were stored at -80°C until further analysis.

RNA was extracted from frozen tissue samples using Isol-RNA Lysis Reagent (Cat. No. 2302700, 5 Prime). Genomic DNA was eliminated and cDNA was synthesized from extracted RNA (1000ng/µL) using the QuantiTect® Reverse Transcription Kit (Cat. No. 205314, Qiagen). Relative gene expression was quantified by real-time PCR using the RealMasterMix™ Fast SYBR Kit (Cat. No. 2200830, 5 Prime) in 10µL reactions on a CFX96Touch real time PCR machine. Ribosomal protein large P1 (RPLP1), Interleukin-6 (IL-6), and complement C3 were analyzed using a QuantiTect® Primer Assay (Cat. No. QT00182896) and diluted according to protocol. All other primers were ordered through Integrated DNA Technologies and diluted to a final concentration of 0.13 µM (IL-1β, BDNF, Cx3cl1, Cx3cr1, CD68, and CD47; See **Table 2.1** for a list of primer sequences). RPLP1 was used as the

housekeeping gene for all experimental groups as it did not differ significantly across any groups. Samples were numbered and blinded to treatment group and run in duplicate on real-time PCR plates. For each reaction, the average quantitative threshold amplification cycle number (C_q) value was determined from each duplicate, and the $2^{-\Delta\Delta C_q}$ method was used to calculate the relative gene expression for each gene of interest relative to the housekeeping gene.

Table 2.1: Rat primers used for quantitative real-time PCR.

Gene	NCBI Sequence	Primers
IL-1β	NM_031512.2	F: GAAGTCAAGACCAAAGTGG R: TGAAGTCAACTATGTCCCG
BDNF	NM_012513.4	F: ATCCCATGGGTTACACGAAGGAAG R: AGTAAGGGCCCGAACATACGATTG
Cx3cl1	NM_134455.1	F: TCCAGGGCTGTCCCCGCAAA R: ACAGGCAGGCAAGCAGGCAG
Cx3cr1	NM_133534.1	F: TTCCTCTTCTGGACGCCTTA R: TAAACGCCACTGTCTCCG
CD68	NM_001031638.1	F: GCTTCTGTTGCGGAAATAC R: AGATTGGTCACTGGCGCAA
CD47	NM_019195.2	F: CTCGCCAAGCACTAACCT R: CCAGAAGTGGCAGTCGTT

2.5 Immunohistochemistry

Two, four, eight, and twenty-four hours after the saline or LPS injections on P21, rats were euthanized by administration of an overdose of Euthasol® (ANADA

200-071) via i.p. injection. Once anesthetized, rats were perfused via cardiac puncture with ice-cold, 0.9% saline solution to remove blood and peripheral immune cells from the brain. Half brains were collected on ice and immediately fixed in 4% ice-cold paraformaldehyde (PFA) for 24 hours at 4℃. Half brains were then transferred to fresh 4% PFA, then cryoprotected in 30% sucrose solution, and finally fresh 30% sucrose solution at 24-hour intervals and stored at 4℃.

Half brains were sliced at 20µm on a Leica cryostat at -25℃ into wells containing 0.001% Sodium Azide Solution. Sliced brains were stored at 4° C until staining. Ionized calcium-binding adaptor molecule (Iba)-1 (Schwarz et al., 2012), PSD95 and SNAP25 (Paolicelli et al., 2011) were chosen as the target proteins for staining. Briefly, sections were washed 3 times (at least 5 minutes each) with phosphate-buffered saline (PBS) and then incubated for 1 hour with a buffer solution containing PBS, normal goat serum (Vector Laboratories), and 30% Triton X (Fisher Scientific). Sections were then washed again (3 times for at least 5 minutes each) and incubated with primary antibodies (rabbit anti-Iba1, 1:1000; Wako Chemicals; mouse anti-PSD95, 1:200; Sigma; goat anti-SNAP25, 1:800; Sigma) overnight at 4° C. On day 2, sections were washed (three times for at least 5 minutes each) and incubated with fluorescent secondary antibodies (Alexa Fluor™ 647goat anti-rabbit IgG , 1:500; Invitrogen; Alexa Fluor™ Plus 488 goat anti-mouse IgG, 1:500; Invitrogen; Alexa Fluor™ 594 chicken anti-goat IgG, 1:500; Invitrogen) for 2 hours at room temperature in the dark. Sections were washed and incubated with Hoescht 33342 (1:3000; Invitrogen) for 15 minutes at room temperature in the dark. Finally, sections were washed for 30 minutes then washed overnight in the dark. The following day, sections were transferred to fresh PBS, covered with foil, and stored at 4° C until mounting.

Sections were mounted on Superfrost++ Micro Slides (Fisher Scientific), cover slipped (1.5; VWR) with ProLong Diamond Antifade mounting medium (Thermo Fisher Scientific) in the dark and stored at -20° C until analysis.

2.6 Image Acquisition and Three-dimensional Rendering of Microglia

Confocal fluorescent images were acquired on a Nikon CSU-W1 confocal microscope equipped with 405, 488, 561, and 647 lasers and a 40x (1.3 NA) oil-immersion objective using Nikon Elements software. Confocal z stacks with 2 μm z intervals were acquired using a 40x objective and used for three-dimensional rendering of microglia and synaptic proteins. Imaged z stacks were reconstructed in Imaris (BitPlane) as described in (Schafer et al., 2014). Individual microglia cells were reconstructed using the surfaces module to create a volumetric boundary of the cell. Reconstructed microglia surfaces were then used as a mask to process the channels containing the synaptic proteins to be co-localized. After masking was performed for each microglia cell of interest, the surface module was used again to determine the volumes of the co-localized synaptic proteins.

2.7 Quantification of Microglia Engulfment of Synaptic Proteins

The total volume of each synaptic protein that was co-localized within the boundary of a reconstructed microglia cell were calculated in Imaris (BitPlane). After the surface module was used to render the volumes for each protein within a cell, the unify function was used to group all of the individual particles of the given protein to give a total volume. All data are normalized to the individual cell volume. Data from 10 cells within each of the three hippocampal regions (i.e. CA1, CA3, DG) for each rat were chosen for analysis.

2.8 Acquisition of Neuronal Morphology and Spine Density

On P24, rats were euthanized by administration of an overdose of Euthasol® (ANADA 200-071) via i.p. injection following saline or LPS injections on P21. Once anesthetized, rats were rapidly decapitated and whole brains were collected and rinsed for 1 minute with ice-cold Millipore water in the dark. Brains were then processed according to the FD Rapid GolgiStain™ Kit (FD NeuroTechnologies, Inc) protocol. Immediately following completion of the protocol, brains were sliced at 200μm on a Leica cryostat at -25ºC and directly mounted onto 75 x 25 mm Gelatin-Coated Slides (Cat# PO101) from FD NeuroTechnologies, Inc. and cover slipped using Permount in the dark. Slides were stored in the dark at room temperature until the time of analysis.

2.9 Quantification of Neuronal Morphology and Spine Density

Neuronal tracings were acquired using a Zeiss Axio Imager M2 and a 63x (1.4 NA) oil-immersion objective. All tracings were done using the Neurolucida software program. Five cells per hippocampal region of interest (i.e. CA1, CA3, DG) were traced for each rat. The Neurolucida Explorer software was used to extract the measures of interest for each cell. Measures collected for all cells were total lengths for primary, secondary, and tertiary branching, spine density for each branch order number of branching points, total branching order, total branching length, and total number of spines. When applicable, apical versus basal branching is distinguished for each measure.

2.10 Statistical Analysis

Data were analyzed using the statistical software program SPSS (IBM). Data from Experiments 1.1, 1.2, 2.1b and 2.2 were analyzed using a 2x2 analysis of variance (ANOVA) with P21 Treatment (saline vs LPS) and Sex (male vs female) as

the between-subjects factors. Additionally, a two-tailed independent samples t-test was used to compare the behavioral data from experimental and control groups (i.e. Context A vs ISD) to confirm learning in the CPFE paradigm. Data from Experiment 1.3 were analyzed using a 2x2x2 ANOVA with P21 Treatment (saline vs LPS), Sex (male vs female) and Minocycline Treatment (minocycline vs water) as the between subjects factors. Data from Experiment 2.1a were analyzed using a 2x2x4 ANOVA with P21 Treatment (saline vs LPS), Sex (male vs female), and Time (2hr, 4hr, 8hr, or 24hr) as the between subjects factors. Data from Experiment 2.1b were analyzed using a 2x2 ANOVA with P21 Treatment (saline vs LPS) and Sex (male vs female) as the between-subjects factors. Additionally, a one-way ANOVA with Region (CA1 vs CA3 vs DG) as the between subjects factor was used to determine whether microglia volume differed between regions. Data from Experiment 2.2 were analyzed using a 2x2 ANOVA with P21 Treatment (saline vs LPS) and Sex (male vs female) as the between-subjects factors. When appropriate, post hoc pairwise comparison tests using the Bonferroni correction were used to examine significant interactions and between group differences. The accepted significance level was $p < 0.05$. All data are presented as the mean +/- the SEM.

Chapter 3

LPS ON POSTNATAL DAY 21 INDUCES DEFICITS IN THE EMERGENCE OF LEARNING IN JUVENILE MALES AND FEMALES

3.1 Introduction

Early-life exposure to immune activation can have significant, lasting consequences for cognition and behavior later in life. This idea has been best demonstrated using a “two-hit” model of immune activation whereby neonatal immune activation produces, (1) learning deficits following a subsequent, very mild immune challenge in adulthood (Bilbo et al., 2006), and (2) exaggerated cytokine production and enhanced cognitive decline associated with aging (Bilbo, 2011); however, very little research has investigated whether early-life immune activation can affect the *emergence* of cognitive function at earlier ages. Within this context, there is a lack of understanding of how dynamic changes in neuroimmune function mediate transitions from normal brain function to the early stages of cognitive dysfunction, or how changes in immune signals are integrated into developing neuronal networks. Previous experiments determined that P24 is the earliest age at which rats reliably and robustly learn in the CPFE paradigm (Jablonski et al., 2012). Thus, using the same "two-hit" model of immune activation described earlier, we treated male and female rats with *E.coli* ("first hit") on P4 and again with LPS ("second hit") on P24 to determine whether this model also precipitates learning deficits, earlier in development, in *juvenile* rats (Osborne et al., 2017). Contrary to our predictions, we found that juvenile rats did not show learning deficits in the CPFE task following the

"two-hit" model of immune activation (**Figure 3.1A**); however, we did find an exaggerated response of IL-1β gene expression in the hippocampus on P24, indicative of the "glial priming" associated with early-life programming of immune function (**Figure 3.1B**). Specifically, this exaggerated cytokine response is similar to what has been observed in adults and aged animals, indicating that the young hippocampus is still vulnerable to the early-life programming effects of neonatal infection, though interestingly, this did not result in learning deficits in the context of the "two-hit" model at this age.

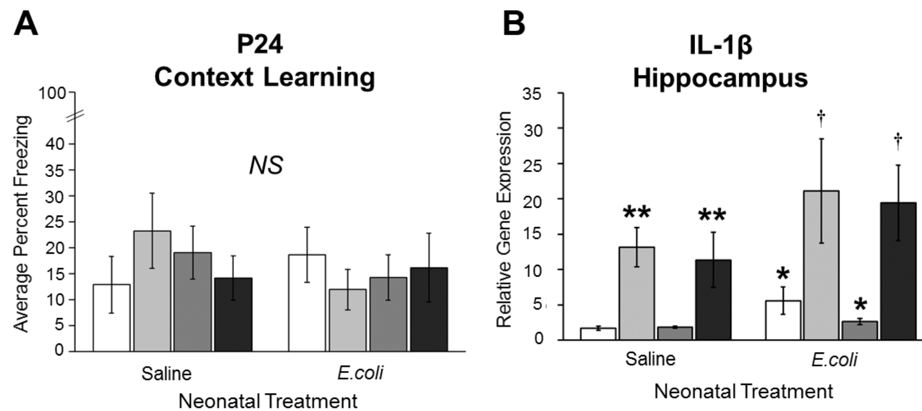


Figure 3.1. Impact of neonatal infection on hippocampal-dependent learning and memory and pro-inflammatory gene expression in the hippocampus in juvenile male and female rats following a mild immune challenge of LPS (25µg/kg). (A) The average percent freezing on day 3 of the CPFE paradigm. (B) Relative gene expression of the pro-inflammatory cytokine IL-1β in the hippocampus. CPFE: N = 11-12/group. Gene Expression: N = 7-9/group. Error bars represent the ±SEM. *$p < 0.05$ indicates the main effect of neonatal infection causing increased baseline IL-1β expression. **$p < 0.01$ indicates the main effect of LPS causing increased IL-1β expression in juvenile male and female rats regardless of neonatal infection status. $^{\dagger}p < 0.05$ indicates post hoc tests showing the additive effect of neonatal infection and LPS causing exaggerated IL-1β expression in juvenile male and female rats. *NS* indicates no statistically significant differences.

In addition to finding that P24 is the earliest age at which rats learn in the CPFE paradigm, Jablonski and colleagues showed that on P21 rats begin to show modest learning in the CPFE. Given these data, we hypothesized that the P21 may be a “sensitive” period in which the hippocampal neural circuits that support this type of learning may be undergoing synaptic remodeling to allow for the emergence of learning days later, on P24. Therefore, we sought to determine whether immune activation during this uniquely "sensitive" period of hippocampal development, between P21 and P24, would disrupt the emergence of this type of hippocampal-dependent learning in juvenile rats.

3.2 Results

Experiments 1.1 (CPFE: N = 10/group and ISD: N = 6/group) and 1.2 (N = 12/group) addressed the hypothesis that immune activation on P21 would produce deficits in the emergence of hippocampal-dependent learning on P24 in the CPFE task. Consistent with our prediction, a 2x2 ANOVA with P21 Treatment (saline vs LPS) and Sex (male vs female) revealed a significant main effect of P21 Treatment ($F(1,27) = 6.001$, $p = 0.021$). Rats that received LPS on P21 showed significantly less freezing behavior in the CPFE task on P24 compared to saline controls indicating a deficit in learning as a result of LPS on P21 (**Figure 3.2A**). No significant effects of Sex were found.

Furthermore, a 2x2 ANOVA with P24 Treatment (saline vs LPS) and Sex (male vs female) revealed no significant main effects or interactions of P24 Treatment ($F(1,20) = 0.399$, $p = 0.535$) or Sex ($F(1,20) = 0.335$, $p = 0.569$) on freezing behavior on P27 (**Figure 3.2B**). These results are consistent with our prediction that immune

activation on P24, once the neural circuits necessary to support learning in the CPFE have been formed, would not produce learning deficits. Together, these results demonstrate that P21 is a uniquely "sensitive" period of hippocampal development whereby immune activation with LPS can disrupt the emergence of hippocampal - dependent learning on P24 in male and female rats.

Additionally, we were able to validate our parameters for the CPFE by running a behavioral control paradigm, the Immediate Shock Deficit (ISD) task. All of our juvenile rats could learn the CPFE task relative to behavioral controls ($t_{29} = -2.350$, $p = 0.026$; **Figure 3.2C**) confirming that freezing on test day was a result of a hippocampal-dependent, learned-association between the context on day 1 and the shock on day 2, rather than a more generalized form of fear learning to the shock alone (Rudy et al., 2004; Fanselow, 1990).

