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# Exposure of neonatal rats to alcohol has differential effects on cytokine levels in the cerebellum and hippocampus : potential role of microglia and astrocytes

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# EXPOSURE OF NEONATAL RATS TO ALCOHOL HAS DIFFERENTIAL EFFECTS ON CYTOKINE LEVELS IN THE CEREBELLUM AND HIPPOCAMPUS: POTENTIAL ROLE OF MICROGLIA AND ASTROCYTES

**by**

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**DISSERTATION**

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# Exposure of Neonatal Rats to Alcohol has Differential Effects on Cytokine Levels in the Cerebellum and Hippocampus: Potential Role of Microglia and Astrocytes

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## Abstract

Drinking during pregnancy can result in fetal alcohol spectrum disorders (FASD), an umbrella term that encompasses a wide array of symptoms ranging from neurocognitive deficits to physical malformations. The mechanism for FASD is not yet fully understood however recently, activation of the neuroimmune system has been suggested to play a role. Neuroinflammation, characterized by increased pro-inflammatory cytokine production and glial cell activation, has been observed following exposure to alcohol during the neonatal period. Furthermore, neuroimmune activation has been implicated in alcohol-induced neuronal death and/or damage. The studies herein investigate several components of the

relationship between neuronal damage following developmental alcohol exposure, and neuroimmune system activation.

This dissertation is broken down into two main studies. In the first study, we focus on the relatively uncharacterized effects of moderate exposure to alcohol during development. Specifically, re-programming of neuroimmune cells has been demonstrated following exposure to insult during the neonatal period. This “primed” neuroimmune system then over-reacts to subsequent insults and results in exaggerated production of pro-inflammatory molecules, which can cause behavioral deficits. We hypothesized that exposure to alcohol could elicit similar effects, and lead to neuroimmune system priming.

In the second study we used a high dose, binge-like exposure paradigm, during the neonatal period. While this type of exposure has been shown to result in robust increases in pro-inflammatory cytokine production and microglial activation, evidence for an anti-inflammatory component also exists. Neuroimmune system activation has recently been shown to consist of multiple pathways, known as M1 and M2. M1 activation is considered pro-inflammatory and can be neurotoxic, while M2 is generally anti-inflammatory and neuroprotective. This study sought to test the hypothesis that alcohol exposure in the neonatal period would initially produce a M1-type response followed by a regenerative M2 period.

The overall findings suggest that high dose, binge-like exposures are much more disruptive to neuroimmune cells than more moderate, yet longer-term

exposures. This dissertation reports that withdrawal periods may be more detrimental to neurons than the alcohol exposure itself, and that there is an important role of astrocytes in mediating this response, that is distinct from microglia.

# Table of Contents

# List of Figures

# List of Tables

# 1. Introduction

## 1.1 Fetal Alcohol Spectrum Disorder Overview

Drinking alcohol during pregnancy is one of the leading causes of teratogenic birth defects and can result in a child being born with fetal alcohol spectrum disorder (FASD). FASD is an umbrella term and includes a number of disorders with symptoms ranging from subtle alterations to debilitating mental retardation. The most severe form of FASD is fetal alcohol syndrome (FAS), characterized in part by physical malformations, such as subnormal weight and height, small eyes, a flattened philtrum, and thin upper lips. In addition, children with FAS suffer from severe cognitive dysfunction, behavioral abnormalities, and impaired social skills[1]. While the incidence of FAS in the U.S. is relatively low, at approximately 0.1% of live births[2], the CDC estimates that the cost of care of FAS individuals to the U.S. is $4 billion annually. Conversely, FASD is much more prevalent, occurring in an estimated 2-5% of births in the U.S[3], and includes less severe forms such as Alcohol-Related Neurodevelopmental Disorder (ARND), which encompasses subtler neurocognitive deficits. Symptoms of ARND are often similar to those in other developmental conditions, particularly attention-deficit/hyperactivity disorder, and as a result ARND is often misdiagnosed.

When a pregnant woman drinks, the developing fetus is exposed to levels of alcohol similar to the mother's blood alcohol concentration (BAC)[4]. While the teratogenic effects of alcohol have been known for decades, and despite public awareness campaigns including warnings issued by the Surgeon General, 1 in

13 women in the U.S. continue to drink while pregnant (CDC). In many instances, fetal alcohol exposure (FAE) occurs accidentally, since the majority of pregnancies are unplanned. According to the CDC, 51.5% of women of childbearing age report drinking alcohol within the last 30 days, and some of these women could be pregnant and be aware of it. In this case, women typically abstain from drinking after becoming aware that they are pregnant. As a result, alcohol consumption limited to the first trimester is the most common pattern of drinking during pregnancy[5]. The second most common pattern is drinking throughout the entire pregnancy, and the third is drinking solely during the third trimester[5] as many women incorrectly believe that the brain and other organs are fully formed at this point. Additionally, some of these women suffer from alcoholism and are unable to quit drinking. In this case, their fetuses can be exposed to very high levels of alcohol. A recent example includes a woman whose BAC was measured to be 266 mg/dL (3.3 times the legal limit) at the time that she gave birth[4].

Equally worrisome is the amount of misinformation circulating through magazines, social media, and even by doctors about whether or not drinking during pregnancy is safe. A number of factors can influence whether or not a fetus exposed to alcohol will develop FASD, such as the mother's diet, her lifestyle and stress levels, genetics, co-exposure to other drugs, and the mother's body weight and metabolism, which determine her resulting BAC after each drink and the rate at which it is cleared[6,7]. Therefore, it is possible that if two pregnant women consume the same amount of alcohol, one could have a child with FASD

and the other a child who is unaffected. As a result, there is considerable disagreement between even medical professionals about whether or not drinking low levels of alcohol during pregnancy is dangerous.

There exists little in the way of treatment options for FASD patients and almost all are based on behavioral, educational, or social interventions[2]. Some of the most promising potential therapeutics includes enriched environments, which has been shown to improve neurogenesis in animal models of FASD. Additionally, nutritional supplementation with choline has been shown to improve some behavioral outcomes[8]. At present, no medications have been approved specifically to treat FASD.

## 1.2 Animal Models of FASD

To investigate the mechanism for how FAE negatively affects brain development and to develop potential therapeutic options, animal models are typically utilized to study this disorder. Rodents, either mice or rats, are the most common type of animal used in FASD research, however rodents do not willingly ingest significant levels of alcohol. Instead there are several ways to administer alcohol to rodents. Some of the most prevalent are via oral gavage into the stomach, an intraperitoneal (i.p.) injection, mixing the alcohol into food or sweetened water, or vapor inhalation. Each method has accompanying advantages and disadvantages.

Mixing the alcohol into the animal's food or water source is typically considered lower stress than other methods, and alcohol is ingested similarly to

normal human consumption. This method has several limitations in that it is difficult to control dosage and often cannot be used to achieve high BACs, in part, because alcohol has an aversive taste to rodents. Conversely, alcohol administration via I.P. injection or gavage can be used to reach high BACs and offer more control over dosing. Unfortunately, these methods are very stressful for the animals and result in an unrealistically sharp rise in BACs that peak within 30 minutes[9]. Finally, vaporizing the alcohol so it can be inhaled has the advantage of reducing animal handling and, in the case of postnatal exposure, allows pups to remain with their mothers. BACs rise more gradually than in gavage and I.P., and high BACs can be achieved this way. However, in inhalation, the alcohol bypasses the first pass metabolism that would normally take place in the liver and stomach, in which the enzyme alcohol dehydrogenase breaks alcohol down into acetaldehyde[10]. Instead, the alcohol enters the bloodstream through the lungs, making it a less realistic administration model. Additionally, the intense smell of the vaporized alcohol likely introduces an alternative form of stress.

Another consideration when using rodent models is in the differences between the rodent and human gestational periods. Specifically, the rodent gestational period comprises only the stages of development that would take place in a human pregnancy during the first and second trimester. The third trimester-equivalent occurs during the first two postnatal weeks in a rat pup[11]. As a result, FASD research has typically focused on either alcohol exposure during the first and second trimesters (prenatal) or the third trimester (postnatal), but not

in all three. Studies in prenatal and postnatal models can often show different outcomes, highlighting the heavy influence the timing of exposure has on the effects seen in both rodents and humans. For example, early postnatal ages in rodents may be particularly sensitive to neurocognitive damage as the third trimester-equivalent is when many important processes of central nervous system (CNS) development occur, including synaptogenesis; a period known as the brain growth spurt[11].

Lastly, along with the factors listed above, dose and pattern of exposure are two of the most important elements of an exposure paradigm. There are many different patterns of normal human alcohol consumption. While binge exposures more realistically model types of drinking observed in college-age women and alcoholics, moderate and low dose exposures are more reflective of women with a higher socio-economic status[12]. As stated above, there is no safe level of alcohol during pregnancy and studies utilizing low or moderate alcohol doses have found a number of negative consequences, which will be reviewed in greater depth in the following sections. However, binge alcohol exposure is widely accepted as the most harmful type of fetal exposure[13-15]. The NIAAA defines binge exposure as a pattern of drinking that induces BACs above the legal limit of intoxication within 2 hours (0.08 g/dL, around 4 drinks in an average woman). Importantly, to mimic this type of exposure in rodents it may be necessary to use even higher BACs, as rodents have been shown to be more resistant to alcohol than humans[16,17], in part due to their elevated metabolic rate. It is critical that studies continue to investigate the array of effects generated by

different doses and patterns of drinking, as ideally future FASD treatments will consider these factors on an individual patient basis in order to prescribe the most efficacious treatment option.

