

**THE ROLE OF DNA METHYLATION IN MODULATING
CHANGES IN EXPRESSION OF NEUROTROPHIC FACTORS
AND MARKERS OF MICROGLIAL ACTIVITY FOLLOWING EARLY-LIFE
IMMUNE ACTIVATION IN JUVENILE AND ADULT RATS.**

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the Bachelor of Science Degree in Neuroscience with Distinction

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RATS FOLLOWING EARLY-LIFE IMMUNE ACTIVATION.**

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ABSTRACT

Immune activation during early development can have profound effects on cognitive and behavioral outcomes in adulthood. Epidemiological data indicate a strong correlation between early-life immune activation and later-life diagnosis with certain neurodevelopmental disorders including as autism, schizophrenia, and depression (Maezawa et al., 2011; Frick et al., 2013; O'Connor et al., 2014; Leckman, 2014). Microglia are the resident immune cells of the brain. They are responsible for phagocytosing cellular debris following infection or injury, and have an active role in responding to environmental stimuli through the release of various pro- and anti-inflammatory cytokines and neurotrophic factors. Recent findings from our laboratory have shown that robust immune activation of a juvenile rat (postnatal day 21) produces a peak response in the expression of the pro-inflammatory cytokine, Interleukin-1 β (IL-1 β), in the hippocampus of juvenile rats within 4 hours. Notably, this cytokine response is completely resolved within 24 hours; *however, rats still exhibit significant cognitive deficits several days later on postnatal day (P) 24*. The purpose of the experiments is to determine whether changes in the expression neurotrophic factors in the hippocampus are responsible the cognitive deficits we have observed on P24, following early-life immune activation, and whether these changes persist into adulthood. Ongoing experiments will examine whether DNA methylation could be an underlying mechanism responsible for lasting changes in the expression of neurotrophic factors and associated cognitive deficits.

Chapter 1

INTRODUCTION

1.1 Early-Life Immune Activation Produces Behavioral Deficits in Adulthood

The principals of adaptation account for an organism's ability to endure adversity and prepare for similar, subsequent challenges. The immune system and its effect on behavior are an excellent and well-characterized example of an adaptive strategy that aids in host defense by enabling an organism to clear a pathogenic infection by diverting energy towards an essential immune response, and away from activities that otherwise consume energy. The immune defense mechanisms include a range of sickness behaviors that paradoxically contribute to host survival. These sickness behaviors were initially characterized by Benjamin Hart (1988), and include the onset of reduced appetite, social interactions, and overall state of activity, as well as altered states of cognitive function and impaired learning. These hallmark sickness behaviors can act as a "double-edged sword", as they put the animal at short-term risk (via malnourishment or risk of attack by predator), while the animal fights the infection; but these behaviors are seen as adaptive because they ultimately aid in the long-term survival of the animal and thus the species (1988).

The concept of *experience-expectant* development posits that there are distinct developmental windows during which the developing brain is dependent upon on the organism interacting with a nourishing environment in order for the brain to properly develop, and thus effectively function (Shonkoff & Phillips, 2000; Sweatt, 2009). These environmental stimuli can influence on-going, pre-programmed cellular events

in the brain including neurogenesis, cell migration and differentiation, as well as the formation and elimination of synapses and myelination (Hagberg & Mallard, 2005). In line with this theory, a variety of experiments indicate that exposure to adverse experiences during the perinatal period can upset these delicate developmental processes and thus have a causal role for the subsequent decline in cognitive behavioral outcomes into adulthood (Pardo et al., 2009).

The role of the immune system in neurodevelopment and its potential effects on brain and behavior in adulthood remains relatively unexplored. However, increasing evidence suggests that exposure to infectious agents during critical developmental periods can increase the vulnerability to cognitive dysfunction, such as deficits in learning or memory, deficits in social behavior, and attention disorders (Benveniste et al., 2001; Adams-Chapman & Stoll, 2006). As a primary example, Bilbo et al. (2006) originally found that neonatal infection of rat pups with *Escherichia coli* resulted in memory impairments *in adulthood* when the hippocampal-dependent learning is also paired with a second, low-dose immune challenge. Notably, this same low-dose immune challenge had *no effect* on learning and memory in adult rats that had never had a previous neonatal infection (Bilbo et al., 2006). These findings were some of the first to suggest that early-life immune activation during a sensitive period of life can disrupt subsequent immune function and lead to cognitive dysfunction, in this case, a selective impairment in the consolidation of memories that depend on hippocampus in adulthood (Bilbo et al., 2006). ***Moreover, these deficits observed in context-dependent memory suggest that neonatal infection can alter the developmental trajectory of the immune system and the brain, and thus serve as a negative factor that can perturb experience-expected development.***

1.2 Microglia are the Resident Immune Cells in the Brain

The primary immune cells of the brain are microglia. These immunocompetent cells have held significant interest throughout history, particularly for their role in host defense of the brain. Microglial cells are responsible for phagocytosing cellular debris following infection or injury, and responding to infectious environmental stimuli through release of various inflammatory cytokines and the signaling pathways involved in release of neurotrophic factors. However, the concept of activated microglia contributing to brain dysfunction has only recently emerged in scientific literature (Nguyen et al., 2002; Rock et al., 2004; Dantzer & Kelley, 2007). The origins, distribution, and function of microglial cells are important considerations for understanding the unique influence the immune system has on the trajectory of normal brain development and subsequent behaviors that emerge later in life.

There has been considerable debate about the origins of the microglial cells. It is now accepted that microglial cells are mononuclear cells that arise from the mesodermal germ layer, and migrate and infiltrate the parenchyma of the brain from the periphery beginning around embryonic day (E)14 in rodents (Harry, 2013). At this point in early embryonic development, microglial cells exhibit a distinct amoeboid morphology, with short, rounded processes. This shape is characteristic of an activated state of microglial activity that is responsible for surveying the environment of the developing embryonic brain in order to eliminate a large number of cells in the neocortex that have been deemed cellular waste (Graeber et al., 2010). The distribution of these amoeboid microglia are found consistently throughout the embryonic brain (Reemst et al., 2016).