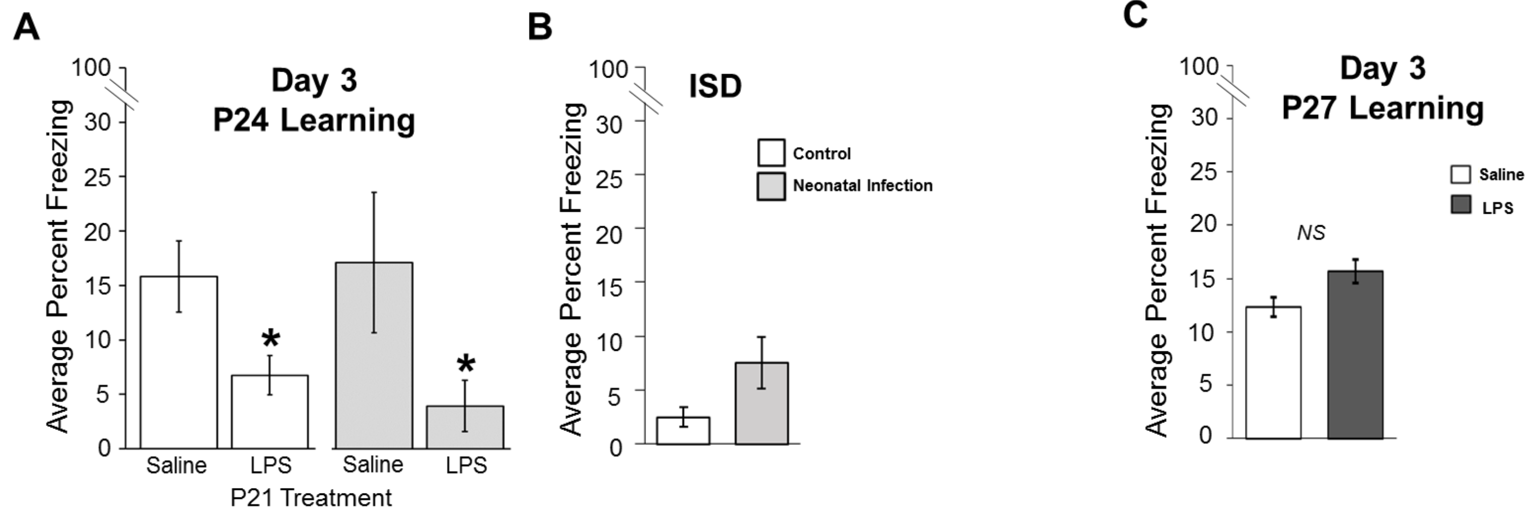


Figure 3.2. Learning in the Context Pre-exposure Facilitation Effect (CPFE) paradigm at P24. (A) On Day 3, only rats that received LPS treatment (100ug/ml/kg) on P21 show significantly less freezing compared to saline treated rats, regardless of previous neonatal immune activation. (B) Rats show the "immediate shock deficit" (ISD) after receiving no pre-exposure to the context on Day 1 of the CPFE. (C) On Day 3, rats that received LPS treatment (100ug/ml/kg) on P24 do not show significantly less freezing compared to saline treated rats. Context A: N = 10-12/group. ISD: N = 5-6/group. Error bars represent the ±SEM. *$p < 0.05$ indicates the main effect of P21 treatment causing rats treated with LPS to show significantly less freezing, regardless of neonatal infection. *NS* indicates no statistically significant differences.

3.3 Discussion

Here we show that immune activation on P21, immediately preceding the emergence of the ability to learn in the hippocampal-dependent CPFE task, produces learning deficits. Subsequently, we also showed that immune activation once learning emerges on P24 *does not* produce learning deficits. Our results support the notion that P21 is a "sensitive" period in hippocampal development that is uniquely vulnerable to immune activation. Specifically, our data identify a discrete window during the first few weeks of postnatal development in which immune activation is likely disrupting the formation or maturation of the underlying neural circuits in the hippocampus that allow for the emergence of this type of context learning.

While others have examined the effects of immune activation on this type of spatial learning (Bilbo et al., 2006), our novel data provide important insights into how immune activation can disrupt hippocampal-dependent cognitive function which have not been previously addressed. The two-hit model of immune activation has been widely used to show that immune activation in the neonatal period (the "first hit") followed by subsequent immune activation in adulthood or aging (the "second hit') results in a host of cognitive deficits which are driven largely by an exaggerated neuroimmune response following the second hit (Bilbo et al., 2006). While this model has been enormously informative, we have found that it does not lead to hippocampal-dependent learning deficits in younger animals. Using the same two-hit model that had previously produced learning deficits in adult male rats, Osborne and colleagues (2017) found no effect on learning in juvenile rats on P24. Thus we shifted the "second hit" to P21, because we hypothesized that this age, just prior to the onset of hippocampal-dependent learning, would be vulnerable to immune activation.

Interestingly, we found that the two-hit model was not necessary to produce learning deficits, rather the "second hit" on P21 *alone* was sufficient to produce learning deficits in this hippocampal-dependent task (**Figure 3.2A**). Moreover, we found that rats treated with LPS on P24, an age when rats were already able to learn the CPFE, did not have learning deficits. Our data suggest that a mechanism other than microglia "priming" and its associated *exaggerated* inflammation underlie the disruptions in the emergence of cognitive function resulting from immune activation in developing animals. Rather, these deficits in learning likely represent the unique vulnerability of the hippocampus during this period of neural circuit maturation such that even low dose inflammation may precipitate a host of dysregulation in microglia-neuron interactions in the juvenile brain. This hypothesis will be tested in the next chapters of this thesis.

Chapter 4

LPS ON POSTNATAL DAY 21 PRODUCES ROBUST INFLAMMATION AND ALTERS EXPRESSION OF MICROGLIA-NEURON COMMUNICATION MOLECULES IN THE JUVENILE HIPPOCAMPUS

4.1 Introduction

As the immune cells of the brain, microglia respond to a number of noxious stimuli by increasing their release of pro- and anti-inflammatory cytokines and chemokines and altering their morphology. This neuroimmune response has been well characterized in adults and is adaptive and necessary to bring the brain back to homeostasis; however, neurons are particularly sensitive to these immune molecules, and thus a prolonged or exaggerated inflammatory response can be detrimental to cognitive function in adults (Yirmiya et al., 2015; Williamson et al., 2011). Despite knowing quite a bit about the function of mature microglia cells in the adult brain, we know very little about the development of these cells and how they respond to immune activation during brain development. Further, recent research indicates that the role of microglia during brain development is more multifaceted than once thought. In addition to being the primary immune cell of the brain, microglia are integral to processes such as synaptic pruning, synaptic plasticity, and engulfing newborn cells in order to establish the proper formation of neural circuits (Bolós et al., 2018; Nelson, Warden, & Lenz, 2017; Schafer et al., 2012; Paolicelli et al., 2011). When the ability

of microglia to perform these functions is compromised, rodents exhibit disruptions in the establishment and maturation of neural circuits and synapses and deficits in cognitive and social behaviors (Nelson & Lenz, 2017; Zhang et al., 2012).

Thus, we sought to characterize the microglial response to immune activation at this juvenile age, specifically during a "sensitive" period of hippocampal development. We also examined the response of molecules important for microglia-neuron communication to determine whether immune activation disrupts these signals, which may lead to deficits in the emergence of the associative fear learning that is necessary to perform the CPFE. The goal of this experiment is to gain a better understanding of how dynamic interactions among multiple cell types involved in neuroimmune response mediate the transition from normal CNS development and function to disorder.

4.2 Results

Here we addressed the hypothesis that immune activation with LPS on P21 will produce a robust inflammatory response thus altering subsequent microglia-neuron communication in the hippocampus. We characterized the type and time course of the inflammatory response necessary to produce learning deficits in our juvenile rats (N = 12/group). A 2x2x4 ANOVA with P21 Treatment (saline vs LPS), Sex (male vs female), and Time (2hr, 4hr, 8hr, 24hr) post-LPS as the between-subjects factors revealed a significant interaction of P21 Treatment x Sex x Time ($F(3,61)$ =

3.478, $p = 0.021$) for IL-1β expression in the hippocampus (**Figure 4.1A**). Post hoc tests showed that while both males and females had a significant increase in IL-1β expression following LPS, males had a more rapid response with levels being significantly above that of control levels at 2hrs ($p = 0.004$; **Figure 4.1B**), whereas females did not show a significant increase until 4hrs ($p = 0.000132$; **Figure 4.1C**). Additionally, IL-1β expression levels were not significantly different from saline control levels at 24hrs indicating the resolution of this inflammatory response (males: $p = 0.821$, females: $p = 0.995$; **Figure 4.1A**). We found a significant interaction of P21 Treatment x Sex x Time for IL-6 expression ($F(3,61) = 3.082$, $p = 0.034$; **Figure 4.1D**). Post hoc tests revealed that, like IL-1β, males had a more rapid IL-6 response to LPS with significantly increased expression in the LPS-treated rats at 2hrs compared to controls ($p = 0.025$; **Figure 4.1E**), while LPS-treated females did not show a significant increase in IL-6 until 4hrs compared to controls ($p = 0.002$; **Figure 4.1F**). The IL-6 response to LPS was resolved back to levels that were not significantly different from control levels by 8hrs post-LPS (males: $p = 0.825$, females: $p = 0.242$) which was maintained at 24hrs (males: $p = 0.814$, females: $p = 0.893$; **Figure 4.1D**) indicating the resolution of this inflammatory response.

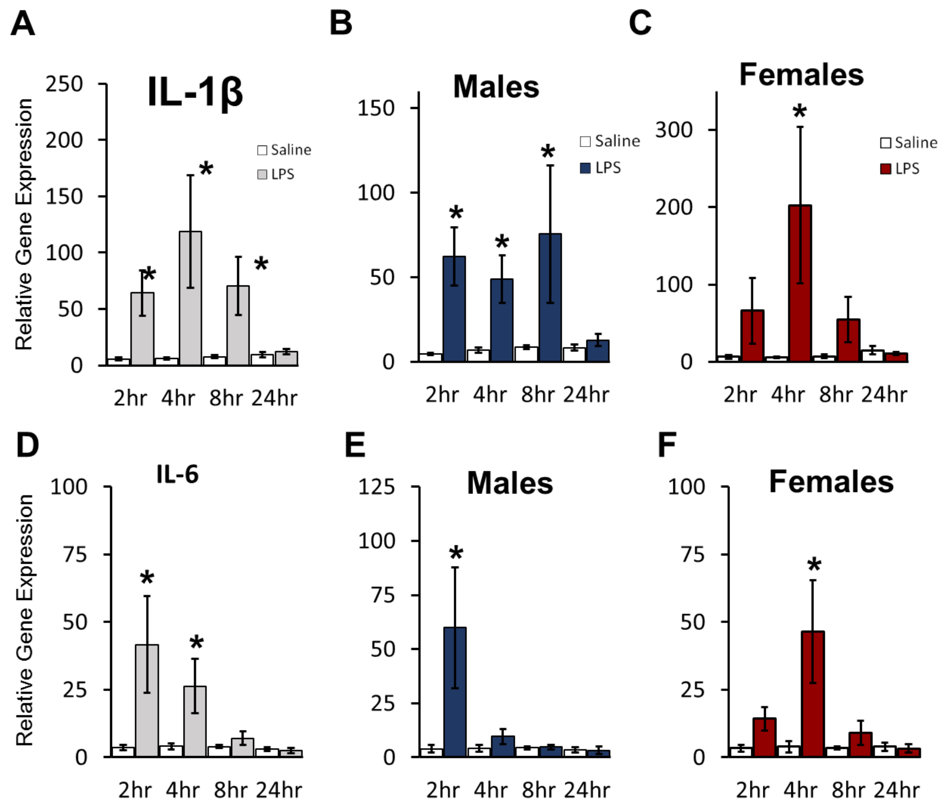
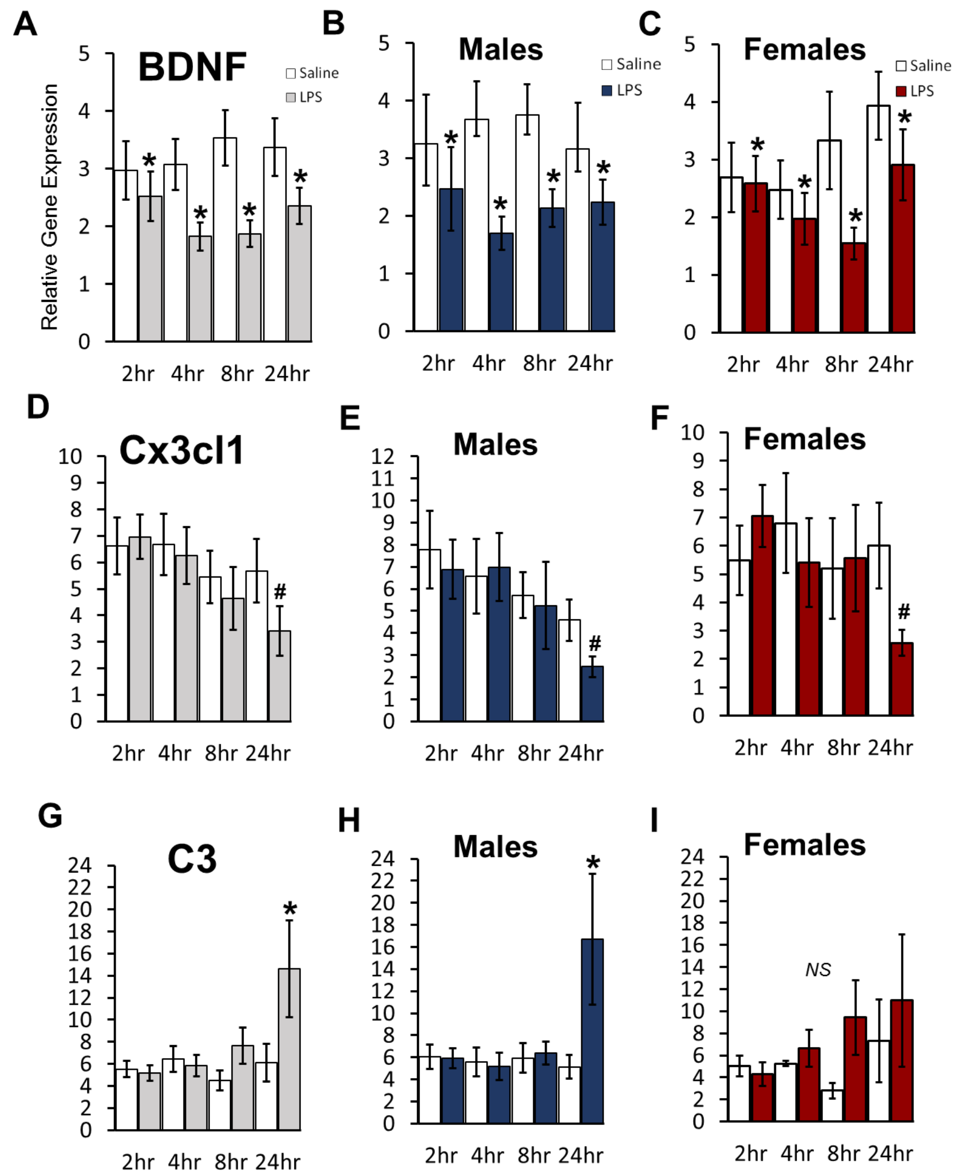


Figure 4.1. The inflammatory response in the hippocampus following treatment with LPS on P21. (A) IL-1β gene expression shows a robust increase two hours after LPS injection and is resolved to baseline expression levels by 24 hours. (B) IL-1β gene expression in males is significantly increased two hours following LPS, remains significantly elevated at four and eight hours, then decreases to baseline expression levels by 24 hours. (C) IL-1β gene expression in females is not significantly increased above baseline expression levels until four hours and decreases to baseline expression levels by 24 hours. (D) IL-6 gene expression shows a robust increase starting two hours after LPS injection and is resolved to baseline expression levels by 24 hours. (E) IL-6 gene expression in males is significantly increased two hours following LPS and is decreases to baseline expression levels by fours. (F). IL-6 gene expression in females is significantly increased four hours following LPS and decreases to baseline expression levels by eight hours. N = 10-12/group. Error bars represent the ±SEM. *$p < 0.05$ indicates significant post hoc effects the using bonferroni correction for multiple comparisons for the significant interaction of P21 treatment x time x sex.