To sum, there are many important aspects to consider when designing an FASD paradigm in an animal model, emphasizing the complexity of this disorder. While it is essential that the individual effects of each factor be assessed, differences in exposure paradigms can lead to contrasting results and make it difficult to compare conclusions reached across studies. In the next sections, findings from different FASD models will be discussed with regard to the hippocampus and cerebellum; two brain regions that have been shown to be particularly susceptible to insult by FAE.

## 1.3 The Hippocampus: Function, Anatomy and Development

The hippocampal formation is part of the limbic system and is responsible for many important CNS functions, most notably learning and memory. The structure is divided into sub regions, consisting of the dentate gyrus (DG) and the Cornu Ammonis (CA) areas, which are considered the hippocampus proper and of which the CA1 and the CA3 are the best characterized[18]. The primary cell type of the DG and the CA sub regions are the granule cells and pyramidal neurons, respectively, which are both excitatory and organized into distinct layers. There is also a highly diverse population of GABAergic interneurons throughout the hippocampal formation, including the axo-axonic cells, basket cells, and oriens-lacunosum moleculare cells[18].

The major input to the hippocampus comes from layer 2 of the entorhinal cortex (EC), which projects primarily into the DG, along the perforant path. The granule cells then relay this information through the mossy fiber pathway, which synapses onto the CA3 pyramidal neurons[18]. From the CA3, information flows to the CA1 pyramidal neurons along the Schaffer collateral pathway. This DG-CA3-CA1 loop is known as the tri-synaptic circuit (Figure 1.1). Importantly, the CA3 pyramidal cells also receive a high amount of input/output from neighboring cells, as this region is highly interconnected by the associational-commissural (AC) pathway[19]. CA1 neurons are the major output for the hippocampus and project back out to the EC, which is then relayed to other parts of the cortex. The hippocampus also has additional output pathways to the fornix and the amygdala[20].

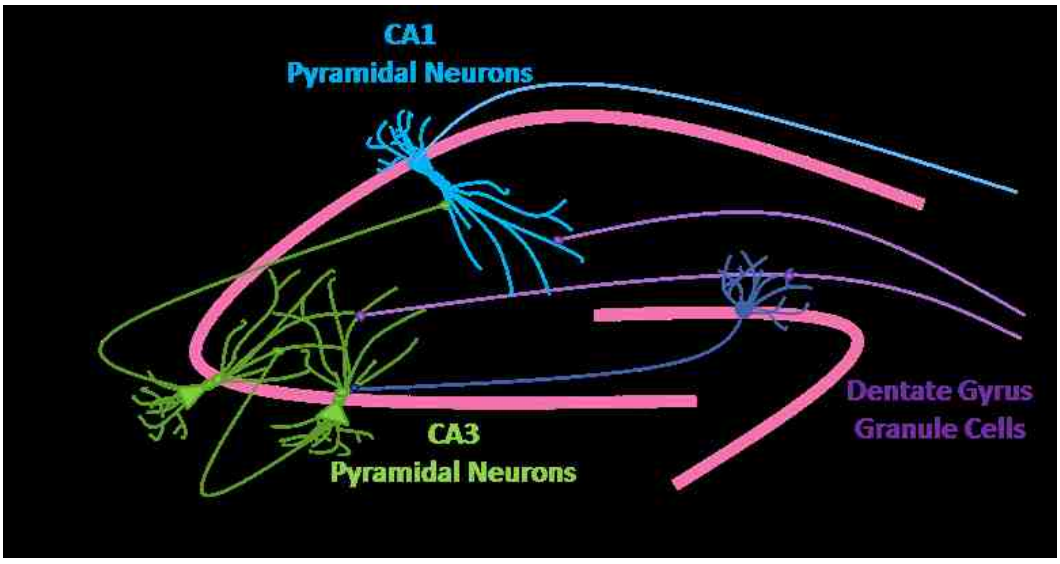


**Figure 1.1: Structural Organization of the Hippocampus**

Neurogenesis occurs in waves in the hippocampus, beginning as early as embryonic day (E)10 and continuing into the postnatal period[19] (and adulthood in the DG). In the CA3, neuronal birth peaks at E14 while in the CA1 it is most pronounced slightly later at E15. In the DG neurogenesis occurs over a longer timescale, peaking at E16 and continuing until the first postnatal week in the DG. Within a couple of days after their birth, neurons begin migrating to their respective cell layers, guided by glial cells. Synaptogenesis occurs primarily during the 3rd trimester-equivalent of development along with synaptic pruning, although refinement of synapses can continue into adolescence. Importantly, these stages of development can occur on slightly different timescales in the individual sub regions[19]. However, recent studies have discovered massive increases in cell number from postnatal day (P)3-7, followed by a period of cell loss, throughout many regions of the brain including the hippocampus[21]. These findings are at odds with others in the literature, and suggest that neurogenesis and gliogenesis transpire heavily in the rodent postnatal period and that the majority of cells in the brain are generated during this time. The important functions of the hippocampus, combined with its noteworthy configuration and unique characteristics have made this one of the most studied regions of the brain, including in the pathophysiology of FASDs.

## 1.4 The Hippocampus and FASD

It is well established that fetal exposure to alcohol can result in deficits in hippocampal-dependent behavior. There exist several behavioral paradigms to

assess hippocampal function, including the Morris Water Maze (MWM), variations of fear conditioning paradigms, and the radial arm maze, each of which are tests of the rodent's learning and memory abilities. The MWM, a measure of spatial memory, is one of the best characterized with respect to developmental alcohol exposure and deficits have been observed in both pre- and postnatal models[22-25], as well as in human FAS subjects when tested on a virtual version of this task[26]. Similarly, FAE has been shown to impair performance on variations of the fear conditioning task and/or the radial arm maze[27-30].

The hippocampus is a site of considerable neuronal plasticity and the only brain region in humans that undergoes neurogenesis in adulthood. FAE can interfere with these processes, which have been linked to the deficits in learning and memory described above[31]. Developmental alcohol exposure has been shown to affect synaptic transmission and plasticity, although the mechanism likely differs between prenatal and postnatal models. In prenatal exposure models, NMDA receptors seem to be particularly sensitive to insult. One study found reduced neuronal plasticity and changes in NMDA receptor subunit composition in the DG[32]. A comparable alcohol exposure model impaired NMDAR activation of ERK signaling, also in the DG[33]. Finally, prenatal alcohol decreased NMDA receptor binding sites, in multiple sub regions of the hippocampus, into adolescence[34].

Similarly, postnatal alcohol exposure has been shown to inhibit synaptic plasticity in the CA1[35] and CA3 sub regions[36]. However, an acute application of alcohol to neonatal slices suggested that, at this age, alcohol interferes with

hippocampal transmission through inhibition of presynaptic voltage-gated $Ca^{2+}$ channels and affects both AMPA and NMDA receptors[37]. In this study, the authors suggest that differences in the receptor subunits expressed during development, and the phosphorylation states, could explain the increased sensitivity to alcohol at this time.

FAE has also been shown to interfere with neurogenesis[31]. The generation of adult-born neurons occurs specifically within the DG and is thought to be the basis of new memory formation, as increases in neurogenesis correlate with learning, as well as with improved performance on some learning and memory tasks. However, exactly how neurogenesis contributes to memory formation is not fully understood, and the concept remains controversial[38]. A model of postnatal binge exposure reduced the survival of adult born neurons and prevented incorporation into the neuronal network[39]. Similarly, exposure to moderate levels of alcohol during the prenatal period has been shown to blunt the normal neurogenic response to an enriched environment[40] and alter the expression of genes involved in neurogenesis[41].

Finally, one of the most detrimental effects of postnatal alcohol exposure is neuronal loss, which is seen in the hippocampus after high dose, binge exposures encompassing periods late in the first postnatal week[13,42,43]. Importantly, when exposure is limited to early in the postnatal week (~P3-4), no cell loss is observed[44,45], suggesting that there is a specific window of development during which the hippocampus is susceptible to neuronal loss.

## 1.5 The Cerebellum: Function, Anatomy and Development

The cerebellum is best characterized for its motor functions[46,47], in which it is critical for maintaining balance, motor learning, and coordinating voluntary movements. Additionally, several human studies have suggested the cerebellum has a role in a number of other neurocognitive processes, such as emotion [48,49] memory retrieval[50], and attention[51]. It has also been theorized to play a role in psychiatric disorders[52].

Structurally, the cerebellum consists of two hemispheres, divided by a mid-line region known as the vermis[52]. The cerebellum is further organized into distinct lobules that increase in number toward the midline and in the adult vermis there are 10 lobules. Importantly, in the developing brain the lobules mature at different rates[53].