In the early postnatal period, however, the amoeboid microglia alter their structure and state of activity quite rapidly (Chan et al., 2007). Over the first few

weeks of life, the microglial cell morphology appears more elongated, and the cells grow out thinner dendritic processes compared to the rounded amoeboid embryonic counterpart. During this postnatal period, the microglial cells become quiescent in nature, but can rapidly transition into an activated amoeboid state in response to infection or injury (Chan et al., 2007). There is a high density of ramified microglia within the early postnatal rat hippocampus (Bilbo & Schwarz, 2009). This is a brain region that is well characterized for its role in learning and memory, and thus taken together; these data suggest that the developing hippocampus may be particularly vulnerable to immune activation during postnatal development in the rodent.

The varying degree of microglial morphologies and developmental stages, as well as the variety of potentially hazardous environmental conditions that can activate the immune system (pathogens, environmental toxicants, stress, and even drugs of abuse) may ultimately contribute significantly to the processes that disrupt proper brain development. To that end, researchers are particularly interested in understanding the cellular and molecular interactions of microglial and developing neural cells, as these interactions likely have important implications for the etiology of various neurodevelopmental disorders.

1.3 Previous Findings

1.3.1 Context Preexposure Facilitation Effect Paradigm

The impact of early life immune activation on hippocampal-dependent learning in juvenile male and female rats has been an important point of investigation in our previous experiments (Schwarz & Bilbo, 2014; Osborne et al., 2017). A version of contextual fear conditioning known as the context preexposure facilitation effect

(CPFE) has been used in order to assess hippocampal-dependent learning. This behavioral paradigm is a variant of classical contextual fear conditioning in which the context learning and the context-shock association occur on separate days (Rudy et al., 2004). This method allows the researcher to exclusively manipulate and specifically determine the mechanisms underlying hippocampal-dependent learning of the context on one day, independent of the context-shock association that occurs on the second day. Previous findings indicate that the CPFE paradigm is a form of context learning that depends on appropriate hippocampal development, plasticity and function. Notably, this type of learning first emerges at P24 (Jablonski et al., 2011).

Our recent data indicate that a juvenile period of development (ages P21-24) is an important period of hippocampal development, and thus a period of increased vulnerability to immune activation. Specifically, immune activation at P21 results in hippocampal-dependent learning deficits that are manifested days later, on P24 (**Figure 1A**). In contrast, immune activation on P24, after the emergence of learning, does not result in subsequent learning deficits several days later (**Figure 1C**) indicating that the hippocampal circuits underlying this type of learning may be formed or undergoing important synaptic remodeling between the ages of P21 and P24.

These findings indicate that immune activation during a critical period of development (P21) significantly impairs the rat's ability to learn and construct a memory days later. It is during this time of brain development where microglia in the hippocampus are still undergoing significant development, which may coincide with and potentially interfere with the neurodevelopmental processes underlying the CPFE task.

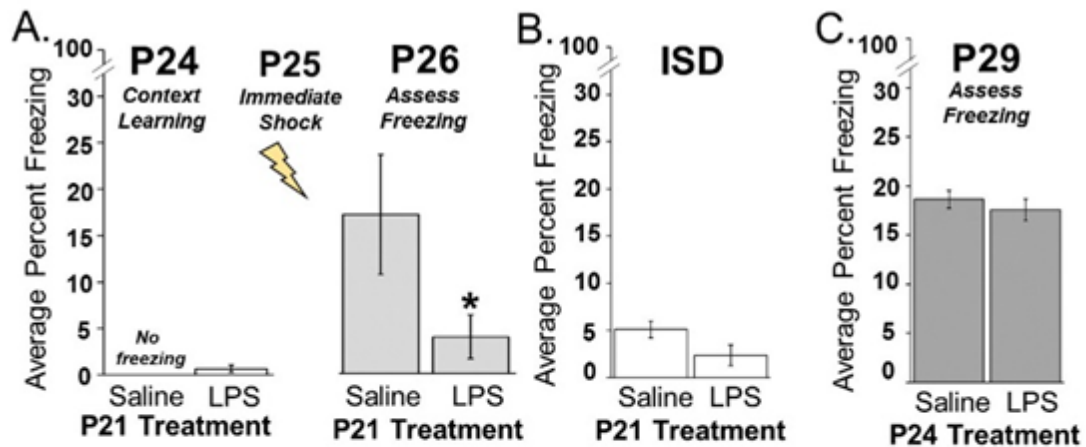


Figure 1. Learning in the Context Pre-Exposure Facilitation Effect (CPFE) paradigm. **A.** On Day 1 of the CPFE (P24) rats explore the context for 5 minutes and all rats show no, or almost no, freezing behavior or lethargy (three days post LPS administration on P21). **B.** Rats show the “immediate shock deficit” (ISD) during the 5 minute test on Day 3 after receiving only the immediate shock on Day 2 of the CPFE, indicating that the learning on P24 is context-hippocampal-dependent. **C.** Rats treated with LPS on P24 (after the full emergence of learning show no learning deficits when tested in the chamber 3 days later (P26 context exposure – P29 test freezing).

1.3.2 Does the Peak and Resolution of the Immune Response on P21 Persist Until P24, at the Time of Learning?

The results of these recent experiments in which we have observed significant behavioral impairments as a result of early life infection have pushed us to examine the molecular and cellular mechanisms of these impairments in learning and memory during this critical developmental period (between P21-24). Microglial cells are important regulators of neuronal development because of their role in pruning and maturation of synapses which are necessary for development of mature neuronal circuits that underlie behavior (Tremblay et al., 2010; Schafer et al., 2012).

We hypothesize that the ability of the microglial cells to actively engage in the maturation of these neuronal circuits is compromised when the cells are presented with an immune challenge during this time of development.

Moreover, because of the immune challenge, the microglial cells may divert their activity towards releasing pro-inflammatory cytokines and chemokines to eliminate the pathogen, but in doing so, may ultimately reduce their expression of important molecular signals or reduce critical phagocytic activity of developing synapses. Thus, an important point of investigation for our previous experiments has been to examine the peak and duration of the microglial response to an immune challenge within the sensitive period of neurodevelopment, on P21 (**Figure 2**), in order to see if it has the potential to *directly* interfere with the learning deficits measured on P24 (**Figure 1**). Examination of this profile of microglial-mediated activity suggests that there exists a distinct peak and resolution profile of certain pro-inflammatory cytokines, chemokines, and neurotrophic factors (**Figure 2**). Most notably, we found that many of the primary pro-inflammatory cytokines showed a peak in expression within 2-4 hours post-LPS treatment, but that their expression had resolved within 24 hours post-LPS treatment (by P22). Brain Derived Neurotrophic Factor (BDNF) was downregulated at 4 hours post LPS-treatment, and this effect was maintained until 24 hours post immune activation, after the primary cytokine response had resolved (on P22). In addition, we found a significant decrease in the neuronal cytokine, Cx3cl1 (fractalkine) and an increase in the phagocytic marker, complement C3, only at 24 hours post-LPS treatment (on P22). These findings suggest that classical pro-inflammatory cytokines are no longer up-regulated at 24 hours post immune activation; however, fractalkine (Cx3cl1), a neuronal chemokine that directs microglia

towards developing synapses (Hoshiko et al., 2012, is significantly decreased at this time, and complement C3, an immune signal of phagocytosis, is significantly upregulated at this time (**Figure 2**). *Importantly, these data suggest that beyond the initial pro-inflammatory response, changes are occurring across multiple cell types and multiple genes to initiate dysfunction in the developing neural circuits even beyond P22. This has been the primary focus on my thesis research.*