For the analysis of BDNF, a 2x2x4 ANOVA with P21 Treatment (saline vs LPS), Sex (male vs female), and Time (2hr, 4hr, 8hr, 24hr) post-LPS as the between subjects factors revealed a significant main effect of P21 Treatment such that all rats that received LPS showed decreased BDNF expression at all four times post-LPS ($F(1,61) = 9.948$, $p = 0.002$; **Figure 4.2A**). No significant effects of sex were observed (**Figure 4.2B-C**). We found a significant main effect of Time for Cx3cl1 ($F(3,76) = 4.500$, $p = 0.006$; **Figure 4.2D**). Post hoc tests revealed that at 24hrs rats treated with LPS had a marginally significant decrease in Cx3cl1 expression compared to saline controls ($p = 0.080$). No significant effects of sex were observed (**Figure 4.2E-F**). Finally, we found a significant main effect of Time for C3 ($F(3,72) = 2.666$, $p = 0.054$; **Figure 4.2G**). Post hoc tests revealed that at 24hrs males, but not females, treated with LPS had a significant increase in C3 expression ($p = 0.003$; **Figure 4.2H-I**).



A
BDNF
Saline
LPS
Relative Gene Expression
2hr 4hr 8hr 24hr
B
Males
Saline
LPS
C
Females
Saline
LPS
D
Cx3cl1
E
Males
F
Females
G
C3
H
Males
I
Females
NS

Figure 4.2. Characterization of changes in BDNF, Cx3cl1, and C3 in the hippocampus following treatment with LPS on P21. (A) BDNF gene expression significantly decreases at all time points following LPS treatment. (B) LPS significantly decreases BDNF gene expression in males at all time points. (C) LPS significantly decreases BDNF gene expression in females at all time point. (D) LPS produces a marginally significant decrease in Cx3cl1 gene expression at twenty-four hours. (E) LPS produces a marginally significant decrease in Cx3cl1 gene expression in males at twenty-four hours. (F) LPS produces a marginally significant decrease in Cx3cl1 gene expression in females at twenty-four hours. (G) LPS significantly increases C3 gene expression at twenty-four hours. (H) LPS significantly increases C3 gene expression in males at twenty-four hours. (I) LPS does not significantly increase C3 gene expression at any time point in females. N = 10-12/group. Error bars represent the ±SEM. $^{*}p < 0.05$ indicates significant post hoc effects the using bonferroni correction for multiple comparisons for the significant main effect of time. $^{\#}p < 0.05$ indicates marginally significant post hoc effects the using bonferroni correction for multiple comparisons for the significant main effect of time. *NS* indicates no statistically significant differences.

Finally, to examine whether LPS on P21 led to any *intrinsic* changes in microglia in the hippocampus we measured several genes indicative of microglia phenotype. A 2x2x4 ANOVA with P21 Treatment (saline vs LPS), Sex (male vs female), and Time (2hr, 4hr, 8hr, 24hr) post-LPS revealed no significant changes for Cx3cr1 (**Figure 4.3A-C**), CD68 (**Figure 4.3D-F**) nor CD47 (**Figure 4.3G-I**).

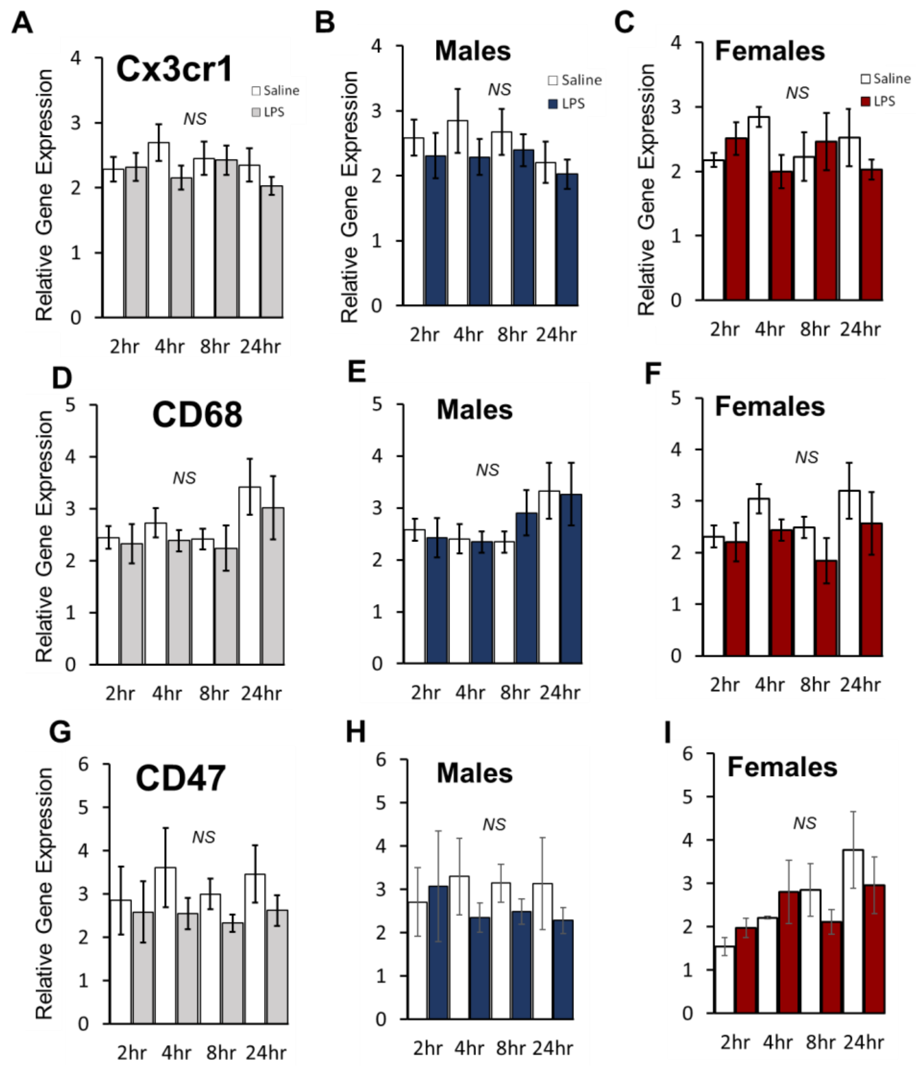

A
Cx3cr1
Relative Gene Expression
Saline
LPS
NS
2hr 4hr 8hr 24hr
B
Males
Saline
LPS
NS
Relative Gene Expression
2hr 4hr 8hr 24hr
C
Females
Saline
LPS
NS
Relative Gene Expression
2hr 4hr 8hr 24hr
D
CD68
Relative Gene Expression
NS
2hr 4hr 8hr 24hr
E
Males
NS
2hr 4hr 8hr 24hr
F
Females
NS
2hr 4hr 8hr 24hr
G
CD47
Relative Gene Expression
NS
2hr 4hr 8hr 24hr
H
Males
NS
2hr 4hr 8hr 24hr
I
Females
NS
2hr 4hr 8hr 24hr

Figure 4.3. Characterization of changes in Cx3cr1, CD68, and CD47 in the hippocampus following treatment with LPS on P21. (A) LPS does not produce any significant changes in Cx3cr1 gene expression at any time point. (B) LPS does not produce any significant changes in Cx3cr1 gene expression in males at any time point. (C) LPS does not produce any significant changes in Cx3cr1 gene expression in females at any time point. (D) LPS does not produce any significant changes in CD68 gene expression at any time point. (E) (LPS does not produce any significant changes in CD68 gene expression in males at any time point. (F) LPS does not produce any significant changes in CD68 gene expression in females at any time point. (G) LPS does not produce any significant changes in CD47 gene expression at any time point. (H) LPS does not produce any significant changes in CD47 gene expression in males at any time point. (I) LPS does not produce any significant changes in CD47 gene expression in females at any time point. N = 10-12/group. Error bars represent the ±SEM. *NS* indicates no statistically significant differences.

4.3 Discussion

As predicted, immune activation with LPS caused a significant increase in the expression of two ubiquitous pro-inflammatory cytokines, IL-1β and IL-6. While both males and females showed a robust neuroimmune response, males showed a more rapid onset than females with cytokine expression levels being significantly increased by 2hrs post-LPS compared to controls. Regardless of these differences, the inflammatory response was resolved within 24hrs in both sexes. The resolution of the inflammatory response by 24hrs post-LPS is significant because it confirms that the learning deficits we observed on P24 in Experiment 1 are not the result of lingering inflammation, rather they are likely the result of changes to the hippocampal milieu that were precipitated by the inflammatory response on P21.

We found that LPS significantly decreased the expression of BDNF compared to controls at all time points examined. Similarly, others have shown decreases in

BDNF throughout early development following exposure to LPS *in utero* in offspring (Lin & Wang, 2014; Golan et al., 2005), while others have found increases in BDNF in the hippocampus following *in utero* exposure to *E.coli* in adults (P40) (Jiang et al., 2013). In our model, BDNF gene expression levels significantly decrease at the beginning of the inflammatory response and begin to return to normal at 24hrs, once the inflammatory response is resolved, suggesting that this decrease in BDNF may be a transient response to the active inflammation that follows LPS administration. BDNF is critical to the survival and differentiation of neurons during development (Huang & Reichardt, 2001). There is also an abundance of evidence showing that BDNF regulates the structure and functions of neural circuits across the lifespan (Matsuda et al., 2009; Tyler et al., 2002). Furthermore, BDNF is well known to be a crucial factor in regulating learning and memory processes such as consolidation of context fear memories (Choi et al., 2010) thus downregulation of BDNF could certainly be contributing to the learning deficits we see here.

LPS produced a marginally significant decrease in the expression of the fractalkine ligand, Cx3cl1, in males and females at 24hrs post-LPS. Previous studies indicate that fractalkine is highly expressed in brain regions such as the hippocampus and cortex and that the principal cell type expressing it is neurons (Harrison et al., 1998). It is not clear exactly how Cx3cl1 is involved in the inflammatory response. Its function is highly context dependent, and it is involved in many diseases from cancer to age-related macular degeneration (for a review see Liu et al., 2016). For example, models of various neurodegenerative disorders have shown that decreased Cx3cl1 leads to dysregulated microglia and neuroinflammation (Chen et al., 2016). Furthermore, Sheridan and colleagues (2014) showed that Cx3cl1 is upregulated

during memory-associated synaptic plasticity in the rat hippocampus. Moreover, chronic injection of Cx3cl1 rescues hippocampal-dependent memory deficits in the BDNFVal66Met mouse model (Lin & Wang, 2014). These data suggest that a decrease in Cx3cl1 could lead to dysregulation of microglia function and/or an inflammatory response and possibly produce deficits in learning and memory. Indeed, Ragozzino and colleagues (2006) found that Cx3cl1 negatively regulates AMPA receptor function at active glutamatergic synapses, potentially by altering Ca2+ influx. Given that our model produces a decrease in Cx3cl1 24hrs after LPS on P21, it is possible that this decrease is leading to changes in AMPA receptor activity that interferes with memory consolidation, thus leading to learning deficits we observed in the CPFE on P24.

Finally, we determined that LPS significantly increased the expression of C3 in males, but not females 24hrs following LPS on P21. This sex difference is in line with previous research showing the role of C3 in sculpting neural circuits in the developing nucleus accumbens in male rats, but not female rats during adolescence (Kopec et al., 2018). In their model, Kopec and colleagues (2018) determined that dopamine D1 receptors in the nucleus accumbens are developmentally refined via microglia and complement C3-mediated phagocytic elimination in males and that this process regulates the developmental changes in play behavior in males, but not females. This study is the first to investigate the role of microglia and complement C3-mediated phagocytosis in sculpting neural circuits that drive behavior in the adolescent period. Our data here provide a novel contribution by investigating another type of behavior, the emergence of hippocampal-dependent context fear learning following early-life immune activation. Our finding that C3 is upregulated following an acute

inflammatory response in males, but not females suggests that microglia and C3 signaling may be a mechanism that is utilized predominately in males for experience-dependent synaptic pruning during juvenile/adolescent development. It is also possible that this mechanism is more vulnerable to disturbance by immune activation in males than in females. A point of consideration, however, is that we did not find any sex differences in baseline expression of C3 on P21, thus, it is not clear exactly how this mechanism may function differently between males and females for the refinement of synaptic circuits during juvenile development.

Surprisingly, we found no significant changes in Cx3cr1, CD68, or CD47 gene expression. Together, these results indicate that immune challenge on P21 is likely not changing the constitutive properties of microglia; rather, our results indicate that immune challenge alters the molecules Cx3cl1 and C3 likely impairing communication between microglia and neurons.

Chapter 5

LPS ON POSTNATAL DAY 21 ALTERS MICROGLIA-MEDIATED PHAGOCYTOSIS OF PRE- AND POST-SYNAPTIC PROTEINS IN THE JUVENILE HIPPOCAMPUS

5.1 Introduction

Given our finding that immune activation on P21 alters Cx3cl1 and C3 expression in the hippocampus 24hrs after immune activation, we next sought to determine whether these changes led to alterations in microglia-mediated phagocytosis of synapses in the hippocampus. Indeed, previous studies have shown that immune signaling molecules are important for microglia-mediated phagocytosis of synapses during early development. For example, C3 tags synapses for phagocytic elimination by microglia in the developing visual system of male mice (Stevens et al., 2007). Furthermore, when either the components of complement signaling or microglia-specific signaling are missing, an increase in synaptic number is observed (Schafer et al., 2012; Paolicelli et al., 2011). **Importantly, most of what we know about microglia and immune-mediated phagocytosis of synapses comes from examining the early postnatal period.** Moreover, these models have examined this phenomenon within the context of either typical development of neural circuits, namely visual circuits, and/or genetic mouse knockout models. Thus, our work is unique because we can extend upon this work by examining these processes during a sensitive period of hippocampal development in *juvenile* rats, and within the context of early-life immune activation (rather than via genetic manipulations in mice).

Inflammation resulting from microglia activation is a naturally occurring response to infection or injury, but when it occurs early in life, it has the potential to produce life-long deviations in normal cognitive development. While this phenomenon is well known, the mechanisms underlying such vulnerability are not clear. In this experiment, we aimed to determine whether the changes in Cx3cl1 and C3 expression that we measured at 24hrs following immune activation could lead to altered phagocytic elimination of synapses by microglia.

5.2 Results

To determine whether immune activation altered microglia-mediated phagocytosis of synapses in the juvenile hippocampus we used fluorescent immunohistochemistry to examine co-localization of the pre- and post-synaptic proteins SNAP25 and PSD95 within microglia 24hrs post-LPS (**Figures 5.1A, 5.2A, 5.3A;** N = 12/group). A 2x2 ANOVA with P21 Treatment (saline vs LPS) and Sex (male vs female) as the between subjects factors was used to analyze all three regions of interest (i.e. CA1, CA3, DG). Within CA1 we found a significant main effect of Sex for PSD95 with males showing more engulfed PSD95 protein compared to females regardless of treatment ($F(1,15) = 5.035$, $p = 0.040$; **Figure 5.1B**). There were no significant differences found for SNAP25 in CA1 (**Figure 5.1B**).