The principal neuron of the cerebellum is the Purkinje cell (PC)[54]. Their axons provide the sole output from the cerebellar cortex and project to the deep cerebellar nuclei. From the deep cerebellar nuclei information is relayed to target regions (e.g., cortical and spinal motor neurons) via brainstem and thalamic neurons. PCs are GABAergic neurons, though there exist a number of other inhibitory interneurons in the cerebellum, such as the Golgi, stellate and basket cells. PCs receive inhibitory input from other PCs through the recurrent collaterals, and from the surrounding interneurons. The major excitatory cell type in the cerebellar cortex is the granule cell, which projects to PCs via the parallel fibers. Excitatory input also comes from inferior olive neurons (climbing fibers),

which synapse onto the PCs, and the precerebellar nuclei (mossy fiber pathway) which provides input to granule cells. While granule cells are some of the smallest neurons in the brain, PCs are some of the largest[54]. The cellular structure of the cerebellum is well organized into specific layers. In the adult, the outermost layer is the molecular layer, which contains the parallel fibers and stellate cells. The next is the PC layer (PCL), which contains the PC soma. The innermost layer is the granule cell layer which houses the granule cells and Golgi cells[55].

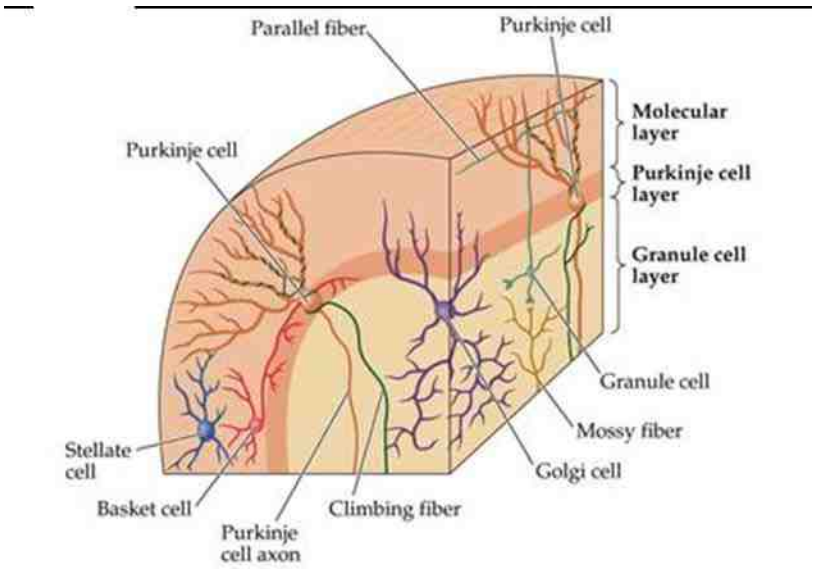


**Figure 1.2: Structural Organization of the Cerebellum.**

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During development, PCs of the cerebellum are generated around E10-13, while granule cell precursors emerge from E12-17, before migrating along glial cells to their respective layers. Initially, granule cells make their way to the external granule layer (EGL) which is a temporary cell layer consisting of proliferating neurons. During the postnatal period, cells in this layer migrate

through the PCL and into the internal granule layer (IGL), which is equivalent to the granule cell layer in the mature brain. In the late postnatal period, and for a few weeks following, there is a period of programmed cell death in which the total number of cerebellar neurons is diminished. During the postnatal period is also when the majority of interneurons are generated[56] and large increases in the number of neuronal and non-neuronal cells continues to occur during the first postnatal months[21]. During the 3rd trimester-equivalent the neuronal network is established in the cerebellum, including refinement of climbing fiber synapses[57] and formation of the mossy fiber pathway[58].

## 1.6 The Cerebellum and FASD

A number of behavioral tests can be used to assess cerebellar function. Some of the most common include the runway assay, which measures alterations in gait, and the rotarod, which is a measure of balance and motor coordination. Another common test is called eyeblink conditioning, in which the subject receives a conditioned stimulus, such as an auditory tone, before a puff of air is released into their eye. Eventually the subject will adopt a conditioned response and blink in response to the conditioned stimuli, before the puff occurs, the development of which has been shown to be cerebellar-dependent[59]. Prenatal alcohol exposure has been shown to result in deficits in all three tests[60]. Similarly, exposure to alcohol during the 3rd trimester-equivalent has been shown to impair performance in all of the tests listed above, and some additional cerebellar-dependent tasks[61-64].

There are several potential mechanisms by which FAE may be contributing to these functional deficits. First, FAE has been shown to interfere with several components of cerebellar maturation. When rat pups were exposed to alcohol through gestation and weaning, the migration of granule cells was delayed and they exhibited an enlarged cellular morphology. Additionally, in that study Bergmann glia exhibited structural alterations indicative of immaturity, which may explain the obstructed granule cell migration as the glia are important for providing guidance during this process[65]. Similarly, a single injection of alcohol in the postnatal period (P10) was enough to inhibit granule cell migration, potentially by interfering with signaling pathways[66]. Furthermore, postnatal alcohol exposure decreased synaptogenesis in the cerebellum[67].

Second, FAE may be affecting synaptic plasticity and transmission. In a prenatal model, Servais and colleagues found that alcohol decreased the voltage gated calcium currents in PCs and led to increased firing. Furthermore, parallel fiber synaptic plasticity, was switched to from long-term depression (LTD) to long-term potentiation (LTP). They were able to link these changes to deficits in cerebellar-dependent behavior[60].

A third mechanism for FAE-induced deficits in cerebellar function is cell loss. As in the hippocampus, neurodegeneration can occur after exposure to alcohol during the postnatal period[68]. However, while hippocampal cell loss is seen only after alcohol exposure late in the first postnatal week (P7-9), the window of development in which the cerebellum is most vulnerable is comparatively earlier (~P4)[69-73] although exposure throughout all three trimesters

worsens this effect[74]. Additionally, the lobules themselves exhibit diverse susceptibilities, with the earlier maturing lobules (I-III and IX-X) showing increased neuronal loss after postnatal alcohol exposure[53]. Interestingly, 3rd trimester-equivalent exposure can also cause neuronal loss in the deep cerebellar nuclei, which receives output directly from PCs[62].

While the mechanism is not fully understood for how FAE damages the cerebellum and hippocampus, particularly with respect to neuronal loss, recently activation of the neuroimmune system has been suggested to play a role. The following sections will address this possibility.

## 1.7 Neuroimmune Sytem Overview

The nervous system is isolated from the rest of the body by the blood brain barrier (BBB), an additional lining around the blood vessels that prevents passage of hydrophilic or large molecules into the parenchyma. The innermost layer of the BBB consists of closely packed endothelial cells interfacing at tight junctions. The next layer is an extracellular matrix known as the basal lamina. Outermost are the feet of astrocytes, which provide further structural support. Pericytes, microglia, and perivascular macrophages can also contribute to BBB integrity. The BBB is a selectively permeable barrier; small gaseous molecules and lipophilic substances (such as ethanol) can diffuse across. Additionally, there are selective transport mechanisms to allow passage of certain nutrients. However, the entry of hydrophilic and/or large molecules is restricted, as well as the migration of cells of the peripheral immune system[75]. As a result, the brain

has its own highly specialized immune system, known as the neuroimmune system, which protects the CNS.

Although the brain has traditionally been thought of as immunoprivileged, it is important to note that recent studies are finding more and more that there is an intense interaction and interconnection between cells of the nervous system and the peripheral immune system. For example, neurons can regulate peripheral innate immune responses through secretion of soluble factors or even direct interaction with peripheral immune cells[76]. Furthermore, very recent research has identified a novel lymphatic system that allows passage of peripheral immune cells into the CNS[77], which could potentially implicate these cells in many CNS processes.

## 1.8 Cells of the Neuroimmune system

The best-characterized cells with respect to the neuroimmune system are microglia, which are the resident macrophage of the CNS. Microglia were first described in 1932 by Pio del Rio-Hortega. Under normal conditions, he noted that microglia have small cell bodies and long dendritic processes, a morphology that has been termed “resting” as the cell bodies do not move around freely[78]. However, strong evidence has proven that microglia in a “resting” morphology are anything but that, with their processes constantly surveying the environment and making contact with nearby cells[79]. A number of cues, including changes in their surrounding environment, can cause the microglia to rapidly change their phenotype, which is often accompanied by a morphological change[80]. This

morphological change occurs along a spectrum, as the microglia distend their processes and their cell body swells. At the far-end of this spectrum the microglia exist in what is called an “amoeboid” morphology or, until recently an “activated” morphology, as microglia in this state have been shown to release molecules that can be neurotoxic and exhibit phagocytic activity[81]. However, there is now mounting evidence to show that changes in microglia morphology, while a good marker, are not always indicative of phenotype or activity[80].

A number of new roles for microglia have been discovered, changing the viewpoint on the importance of these cells in the CNS, and will be discussed in more depth in later sections. Briefly, rather than being strictly neuroinflammatory cells, microglia have recently been shown to have important functions in homeostasis and during development. Additionally, one of the most controversial and highly debated subjects with respect to microglia pertains to a potential role in mediating toxicity in cases of neurodegeneration.

Another important cell type of the neuroimmune system is the astrocyte. Astrocytes are star-shaped glial cells with many important functions in the CNS, including providing structural support to the BBB as previously mentioned. In response to CNS insult, astrocytes assume a structural change that includes thickening of their processes. These “reactive” astrocytes mediate astrogliosis, or “reactive gliosis” (proliferation of astrocytes), and up-regulate intermediate filament proteins, in particular glial fibrillary acidic protein (GFAP). This elevated GFAP production is highly associated with increased gene expression and an “activated” phenotype[82]. Astrocytes are thought to communicate largely through

calcium waves, which can be transmitted to other cells across gap junctions or by the release of gliotransmitters, such as ATP, and represent a potential for rapid communication with distant astrocytes[83].