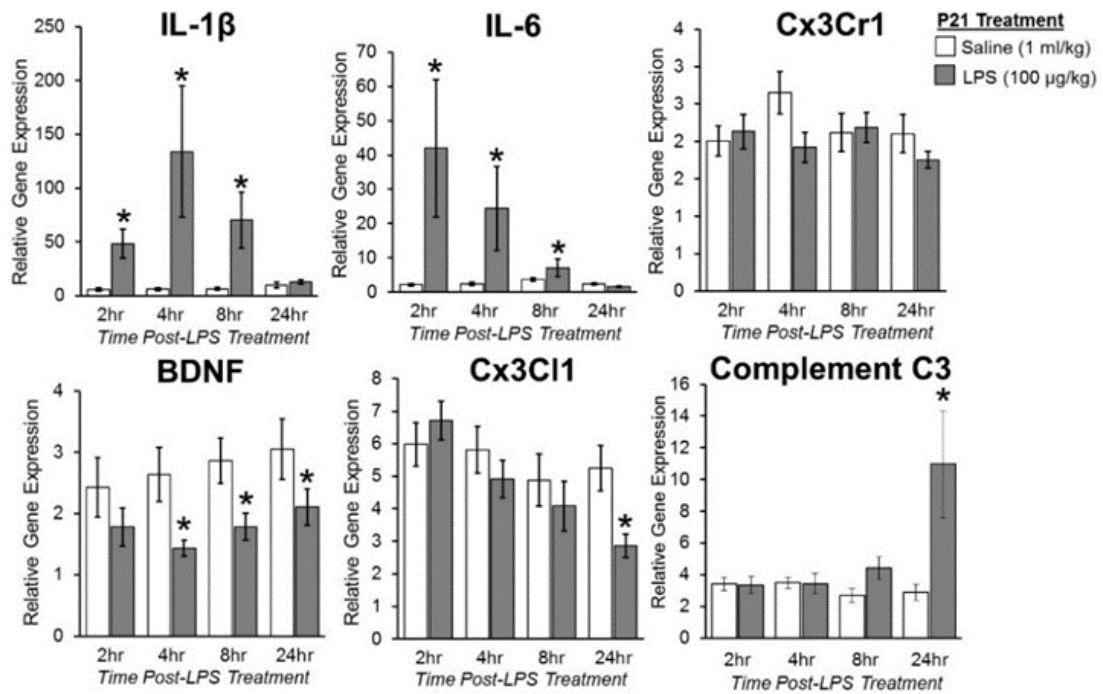


Figure 2. LPS induces a significant increase in **IL-1 β** and **IL-6** in the hippocampus, an effect that is resolved within 24 hours post-treatment. LPS produces no change in the fractalkine receptor expression, **Cx3Cr1**. Interestingly, however, LPS-induced immune activation also produces a significant decrease in the expression of Brain Derived Neurotrophic Factor (**BDNF**) that coincides with the peak in **IL-1 β** expression and is *not* resolved within 24 hours post-treatment. Fractalkine (**Cx3CI1**, a cytokine produced by neurons) is significantly decreased by LPS, while **complement C3**, a phagocytic marker found on neurons is significantly increased by LPS, but both effects occur *after* the resolution of the primary immune response, at 24 hours post immune activation.

1.4 Looking Beyond the Initial Inflammatory Response: A Potential Role of Epigenetics

The immune response described in our previous experiments is the direct result of exposure to an immunogenic molecule (LPS) early in development. A study conducted by Barrientos et al. (2004) investigated the effects of intrahippocampal injection of IL-1 β , a proinflammatory cytokine released by microglial cells, on the expression of BDNF mRNA following contextual fear conditioning, a task that is associated with a timely increase in the expression of BDNF in the hippocampus. Barrientos et al. found that intrahippocampal administration of IL-1 β blocked a learning-induced increase in BDNF mRNA within the hippocampus (Barrientos et al., 2004). These findings indicate that the presence of proinflammatory cytokines *alone* can have a causal role in modulating the expression of genes that are important for learning and memory. Furthermore, these findings support our data (**Figure 2**) that indicate early-life immune activation on P21 produces significant increase in expression of pro-inflammatory cytokines (e.g. IL-1 β) and a similarly-timed decrease in neurotrophic factors (e.g. BDNF). It is well-known that immune activation such as this can result in changes in cognitive function and behavior *at the time of the* inflammatory response. As we mentioned, sickness behavior was initially characterized by Benjamin Hart decades ago (Hart, 1988). The question remains how behavior can become aberrant for longer periods of time, after the initial immune challenge has resolved itself, such as that seen in our proposed model (e.g. after P22). Understanding *this* process is essential to understanding the link between immune activation and the subsequent etiology of many neurodevelopmental and mental health disorders.

The field of epigenetics aims to understand how exposure to various environmental stimuli, such as early-life immune activation, can produce changes in gene expression without directly modifying the DNA (Waddington, 1957). There are a number of post-translational modifications to the genome that have been of intense focus in recent years, and among these modifications includes that of DNA methylation (Fagiolini et al., 2009). DNA methylation refers to the process by which cytosine are chemically modified through the addition of methyl groups by a specialized class of enzymes known as DNA methyltransferases (DNMTs). It is important to note that not all cytosine sites within the genome are similarly methylated. Rather, methyl groups are added to cytosines that are followed by a guanine nucleotide. This dinucleotide arrangement is referred to as a “CpG” site. It has been reported that approximately 70% (or most) of the CpG sites that are present within the genome are methylated, and the rest are typically found to be unmethylated, and these ones are located in special “CpG islands” throughout a given gene sequence (Cooper & Krawczak, 1989). These CpG islands are therefore of great interest as they are the regions that are more susceptible to varying levels of methylation (Bird, 1986). In most empirical analyses that have been conducted so far, methylation of DNA is typically associated with *suppression of gene transcription* as it causes a physical rearrangement of chromatin structure that prevents transcriptional machinery from properly binding. However, other investigations have indicated that DNA methylation can also be associated with transcriptional activation (Sweatt, 2009).