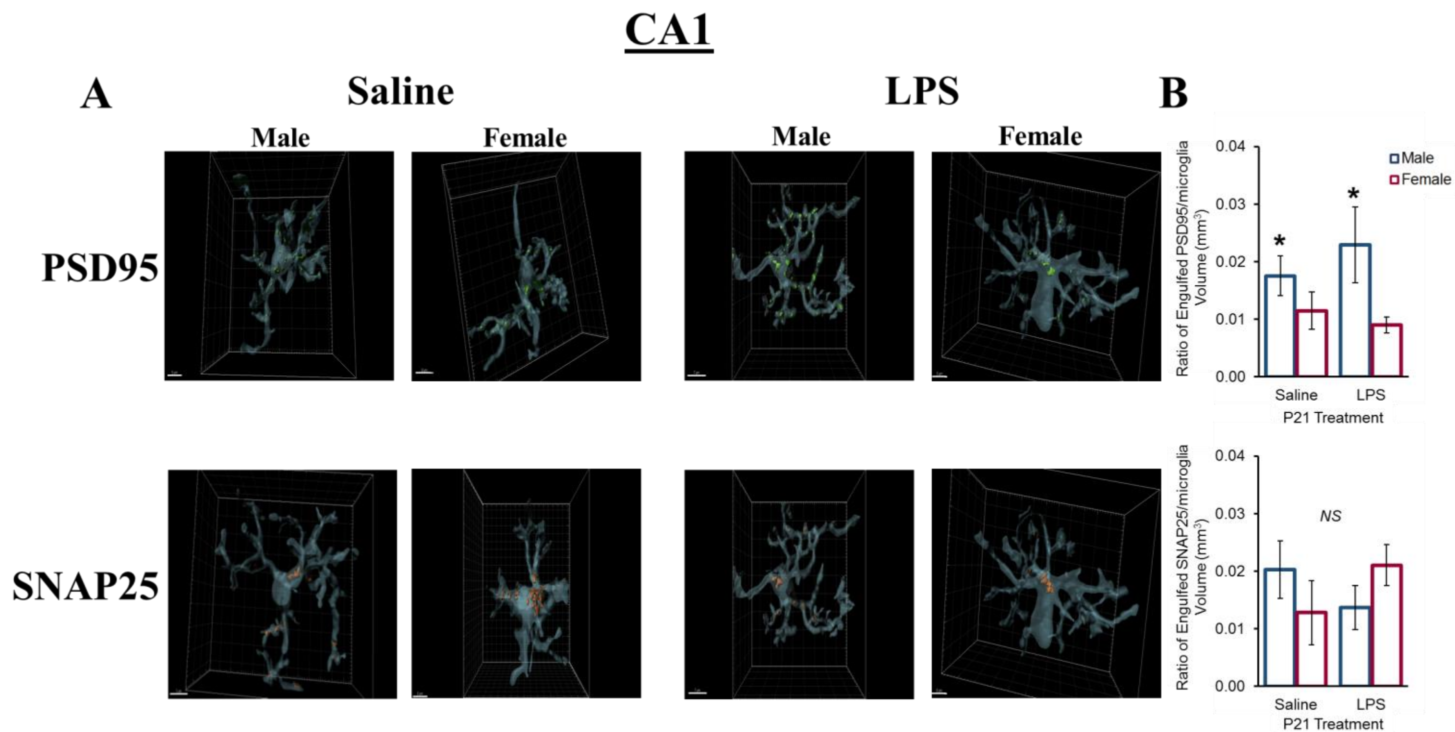


Figure 5.1. Juvenile males have a higher rate of microglia-mediated phagocytosis of PSD95 than females in CA1. (A) Representative images of the engulfment of synaptic proteins by microglia for males and females in each treatment condition. (B) Quantification of the engulfment of PSD95 and SNAP25 proteins. Males show significantly higher rates of engulfment of PSD95 compared to females. No significant differences were observed for SNAP25. N = 4-6/group. Error bars represent the ±SEM. $^*p < 0.05$ indicates the main effect of sex. *NS* indicates no statistically significant differences.

Within CA3 we found a significant P21 Treatment x Sex interaction ($F(1,13) = 9.670$, $p = 0.008$; **Figure 5.2B**) for PSD95. Post hoc tests determined that LPS treatment significantly decreased the amount of PSD95 protein engulfed by microglia in males ($p = 0.000499$), but not females. There was no difference in the amount of PSD95 protein engulfed by female microglia based on treatment. Furthermore, we found a main effect of Sex for SNAP25 in CA3 ($F(1,15) = 5.918$, $p = 0.028$; **Figure 5.2B**) such that male microglia are engulfing significantly more SNAP25 protein than female microglia regardless of treatment.

Finally, in the DG we found a significant main effect of Sex for both PSD95 ($F(1,15) = 6.586$, $p = 0.021$; Figure 5.3B) and SNAP25 ($F(1,15) = 8.425$, $p = 0.011$; Figure 5.3B such that male microglia are engulfing more of oth synaptic proteins than female microglia regardless of treatment.

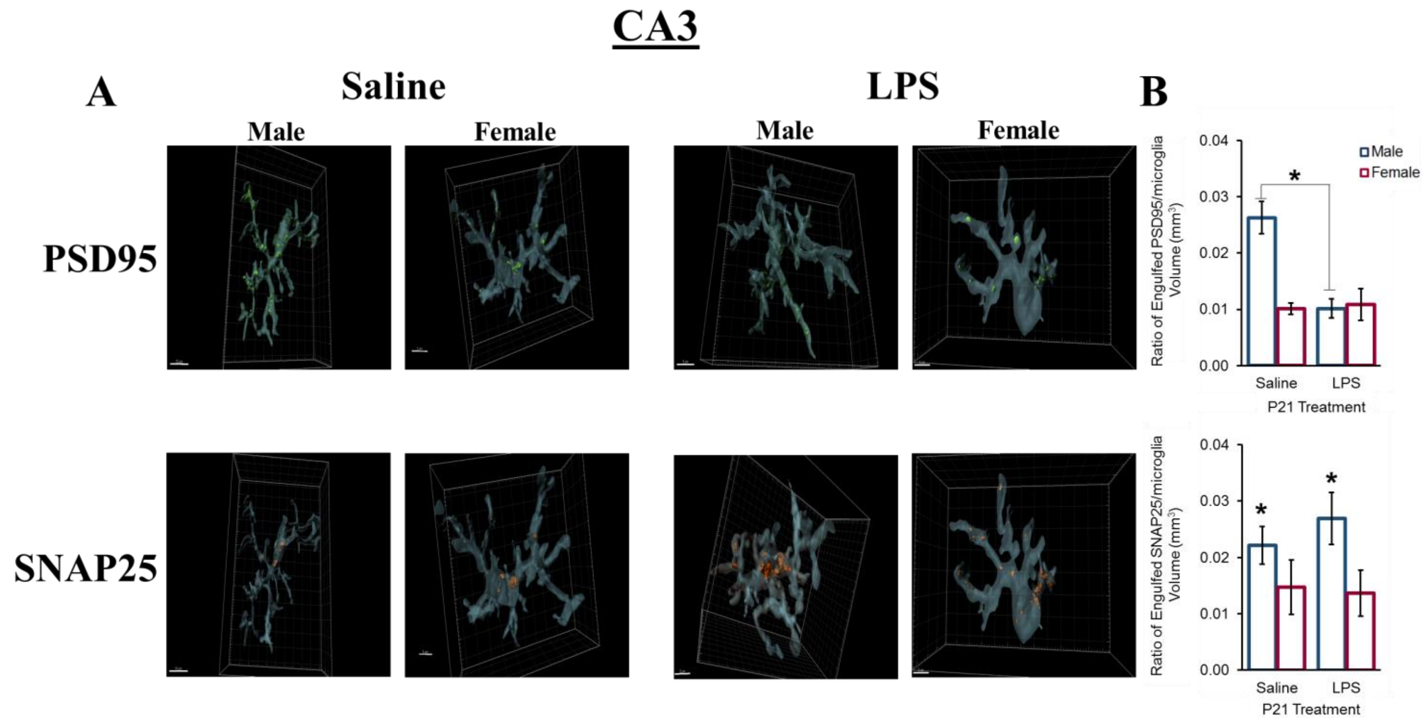


Figure 5.2. LPS on P21 significantly decreases microglia-mediated phagocytosis of PSD95 in CA3 of juvenile males. (A) Representative images of the engulfment of synaptic proteins by microglia for males and females in each treatment condition. (B) Quantification of the engulfment of PSD95 and SNAP25 proteins. Males show significantly lower rates of engulfment of PSD95 24hrs following LPS. Males have higher rates of engulfment of SNAP25 compared to females regardless of treatment. Error bars represent the ±SEM. $*p < 0.05$ indicates the significant P21 treatment x sex interaction for PSD95 and the main effect of sex for SNAP25.

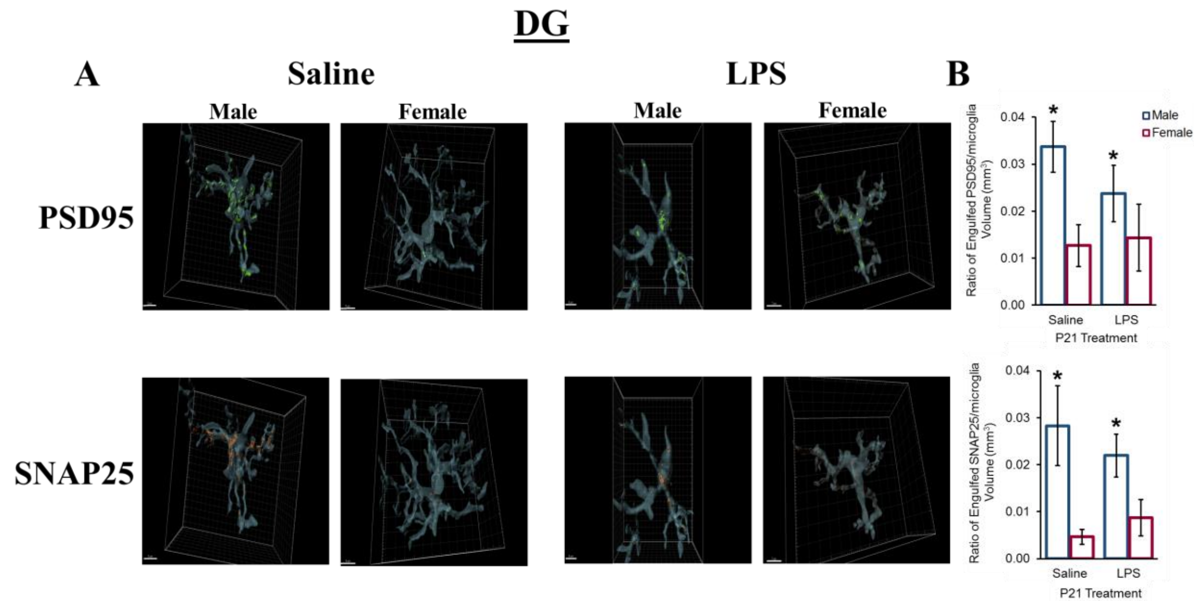


Figure 5.3. Juvenile males have significantly higher rates of microglia-mediated phagocytosis of synaptic proteins compared to females in the DG. (A) Representative images of the engulfment of synaptic proteins by microglia for males and females in each treatment condition. (B) Quantification of the engulfment of PSD95 and SNAP25 proteins. Males show significantly higher rates of engulfment of both PSD95 and SNAP25 compared to females regardless of treatment. N = 4-6/group. Error bars represent the ±SEM. *$p < 0.05$ indicates the main effect of sex for PSD95 and SNAP25.

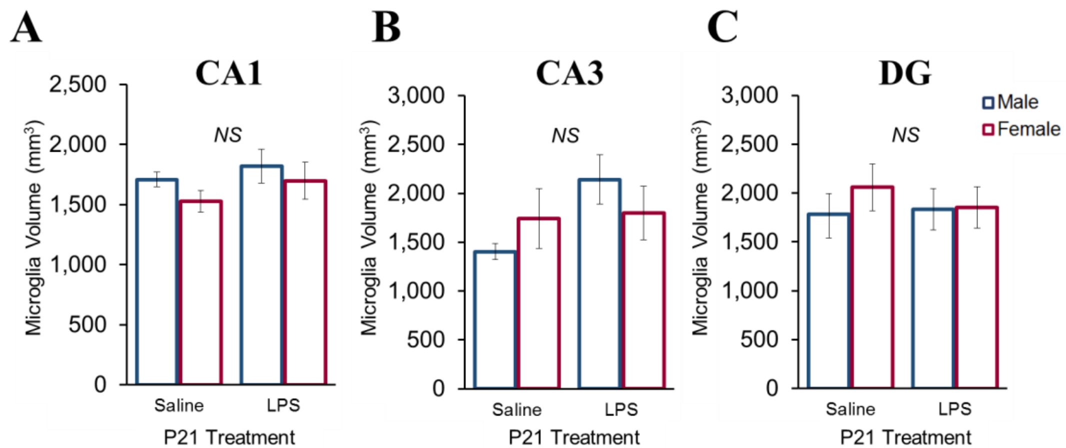


Figure 5.4. Microglia volumes for each region of interest in the juvenile hippocampus 24hrs following LPS treatment of P21. (A) Microglia volumes are not different following LPS treatment or between sexes in the CA1. (B) Microglia volumes are not different following LPS treatment or between sexes in the CA3. (C) Microglia volumes are not different following LPS treatment or between sexes in the DG. Additionally, there were no significant differences in microglia volumes between regions. N = 4-6/group. Error bars represent the ±SEM. *NS* indicates no statistically significant differences.

5.3 Discussion

Here we identify a novel mechanism by which immune activation may result in learning deficits observed several days after the initial inflammatory response. Specifically, our data suggest that decreases in microglia-mediated engulfment of PSD95 in the CA3 may underlie learning deficits observed in juvenile males following immune activation on P21. CA3 is thought to be important for the encoding of new spatial information, particularly for the retrieval of a context memory through the process of pattern completion (Kesner, 2007; Rudy & O'Reilly, 2001) . Additionally, others have shown that the ability to perform the CPFE requires hippocampal NMDA receptor activity during the pre-exposure phase. It is possible that decreasing synaptic pruning in CA3, by decreasing microglia-mediated engulfment of PSD95 protein,

creates an excess of excitatory synapses, thus leading to neuronal dysfunction. Consistent with our findings, using a Cx3cr1 knockout mouse model, Paolicelli and colleagues (2011) found a similar reduction in microglia-mediated synaptic pruning which led to a *delay* in the maturation of neural circuit development in the hippocampus. Further research is necessary to determine whether our model is producing permanent disturbances of the hippocampal neural circuits that support learning in the CPFE or if we are simply delaying the maturation of these circuits.

Paolicelli and colleagues (2011) also found that mice lacking Cx3cr1 have a reduction in microglia density within the hippocampus, and thus a decrease in microglia surveillance of surrounding neurons. The authors of this study posit that this is what ultimately resulted in the decrease in synaptic pruning during this period of juvenile, activity-dependent neural circuit formation. In contrast to these data, our data have identified no differences in microglia density at baseline or as a result of immune challenge. Instead, taken in conjunction with the results from our gene expression analyses, our results suggest that rather than a *direct* effect of immune activation on microglia number or phenotype, immune activation and the subsequent production of cytokines *indirectly* alters signaling molecules that are necessary for neurons to communicate with microglia to guide their developmentally appropriate function.

Chapter 6

LPS ON POSTNATAL DAY 21 DECREASES BASAL DENDRITIC SPINE DENSITY IN CA3 PYRAMIDAL CELLS ON POSTNATAL DAY 24 IN THE JUVENILE HIPPOCAMPUS

6.1 Introduction

The role of microglia in synaptic remodeling and plasticity in the healthy brain is multifaceted. Microglia are now recognized as an integral part of neural networks that modulate synaptic function throughout brain development (Schafer et al., 2012; Paolicelli et al., 2011; Wake et al., 2009; Davalos et al., 2005; Nimmerjahn et al., 2005). Remodeling of neural circuits is achieved through several mechanisms, a primary mechanism being the phagocytic elimination of axonal terminals and dendritic spines by microglia (Hong et al., 2016; Schafer et al., 2012; Zhang et al., 2012). Dendritic spines serve as the postsynaptic sites for the majority of excitatory synapses (Harris, 1999). During the first few weeks of postnatal development in rodents, up to 40% of spines are selectively eliminated and the remaining spines mature, changing their morphology from long and thin to short and stubby (Zuo et al., 2005; Grutzendler et al., 2002; Rakic et al., 1986; Peter, 1979; Marin-Padilla, 1967). Moreover, this elimination and maturation process of synaptic elements is necessary for the proper establishment and refinement of mature neural circuits (Ethell & Pasquale, 2005; Mataga et al., 2004; Churchill et al., 2002).