While microglia and astrocytes are the primary cells of the neuroimmune system, there exist a host of other cells that can play a role in mediating this response. A great deal of communication occurs between neurons and glia cells, and neurons can guide many of their responses (as discussed in the next section). Additionally, some peripheral immune cells (mostly T cells) exist within the healthy brain at low levels and contribute to surveillance[84]. Under inflammatory conditions, peripheral cells infiltrate the brain and assist in defending the CNS.

## 1.9 Neuroimmune Signaling

### 1.9.1 Neuronal-Glial Communication

There is a great deal of neuroimmune signaling that occurs between the cells of the neuroimmune system under both resting and pathological conditions, and the activation of each cell is tightly regulated by this network. Neurons interact closely with glial cells at multipartite synapses and have been shown to regulate neuroinflammation a number of different ways. One of the well-studied mechanisms is through the constitutive expression of the chemokine, fractalkine, whose receptor is expressed primarily on microglia. Fractalkine has an important role in chemotaxis. It was initially thought of as recruiting and activating microglia, although more recent studies have shown that fractalkine likely restricts

microglial activation in the healthy brain[85,86]. Similarly, glial cells express receptors for a multitude of other substances released from neuronal terminals such as substance P, neurotransmitters, and ATP[78,87,88]. Glutamate, GABA, and ATP were all shown to increase superoxide production in cultured microglia, although phenotype varied based on the stimulant [89]. Histamine and substance P were also found to increase microglial production of pro inflammatory cytokines and ROS generation[90]. Interestingly, it has been suggested that specific subsets of microglial populations have different sensitivities to these molecules[91]. Finally, microglia can respond to episodes of increased neuronal firing, through an ATP-dependent mechanism[92], suggesting that neuroimmune responses could be activity-dependent.

Neurons can also heavily influence surrounding astrocytes. In one experiment, stimulation of the Schaffer collateral pathway in the hippocampus induced intracellular calcium increases in astrocytes[93]. The authors further demonstrated that this effect was likely mediated by glutamate, released from neuronal terminals, binding to metabotropic glutamate receptors on nearby astrocytes. Indeed, increased calcium signaling in astrocytes in response to neuronal activity or neurotransmitters has been widely observed[94-96], and blocking astrocytic calcium signaling has been shown to interfere with types of neuronal transmission and plasticity[97-99]. These findings suggest that neuron-astrocyte intercommunication is essential for normal brain function, yet the implications of astrocytic calcium waves are still controversial[100]. Both microglia

and astrocytes can also influence neurons, and each other, through secreted factors such as cytokines, which will be discussed more in the next section.

### 1.9.2 Cytokine Secretion

Cytokines are small, protein signaling molecules that can have autocrine, paracrine, or some times endocrine effects. In the CNS, they are produced by neurons and glial cells[101], however they can also be sourced from peripheral immune cells and cross the BBB. Most cytokines are produced in their proform and must be cleaved into an activated state before binding to their receptor[102]. While cytokines were originally characterized as being immune modulators, they have since been shown to play important roles in many other biological processes and are commonly considered neuroimmune signaling molecules. Receptors for a multitude of cytokines are constitutively expressed on both glial cells and neurons[102], although receptor expression is also inducible under pathological conditions[103,104]. Cytokines are typically classified as being either pro- or anti-inflammatory depending on their effects, however many cytokines can elicit dual functions.

Two of the best-characterized pro-inflammatory cytokines in the CNS are tumor necrosis factor α (TNFα) and interleukin 1β (IL1β). TNFα is a member of the TNF superfamily of ligands and can be produced by glial cells and certain neurons. It has two receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). In the brain, TNFR1 is expressed in most cells including astrocytes, microglia, neurons, and oligodendrocytes[105,106], whereas TNFR2 is primarily

expressed in microglia and other immune cells[107,108]. Ligand binding to the two receptors has been shown to have differential outcomes. Activation of TNFR1 is generally cytotoxic, while TNFR2 has been shown to be neuroprotective in some cases[109,110].

TNFR1 works as a signaling complex with TNF receptor associated death domain and other adaptor proteins, such as receptor interacting protein and TNF receptor associated factor 2[107]. Binding to TNFR1 can lead to activation of a number of pathways, including ERK, JNK, and p38 MAP kinase, and activation of transcription factors such as NF-kB. Additionally, in some cases TNFR1 is internalized after TNF binding, at which point it can trigger apoptosis by association of Fas-associated death domain, activation of caspases, and formation of the death-inducing signaling complex. As a result, TNF signaling through TNFR1 can have a variety of outcomes, including proliferation and/or activation that likely depend on environmental factors and on in which cell type the receptor is expressed[107].

TNFR2 signaling shares some similarities to TNFR1; signaling through this receptor involves TRAF2 and can lead to increased transcription by NF-kB. However, TNFR2 does not have a death domain. Additionally, TNFR2 is preferentially activated by membrane-integrated TNF, rather than the soluble form, whereas TNFR1 is activated by both forms. Interestingly, there is some evidence to suggest that the TNFR1 and TNFR2 receptors have a degree of crosstalk that influences signaling outcomes, further diversifying the types of responses generated by TNF binding[111].

Conversely, IL-1β, one of the most important pro-inflammatory cytokines, primarily binds to one receptor, the IL1 receptor type I (IL-1R1), although there is a type 2 II1 receptor that is thought to act as a decoy. IL-1R1 has differential expression levels throughout the brain, with some of its highest densities being in the granular layer of the DG and granule cell layer of the cerebellum. It is primarily expressed in non-neuronal cells, but there is evidence to suggest that it is expressed in neurons as well[112]. Activation of IL-1R1 in neurons has been implicated in both neuronal survival and death[113,114]. IL-1R1 is dependent on myeloid differentiation primary-response protein 88 (MYD88) and acts through recruitment of interleukin-1 receptor-associated kinases (IRAKs). In some cases, binding to IL-1R1 leads to NF-kB activation, and in others it acts through the p38 or MAPK pathways. IL-1β elicits different responses in different cell types, likely because of different IL-1R1 signaling pathways becoming activated. One cell culture study showed that the IL-1R1 signaling pathway that became activated after ligand binding was different in neurons than in glial cells[115].

There are also anti-inflammatory cytokines which can mediate glial cell activation and inhibit the release of pro-inflammatory cytokines[116]. Two common anti-inflammatory cytokines are transforming growth factor beta (TGFβ) and interleukin 10 (IL-10). TGFβ is produced by many cell types, including glial cells and neurons[117,118]. There exist three isoforms of TGFβ (1, 2, 3) and two of its receptors (type I and type II). TGFβ receptor signaling is mediated either by SMAD proteins, which can act as transcription factors and regulate gene expression[119], or by activating anti-apoptotic NF-kB pathways[120]. In neurons,

TGFβ is important for cell survival[121] and was recently found to act as a "switch" critical for neurogenesis[122].

IL-10 is a powerful anti-inflammatory cytokine that is being investigated as a potential therapeutic for many inflammatory diseases[123-125]. Astrocytes and microglia both express IL-10 receptor and produce IL-10[126]. IL-10 receptor signaling in immune cells has been shown to be mediated by the JAK-STAT pathway[127]. The role for IL-10 and its receptor is more controversial in neurons, though IL-10 has been shown to have important roles in neuronal survival. In a cell culture model, the IL-10 receptor was found to be expressed on spinal neurons and directly contributed to cell survival[128]. However, a similar study showed IL-10 signaling was mediated in neurons by PI3K and STAT-3 pathways[129]. To my knowledge, IL-10 receptor expression has not yet been confirmed in neurons *in-vivo*.

## 1.10 Neuroimmune Functions in Inflammation

A number of different stimuli can generate neuroinflammation including invasion of pathogens or harmful molecules, excitotoxicity, debris from dead neurons, and brain trauma. The type of response generated depends heavily on the insult, factors in the environment, and interactions between the cells involved. Typically, neuroinflammation is characterized by increased cytokine production, production of reactive oxygen species (ROS) and other molecules, and morphological/phenotypic changes in glial cells.

Microglial activation is being discovered to be a complex and controversial subject. We understand that microglial activation is a “rolling” or graded process[130] that can differ depending on the situation or insult. Microglia can become only partially activated or they can become fully amoeboid, a state that is classified by the microglial cell body becoming mobile and by phagocytic activity. Phagocytosis by microglia is critical for helping clean up debris and dead neurons. However, in some cases it has been shown that microglia can also kill viable neurons by phagocytosing them[131].

Recently, the discovery of M1 and M2 activation has introduced another element into microglial activation. First described in macrophages, recent studies have identified that microglia also express different phenotypes of activation[132]. The M1 phenotype designates an activated state in microglia that is heavily pro-inflammatory and, when prolonged, neurotoxic. Conversely, M2 activation is considered anti-inflammatory and neuroprotective. Even beyond that, M2 activation is being broken down into further subsets including M2a (anti-inflammatory, alternative activation) and M2b/c (deactivation/wound healing and repair), which are classified by distinct markers and cytokines[133,134]. Due to the cytotoxic effects of prolonged M1 neuroinflammation, blocking neuroimmune activation has long been considered as a potential therapeutic intervention against a number of diseases. However, the discovery of distinct functions for these two types of activation, with M2 having many beneficial functions in regeneration[135,136], has led to some focus on attempting to alter the polarization of microglia (M1->M2), rather than blunting activation altogether.