There is growing interest in the potential role that epigenetic modifications of certain genes may have particularly following an early-life immune challenge and the subsequent susceptibility to neurodevelopmental disorders (Borrelli et al., 2008;

Bayarsaihan, 2011). We hypothesize that the changes observed in expression of genes that are markers of microglial activity or that maybe critical for learning and memory (e.g. neurotrophic factors) may be targets of epigenetic modifications that are the result of early-life immune activation. Surprisingly, the literature on epigenetic mechanisms of or following inflammatory responses is quite limited. *The aim of this experiment is to understand whether exposure to early-life immune activation may have a long-term impact on the expression of genes that determine behavioral outcomes later in life, and whether some of these genes may also be epigenetically modified.*

1.5 Current Study, Purpose, and Significance

The first experiment of the current study sought to determine the long-term effect of a P21 immune challenge (using our previously described model) on the expression of neurotrophic factors and markers of microglial activity. To this end, we collected hippocampal tissue from P24 and P60 male and female Sprague Dawley rats that were treated with LPS (100 $\mu\text{g}/1\text{mL}/\text{kg}$) on P21 and examined the relative expression of proinflammatory cytokines, neurotrophic factors, and markers of microglial mediated activity at these later time points using quantitative real-time PCR.

The second experiment sought to determine whether the observed changes in gene expression may be the result of epigenetic modifications, specifically DNA methylation, and whether or not these epigenetic prints persist into adulthood. To this end, we examined the relative levels of DNA methylation of the brain derived neurotrophic factor (BDNF) and the glial-cell derived neurotrophic factor (GDNF) gene. The same samples used to analyze gene expression in the first experiment were

also used for the DNA methylation analysis. The relative levels of methylation were obtained by designing primers that allowed for amplification of DNA that contained rich CpG sites. These sites were located on electropherograms and peak values were obtained from each site ($G/(G+A)$).

It is important that we understand the mechanisms that are responsible for initiating a robust neuroimmune response that subsequently become aberrant for longer periods of time, after the initial immune challenge has resolved itself. By investigating these mechanisms scientists may one-day lead to useful insights that give a more complete picture of how the immune system is able to play a critical role in determining cognitive and behavioral outcomes later in life.

Chapter 2

METHODS AND MATERIALS

2.1 Animals and Breeding

Male and female Sprague Dawley rats were ordered from Envigo Laboratories in Indianapolis, Indiana. The rats were housed in same sex pairs in clear, polyethylene cages in the animal facility of McKinly Laboratory at the University of Delaware. They were 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 0700 hr.) 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). Two days prior to the calculated date of birth, postnatal day zero (P0), pregnant females were housed individually, allowing them to give birth undisturbed. Litter sizes and male to female ratios were not adjusted at the time of birth. 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 (IACUC) as approved in AUP#1239.

2.1.1 Experiment 1: Examination of Long-Term Changes in Gene Expression Following Early-Life Immune Activation

A total of 32 rats were used for tissue collection in Experiment 1 (n=16/group, 8M, 8F). There were two groups that were treated with either saline or an immune challenge (saline or LPS, 100 μ g/kg) on postnatal day 21 (P21), and these treatment groups were subsequently divided into two additional groups, each dependent upon

the age at time of tissue collection (P24 or P60). At the time of euthanasia and tissue collection, the medial prefrontal cortex, dorsal hippocampus, and amygdala were collected in order to characterize relative levels of gene expression of Interleukin 1 (IL-1) β , Brain Derived Neurotrophic Factor (BDNF), Glial Derived Neurotrophic Factor (GDNF), the neuronal cytokine - CX3CL-1 (fractalkine), and Glial fibrillary acidic protein (GFAP). See **Table 1** for a list of genes and their associated functions.

Table 1: Functions of genes used for gene expression analysis.

<i>Gene</i>	<i>Function</i>
<i>IL-1β</i>	Interleukin 1 beta; a proinflammatory cytokine released in response to injury or infection.
<i>BDNF</i>	Brain Derived Neurotrophic Factor; a neurotrophic factor implicated in learning and memory.
<i>GDNF</i>	Glial Derived Neurotrophic Factor; a neurotrophic factor important in neuronal-glia communication and neural development.
<i>CX3CL-1</i>	Fractalkine ligand; a neuronal chemokine implicated in microglial-neuronal immune response (decreasing microglial activation)
<i>GFAP</i>	Glial Fibrillary Acidic Protein; expressed in astrocytes, marker of immune activity

2.1.2 Experiment 2: Examination of the Levels of DNA Methylation in Important Neurotrophic Factors Following Early-Life Immune Activation

The same 32 rats used in Experiment 1 were also used for tissue collection in Experiment 2 (n=16/group, 8M, 8F). There were two treatment groups based on postnatal day 21 (P21) treatment (saline or LPS, 100 μ g/kg) and age at time of tissue collection (P24 or P60). The medial prefrontal cortex, dorsal hippocampus, and amygdala were collected in order to characterize relative levels of DNA methylation of BDNF and GDNF.

2.2 Treatment

2.2.1 Lipopolysaccharide Preparation

All experiments used lipopolysaccharide (LPS) which is a bacterial cell wall protein derived from *E. coli* 0111:B4 obtained from Sigma Aldrich (Cat. No. L2630). It is Sterile, pyrogen-free DPBS was used to dilute the stock concentration of LPS (2,500 μ g/mL) to a final concentration of 100 μ g/mL for injections. On P21, rats in all experiments received an intraperitoneal (i.p.) injection of either 1mL/kg or 100 μ g/kg of LPS or the equivalent volume of sterile pyrogen-free DPBS as a control.

2.2.2 Cage Mate Assignment

All treated rats received an untreated cage mate from the same litter of the same sex. The designated cage mate was undisturbed. Those that received treatment of saline or LPS were marked on the base of their tail with marker to distinguish it from the cage mate. The pairs were housed together with food and water placed *ad-libitum*.