Given the critical role that proper neural circuit formation and maturation has in shaping cognitive function we wanted to investigate whether altered neuronal

morphology, and thus, likely neural circuit formation, is a mechanism underlying the learning deficits we observed on P24. Importantly, CA3 is particularly important for processing and encoding spatial information (Mankin et al., 2012), thus we anticipated that we may see more changes in neuronal morphology in this region compared to CA1 or DG. Furthermore, given our findings in the previous experiment, the goal of this final experiment was to determine whether the changes we have observed in microglia neural signaling and engulfment of synaptic proteins 24hrs following LPS lead to changes in dendritic morphology and spine density in the hippocampus on P24.

6.2 Results

Here we addressed the hypothesis that immune activation on P21 leads to changes in neuronal morphology on P24 in the hippocampus (N = 6-8/group). A 2x2 ANOVA with P21 Treatment (Saline vs LPS) and Sex (male vs female) as the between-subjects factors revealed a significant interaction of P21 Treatment x Sex for total branching order of basal dendrites in the CA1 ($F(1,8) = 8.793$, $p = 0.018$; **Figure 6.1H**). Post hoc tests showed that LPS decreased total branching order of basal dendrites in the CA1 of males, but not females ($p = 0.039$). No other significant effects were found in the CA1(**Figure 6.1A-G, I-J**).

CA1

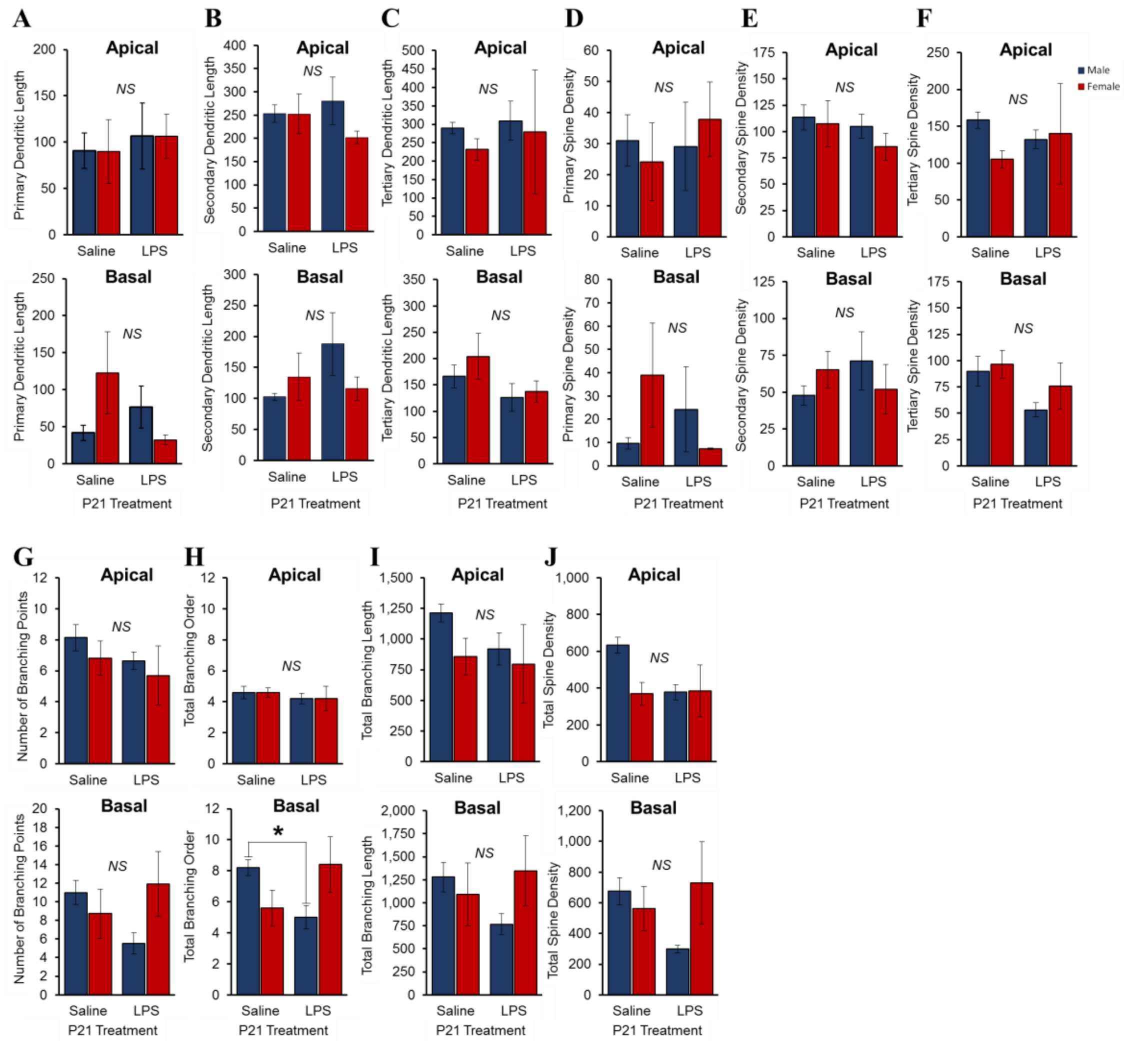


Figure 6.1. Neuronal morphology and dendritic spine density of pyramidal cells in the CA1 of juvenile males and females on P24. (A) The total length of primary apical and basal dendrites is not different following LPS on P21 or between sexes. (B) The total length of secondary apical and apical dendrites is not different following LPS on P21 or between sexes. (C) The total length of tertiary apical and basal dendrites is not different following LPS on P21 or between sexes. (D) The total number of dendritic spines on primary apical and basal dendrites is not different following LPS on P21 or between sexes. (E) The total number of dendritic spines on secondary apical and basal dendrites is not different following LPS on P21 or between sexes. (F) The total number of dendritic spines on tertiary apical and basal dendrites is not different following LPS on P21 or between sexes. (G) The total number of branching points of apical and basal dendrites is not different following LPS on P21 or between sexes. (H) The total branching order apical dendrites is not different following LPS on P21 or between sexes. The total branching order of basal dendrites is significantly decreased in males that received LPS on P21. (I) The total branching length of apical and basal dendrites is not different following LPS on P21 or between sexes. (J) The total number of dendritic spines on apical and basal dendrites is not different following LPS on P21 or between sexes. N = 3-4/group. Error bars represent the ±SEM. *$p < 0.05$ indicates the significant interaction of P21 treatment x sex. *NS* indicates no statistically significant differences.

In the CA3, we found a significant main effect of P21 Treatment on primary basal dendrite length such that rats treated with LPS had significantly longer primary basal dendrites than saline controls ($F(1,7) = 5.572$, $p = 0.050$; **Figure 6.2A**). We found a significant interaction of P21 Treatment x Sex on the spine density of primary basal dendrites ($F(1,6) = 9.927$, $p = 0.020$; **Figure 6.2D**). Post hoc tests showed that LPS significantly increased spine density on primary basal dendrites in males, but not females ($p = 0.007$). A significant main effect of Sex was observed for spine density on secondary apical dendrites ($F(1,8) = 7.928$, $p = 0.023$; **Figure 6.2E**) with males

having significantly more spines on secondary apical dendrites than females. No other significant effects were found in the CA3 (**Figure 6.2B-C, F-J**).

CA3

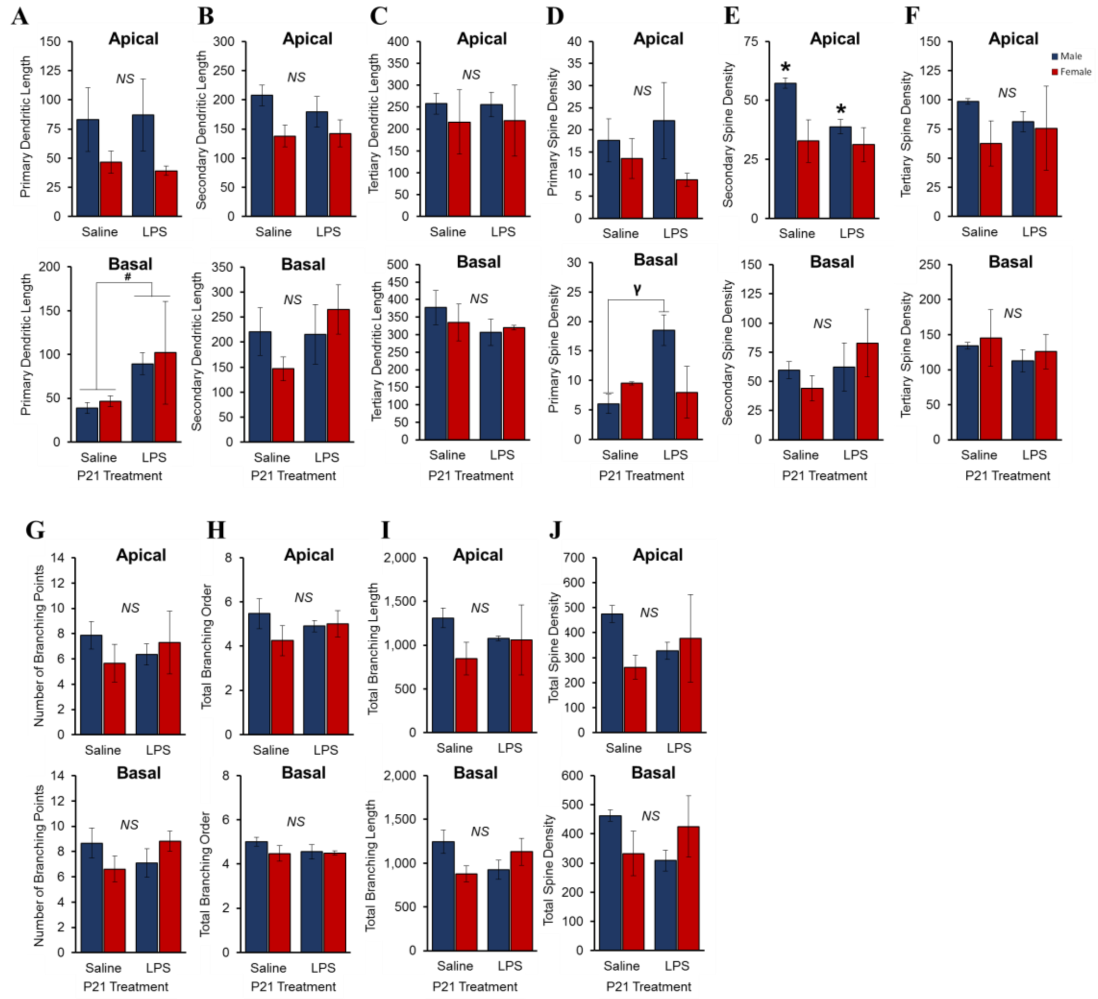


Figure 6.2. Neuronal morphology and dendritic spine density of pyramidal cells in the CA3 of juvenile males and females on P24. (A) The total length of primary apical dendrites is not different following LPS on P21 or between sexes. LPS significantly increases the total length of primary basal dendrites in males and females. (B) The total length of secondary apical and basal dendrites is not different following LPS on P21 or between sexes. (C) The total length of tertiary apical and basal dendrites is not different following LPS on P21 or between sexes. (D) The total number of dendritic spines on primary apical dendrites is not different following LPS on P21 or between sexes. LPS significantly increases the total number of dendritic spines on primary basal dendrites in males, but not females. (E) Males have significantly more dendritic spines on secondary apical compared to females regardless of treatment condition. The total number of dendritic spines on secondary basal dendrites is not different following LPS on P21 or between sexes. (F) The total number of dendritic spines on tertiary apical and basal dendrites is not different following LPS on P21 or between sexes. (G) The total number of branching points of apical and basal dendrites is not different following LPS on P21 or between sexes. (H) The total branching order of apical dendrites is not different following LPS on P21 or between sexes. The total branching order of basal dendrites is significantly decreased in males that received LPS on P21. (I) The total branching length of apical and basal dendrites is not different following LPS on P21 or between sexes. (J) The total number of dendritic spines on apical and basal dendrites is not different following LPS on P21 or between sexes. N = 3-4/group. Error bars represent the ±SEM. #$p < 0.05$ indicates the significant main effect of P21 Treatment. $^{\gamma}p < 0.05$ indicates the significant interaction of P21 treatment x sex. *$p < 0.05$ indicates the significant main effect of sex. *NS* indicates no statistically significant differences.

No significant main effects or interactions were found for any measure of neuronal morphology or spine density in the DG (**Figure 6.3A-J**).

DG

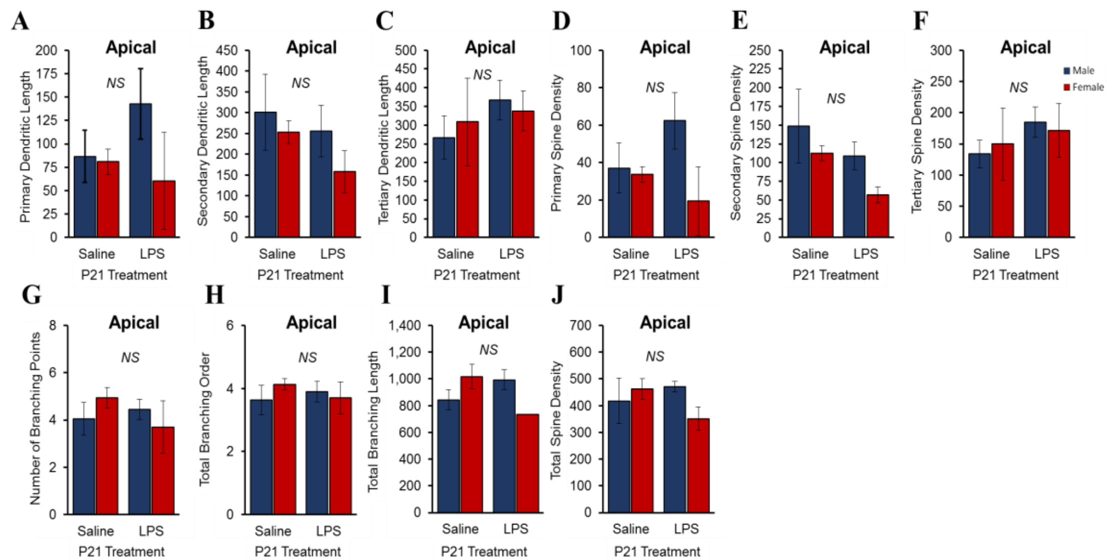


Figure 6.3. Neuronal morphology and dendritic spine density of granule cells in the DG of juvenile males and females on P24. (A) The total length of primary apical dendrites is not different following LPS on P21 or between sexes. (B) The total length of secondary apical dendrites is not different following LPS on P21 or between sexes. (C) The total length of tertiary apical dendrites is not different following LPS on P21 or between sexes. (D) The total number of dendritic spines on primary apical dendrites is not different following LPS on P21 or between sexes. (E) The total number of dendritic spines on secondary apical dendrites is not different following LPS on P21 or between sexes. (F) The total number of dendritic spines on tertiary apical dendrites is not different following LPS on P21 or between sexes. (G) The total number of branching points of apical dendrites is not different following LPS on P21 or between sexes. (H) The total branching order of apical dendrites is not different following LPS on P21 or between sexes. (I) The total branching length of apical dendrites is not different following LPS on P21 or between sexes. (J) The total number of dendritic spines on apical dendrites is not different following LPS on P21 or between sexes. N = 3-4/group. Error bars represent the ±SEM. *NS* indicates no statistically significant differences

6.3 Discussion

These results support the idea that immune activation and subsequent cytokine production during a sensitive period of hippocampal development produces significant remodeling of neuronal morphology and spine densities several days later. Interestingly, rather than large-scale changes throughout the whole hippocampus, our results show that inflammation caused by LPS produces more targeted and nuanced alterations to neurons that are dependent upon the hippocampal sub-region. For example, we found that LPS decreased the total branching order of basal dendrites of neurons in the CA1 of both males and females, while all of the other measures we examined in CA1 remained unchanged. While it is possible that this change in CA1 basal dendrites is responsible for the learning deficits we observed, it seems unlikely as it has been demonstrated that cells in CA1 are primarily important for encoding temporal context (for a review see Wang & Diana, 2016). Indeed, studies have shown that CA1 "time cells" change their pattern of firing as a function of time (Kraus et al., 2013; Mankin et al., 2012; MacDonald et al., 2011) and that they seem to be particularly important for encoding preceding events, possibly in an effort to give temporal context to an event (MacDonald et al., 2011).