Like microglia, astrocytes have their own unique roles in neuroinflammation. In the case of severe CNS injury, such as lesions, astrocytes react by walling off the affected area and forming a glial scar. This barrier consists primarily of reactive astrocytes and is critical for stopping the spread of inflammation and in neural repair[137]. However, astrocytic scars can also prevent axon regeneration, and recent attention has focused on ways to circumvent this obstruction[138].

While the orchestration of neuroimmune responses has typically been attributed to microglia, astrocytes have been shown to regulate microglial activation and to have their own independent functions[139,140]. This suggests that astrocytes may have a prominent role in directing neuroinflammation. Additionally, like microglia, astrocytes can be either pro- or anti-inflammatory, depending on the situation and type of insult[137].

Finally, in certain inflammatory conditions, peripheral immune cells are recruited across the BBB to aid in the neuroinflammatory response[141,142]. Chemokines, a type of chemotactic cytokine involved in immune cell recruitment, are particularly important to this process. Chemokines work by binding to G protein-coupled receptors and inducing chemotaxis. Furthermore, chemokine secretion can up-regulate integrins and adhesion molecules, such as ICAM and VCAM, on the endothelial cells of the BBB. These integrins bind to the immune cells and arrest their movement, allowing them to squeeze between the tight junctions of the endothelial cells and into the perivascular space. Beyond that, these cells likely traverse the barrier formed by the astrocyte end feet (glia

limitans) in a manner dependent on matrix metalloproteases[141,142]. Inflammation can also lead to BBB breakdown and less closely associated endothelial cells, which increases permeability and allows peripheral cells to infiltrate the CNS more easily, although the mechanism is not completely understood[143]. After the inflammation resolves it is thought that many of the peripheral cells die[144], although there is some evidence that macrophages in the brain will differentiate into resident microglia[145].

## 1.11 Neuroimmune Functions in Homeostasis

Under normal conditions, microglia and astrocytes have many important roles in maintaining homeostasis. Astrocytes are well known for their function in providing neuronal support, particularly in removing excess neurotransmitter from the synaptic cleft to maintain neuronal excitability while preventing excitotoxicity. They are also thought to provide energy by taking up glucose, producing lactate, and shuttling it to neurons. They have many neuroprotective functions, including providing neurons with precursors for the powerful antioxidant and ROS scavenger, glutathione[146]. Under neuroinflammatory conditions, when astrocytes become reactive, they reduce some of these support processes[147]. Finally, astrocytes are important players in mediating synaptic plasticity[148].

Microglia also provide support for neurons[149] and regulate synaptic density and function[150]. In addition, microglia stimulate learning-related synapse formation by secretion of brain-derived neurotrophic factor (BDNF) [151] and may

also promote synapse formation through the release of IL-10[152]. Finally, there is evidence that microglia control synaptic strength[153,154].

Similarly, cytokines have been shown to regulate synaptic plasticity and transmission. In one study, deleting the TNFR1 in neurons reduced the number of synaptic AMPA receptors and inhibited transmission[108]. Similarly, increasing the levels of pro-inflammatory cytokines has been shown to affect synaptic transmission. Increasing IL-6 enhanced synaptic transmission in the hippocampus, but reduced some forms of plasticity in an age-dependent manner[155]. Similarly, application of pro-inflammatory chemokine CCL2 increased synaptic currents and neuronal firing in the hippocampus[156], while increased IL-1β blocks BDNF-dependent long-term plasticity in the same region[157]. Interestingly, IL-1R expression is densest at synapses and IL-1R interacts with NMDA receptors, indicating a potential role for IL-1β in modulating NMDA receptor function[158].

## 1.12 Neuroimmune Functions during CNS Development

Many important CNS developmental processes occur during the third trimester-equivalent to human pregnancy. This is the period in which synaptogenesis, network formation, and potentially even neurogenesis, transpire. Additionally, the majority of gliogenesis and glial cell maturation takes place, concurrent with these processes.

Microglial precursors originate from the bone marrow, however the process of entry into the brain as monocytes and eventual differentiation is

controversial. Fate mapping studies suggest that CNS infiltration of the microglial progenitors occurs in waves at different developmental time points, with the first wave of hematopoiesis into the CNS occurring between E7-7.5 from the extra-embryonic yolk sac[159]. Specifically, microglial progenitors are detected in the cerebellum as early as E11[160]. Interestingly, one study showed that when microglia were depleted form the adult brain, the microglial population was replenished within 1 week, likely by microglial progenitor cells residing in the CNS[161].

During the postnatal period, microglial proliferation peaks[162] and microglia begin to mature. However, during the early postnatal period microglia are in an immature state that resembles the amoeboid morphology associated with activation in adulthood. It is during the first two weeks of postnatal life in rodents that microglia adopt the ramified morphology seen under normal conditions in adulthood[163]. Interestingly, microglial maturation has been shown to be brain region- and sex-dependent. Males have more microglia during the early postnatal period than females, while females have more amoeboid microglia in adolescence and as young adults[164]. Furthermore, there is evidence to suggest that microglia interact with the nervous and endocrine systems during development to “masculinize” the brain, contributing to sex differences in behavior[165]. Additionally, microglial maturation has been shown to occur on a slightly delayed timescale in the cerebellum than hippocampus[166].

Like most cells of the CNS, astrocytes differentiate from neural stem cells and are thought to arise initially from progenitors in the subventricular zone[167].

During the first few weeks of postnatal life, there is a period of massive gliogenesis, in which the population of astrocytes increases by 6-8 fold. Recent studies have shown that the majority of cortical astrocytes likely arise from this local gliogenesis, rather than infiltrating cells[168]. However, astrocyte gliogenesis occurs in other brain regions from E17 or earlier. Similar to microglia, astrocytes also mature during the postnatal period, adopting their long, fine processes and beginning to express genes important for proper functioning, including in neuronal support[169].

Despite the fact that these cells are undergoing their own maturation during this time, both microglia and astrocytes have important functions in CNS development. Recently, microglia were shown to be critical for synaptic pruning during development, actively engulfing and removing unnecessary synapses thereby fine-tuning neuronal networks[170]. Additionally, astrocyte regulation of glutamatergic signaling, including removal of glutamate from synapses, is critical for proper neurite outgrowth, neuronal migration, and synaptogenesis[169].

Cytokine secretion also plays an important role in development. Cytokines have been shown to exist at different levels in the postnatal brain than in adults, suggesting an important role in development, and occurring in a sex-specific manner[132]. Specifically, cytokines and chemokines have important roles in neuronal differentiation, successful migration of progenitors, and synaptic strength and refinement[171].

Given these important roles, it is not surprising that dysfunction of the neuroimmune system has been implicated in a number of neurodevelopmental disorders. Increased astrogliosis has been observed in the brains of autism patients[172]. Additionally, neurons had stunted dendritic outgrowth when co-cultured with astrocytes derived from a rodent model of Rett syndrome, suggesting that improper functioning of these astrocytes contributes to this disorder[169]. Similarly, compelling research implicates early-life infection and subsequent microglial activation in the development of schizophrenia[173]. Furthermore, anti-psychotic drugs were shown to decrease neuroinflammation[174], suggesting that reduction of neuroimmune responses may alleviate some of these symptoms.

Finally, seminal work by Dr. Bilbo's lab has shown that developmental insults that activate the neuroimmune system (such as infections) can lead to subtle yet long-term changes in microglia that alters their programming into adulthood. These "primed" microglia appear normal, but over-react to secondary insults in adulthood, resulting in exaggerated cytokine production and secretion, and contributing to behavioral deficits[163].

## 1.13 The Neuroimmune System and Alcohol

A number of studies have provided evidence for a central role of the neuroimmune system in mediating alcohol's effects on the brain, particularly in adults. Post-mortem analysis of adult alcoholics revealed brain-region specific increases in the microglial marker, ionized calcium-binding adaptor molecule 1

(IBA-1), indicating that microglia are activated and/or increased in number. Additionally, expression of the pro-inflammatory chemokine monocyte chemoattractant protein-1 (MCP-1, also known as CCL2) was elevated[175]. These findings were confirmed in a rodent model, in which long-term alcohol use elevated MCP-1 expression and microglial activation, and the effects were correlated with impairment of spatial memory and increased BBB permeability[176]. Acute alcohol was also shown to change the expression of hundreds of proteins, some involved in the neuroimmune system, when applied to cultured microglia[177]. Similarly, when alcohol was applied to cultured astrocytes, increased NF-kB nuclear translocation was observed[178] and there was increased production of pro-inflammatory iNOS, COX-2 and IL1β[179]. Finally, binge-alcohol exposure has been shown to cause neuronal death in parallel with large-scale activation of microglia and astrocytes[180], suggesting there may be a relationship between glial cells and neuronal loss. However, Marshall and colleagues utilized a model of heavy binge exposure, that was previously shown to result in neuronal loss, and called into question some of these previous findings. In this study they found increased microglial activation, but no evidence of pro-inflammatory cytokine production or BBB breakdown. Instead they found small increases in anti-inflammatory IL10 and TGFβ, suggesting that there may be a contribution of M2-type activation in alcohol-induced neuroimmune responses[181].