2.3 Euthanasia for Tissue Collection Procedure

The rats were euthanized by administration of an overdose of Euthasol[®] (ANADA 200-071) via i.p. injection at P24 (3 days post LPS administration) or at P60 (39 days post LPS administration). The rat was determined to be unconscious when pinching their hind feet with forceps did not produce a leg-muscle retraction response.

The unconscious rat was placed on the surgical tray, where the lower abdomen was pinched with forceps and an incision with surgical scissors was made into the abdomen muscle. The cut extended to each side and continued up both sides of the cavity until they reached just below the shoulders, exposing the heart. A 1.0mL syringe and 25-gauge needle tip was used to extract approximately 1.0mL blood via

cardiac puncture from the left ventricle of the heart. The blood was put into a sterile 1.5mL tube and placed directly onto wet ice.

Rats were then perfused via this same cardiac puncture with ice-cold, 0.9% saline solution to remove blood and peripheral immune cells from the brain. A needle attached to a saline pump was inserted into the left ventricle of the heart, and a cut was made on the ascending aorta. Perfusions were determined to be completed when the folds of the liver as well as liquid exiting the incised aorta were cleared of blood. A small segment of the inner portion of the spleen was removed for analysis of possible peripheral immune function if needed.

A 1mm brain block matrix was used to make consistent anatomical 2mm slices from the rat brain. The medial prefrontal cortex dorsal hippocampus, and amygdala were collected and immediately flash frozen on dry ice. Samples were stored at -80°C until further analysis.

The assigned, untreated cage mates were placed in a CO₂ chamber until there were definitive signs of unconsciousness. They were then sacrificed using a guillotine.

2.4 Gene Expression Analysis

2.4.1 RNA Extraction and cDNA Synthesis

The AllPrep[®] DNA/RNA Mini Kit (Qiagen Inc., Valencia, CA) was used to extract mRNA from the dorsal hippocampus samples. The samples were first lysed and homogenized with a guanidine-isothiocyanate buffer. The resulting lysate was then passed through a series of spin columns that allow for selective binding and separation of genomic DNA from RNA.

cDNA was synthesized from the clean, extracted RNA (100 ng/ μ L) using the QuantiTect[®] Reverse Transcription Kit (Cat. No. 205314, Qiagen).

2.4.2 Quantitative Real Time PCR

Relative gene expression was quantified by real-time PCR using the RealMasterMix[®] Fast SYBR Kit (Cat. No. 2200830, 5 Prime) in 10 μ L reactions on a CFX96Touch real time PCR machine. All other primers were ordered through Integrated DNA Technologies and diluted to a final concentration of 0.13 μ M (IL-1 β ; BDNF, GDNF, CX3CL-1, and GFAP). See **Table 2** for a list of primer sequences. RPLP-1 was analyzed using a QuantiTect[®] Primer Assay (Cat. No. QT00365561) and diluted according to protocol. RPLP-1 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.

Table 2: List of primer sequences used for qPCR gene expression analysis.

	<i>Primer Sequence</i>
<i>IL-1β</i>	F: GAAGTCAAGACCAAAGTGG R: TGAAGTCAACTATGTCCCG
<i>BDNF</i>	F: ATCCCATGGGTTACACGAAGGAAG R: AGTAAGGGCCCGAACATACGATTG
<i>GDNF</i>	F: ATTCAAGCCACCATCAAAAG R: TCAGTTCCTCCTTGGTTTCG
<i>CX3CL-1</i>	F: TCCAGGGCTGTCCCGCAAA R: ACAGGCAGGCAAGCAGGCAG
<i>GFAP</i>	F: AGGGACAATCTCACACAGG R: GACTCAACCTTCCTCTCCA

2.4.3 Statistical Analysis

For each reaction, the average quantitative threshold amplification cycle number (Cq) value was determined from each duplicate, and the $2^{-\Delta\Delta Cq}$ method was used to calculate the relative gene expression for each gene of interest relative to the housekeeping gene.

Gene expression data (Experiment 1) and CpG Methylation data (Experiment 2) were analyzed using the statistical software program SPSS (IBM). Data from all experiments were analyzed using 2x2 ANOVAs with P21 Treatment (Saline vs LPS), and Age (P24 vs P60) as the between-subjects factors. Significant interactions were followed up with post hoc pairwise comparison tests using the Bonferroni correction to control for multiple comparisons and examine between group differences. The α -level for all experiments was 0.05.

2.5 DNA Methylation Analysis

The AllPrep DNA/RNA Mini Kit (Qiagen Inc., Valencia, CA) was also used to extract DNA from the dorsal hippocampus samples. Quantification and assessment of nucleic acid quality from samples were determined using spectrophotometry (NanoDrop 2000). The EpiTect[®] Bisulfite Kit (Qiagen Inc., Valencia, CA) was used to bisulfite convert the samples. The bisulfite-modified DNA was assessed via direct bisulfite DNA sequencing (BSP, on Bio-Rad CFX96 system). Using NCBI BLAST (<https://blast.ncbi.nlm.nih.gov/Blast>.) and MethPrimer (<http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi>) sites, specific forward and reverse primers were designed for amplification of our bisulfite treated samples (see **Table 3**). PCR products were purified using Diffinity RapidTips[®] (Diffinity Genomics) and verified using gel electrophoresis. Samples were then sequenced at the Delaware

Biotechnology Institute (<http://www.dbi.udel.edu/core/dnasequencing>). The electropherogram was read on Chromas software, where the percent methylation of each CG site was determined by the ratio between peak values of G and A (G/[G+A]).

Table 3: List of specifically designed primer sequences (MethPrimer) for amplification of bisulfite converted DNA. Reverse primer used for sequencing.

<i>Primer Sequence</i>	
<i>GDNF</i>	F: TTAGTTTTTTTGGGATTTAAGAGGT R: TAAACCCCAATTAAACATTAAGTC
<i>BDNF</i>	F: GGTAGAGGAGGTATTATATGATAGTTTA R: TACTCCTATTCTTCAACAAAAAATTAAT

Chapter 3

RESULTS

3.1 Experiment 1: Examination of Changes in Gene Expression Following Early-Life Immune Activation

The results of Experiment 1, examining changes in gene expression for IL-1 β , BDNF, CX3CL-1, GFAP and GDNF are shown in **Figure 3**.