In contrast to CA1, CA3 neurons exhibit highly reproducible firing patterns over time regardless of temporal context (Mankin et al., 2012). CA3 has been demonstrated to be important for aspects of context learning such as pattern completion and binding spatial features (Kesner, 2007; Rudy & O'Reilly, 2001). Here, we determined that LPS leads to an increase in primary basal dendrite length in both sexes and an increase in the number of spines on primary basal dendrites, in males. We also found that LPS results in decreased microglia-mediated engulfment of PSD95 in the CA3 of males (Chapter 5). Taken together, our results suggests that, 1)

decreased microglia-mediated engulfment of synapses is quite likely a mechanism underlying the *increase in spine density observed on primary basal dendrites of CA3 neurons in males,* and 2) the increase in primary basal dendrite length is likely mediated by a mechanism that is independent of microglia, as this change was seen in both sexes. Furthermore, if the changes in spine density of CA3 neurons on P24 is, in fact, leading to learning deficits then it is likely that LPS impairs learning in females via a different mechanism as they *do not* show the same increase in spine density nor the decrease in microglia-mediated engulfment of PSD95. These results are in line with recent data from Kopec and colleagues (2018) showing that, while D1r downregulation in the adolescent nucleus accumbens occurs in both males and females, this is achieved through C3/microglia-mediated phagocytic engulfment in males only. The mechanism mediating this downregulation in females has yet to be identified. Similarly, our results suggest that the mechanism by which LPS alters neuronal structure in the CA3 and leads to learning impairments in the CPFE of females has yet to be determined.

Chapter 7

DISCUSSION

7.1 A Sensitive Period of Neural Circuit Remodeling May Explain Hippocampal Vulnerability to Inflammation during Juvenile Development

In these experiments we tested the hypothesis that immune activation on P21 would produce deficits in the emergence of context learning on P24 in male and female rats. ***Our results support this overall hypothesis***. Rats that received LPS on P21 showed significantly less freezing in the CPFE task on P24 compared to their control counterparts. Previous work identified P24 as the youngest age at which rats reliably learn and perform the CPFE (Jablonski et al., 2012). Additionally, Raineki and colleagues (2010) showed that c-Fos activity in CA1, CA3, and dentate gyrus occurred only on P24 once rats perform context learning and not in younger rats (P21) that do not show context learning. Furthermore, muscimol infusion into the hippocampus impaired context learning, but not cue or aversion to odor learning on P24. These results indicate that the hippocampus supports emerging contextual learning that occurs around P24 of juvenile development.

Our results indicate that, in addition to the traditionally defined critical periods of brain development (e.g. sensory and motor systems, sexual differentiation), there exists "sensitive periods" of brain development in which behaviors and the neural circuits that underlie them are especially susceptible to particular types of stimuli. Indeed, this concept has gained quite a deal of attention recently. Studies using the visual system have elucidated a relatively advanced understanding of the mechanism

that control the initiation, closure, and reinstatement of sensitive periods (for a review see Hartley & Lee, 2015). As one example, the onset of GABA signaling initiates the opening of a critical period for the formation of ocular dominance columns (Bavelier et al., 2010; Takao & Hensch, 2004), and preventing this increase in GABA signaling delays the onset of this critical period necessary for the formation of ocular dominance. Moreover, treatment with benzodiazepines, which potentiates GABA signaling, can reinstate this critical period of plasticity - even in adult animals (Hensch et al., 1998). Furthermore, there is now evidence suggesting that the existence of sensitive periods generalizes to neural circuits in other modalities (Nabel & Morishita, 2013). One such modality is affective learning, specifically that emotional responses are learned during a sensitive period of development. In humans, we know that brain regions important for regulating affect, including the hippocampus, amygdala, and the prefrontal cortex continue to mature well into young adulthood (Gogtay et al., 2004) suggesting an extended period of neuroplasticity in these circuits that control emotion and affect.

Here we examined whether the neural circuits critical to associative and emotional learning (i.e. context fear learning) in the hippocampus are vulnerable to an environmental insult such as immune activation during a sensitive period of development. Our results show, for the first time, that neural circuits that support contextual learning in the juvenile hippocampus are vulnerable to immune activation during a discrete window of increased plasticity (i.e. a sensitive period). Still, many questions remain. How long does this sensitive period last? We present evidence that this sensitive period ends on P24, after the emergence of the behavior itself, however, it remains unclear if the hippocampus could still be vulnerable in the days following

P21 – P24. What are the mechanisms that initiate, close, and reinstate this sensitive period? Are there similar sensitive periods for other types of hippocampal-dependent learning? Does increased susceptibility to immune activation produce transient or long-term consequences? While much research is needed to address these questions, our findings are beginning to unravel the mystery of how early-life challenges can affect behavioral and cognitive outcomes.

7.2 Complement C3 and Fractalkine Cx3cl1 Signaling May be Key Mechanisms Underlying Microglia-Mediated Phagocytosis of Synapses that Lead to Abnormal Neuronal Remodeling in CA1 and CA3 following Immune Activation

Based on our finding that immune activation on P21 impaired the emergence of hippocampal-dependent learning on P24, we aimed to determine the possible mechanism underlying these deficits. (i.e. how do we get from immune activation to cognitive dysfunction days later?). We determined that 24hrs after the immune challenge, the expression of pro-inflammatory cytokines IL-1β and IL-6 had returned to baseline levels, but two immune molecules critical to microglia-neuron communication had only just begun to exhibit changes in their expression. Cx3cl1 is expressed at high levels in the hippocampus and other cortical regions in the brain (Harrison et al., 1998). Cx3cl1 is primarily released by neurons and binds its receptor, Cx3cr1, which is almost exclusively expressed on microglia in the brain (Harrison et al., 1998). Cx3cl1 exists in two forms – membrane bound and cleaved. The membrane bound form acts as a cell adhesion molecule for cells that express its receptor and the cleaved form functions as a chemoattractant similar to other chemokines. During an inflammatory response, the mucin stalk of the membrane bound form adheres to microglia (Hermand et al., 2008). Evidence suggests that this fractalkine can be

neuroprotective under conditions of neurodegeneration such as Alzheimer's disease (Nash et al., 2015). We found no change in Cx3cl1 expression coinciding with the robust inflammatory response we observed following LPS; however, the decrease in Cx3cl1 expression at 24hrs post-LPS suggests the possibility of neuronal damage and dysregulation of microglia (Cardona et al., 2006).

In addition to Cx3cl1, complement C3 expression was found to be increased in males, but not females, 24hrs post-LPS. Much of what we know about the role of C3-mediated microglia-neuron interactions comes from studying the developmental refinement of the visual system. In this lateral geniculate nucleus of the visual system, C3 tags weak synapses for elimination necessary for the formation of appropriate visual circuits (Schafer et al., 2012). Our data suggest that, following inflammation during this sensitive period of hippocampal development, C3 may be upregulated in response to inflammation in males, however, microglia do not appear to be capable of responding to C3 appropriately. In line with this idea, we saw a decrease in microglia-mediated engulfment of PSD95 in the CA3 of males immediately following the inflammatory response. Furthermore, our neuronal morphology data support this by showing a significant increase in spine density of primary basal dendrites in the CA3 of males, but not females. This increase in spine density could certainly underlie the learning deficits we observed as this would increase the presence of excitatory synapses resulting in immature connectivity (Paolicelli et al., 2011). Interestingly, CD47 has been shown to protect against excessive synaptic pruning by microglia (Lehrman et al., 2018), thus, we may expect to find increases in CD47 in response to the increase in C3, however we found no change in CD47 expression at any time point examined. These results, again suggest that abnormal expression of signaling

molecules leads to dysfunctional microglia, rendering them incapable of responding to changes in their microenvironment.

Our findings are in line with previous data showing that the complement C3 pathway is responsible for sex-specific pruning of synapses during juvenile development (Kopec et al., 2018). These experiments showed that C3 was responsible for the downregulation of D1r receptors in the nucleus accumbens that coincided with changes in social behavior in males. While our model does not show evidence for sex differences in associative fear learning necessary for learning the CPFE, others have similarly shown sex differences in microglia function in the hippocampus during early development. Nelson and colleagues (2017) found that neonatal microglia in the hippocampus of females phagocytosed more neural progenitor cells and healthy neural cells and had more phagocytic cups than males. Our data from juvenile males and females also show a sex difference in microglia function; however, at this age microglia from males show higher rates of engulfment of pre- and post-synaptic proteins than females. Together, these results indicate that the specific sex differences observed are likely going to depend on a number of factors including age and function being examined and are important when considering the long-term implications of microglia function on brain development in both males and females.

Finally, there are many other factors that could potentially be influencing the developmental regulation of neural circuit maturation that could be affected by LPS which we did not examine in these experiments. For example, it has recently been proposed that miRNAs in astrocyte-derived exosomes may mediate neuronal morphology and synaptic transmission (Lafourcade etal., 2016). We also are not certain how the incorporation of new cells into the existing circuits could be affected

by immune activation with LPS in our model, but evidence has shown that inflammation can alter neurogenesis leading to long-term behavioral changes (Borsini et al., 2015; Green & Nolan, 2014). Further research is needed to create a cohesive understanding of the mechanisms that underlie the effects of immune activation during this sensitive period of hippocampal development on deficits in the emergence of associative emotional learning in juvenile males and females.

7.3 Broader Impact

Two fundamental principles underlie these current experiments which sought to explore the causes and mechanisms underlying neurodevelopmental disorders that are associated with early-life immune dysregulation. **First,** models of neurodevelopmental disorders should be grounded in a contemporary knowledge of normal developmental processes and their neurobiological mechanisms. Context and space are foundations upon which humans and rodents learn most information (Knierim et al., 2013; Burgess et al., 2002). Even children (age 3-4) learn best when information is presented in a book in a previously learned spatial orientation (McCrink et a., 2014). In addition, many context learning tasks that examine the rodent hippocampus and associated brain regions are well-adapted to understand human spatial learning and memory (Bohbot et al., 2002), which taken together, is why we have focused on the hippocampus and context learning in the current experiments. **Second,** similar causal mechanisms, such as an early-life immune activation or dysregulation, may play a role in and lead to markedly varying degrees of neurodevelopmental disorders (from simple learning disabilities to severe forms of autism) depending on the severity and timing of onset. Many mental health disorders have been linked to early-life immune activation or immune dysregulation.

Furthermore, many developmental and neuropsychiatric disorders are characterized by dysregulation of associative and emotion processing, which are controlled by brain regions like the hippocampus (Kessler et al., 2005).

These experiments aimed to determine the impact of early-life immune activation during a "sensitive" period of hippocampal synaptic development on microglia function, neuronal development, and the risk for learning disorders. We found that immune activation during a sensitive period of hippocampal development in juveniles impairs the emergence of associative and emotional learning (i.e. context fear learning). These deficits are likely the result of the abnormal microglia-neuron communication and decreased microglia-mediated phagocytosis which led to increased synaptic spine density on CA3 neurons. Thus, we feel these experiments have the potential to significantly advance our understanding of the mechanisms underlying a variety of neurodevelopmental disorders. In closing, neurodevelopmental disorders have become one of the largest sources of years lived with disability and our findings significantly advance our understanding of how early-life immune activation or dysregulation can impact the long-term risk of human mental health and cognitive disorders.

REFERENCES

Abbott, N. J., Patabendige, A. A. K., Dolman, D. E. M., Yusof, S. R., & Begley, D. J. (2010). Structure and function of the blood–brain barrier. *Neurobiology of Disease*, *37*(1), 13–25. https://doi.org/10.1016/j.nbd.2009.07.030

Agorastos, A., Pervanidou, P., Chrousos, G. P., & Baker, D. G. (2019). Developmental Trajectories of Early Life Stress and Trauma: A Narrative Review on Neurobiological Aspects Beyond Stress System Dysregulation. *Frontiers in Psychiatry*, *10*, 118. https://doi.org/10.3389/fpsyt.2019.00118

Anderson, A. C., Anderson, D. E., Bregoli, L., Hastings, W. D., Kassam, N., Lei, C., … Hafler, D. A. (2007). Promotion of Tissue Inflammation by the Immune Receptor Tim-3 Expressed on Innate Immune Cells. *Science*, *318*(5853), 1141–1143. https://doi.org/10.1126/science.1148536

Bavelier, D., Levi, D. M., Li, R. W., Dan, Y., & Hensch, T. K. (2010). Removing brakes on adult brain plasticity: from molecular to behavioral interventions. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *30*(45), 14964–14971. https://doi.org/10.1523/JNEUROSCI.4812-10.2010

Bayer, T. A., Buslei, R., Havas, L., & Falkai, P. (1999). Evidence for activation of microglia in patients with psychiatric illnesses. *Neuroscience Letters*, *271*(2), 126–128. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10477118

Bilbo, S. D. (2010). Early-life infection is a vulnerability factor for aging-related glial alterations and cognitive decline. *Neurobiology of Learning and Memory*, *94*(1), 57–64. https://doi.org/10.1016/j.nlm.2010.04.001

Bilbo, S. D., Levkoff, L. H., Mahoney, J. H., Watkins, L. R., Rudy, J. W., & Maier, S. F. (2005). Neonatal Infection Induces Memory Impairments Following an Immune Challenge in Adulthood. *Behavioral Neuroscience*, *119*(1), 293–301. https://doi.org/10.1037/0735-7044.119.1.293

Bilbo, S. D., Rudy, J. W., Watkins, L. R., & Maier, S. F. (2006). A behavioural characterization of neonatal infection-facilitated memory impairment in adult rats. *Behavioural Brain Research*, *169*(1), 39–47. https://doi.org/10.1016/j.bbr.2005.12.002

Bland, S. T., Beckley, J. T., Young, S., Tsang, V., Watkins, L. R., Maier, S. F., & Bilbo, S. D. (2010). Enduring consequences of early-life infection on glial and neural cell genesis within cognitive regions of the brain. *Brain, Behavior, and Immunity*, *24*(3), 329–338. https://doi.org/10.1016/j.bbi.2009.09.012

Blaze, J., & Roth, T. L. (2017). Caregiver maltreatment causes altered neuronal DNA methylation in female rodents. *Development and Psychopathology*, *29*(2), 477–489. https://doi.org/10.1017/S0954579417000128

Blinzinger, K and Kreutzberg, G. (1968). Displacement of synaptic terminals from regenerating motoneurons by microglial cells. *Z Zellforsch Mikrosk Annat*, *85*, 145–157.