In adults, alcohol exposure has been suggested to modify neuroimmune cell programming, similar to priming in neonates, altering the way they react to additional insults. Pre-exposure to alcohol resulted in exaggerated production of

pro-inflammatory cytokines and microglial activation after injections of lipopolysaccharide (LPS), although levels of IL10 were decreased by alcohol[182]. Conversely, pretreatment of cultured microglia with alcohol blunted LPS-induced production of nitric oxide, a marker of M1 activation[183].

Recent studies are beginning to shed light on the mechanism by which alcohol activates glial cells. Deletion of Toll-like receptor 4 (TLR4) blunted alcohol-induced increases in pro-inflammatory cytokines, iNOS, and both microglia and astrocyte activation in an animal model[184]. Importantly, deletion of TLR4 prevented alcohol induced caspase-3 expression, providing further support to the theory that neuroinflammation causes neuronal loss after alcohol[184]. Furthermore, TLR4 knockout mice were resistant to alcohol-induced impairments in behavior[185]. Alcohol activation of microglia may also be dependent on other toll-like receptors including TLR2[186] and/or TLR3[187]. There is also evidence to suggest that alcohol increases mir155 and that this may mediate part of these actions, as deletion of mir155 blunted alcohol-induced production of TNFα and MCP1[188].

The work of Zou and Crews showed that alcohol likely causes a neuroimmune response by first acting on neurons and working through a nuclear protein that has cytokine-like activity, called high-mobility group box 1 (HMGB1). Alcohol was shown to increase HMGB1 secretion from neurons and other cells, which binds to TLR4 on microglia and activates them, leading to increased cytokine production and furthering neuroinflammation[189].

Some work has also been done in adolescents, showing that alcohol partially activates microglia, although in this exposure it did not lead to increases in measured pro-inflammatory cytokines[190]. Conversely, a study in which animals were exposed to alcohol intermittently from adolescence into adulthood found that alcohol decreased neuronal and glial cell numbers and found increases in inflammatory markers[191]. Finally, one study found that alcohol increased GFAP staining in both adults and adolescents, but that pro-inflammatory cytokine production was increased only in adults and these effects were brain-region dependent[192].

Interestingly, it has even been suggested that neuroinflammation may play a role in the actual addiction process. One study showed that injections of LPS cause increased voluntary alcohol consumption in rodents[193]. Similarly, deletion of certain neuroimmune genes reduced voluntary alcohol consumption in a gender-specific manner[194].

## 1.14 Neuroimmune System Implications in FASD

Compared to research in adults and adolescents, the possible role of the neuroimmune system in FASD has been much less studied, however evidence in this emergent field has already strongly implicated the neuroimmune system in this disorder.

### 1.14.1 Studies in 1st and/or 2nd Trimester Models

Early, binge-like alcohol exposure increases the number of microglia in the white matter of the brain[195]. Likewise, exposure to alcohol throughout the first and second trimesters increased the number of astrocytes and microglia in the cerebellum in a dose –dependent manner[196]. A similar study showed an increase in microglial number and that these cells exhibited more immature morphology compared to controls[197]. Conversely, several studies have shown a decrease in astrocyte number after prenatal alcohol exposure[198,199], consistent with cell culture studies which show that treatment with alcohol restricts astrocyte proliferation[200-202]. Cell culture studies have implicated astrocytes, from pups prenatally exposed to alcohol, in several negative outcomes for neuronal plasticity, including reduced dendritic outgrowth[203]. Multiple studies have shown that prenatal alcohol exposure does not seem to prime glial cells and influence pro-inflammatory cytokine production in response to secondary insults[204,205]. Conversely, anti-inflammatory fractalkine was found to be elevated by prenatal alcohol exposure[206].

### 1.14.2 Studies in 3rd Trimester Models

Studies in 3$^{rd}$ trimester models are summarized in Table 1.1 and the overall hypothesis within the field is shown in Figure 1.3. A number of different animal models and exposure paradigms have confirmed that 3$^{rd}$ trimester-equivalent alcohol can induce microglial activation in multiple brain regions[207-211]. Similarly, increased pro-inflammatory cytokine production has been reported in a number of studies, in several brain regions[208,210,212,213]; anti-inflammatory

cytokines have also been shown to be elevated[212-214]. Although most studies have assessed time points within 1 day of the last alcohol exposure, one group has reported cytokine production to be elevated weeks after exposure[212,213]. However, lower BACs (110 mg/dL) do not seem to increase cytokine production, despite inducing microglial and astrocytic responses[207]. Interestingly, in this lower dose study there was significant variability in the outcomes of the fetuses, with one of 6 fetuses being unaffected and the other 5 having differential signs of neuroinflammation and neuronal injury[207]. To the best of my knowledge this study is the only study to use a 3rd trimester model and include an investigation into astrocytes, reporting that signs of astrocyte activation correlated with microglial activation[207].

| Study | Animal | Method | Days | BAC (mg/dL) | Brain Region | Cytokine Increases | Microglial Activation | Astrocyte Activation | Neuronal Damage or Death |
|---|---|---|---|---|---|---|---|---|---|
| Dalitz 2008 | Sheep | I.V. | 3 days | 110 | Cerebellum and Cerebral Cortex | Not affected | X | X | X |
| Drew 2015 | Mice | Gavage | P4-9 | 400 | Hippocampus, Cerebellum, Cortex | X | X | | |
| Kane 2011 | Mice | Gavage | P3-5 | 250-300 | Cerebellum | | X | | X |
| Li 2015 | Mice | I.P. | P4 P4-9 | 350 | Cerebellum | X | X | | X |
| Saito 2010 | Mice | Injection | P7 | ? | Forebrain | | X | | X |
| Sarkar 2007 | Rat | Gavage | P2-6 | 250 | Hypothalamus | X | | | X |
| Tiwari 2011 &2012 | Rat | Gavage | P7-9 | 325 | Hippocampus and Cortex | X | | | X |

**Table 1.1: Summary of Studies Investigating the Neuroimmune Reponse Following a 3rd Trimester-Equivalent Exposure to Alcohol**

The symbol (X) refers to a positive finding, wheras an empty box indicates that this outcome was not investigated.

A study by Li and colleagues compared neuroinflammation resulting from different exposures paradigms, finding that moderate alcohol exposure (BAC = 200 mg/dL) did not produce cytokine secretion or signs of neuronal loss. However, higher doses (BAC = 350 mg/dL) after only one day of alcohol exposure on P4 resulted in robust cytokine secretion and neuronal loss, while that same dose from P4-9 further increased these measures[210]. Another group reported signs of microglial activation within 4 hours of alcohol exposure, with full activation by 16 hours, and that microglia appeared to be engulfing neuronal processes[211].

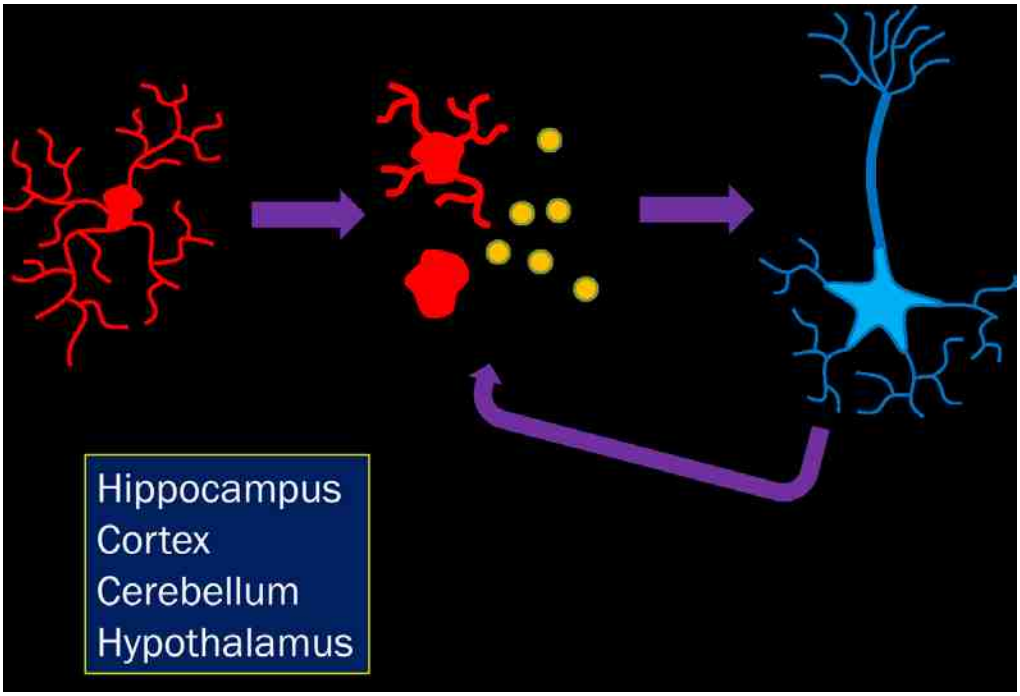


**Figure 1.3: Model of Current Theory of the Neuroimmune System Following 3rd Trimester-Equivalent Exposure.**

Microglia (red) become activated following alcohol exposure and release pro-inflammatory cytokines and chemokines (yellow dots), which may contribute to neuronal damage/death. Neuronal damage may stimulate further microglial activation. This model has been suggested to apply in multiple brain regions.