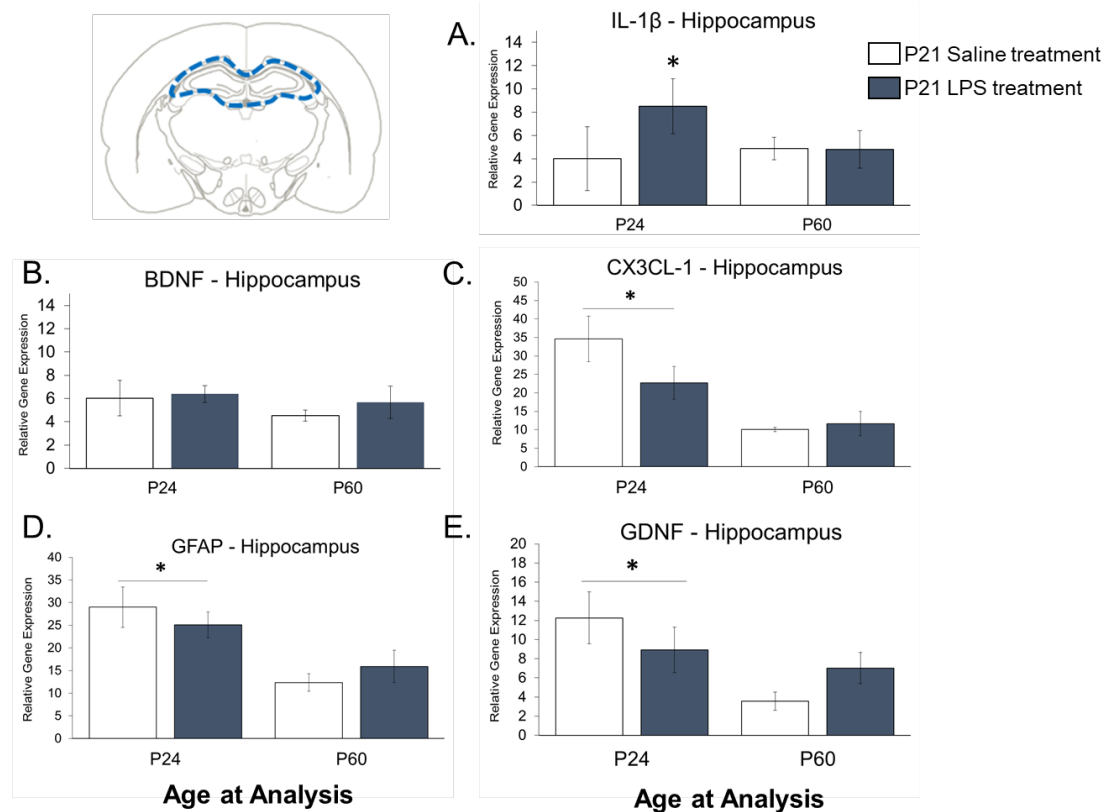


Figure 3: Analysis of gene expression of hippocampus in P24 and P60 rats treated with saline or LPS on P21. **A.** P21 LPS treatment significantly increased **IL-1 β** production in P24 rats compared to saline control. In addition, **IL-1 β** production was significantly higher in P24 than in P60. **B.** There were no significant effects of **BDNF** within P24 and P60 rats. **C, D, E.** P21 saline treated controls produced significantly more **Cx3C11**, **GFAP**, and **GDNF** at P24 than at P60 timepoints.

Specifically, we found a significant interaction of LPS treatment at P21 on the expression of IL-1 β later in life ($F_{3,28}=4.592$; $p = 0.42$; **Figure 3A**) and post hoc tests revealed that IL-1 β was still significantly increased compared to saline treated controls at P24 ($p < 0.01$) in the hippocampus. This is in contrast to the lack of effect we saw on P22 (see **Figure 2**), which indicated that the cytokine response had been resolved in our post hoc tests. We found no main effects or interactions of P21 LPS treatment and age on the expression of BDNF (**Figure 3B**). We found a significant main effect of age on the expression of the neuronal cytokine, fractalkine, Cx3C11, in the hippocampus ($F_{1,28} = 17.97$; $p < 0.001$; **Figure 3C**). Specifically, fractalkine was significantly upregulated at P24 relative to P60 ($p < 0.001$). We also found a significant main effect of age on the expression of GFAP ($F_{1,28} = 1.219$; $p = 0.46$; **Figure 3D**). Post hoc tests revealed that GFAP expression in the hippocampus was significantly higher at P24 than at P60 ($p < 0.05$). Analysis of GDNF in the hippocampus also revealed a main effect of age ($F_{1,26} = 6.00$; $p = 0.22$; **Figure 3E**). As with the other genes, we found that GDNF expression in the hippocampus was significantly upregulated at P24 compared to P60 ($p < 0.01$).

Taken together, these results suggest that **(1)** contrary to our predictions there is still a significant (though small) increase in the expression of the pro-inflammatory cytokine IL-1 β at the time of the context learning, on P24. This effect is not observed later in life, at P60. **(2)** Many of the genes examined are significantly influenced by age, in particular, genes that are important for modulating neuronal-glia communication and neural development are significantly upregulated at P24 relative to P60, and none of these genes was significantly affected by the immune challenge on P21.

3.2 Experiment 2: Examination of the Levels of DNA Methylation Following Early-Life Immune Activation

In Experiment 2, we focused our analysis of DNA methylation on two genes in particular, BDNF and GDNF. BDNF methylation levels were examined because of its important role in learning and memory. GDNF methylation levels were examined because of its important role in neuronal-glia communication and neural development. Unfortunately, the results from our BDNF methylation analysis yielded no analyzable results (*data not shown*). The data from our analysis of 27 CpG sites on the GDNF gene are shown in **Figure 4** and **Figure 5**.