Block, M. L., Zecca, L., & Hong, J.-S. (2007). Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nature Reviews. Neuroscience*, *8*(1), 57–69. https://doi.org/10.1038/nrn2038

Bloomfield, P. S., Selvaraj, S., Veronese, M., Rizzo, G., Bertoldo, A., Owen, D. R., … Howes, O. D. (2016). Microglial Activity in People at Ultra High Risk of Psychosis and in Schizophrenia: An [^{11} C]PBR28 PET Brain Imaging Study. *American Journal of Psychiatry*, *173*(1), 44–52. https://doi.org/10.1176/appi.ajp.2015.14101358

Bohbot, V. D., Jech, R., Růzicka, E., Nadel, L., Kalina, M., Stepánková, K., & Bures, J. (2002). Rat spatial memory tasks adapted for humans: characterization in subjects with intact brain and subjects with selective medial temporal lobe thermal lesions. *Physiological Research*, *51 Suppl 1*, S49-65. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12479786

Bolós, M., Perea, J. R., Terreros-Roncal, J., Pallas-Bazarra, N., Jurado-Arjona, J., Ávila, J., & Llorens-Martín, M. (2018). Absence of microglial CX3CR1 impairs the synaptic integration of adult-born hippocampal granule neurons. *Brain, Behavior, and Immunity*, *68*, 76–89. https://doi.org/10.1016/j.bbi.2017.10.002

Bolton, J. L., & Bilbo, S. D. (2014). Developmental programming of brain and behavior by perinatal diet: focus on inflammatory mechanisms. *Dialogues in Clinical Neuroscience*, *16*(3), 307–320. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/25364282

Borsini, A., Zunszain, P. A., Thuret, S., & Pariante, C. M. (2015). The role of inflammatory cytokines as key modulators of neurogenesis. *Trends in Neurosciences*, *38*(3), 145–157. https://doi.org/10.1016/j.tins.2014.12.006

Bosworth, A. P., & Allen, N. J. (2017). The diverse actions of astrocytes during

synaptic development. *Current Opinion in Neurobiology*, *47*, 38–43. https://doi.org/10.1016/J.CONB.2017.08.017

Boulle, F., Pawluski, J. L., Homberg, J. R., Machiels, B., Kroeze, Y., Kumar, N., … Van den Hove, D. L. A. (2016). Prenatal stress and early-life exposure to fluoxetine have enduring effects on anxiety and hippocampal BDNF gene expression in adult male offspring. *Developmental Psychobiology*, *58*(4), 427–438. https://doi.org/10.1002/dev.21385

Burgess, N., Maguire, E. A., & O'Keefe, J. (2002). The Human Hippocampus and Spatial and Episodic Memory. *Neuron*, *35*(4), 625–641. https://doi.org/10.1016/S0896-6273(02)00830-9

Burman, M. A., Murawski, N. J., Schiffino, F. L., Rosen, J. B., & Stanton, M. E. (2009). Factors governing single-trial contextual fear conditioning in the weanling rat. *Behavioral Neuroscience*, *123*(5), 1148–1152. https://doi.org/10.1037/a0016733

Cardona, A. E., Pioro, E. P., Sasse, M. E., Kostenko, V., Cardona, S. M., Dijkstra, I. M., … Ransohoff, R. M. (2006). Control of microglial neurotoxicity by the fractalkine receptor. *Nature Neuroscience*, *9*(7), 917–924. https://doi.org/10.1038/nn1715

Chen, P., Zhao, W., Guo, Y., Xu, J., & Yin, M. (2016). CX3CL1/CX3CR1 in Alzheimer's Disease: A Target for Neuroprotection. *BioMed Research International*, *2016*, 1–9. https://doi.org/10.1155/2016/8090918

Choi, D. C., Maguschak, K. A., Ye, K., Jang, S.-W., Myers, K. M., & Ressler, K. J. (2010). Prelimbic cortical BDNF is required for memory of learned fear but not extinction or innate fear. *Proceedings of the National Academy of Sciences*, *107*(6), 2675–2680. https://doi.org/10.1073/pnas.0909359107

Christopherson, K. S., Ullian, E. M., Stokes, C. C. A., Mullowney, C. E., Hell, J. W., Agah, A., … Barres, B. A. (2005). Thrombospondins Are Astrocyte-Secreted Proteins that Promote CNS Synaptogenesis. *Cell*, *120*(3), 421–433. https://doi.org/10.1016/J.CELL.2004.12.020

Churchill, J. D., Grossman, A. W., Irwin, S. A., Galvez, R., Klintsova, A. Y., Weiler, I. J., & Greenough, W. T. (2002). A converging-methods approach to fragile X syndrome. *Developmental Psychobiology*, *40*(3), 323–338. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11891642

Davalos, D., Grutzendler, J., Yang, G., Kim, J. V, Zuo, Y., Jung, S., … Gan, W.-B. (2005). ATP mediates rapid microglial response to local brain injury in vivo.

Nature Neuroscience, *8*(6), 752–758. https://doi.org/10.1038/nn1472

Davoust, N., Vuaillat, C., Androdias, G., & Nataf, S. (2008). From bone marrow to microglia: barriers and avenues. *Trends in Immunology*, *29*(5), 227–234. https://doi.org/10.1016/j.it.2008.01.010

Dowlati, Y., Herrmann, N., Swardfager, W., Liu, H., Sham, L., Reim, E. K., & Lanctôt, K. L. (2010). A meta-analysis of cytokines in major depression. *Biological Psychiatry*, *67*(5), 446–457. https://doi.org/10.1016/j.biopsych.2009.09.033

Elkabes, S., DiCicco-Bloom, E. M., & Black, I. B. (1996). Brain microglia/macrophages express neurotrophins that selectively regulate microglial proliferation and function. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *16*(8), 2508–2521. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8786427

Ethell, I. M., & Pasquale, E. B. (2005). Molecular mechanisms of dendritic spine development and remodeling. *Progress in Neurobiology*, *75*(3), 161–205. https://doi.org/10.1016/J.PNEUROBIO.2005.02.003

Fanselow, M. S. (1990). Factors governing one-trial contextual conditioning. *Animal Learning & Behavior*, *18*(3), 264–270. https://doi.org/10.3758/BF03205285

Frank, M. G., Barrientos, R. M., Biedenkapp, J. C., Rudy, J. W., Watkins, L. R., & Maier, S. F. (2006). mRNA up-regulation of MHC II and pivotal pro-inflammatory genes in normal brain aging. *Neurobiology of Aging*, *27*(5), 717–722. https://doi.org/10.1016/j.neurobiolaging.2005.03.013

Frick, L. R., Williams, K., & Pittenger, C. (2013). Microglial dysregulation in psychiatric disease. *Clinical & Developmental Immunology*, *2013*, 608654. https://doi.org/10.1155/2013/608654

Ginhoux, F., & Prinz, M. (2015). Origin of Microglia: Current Concepts and Past Controversies. *Cold Spring Harbor Perspectives in Biology*, *15*. https://doi.org/10.1101/cshperspect.a020537

Gogtay, N., Giedd, J. N., Lusk, L., Hayashi, K. M., Greenstein, D., Vaituzis, A. C., … Thompson, P. M. (2004). Dynamic mapping of human cortical development during childhood through early adulthood. *Proceedings of the National Academy of Sciences*, *101*(21), 8174–8179. https://doi.org/10.1073/pnas.0402680101

Golan, H. M., Lev, V., Hallak, M., Sorokin, Y., & Huleihel, M. (2005). Specific neurodevelopmental damage in mice offspring following maternal inflammation

during pregnancy. *Neuropharmacology*, *48*(6), 903–917. https://doi.org/10.1016/j.neuropharm.2004.12.023

Green, H. F., & Nolan, Y. M. (2014). Inflammation and the developing brain: Consequences for hippocampal neurogenesis and behavior. *Neuroscience & Biobehavioral Reviews*, *40*, 20–34. https://doi.org/10.1016/j.neubiorev.2014.01.004

Grutzendler, J., Kasthuri, N., & Gan, W.-B. (2002). Long-term dendritic spine stability in the adult cortex. *Nature*, *420*(6917), 812–816. https://doi.org/10.1038/nature01276

Hanisch, U.-K., & Kettenmann, H. (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nature Neuroscience*, *10*(11), 1387–1394. https://doi.org/10.1038/nn1997

Harris, K. M. (1999). Structure, development, and plasticity of dendritic spines. *Current Opinion in Neurobiology*, *9*(3), 343–348. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10395574

Harrison, J. K., Jiang, Y., Chen, S., Xia, Y., Maciejewski, D., McNamara, R. K., … Feng, L. (1998). Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(18), 10896–10901. https://doi.org/10.1073/pnas.95.18.10896

Hartley, C. A., & Lee, F. S. (2015). Sensitive periods in affective development: nonlinear maturation of fear learning. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *40*(1), 50–60. https://doi.org/10.1038/npp.2014.179

Hensch, T K, Fagiolini, M., Mataga, N., Stryker, M. P., Baekkeskov, S., & Kash, S. F. (1998). Local GABA circuit control of experience-dependent plasticity in developing visual cortex. *Science (New York, N.Y.)*, *282*(5393), 1504–1508. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9822384

Hensch, Takao K. (2004). CRITICAL PERIOD REGULATION. *Annual Review of Neuroscience*, *27*(1), 549–579. https://doi.org/10.1146/annurev.neuro.27.070203.144327

Hermand, P., Pincet, F., Carvalho, S., Ansanay, H., Trinquet, E., Daoudi, M., … Deterre, P. (2008). Functional adhesiveness of the CX3CL1 chemokine requires its aggregation. Role of the transmembrane domain. *The Journal of Biological Chemistry*, *283*(44), 30225–30234. https://doi.org/10.1074/jbc.M802638200

Hong, S., Dissing-Olesen, L., & Stevens, B. (2016). New insights on the role of microglia in synaptic pruning in health and disease. *Current Opinion in Neurobiology*, *36*, 128–134. https://doi.org/10.1016/j.conb.2015.12.004

Huang, E. J., & Reichardt, L. F. (2001). Neurotrophins: Roles in Neuronal Development and Function. *Annual Review of Neuroscience*, *24*(1), 677–736. https://doi.org/10.1146/annurev.neuro.24.1.677

Jablonski, S. A., Schiffino, F. L., & Stanton, M. E. (2012). Role of age, post-training consolidation, and conjunctive associations in the ontogeny of the context preexposure facilitation effect. *Developmental Psychobiology*, *54*(7), 714–722. https://doi.org/10.1002/dev.20621

Jiang, R., Brummett, B. H., Babyak, M. A., Siegler, I. C., & Williams, R. B. (2013). Brain-derived neurotrophic factor (BDNF) Val66Met and adulthood chronic stress interact to affect depressive symptoms. *Journal of Psychiatric Research*, *47*(2), 233–239. https://doi.org/10.1016/j.jpsychires.2012.10.009

Jung, S., Aliberti, J., Graemmel, P., Sunshine, M. J., Kreutzberg, G. W., Sher, A., & Littman, D. R. (2000). Analysis of Fractalkine Receptor CX3CR1 Function by Targeted Deletion and Green Fluorescent Protein Reporter Gene Insertion. *Molecular and Cellular Biology*, *20*(11), 4106–4114. https://doi.org/10.1128/MCB.20.11.4106-4114.2000

Katz, L. C., & Shatz, C. J. (1996). Synaptic activity and the construction of cortical circuits. *Science (New York, N.Y.)*, *274*(5290), 1133–1138. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8895456

Kesner, R. P. (2007). Behavioral functions of the CA3 subregion of the hippocampus. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *14*(11), 771–781. https://doi.org/10.1101/lm.688207

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Merikangas, K. R., & Walters, E. E. (2005). Lifetime Prevalence and Age-of-Onset Distributions of DSM-IV Disorders in the National Comorbidity Survey Replication. *Archives of General Psychiatry*, *62*(6), 593. https://doi.org/10.1001/archpsyc.62.6.593

Kettenmann, H., Hanisch, U.-K., Noda, M., & Verkhratsky, A. (2011). Physiology of Microglia. *Physiological Reviews*, *91*(2), 461–553. https://doi.org/10.1152/physrev.00011.2010

Klintsova, A., Hamilton, G., & Boschen, K. (2012). Long-Term Consequences of Developmental Alcohol Exposure on Brain Structure and Function: Therapeutic Benefits of Physical Activity. *Brain Sciences*, *3*(4), 1–38.

https://doi.org/10.3390/brainsci3010001

Knierim, J. J., Neunuebel, J. P., & Deshmukh, S. S. (2013). Functional correlates of the lateral and medial entorhinal cortex: objects, path integration and local-global reference frames. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *369*(1635), 20130369–20130369. https://doi.org/10.1098/rstb.2013.0369

Kopec, A. M., Smith, C. J., Ayre, N. R., Sweat, S. C., & Bilbo, S. D. (2018). Microglial dopamine receptor elimination defines sex-specific nucleus accumbens development and social behavior in adolescent rats. *Nature Communications*, *9*(1), 3769. https://doi.org/10.1038/s41467-018-06118-z

Kraus, B. J., Robinson, R. J., White, J. A., Eichenbaum, H., & Hasselmo, M. E. (2013). Hippocampal "Time Cells": Time versus Path Integration. *Neuron*, *78*(6), 1090–1101. https://doi.org/10.1016/J.NEURON.2013.04.015

Kreutzberg, G. W. (1996). Microglia: a sensor for pathological events in the CNS. *Trends in Neurosciences*, *19*(8), 312–318. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8843599

Lafourcade, C., Ramírez, J. P., Luarte, A., Fernández, A., & Wyneken, U. (2016). MiRNAs in Astrocyte-Derived Exosomes as Possible Mediators of Neuronal Plasticity. *Journal of Experimental Neuroscience*, *10*(Suppl 1), 1–9. https://doi.org/10.4137/JEN.S39916

Laskaris, L. E., Di Biase, M. A., Everall, I., Chana, G., Christopoulos, A., Skafidas, E., … Pantelis, C. (2016). Microglial activation and progressive brain changes in schizophrenia. *British Journal of Pharmacology*, *173*(4), 666–680. https://doi.org/10.1111/bph.13364

Leckman, J. F. (2014). Commentary: What does immunology have to do with brain development and psychopathology? - a commentary on O'Connor et al. (2014). *Journal of Child Psychology and Psychiatry*, *55*(6), 632–634. https://doi.org/10.1111/jcpp.12259

Lehrman, E. K., Wilton, D. K., Litvina, E. Y., Welsh, C. A., Chang, S. T., Frouin, A., … Stevens, B. (2018). CD47 Protects Synapses from Excess Microglia-Mediated Pruning during Development. *Neuron*, *100*(1), 120-134.e6. https://doi.org/10.1016/j.neuron.2018.09.017

Lenz, K. M., Nugent, B. M., Haliyur, R., & McCarthy, M. M. (2013). Microglia Are Essential to Masculinization of Brain and Behavior. *Journal of Neuroscience*, *33*(7), 2761–2772. https://doi.org/10.1523/JNEUROSCI.1268-12.2013

Lin, Y.-L., & Wang, S. (2014). Prenatal lipopolysaccharide exposure increases depression-like behaviors and reduces hippocampal neurogenesis in adult rats. *Behavioural Brain Research*, *259*, 24–34. https://doi.org/10.1016/j.bbr.2013.10.034

Liu, W., Jiang, L., Bian, C., Liang, Y., Xing, R., Yishakea, M., & Dong, J. (2016). Role of CX3CL1 in Diseases. *Archivum Immunologiae et Therapiae Experimentalis*, *64*(5), 371–383. https://doi.org/10.1007/s00005-016-0395-9

López-Murcia, F. J., Terni, B., & Llobet, A. (2015). SPARC triggers a cell-autonomous program of synapse elimination. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(43), 13366–13371. https://doi.org/10.1073/pnas.1512202112