Several studies have investigated the effect of blunting neuroinflammation of neuronal loss[209,210,212,213]. In all studies, preventing microglial activation and/or cytokine secretion correlated with reduced neuronal loss. One group also reported partial rescuing of behavioral deficits[212,213], suggesting that anti-inflammatory agents may be a potential therapeutic option in the treatment of FASDs.

# 2. Overview of Studies

## 2.1 Study #1 (moderate, long-term exposure)

*"Effect of repeated alcohol exposure during the third trimester-equivalent on messenger RNA levels for interleukin-1b, chemokine (C-C motif) ligand 2, and interleukin 10 in the developing rat brain after injection of lipopolysaccharide".*

2.1.1 Rationale: A number of studies investigating the effect of postnatal alcohol exposure (PAE) have shown that high dose, binge-like exposures can activate the neuroimmune system[208-210,213]. However, whether or not neuroimmune system activation occurs following lower doses of alcohol remains controversial. Instead there has been speculation that lower doses of alcohol during this period could have more subtle "priming" effects on neuroimmune system function. This type of effect has been reported after infections and other insults during the early postnatal period that resulted in permanent alterations in the programming of neuroimmune cells[215,216]. This priming causes the cells to over-react to subsequent stimulation, resulting in dramatically increased cytokine production, and causing deficits in memory and learning. This could be especially relevant in cases of FASD, as FAE increases the susceptibility to infections and severity of inflammation[217-219].

2.1.2 Hypothesis: Exposure to moderate, long-term alcohol during the 3$^{rd}$ trimester-equivalent will not induce a basal increase in neuroinflammation, but will re-program cells to over-react to subsequent insults.

2.1.3 General Approach: We used a vapor chamber inhalation paradigm to model exposure to moderate levels of alcohol during the entire 3rd trimester-equivalent to human pregnancy. To simulate a neonatal infection, pups were injected with LPS or saline one day following the alcohol exposure paradigm. Two hours post-LPS, the cerebellum, dentate gyrus, and cortex were collected. mRNA was analyzed for expression of pro- and anti-inflammatory cytokines in all four groups (control + saline, control + LPS, PAE + saline, PAE + LPS), and the response to LPS was compared between the control and PAE groups. We included both males and females for this study. Based on our hypothesis, we expected to find that cytokine expression would not differ between the PAE and control groups that were injected with saline. Since LPS has been shown to dramatically increase cytokine production, we anticipated that injection of LPS would result in an elevated expression of cytokines in both control and PAE animals. However, we expected that PAE would potentiate this response, resulting in an even larger increase in cytokine production.

2.1.4 Conclusion: As expected, LPS injections dramatically increased expression of pro-inflammatory cytokines. However, we found that, in contrast to our hypothesis, PAE had surprisingly little effect on neuroimmune system function. The only significant difference between the two groups was that PAE blunted the LPS-induced increase of IL1β in the cortex of female pups. Taken together with the literature, these findings indicate that moderate, long-term exposure to alcohol is less disruptive to the neuroimmune system than high-dose, acute exposures. These results are discussed in more detail in chapter 3.

## 2.2 Study #2 (binge-like exposure)

*"Exposure of Neonatal Rats to Alcohol has Differential Effects on Cytokine Levels in the Cerebellum and Hippocampus: Potential Role of Microglia and Astrocytes".*

2.2.1 Rationale: In study #1, we found that moderate alcohol exposure in the postnatal period had little effect on the neuroimmune system. However, binge-like exposure to high doses of alcohol in the postnatal period has been shown to cause both neurodegeneration and robust neuroinflammation, characterized by increased cytokine production and microglial activation [42,208-210,212]. While most studies within this field have focused on pro-inflammatory cytokine production, PAE has also been shown to increase TGFβ[212,214], indicating that there may be an anti-inflammatory component. Recently, the concept of M1 and M2 pathways of microglial activation has been introduced[132]. M1 activation is classified as pro-inflammatory and neurotoxic when prolonged, while M2 polarization is generally anti-inflammatory and can be strongly neuroprotective. Importantly, it has been shown that the activation state of microglia can change during the course of neuroinflammation. Specifically, in a model of multiple sclerosis, Miron and colleagues demonstrated that the initial neuroimmune response consisted of M1 polarized microglia, yet analyses performed at later time points revealed that microglia had switched to a predominantly M2 phenotype, likely to aid in neuronal survival and CNS recovery[135]. Since models of PAE that result in neuroinflammation typically also cause neuronal loss, it is possible that there is

an M2 component to aid with neuronal recovery. The original purpose of this study was to investigate the specific phenotype of neuroimmune system activation during PAE, and to see if it evolved over time.

2.2.2 Hypothesis: Acute binge exposure to alcohol in the 3rd trimester-equivalent initially induces a M1 neuroinflammatory response, which eventually resolves into a M2 anti-inflammatory response that mitigates alcohol-induced damage in some brain regions.

2.2.3 General Approach: We designed and thoroughly characterized a PAE paradigm that would mimic a binge-like exposure and reliably induce both neuroinflammation and neuronal loss. We performed an analysis of both the cerebellum and the hippocampus, to determine if effects were global or brain-region specific, and included behavioral assessments for the functioning of both brain regions. For measurements of neuroinflammation and neuronal loss, we included multiple time points throughout the exposure paradigm to investigate the evolution of the neuroimmune response. Neuroimmune activation was assessed by RT-PCR for expression of mRNAs for pro- and anti-inflammatory cytokines, as well as immunohistochemistry for the morphology of astrocytes and microglia. Neuronal loss was assessed by immunohistochemistry and neuronal number was counted. The study was organized into the following specific aims:

*Aim 1:* Develop and characterize a model of 3$^{rd}$ trimester binge alcohol exposure through vapor inhalation that reliably induces neuroimmune activation.

*Aim 2:* Determine the effect of a binge-like alcohol vapor exposure during the 3rd trimester-equivalent on the neuroimmune system and the type of response at both the level of cytokine expression and microglial activation.

2.2.4 Conclusion: We found that acute binge-like exposure to alcohol induced a robust neuroimmune response within the cerebellum, as well as increased neuronal loss. Conversely, in the hippocampus no neuronal loss was observed, and there was little evidence of microglial activation. Interestingly, astrocyte activation occurred in both brain regions. Furthermore, there did not seem to be a distinct M1 or M2 type response, instead pro and anti-inflammatory cytokines were up-regulated in both brain regions. However, pro-inflammatory cytokine production spiked in periods of withdrawal, and returned to baseline during peak BACs, while anti-inflammatory cytokine production was subtler, yet prolonged. Results of this study are discussed in more detail in chapter 4.

# 3. Effect of repeated alcohol exposure during the third trimester equivalent on messenger RNA levels for interleukin-1β, chemokine (C-C motif) ligand 2, and interleukin 10 in the developing rat brain after injection of lipopolysaccharide

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## 3.1 Abstract

Microglia undergo maturation during the third-trimester of human development (equivalent to the first 1-2 weeks of postnatal life in rodents), during which these cells may be particularly sensitive to insult. Alcohol exposure during this period can activate the neuroimmune system, an effect that may contribute to the pathophysiology of fetal alcohol spectrum disorders. Here, we investigated whether repeated alcohol exposure during the third-trimester-equivalent in rats has a priming effect on the neuroimmune response to injection of bacterial lipopolysaccharide (LPS). Pups were exposed to alcohol in vapor chambers for four hours daily from postnatal day (PD)2 to PD16 (peak blood alcohol concentrations ~ 150 mg/dL). On PD17, rats were injected with either saline or LPS (50 µg/kg) and the frontal cortex, cerebellar vermis, and dentate gyrus were collected 2 hours later. Messenger RNA (mRNA) levels for the pro-inflammatory agents interleukin 1β (IL-1β) and chemokine (C-C) motif ligand 2 (CCL2), as well as levels of the anti-inflammatory cytokine interleukin 10 (IL-10), were measured using reverse transcriptase-polymerase-chain reaction. LPS consistently increased IL-1β and CCL2 mRNA levels in the dentate gyrus, frontal cortex, and cerebellum of both male and female rats. Furthermore, the LPS-induced increase of IL-1β mRNA levels was significantly blunted in the frontal cortex of alcohol-exposed female rats. Conversely, LPS only minimally affected IL-10 mRNA expression and there were no significant differences between air and alcohol-exposed rats. These findings suggest that exposure to comparatively moderate alcohol levels during the third trimester equivalent has relatively subtle effects on

the neuroimmune response after an immune challenge in the developing rat brain.

## 3.2 Introduction

Alcohol consumption during pregnancy is the most common cause of preventable birth defects. The term fetal alcohol spectrum disorders (FASDs) encompasses the molecular and functional consequences resulting from developmental alcohol exposure, ranging from isolated behavioral deficits, particularly in learning and memory, to a constellation of morphological and behavioral alterations characteristic of fetal alcohol syndrome. It is estimated that the prevalence of FASDs in the United States is approximately 2-5%[3]. Recent studies have presented evidence for a role of the neuroimmune system in FASDs[220]

Immune responses in the central nervous system (CNS) are the outcome of interactions between many cell types such as glial cells (microglia, astrocytes, oligodendrocytes), neurons in the surrounding tissue, and invading peripheral components. Of these cells, microglia are the most well-characterized and widely studied with respect to neuroimmune system function. Microglia are dynamic cells that undergo a spectrum of changes in morphology, phenotype, and gene expression upon activation. The response generated by microglia is highly dependent on factors such as type of insult, duration, and individual features of the host including gender and age. Typical microglial activation involves the secretion of cytokines and chemokines which have been identified as powerful modulators of the CNS influencing neuronal plasticity[155,221], receptor activity and expression[222], neurogenesis[216], and maintenance of homeostasis[223,224]. The neuroimmune system plays a particularly critical role during development and

infections in the early postnatal period can impair cognitive functions in adulthood[215,225] and increase susceptibility to neurodevelopmental disorders[226]. Additionally, microglia undergo maturation during the rodent third-trimester equivalent of human pregnancy, which takes place postnatally, possibly leading to an increased vulnerability to insult during this time[163]. It has been postulated that neonatal infection programs an exaggerated response in the developing neuroimmune system that could result in behavioral alterations such as memory impairment[227].