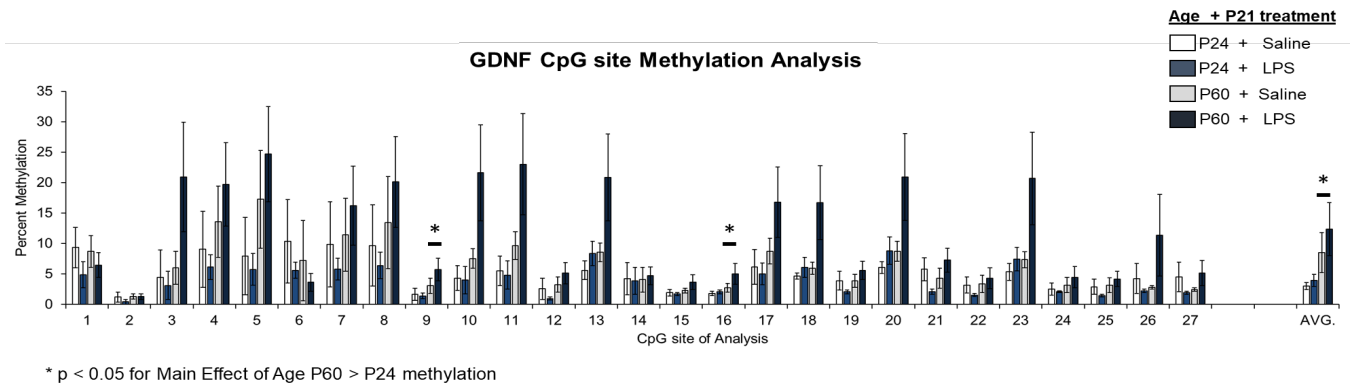


Figure 4: Methylation Analysis of 27 CpG sites along the Glial Derived Neurotrophic Factor Promoter. We found a significant main effect of age at CpG site 9 ($F_{1, 27} = 4.99$; $p = 0.035$) and a trending effect of age at CpG site 16 ($F_{1, 27} = 3.61$; $p = 0.06$). When we averaged the methylation across all CpG sites (see AVG), we found a significant effect of age ($F_{1, 26} = 4.81$; $p = 0.038$). (N = 5 P24 saline treated rats, 3 male, 2 female; N = 7 P24 LPS treated rats, 4 male, 3 female; N = 7 P60 saline treated rats, 4 male, 3 female; N = 7 P60 LPS treated rats, 4 male 3 female). Data represent the mean \pm SEM.

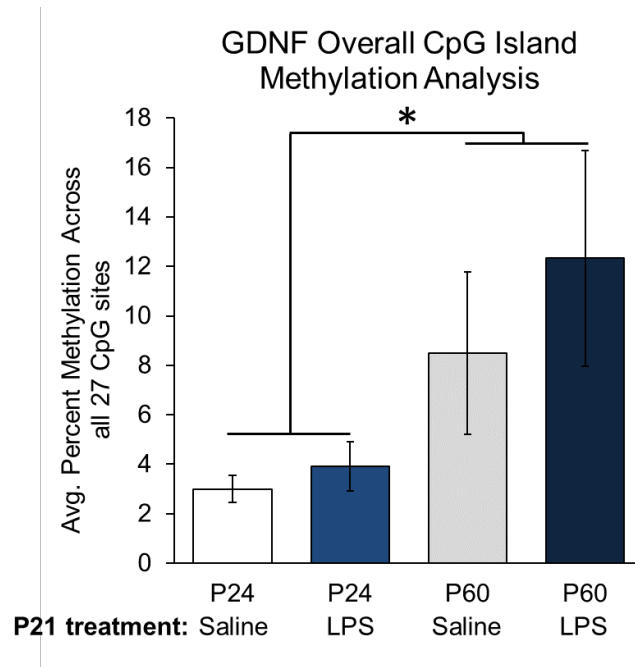


Figure 5: Average methylation across 27 CpG sites along the Glial Derived Neurotrophic Factor Promotor (graphed independently). When we averaged the methylation across all CpG sites (see AVG), we found a significant effect of age ($F_{1,26} = 4.81$; $p = 0.038$). (N = 5 P24 saline treated rats, 3 male, 2 female; N = 7 P24 LPS treated rats, 4 male, 3 female; N = 7 P60 saline treated rats, 4 male, 3 female; N = 7 P60 LPS treated rats, 4 male 3 female). Data represent the mean \pm SEM.

Analysis of percent methylation along 27 CpG sites on the GDNF promoter revealed some interesting trends, and a few significant effects. Notably, we found a significant main effect of age at CpG site 9 ($F_{1,27} = 4.99$; $p = 0.035$) and a trending effect of age at CpG site 16 ($F_{1,27} = 3.61$; $p = 0.06$). When we averaged the methylation across all CpG sites (see AVG in **Figure 4**, or this data lone in **Figure 5**), we found a significant effect of age ($F_{1,26} = 4.81$; $p = 0.038$). Looking more closely at this data as a whole, it appears that there may be an effect of LPS across multiple CpG sites, such that LPS significantly increases the methylation of GDNF at P60; however,

this effect may only be detected with a 2x2x2 repeated-measures ANOVA using CpG site as a within subjects factor, age (P24 vs. P60) as a between subjects factor, and treatment as a between subjects factor. If time permits, we will run this analysis.

Notably, however, these findings support our gene expression data reported in **Figure 3E**. Specifically, we found that GDNF expression was significantly upregulated at the juvenile age of P24 relative to the adult age of P60. Here we find that there is a significant increase in methylation of the GDNF promoter across 27 CpG sites (**Figure 5**), suggesting that increased methylation of this gene across age may contribute directly to the decrease in gene expression measured across age. This conclusion is perhaps more robust given that the data were collected from the same tissue samples, from the same rats.

Chapter 4

DISCUSSION

4.1 Results of Gene Expression Analysis

The results from our preliminary data and Experiment 1 provide insights to the complex neuroimmune response following early-life immune activation on P21. The first interesting finding indicated that P21 LPS treatment significantly upregulated hippocampal proinflammatory cytokine IL-1 β production at P24 relative, though the effect was small. LPS had no effect on IL-1 β expression at the P60 time point.

IL-1 β has been well characterized for its role in mediating hippocampal dependent memory. The presence of IL-1 β within the brain is well characterized for its role to fear conditioning, being up-regulated during this type of learning, and inducing the maintenance of synapses in long-term potentiation (Bilbo et al., 2005). However, an exaggerated IL-1 β response within the brain is associated with a markedly robust display of memory impairment (Williamson et al., 2010). The findings from this experiment support our previous findings that have investigated the effects of early-life immune activation on hippocampal-dependent memory through the behavioral context preexposure facilitation effect (CPFE) paradigm (**Figure 1**). The findings from our previous CPFE experiments indicate that the P24 timepoint is a critical period in which consolidation of memories occurs. The exaggerated production of IL-1 β within this experiment demonstrate that the effects of P21 treatment persist throughout much of this critical period in hippocampal neurodevelopment and behavioral development. Furthermore, the neuroimmune response of the proinflammatory cytokine IL-1 β remains elevated three days post infection, a finding that contrasts with our previously conducted post-hourly tissue collection analysis.