MacDonald, C. J., Lepage, K. Q., Eden, U. T., & Eichenbaum, H. (2011). Hippocampal “Time Cells” Bridge the Gap in Memory for Discontiguous Events. *Neuron*, *71*(4), 737–749. https://doi.org/10.1016/J.NEURON.2011.07.012

Maezawa, I., Calafiore, M., Wulff, H., & Jin, L.-W. (2011). Does microglial dysfunction play a role in autism and Rett syndrome? *Neuron Glia Biology*, *7*(1), 85–97. https://doi.org/10.1017/S1740925X1200004X

Mankin, E. A., Sparks, F. T., Slayyeh, B., Sutherland, R. J., Leutgeb, S., & Leutgeb, J. K. (2012). Neuronal code for extended time in the hippocampus. *Proceedings of the National Academy of Sciences*, *109*(47), 19462–19467. https://doi.org/10.1073/pnas.1214107109

Marin-Padilla, M. (1967). Number and distribution of the apical dendritic spines of the layer V pyramidal cells in man. *The Journal of Comparative Neurology*, *131*(4), 475–490. https://doi.org/10.1002/cne.901310407

Mataga, N., Mizuguchi, Y., & Hensch, T. K. (2004). Experience-Dependent Pruning of Dendritic Spines in Visual Cortex by Tissue Plasminogen Activator. *Neuron*, *44*(6), 1031–1041. https://doi.org/10.1016/J.NEURON.2004.11.028

Matsuda, N., Lu, H., Fukata, Y., Noritake, J., Gao, H., Mukherjee, S., … Poo, M. -m. (2009). Differential Activity-Dependent Secretion of Brain-Derived Neurotrophic Factor from Axon and Dendrite. *Journal of Neuroscience*, *29*(45), 14185–14198. https://doi.org/10.1523/JNEUROSCI.1863-09.2009

McCrink, K., Shaki, S., & Berkowitz, T. (2014). Culturally-Driven Biases in Preschoolers’ Spatial Search Strategies for Ordinal and Non-Ordinal Dimensions. *Cognitive Development*, *30*, 1–14. https://doi.org/10.1016/j.cogdev.2013.11.002

Murawski, N. J., Klintsova, A. Y., & Stanton, M. E. (2012). Neonatal alcohol exposure and the hippocampus in developing male rats: effects on behaviorally induced CA1 c-Fos expression, CA1 pyramidal cell number, and contextual fear conditioning. *Neuroscience*, *206*, 89–99. https://doi.org/10.1016/j.neuroscience.2012.01.006

Nabel, E. M., & Morishita, H. (2013). Regulating Critical Period Plasticity: Insight from the Visual System to Fear Circuitry for Therapeutic Interventions. *Frontiers in Psychiatry*, *4*. https://doi.org/10.3389/fpsyt.2013.00146

Najjar, S., Pearlman, D. M., Alper, K., Najjar, A., & Devinsky, O. (2013). Neuroinflammation and psychiatric illness. *Journal of Neuroinflammation*, *10*(1), 816. https://doi.org/10.1186/1742-2094-10-43

Nash, K. R., Moran, P., Finneran, D. J., Hudson, C., Robinson, J., Morgan, D., & Bickford, P. C. (2015). Fractalkine Over Expression Suppresses α-Synuclein-mediated Neurodegeneration. *Molecular Therapy*, *23*(1), 17–23. https://doi.org/10.1038/mt.2014.175

Nelson, L. H., & Lenz, K. M. (2017). Microglia depletion in early life programs persistent changes in social, mood-related, and locomotor behavior in male and female rats. *Behavioural Brain Research*, *316*, 279–293. https://doi.org/10.1016/j.bbr.2016.09.006

Nelson, L. H., Warden, S., & Lenz, K. M. (2017). Sex differences in microglial phagocytosis in the neonatal hippocampus. *Brain, Behavior, and Immunity*, *64*, 11–22. https://doi.org/10.1016/j.bbi.2017.03.010

Nikodemova, M., Kimyon, R. S., De, I., Small, A. L., Collier, L. S., & Watters, J. J. (2015). Microglial numbers attain adult levels after undergoing a rapid decrease in cell number in the third postnatal week. *Journal of Neuroimmunology*, *278*, 280–288. https://doi.org/10.1016/j.jneuroim.2014.11.018

Nimmerjahn, A., Kirchhoff, F., & Helmchen, F. (2005). Resting Microglial Cells Are Highly Dynamic Surveillants of Brain Parenchyma in Vivo. *Science*, *308*(5726), 1314–1318. https://doi.org/10.1126/science.1110647

O’Connor, T. G., Moynihan, J. A., & Caserta, M. T. (2014). Annual research review: The neuroinflammation hypothesis for stress and psychopathology in children--developmental psychoneuroimmunology. *Journal of Child Psychology and Psychiatry, and Allied Disciplines*, *55*(6), 615–631. https://doi.org/10.1111/jcpp.12187

Osborne, B. F., Caulfield, J. I., Solomotis, S. A., & Schwarz, J. M. (2017). Neonatal

infection produces significant changes in immune function with no associated learning deficits in juvenile rats. *Developmental Neurobiology*, *77*(10), 1221–1236. https://doi.org/10.1002/dneu.22512

Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., … Gross, C. T. (2011). Synaptic Pruning by Microglia Is Necessary for Normal Brain Development. *Science*, *333*(6048), 1456–1458. https://doi.org/10.1126/science.1202529

Peter R., H. (1979). Synaptic density in human frontal cortex — Developmental changes and effects of aging. *Brain Research*, *163*(2), 195–205. https://doi.org/10.1016/0006-8993(79)90349-4

Radewicz, K., Garey, L. J., Gentleman, S. M., & Reynolds, R. (2000). Increase in HLA-DR immunoreactive microglia in frontal and temporal cortex of chronic schizophrenics. *Journal of Neuropathology and Experimental Neurology*, *59*(2), 137–150. https://doi.org/10.1093/jnen/59.2.137

Ragozzino, D., Di Angelantonio, S., Trettel, F., Bertollini, C., Maggi, L., Gross, C., … Eusebi, F. (2006). Chemokine Fractalkine/CX3CL1 Negatively Modulates Active Glutamatergic Synapses in Rat Hippocampal Neurons. *Journal of Neuroscience*, *26*(41), 10488–10498. https://doi.org/10.1523/JNEUROSCI.3192-06.2006

Raineki, C., Holman, P. J., Debiec, J., Bugg, M., Beasley, A., & Sullivan, R. M. (2010). Functional emergence of the hippocampus in context fear learning in infant rats. *Hippocampus*, *20*(9), 1037–1046. https://doi.org/10.1002/hipo.20702

Rakic, P., Bourgeois, J. P., Eckenhoff, M. F., Zecevic, N., & Goldman-Rakic, P. S. (1986). Concurrent overproduction of synapses in diverse regions of the primate cerebral cortex. *Science (New York, N.Y.)*, *232*(4747), 232–235. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3952506

Ransohoff, R. M., & Perry, V. H. (2009). Microglial Physiology: Unique Stimuli, Specialized Responses. *Annual Review of Immunology*, *27*(1), 119–145. https://doi.org/10.1146/annurev.immunol.021908.132528

Risher, W. C., Patel, S., Kim, I. H., Uezu, A., Bhagat, S., Wilton, D. K., … Eroglu, C. (2014). *Astrocytes refine cortical connectivity at dendritic spines*. *3*, 4047. https://doi.org/10.7554/eLife.04047

Rudy, J. W., Huff, N. C., & Matus-Amat, P. (2004). Understanding contextual fear conditioning: insights from a two-process model. *Neuroscience & Biobehavioral Reviews*, *28*(7), 675–685. https://doi.org/10.1016/j.neubiorev.2004.09.004

Rudy, J. W., & O'Reilly, R. C. (2001). Conjunctive representations, the hippocampus, and contextual fear conditioning. *Cognitive, Affective & Behavioral Neuroscience*, *1*(1), 66–82. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12467104

Schafer, D. P., Lehrman, E. K., Heller, C. T., & Stevens, B. (2014). An engulfment assay: a protocol to assess interactions between CNS phagocytes and neurons. *Journal of Visualized Experiments : JoVE*, (88). https://doi.org/10.3791/51482

Schafer, D. P., Lehrman, E. K., Kautzman, A. G., Koyama, R., Mardinly, A. R., Yamasaki, R., … Stevens, B. (2012). Microglia Sculpt Postnatal Neural Circuits in an Activity and Complement-Dependent Manner. *Neuron*, *74*(4), 691–705. https://doi.org/10.1016/j.neuron.2012.03.026

Schafer, D. P., & Stevens, B. (2013). Phagocytic Glial Cells: Sculpting Synaptic Circuits in the Developing Nervous System. *Current Opinion in Neurobiology*, *23*(6), 1034–1040. https://doi.org/10.1016/j.conb.2013.09.012

Schmid, C. D., Sautkulis, L. N., Danielson, P. E., Cooper, J., Hasel, K. W., Hilbush, B. S., … Carson, M. J. (2002). Heterogeneous expression of the triggering receptor expressed on myeloid cells-2 on adult murine microglia. *Journal of Neurochemistry*, *83*(6), 1309–1320. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12472885

Schmid, C., Melchior, B., … K. M.-J. of, & 2009, undefined. (n.d.). Differential gene expression in LPS/IFNγ activated microglia and macrophages: in vitro versus in vivo. *Wiley Online Library*. Retrieved from https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1471-4159.2009.05984.x

Schwarz, J. M., Sholar, P. W., & Bilbo, S. D. (2012). Sex differences in microglial colonization of the developing rat brain. *Journal of Neurochemistry*, *193*(1), 118–125. https://doi.org/10.1016/j.jneumeth.2010.08.011.Autogenic

Sheridan, G. K., Wdowicz, A., Pickering, M., Watters, O., Halley, P., O'Sullivan, N. C., … Murphy, K. J. (2014). CX3CL1 is up-regulated in the rat hippocampus during memory-associated synaptic plasticity. *Frontiers in Cellular Neuroscience*, *8*, 233. https://doi.org/10.3389/fncel.2014.00233

Stevens, B., Allen, N. J., Vazquez, L. E., Howell, G. R., Christopherson, K. S., Nouri, N., … Barres, B. A. (2007). The classical complement cascade mediates CNS synapse elimination. *Cell*, *131*(6), 1164–1178. https://doi.org/10.1016/j.cell.2007.10.036

Tetreault, N. A., Hakeem, A. Y., Jiang, S., Williams, B. A., Allman, E., Wold, B. J., &

Allman, J. M. (2012). Microglia in the cerebral cortex in autism. *Journal of Autism and Developmental Disorders*, *42*(12), 2569–2584. https://doi.org/10.1007/s10803-012-1513-0

Torres-Platas, S. G., Cruceanu, C., Chen, G. G., Turecki, G., & Mechawar, N. (2014). Evidence for increased microglial priming and macrophage recruitment in the dorsal anterior cingulate white matter of depressed suicides. *Brain, Behavior, and Immunity*, *42*, 50–59. https://doi.org/10.1016/j.bbi.2014.05.007

Tyler, W. J., Alonso, M., Bramham, C. R., & Pozzo-Miller, L. D. (2002). From Acquisition to Consolidation: On the Role of Brain-Derived Neurotrophic Factor Signaling in Hippocampal-Dependent Learning. *Learning & Memory*, *9*(5), 224–237. https://doi.org/10.1101/lm.51202

van Berckel, B. N., Bossong, M. G., Boellaard, R., Kloet, R., Schuitemaker, A., Caspers, E., … Kahn, R. S. (2008). Microglia Activation in Recent-Onset Schizophrenia: A Quantitative (R)-[11C]PK11195 Positron Emission Tomography Study. *Biological Psychiatry*, *64*(9), 820–822. https://doi.org/10.1016/j.biopsych.2008.04.025

van Rossum, D., & Hanisch, U.-K. (2004). Microglia. *Metabolic Brain Disease*, *19*(3–4), 393–411. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15554430

van Weering, H. R. J., Boddeke, H. W. G. M., Vinet, J., Brouwer, N., de Haas, A. H., van Rooijen, N., … Biber, K. P. H. (2011). CXCL10/CXCR3 signaling in glia cells differentially affects NMDA-induced cell death in CA and DG neurons of the mouse hippocampus. *Hippocampus*, *21*(2), 220–232. https://doi.org/10.1002/hipo.20742

Wake, H., Moorhouse, A. J., Jinno, S., Kohsaka, S., & Nabekura, J. (2009). Resting Microglia Directly Monitor the Functional State of Synapses In Vivo and Determine the Fate of Ischemic Terminals. *Journal of Neuroscience*, *29*(13), 3974–3980. https://doi.org/10.1523/JNEUROSCI.4363-08.2009

Wang, F., & Diana, R. A. (2016). Temporal context processing within hippocampal subfields. *NeuroImage*, *134*, 261–269. https://doi.org/10.1016/j.neuroimage.2016.03.048

Williamson, L. L., Sholar, P. W., Mistry, R. S., Smith, S. H., & Bilbo, S. D. (2011). Microglia and Memory: Modulation by Early-Life Infection. *Journal of Neuroscience*, *31*(43), 15511–15521. https://doi.org/10.1523/JNEUROSCI.3688-11.2011

Yirmiya, R., Rimmerman, N., & Reshef, R. (2015). Depression as a Microglial

Disease. *Trends in Neurosciences*, *38*(10), 637–658. https://doi.org/10.1016/J.TINS.2015.08.001

Zhang, M., Xu, G., Liu, W., Ni, Y., & Zhou, W. (2012). Role of Fractalkine/CX3CR1 Interaction in Light-Induced Photoreceptor Degeneration through Regulating Retinal Microglial Activation and Migration. *PLoS ONE*, *7*(4), e35446. https://doi.org/10.1371/journal.pone.0035446

Zuo, Y., Yang, G., Kwon, E., & Gan, W.-B. (2005). Long-term sensory deprivation prevents dendritic spine loss in primary somatosensory cortex. *Nature*, *436*(7048), 261–265. https://doi.org/10.1038/nature03715

Appendix A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORMS

University of Delaware
Institutional Animal Care and Use Committee
Request to Amend an Animal Use Protocol

RECEIVED
AUG 21 2017
IACUC

Title of Protocol: Sex Differences in Vulnerability to Neonatal Infection	
AUP Number: 1239-2017-A	**← (4 digits only)**
Principal Investigator: Jaclyn M. Schwarz	

Requested Changes

I am requesting a change to: ***(Check all that apply)***

- ☐ Animal Species *(Complete Section 1)*
- ☒ Animal Numbers *(Complete Section 2)*
- ☒ Animal Procedures *(Complete Section 3)*
- ☐ Therapeutic or Experimental Agents *(Complete Section 4)*
- ☐ Pain Category *(Complete Section 5)*
- ☐ Use of Biological Material, Hazardous Agents or Radiation *(Complete Sections 4 & 6)*
- ☐ Other *(Specify)* Click here to enter text.

(Complete Section 7)

Changes MUST NOT be initiated until IACUC approval is granted

Official Use Only

IACUC Approval Signature: [signature], DVM

Date of Approval: 9/21/17

University of Delaware
Institutional Animal Care and Use Committee
Request to Amend an Animal Use Protocol

RECEIVED
AUG 2 1 2017
IACUC

Title of Protocol: Sex Differences in Vulnerability to Neonatal Infection	
AUP Number: 1239-2017-A	**← (4 digits only)**
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- ☒ Animal Procedures *(Complete Section 3)*
- ☐ Therapeutic or Experimental Agents *(Complete Section 4)*
- ☐ Pain Category *(Complete Section 5)*
- ☐ Use of Biological Material, Hazardous Agents or Radiation *(Complete Sections 4 & 6)*
- ☐ Other *(Specify)* Click here to enter text.

(Complete Section 7)

Changes MUST NOT be initiated until IACUC approval is granted

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Date of Approval: 9/21/17

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