The second results also indicated that expression of genes important for modulating neuronal-glia communication and neural development are significantly upregulated at P24 relative to P60. Many of these genes were not affected by the LPS treatment on P24, however. These findings suggest that early-life infection does not produce treatment effects for a number of important genes, either P24 or P60 rats. Instead, these genes may play a role in neural development as they change across time, from P24 to P60, and are likely not a mechanism by which LPS disrupts learning. That said, these studies only correlate gene expression at the time of learning with the learning that can occur at the same time, thus future experiments may want to manipulate some of these important genes to determine their role in the LPS-induced learning impairments days later.

4.2 Results from DNA Methylation Analysis

The second aim of the experiment examined the overall levels of DNA methylation for the BDNF and GDNF genes in P24 and P60 rats given a P21 treatment of either saline or LPS. Unfortunately, the levels of DNA methylation for the BDNF gene were inconclusive as the samples were unable to be sequenced from DBI due to an unknown source of error with the DNA and primers used. However, the levels of DNA methylation were successfully analyzed for the GDNF gene. Our findings from this analysis indicate that average levels of methylation across the 27 CpG sites within the GDNF gene were significantly upregulated at P60 relative to P24. This finding suggests that the GDNF gene may not be necessarily important in regulating the deficits in learning on P24 that we have previously observed. However, the data provide important insights at the regulation of GDNF across time. It would be interesting to examine epigenetic modifications to genes that mediate the

neuroimmune response—particularly the Cx3CL1 and Complement C3 genes, as they regulate microglial communication and modulation of synapses, respectively.

4.3 Limitations of Experiments and Future Directions

The results of the gene expression analysis revealed that only the proinflammatory cytokine, IL-1 β , was significantly upregulated at P24 relative to P60 as a result of P21 LPS treatment. While we did find that significant changes occurred, we did not investigate the levels of CpG methylation on the IL-1 β gene due to the lack of literature and previous empirical studies examining epigenetic modifications to IL-1 β gene alone. The IL-1 β gene is a small gene, and we were unable to identify CpG islands in its sequence. That said, given that this gene *is* significantly altered in expression following early-life infection, it would be interesting to see whether there is an effect of LPS treatment during this important period of hippocampal development on other types of epigenetic marks associated with the IL-1 β gene (histone methylation or acetylation).

There are a few notable limitations to the assessment of overall DNA methylation in P24 and P60 rats. The DNA used in the methylation assays was bisulfite converted. Treatment with sodium bisulfite converts unmethylated cytosines to uracils which then become thymines in the subsequent PCR amplification, whereas methylated cytosines remain unchanged. The sodium bisulfite treatment thus results in the conversion of the usually undetectable epigenetic information into valuable detectable sequence information at base pair resolution. The chemicals used in the conversion are harsh, and leave room for potential over conversion or under conversion of cytosines (Leontiou et al., 2015). Therefore, it is important to use standardized controls with known methylation values in order to detect whether or not

the conversion did what it was supposed to. The data presented in **Figure 4** and **Figure 5** are shown without analysis of methylated standards. Thus, the findings remain somewhat inconclusive without proper comparison to standard bisulfite converted DNA with known methylation percentages.

An interesting future direction for understanding the role epigenetics may play into the neuroimmune response is to examine changes in histone acetylation as an epigenetic marker of activity. The results of our experiment indicate that the production of proinflammatory neuroimmune molecules in response to early-life infection are resolved at some point beyond that of juvenile age. Thus, it could be that epigenetic treatment effects of a single infection are simply not powerful enough to produce robust methylation marks in response to LPS treatment. Other commonly assessed epigenetic markers include that of histone acetylation, which have been reported to be more labile and mediate regulation of gene expression over shorter periods of time (Borrelli et al., 2008). Thus, it would be another interesting approach to determine whether histone acetylation mediates gene expression of neurotrophic factors and markers of microglial mediated activity following early-life infection. Moreover, it will be important to determine whether the deficits in learning persist past the age of P24 until P60. If we find that these cognitive deficits do not persist into adulthood, this is still fascinating and provides critical insight into the nature of the effects of immune activation during a sensitive period of hippocampal development. There is evidence to suggest that disruption of microglia number or function results in only transient disruptions in hippocampal circuit formation (Paolicelli et al., 2011) and adult spatial learning (Torres et al., 2016). In particular, Paolicelli et al. showed that Cx3Cr1 knockout mice have a transient disruption in

microglia number (~20 days), with an associated transient increase in dendritic spine numbers on hippocampal neurons. Similarly, Torres et al. found that transient depletion of microglia in the adult hippocampus resulted in only temporary deficits in hippocampal dependent spatial learning that lasted approximately 20 days (Torres et al., 2016). Moreover, if we do not see persistent learning deficits into adulthood, future experiments could seek to determine the cellular and molecular mechanisms by which immune activation produces *delays* in the onset of hippocampal-dependent learning at this young age, the results of which will also be important. In addition, future experiments could also examine the cellular and molecular mechanisms by which these cognitive deficits are eventually *reversed* and the role of microglia-neuronal communication in this important process.

Chapter 5

CONCLUSIONS

Thus, it is of great importance to understand the mechanisms that mediate the neuroimmune response following early-life immune activation. Our previous experiments have determined that exposure to infectious agents during early development can have negative implications on the cognitive abilities in juvenile rats later in life. The first aim of the experiment was to characterize the neuroimmune response at two distinct developmental periods, P24 and P60, as it can provide information pertaining to baseline expression of genes implicated in inflammatory response as well as microglial-neuronal communication. The findings from this experiment indicate that the proinflammatory cytokine, IL-1 β , remains significantly upregulated during the critical juvenile period of neurodevelopment. In addition, genes critical for neuronal-microglial interaction are significantly upregulated in the juvenile period relative to adulthood. These findings are particularly important for providing a more complete picture of the neuroimmune response to infection during the complex period of hippocampal neurodevelopment. The second aim of the experiment was to determine whether the neurotrophic factor, GDNF, has been epigenetically modified following early-life immune activation on P21. The results of the analyses indicate that the average percentage of DNA methylation of the GDNF gene across all 27 CpG sites is significantly higher in adulthood than at juvenile timepoints. This finding is consistent with our GDNF gene expression analysis, however, indicates that methylation status is not directly a result of LPS treatment in our model of immune activation on P21.

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