Alcohol intake has been shown to activate microglia, resulting in a neurotoxic increase in pro-inflammatory cytokines[175,187,190] a pattern of activation known as the "classical" M1 response. Conversely, recent research has emphasized the complexity of this system by demonstrating that microglia may, under some conditions, instead assume a neuroprotective role (denoted as "alternative activation" or M2 response)[228] after alcohol exposure, characterized by production of anti-inflammatory factors such as interleukin (IL)-10[181]. Additionally, Kane and colleagues have shown an age-dependent neuroimmune response to alcohol[192], highlighting the importance of discrete developmental stages in neuroimmune function. Additional studies have revealed that gestational alcohol exposure is associated with increases in microglial and astrocyte activation[195-197]. Furthermore, studies focusing on the third-trimester equivalent have shown increases in pro-inflammatory cytokines and microglial activation in several brain regions, including the cerebellum and hippocampus[209,212].

Perturbation of the neuroimmune system by developmental alcohol exposure may contribute to the pathophysiology of FASD in multiple ways. It could directly disrupt CNS synaptic refinement by altering levels of cytokines known to effect neuronal plasticity[155,229] or by changing microglial phenotype, thereby disrupting normal microglial-dependent processes such as synaptic pruning[170]. Conversely, developmental alcohol exposure could act by programming a permanently altered neuroimmune system. In support of this notion, FASD offspring have been shown to have impaired immune responses in adulthood [218]. Furthermore, the prevalence of neonatal infection in FASD patients is increased dramatically, as even small amounts of alcohol during gestation are associated with a 2.5 fold increase in risk for infection, whereas excessive alcohol intake increases the risk by 3-4 times [219]. A neuroimmune system altered by alcohol exposure during development could react differently to a second insult (such as neonatal infection) than that of a normal infant. The relationship between the co-exposure to alcohol and neonatal infection could have critical implications for the pathophysiology of FASD.

The objective of this study was to characterize neuroimmune function in response to repeated third-trimester-equivalent alcohol exposure after a “second insult” in the form of injection of a low dose of bacterial lipopolysaccharide (LPS). We included both male and female pups, and focused on the dentate gyrus (DG) subregion of the hippocampus as well as the cerebellum and frontal cortex. These brain regions have been shown to be susceptible to damage by developmental alcohol exposure[32,230-233] that could be mediated, in part, by

neuroimmune alterations[209,212]. To assess the function of the neuroimmune system, we measured changes in mRNA levels for the pro-inflammatory markers, interleukin-1β (IL-1β) and chemokine (C-C motif) ligand 2 (CCL2) whose expression in the neonatal brain increases after LPS injections[234]. Additionally, to determine if postnatal alcohol exposure (PAE) may induce an anti-inflammatory effect, we measured mRNA levels for IL-10 which has been shown to increase after LPS injections in the hippocampus of male rat pups[234].

## 3.3 Materials and Methods

### 3.3.1 Animal treatments and tissue collection

Animal procedures were approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center and conformed to National Institutes of health guidelines. Time-pregnant Long-Evans rats (gestational day 14-15) were obtained from Harlan Laboratories Inc. (Indianapolis, IN) and allowed to acclimate for approximately one week before giving birth. Two days after litter birth, dams and their respective offspring were housed together in a vapor chamber apparatus. Starting at 10:00 am, litters were exposed to 3.2 ± 0.4 mg/dL alcohol via vapor inhalation for 4 hours daily from postnatal day (PD) 2 until PD 16 as described previously by [235]. Control litters were housed similarly but received only air flow (no alcohol). Litters were culled to 10 pups on PD 5. Blood alcohol concentrations (BACs) were determined using an alcohol dehydrogenase-based assay, as previously described[236]. Rats were given intraperitoneal injections of LPS (50 µg/kg body weight in ~90 µL) (Millipore 0111:B4, lot# 2089863) or endotoxin-free saline (Santa Cruz Biotechnology, sc286637, lot#F0513) ~24 hours after the last alcohol exposure (on PD17). LPS doses were chosen based on previous reports showing significant effects on immune system gene expression[234]. Furthermore, this dose is on the lower end of what is reported in the literature and has been shown to produce submaximal activation of the neuroimmune system [237,238], therefore avoiding possibly masking any effect of PAE on the response to LPS via a “ceiling” effect. Samples were collected 2 hours after LPS injections, as described below. Animals received

ketamine (250 mg/kg) by intraperitoneal injection and brains were removed by decapitation. Brains were placed into ice cold phosphate buffered saline and whole dentate gyri were extracted from both hemispheres as described previously [239]. In addition, frontal cortex and cerebellar vermis samples were collected. A minimum of 6 animals per experimental group were chosen based on an *a priori* power calculation performed using the standard deviation and effect size obtained in a previous study[182]. This experimental sample size is also consistent with previous reports that have detected changes in LPS-induced neuroinflammation after exposure to insult, including alcohol[182,215,227].

### 3.3.2 Real-time PCR analysis

For RNA isolation, tissue was placed into Buffer RLT (Qiagen RNeasy Mini Kit, Cat. No. 74106) containing 40 mM dithiothreitol. Samples were homogenized by sonication and RNA was extracted immediately after tissue removal using an RNeasy mini Kit (SABiosciences/Qiagen) according to the manufacturer's instructions. The RNA was frozen at -20 °C until use. cDNA was synthesized from 1 µg of RNA using the $RT^2$ First Strand Kit (SABiosciences/Qiagen) and stored at -20 °C. Gene expression was measured using real-time PCR. All primers were purchased from SABiosciences/Qiagen including IL-1β (Cat No. PPR06480B) CCL2 (Cat. No. PPR06714B), IL-10 (Cat No. PPR06479A) and hypoxanthine phosphoribosyltransferase 1 (Hprt1; Cat. No. PPR42247F). PCR was carried out according to the $RT^2$ qPCR Primer assay instructions (SABiosciences/Qiagen) in combination with the $RT^2$ SYBR Green qPCR Master Mix. The threshold amplification number ($C_T$) value for each gene

of interest (GOI) was normalized to the housekeeping gene (HKG) Hprt1 following the formula $\Delta C_T = C_T$ (GOI) – $C_T$ (HKG). To validate the use of HPRT1 as a housekeeping gene, its expression was measured and found to be unaffected by vapor chamber treatment (alcohol versus air control), injection (LPS versus saline), or sex (data not shown). To avoid the potential of a floor effect, a cut-off CT value of 35 was used.

### 3.3.3 Statistics

Data analysis was carried out on Prism 6.03 (GraphPad Software, San Diego, CA). Whether the data followed a Gaussian distribution was determined with Shapiro-Wilk or Kolmogorov-Smirnov normality test, and statistics were determined by either parametric tests (one-sample t-test vs. a theoretical mean of 1 and two-tailed student's t-test or by non-parametric tests (Wilcoxon sign test vs. a theoretical mean of 1 and Mann-Whitney test), as appropriate. The unit of determination is defined as the average of results obtained with two male or two female pups per litter.

## 3.4 Results

To model human fetal alcohol exposure during the third-trimester of pregnancy, rat pups were exposed daily to alcohol in vapor inhalation chambers between PD2 and PD16, as previously described [235]. Chamber levels remained relatively stable during exposure and pups were exposed to alcohol vapor levels near 3 g/dL (data not shown). On PD2, prior to culling, approximately equal sized litters were assigned to either the postnatal alcohol exposure (PAE) or air control groups (Control group: 9.769 ± 2.048; PAE group: 11.08 ± 1.935; $p = 0.1072$, $n = 13$, by unpaired *t*-test). With the exception of cage changes on PD2, PD7 and PD14, pups were unhandled and no mortality occurred. Peak BACs immediately following exposure to alcohol were 155 ± 37.59 mg/dL and 164 ± 46.61 mg/dL on PD 6 ($n = 4$ litters) and PD16 ($n = 3$ litters), respectively. These levels reflect a BAC of approximately twice the legal limit of intoxication (80 mg/dL). Analysis of pup weight on PD17 showed a significant decrease in the PAE group compared to air control rats (Control group: 34.74 ± 3.616 g, $n = 12$ litters; PAE group: 32.01 ± 2.131 g, $n = 11$ litters; $p = 0.0123$, by Mann Whitney test).

### 3.4.1 Alcohol exposure does not significantly alter the neuroimmune response to LPS in the DG

On PD 17, 24 hours after completion of alcohol exposure, two pups from each litter (one male, one female) received an LPS injection and two pups (one male, one female) received a saline injection. After 2 hours, mRNA was harvested and the levels of pro-inflammatory cytokine IL-1β and chemokine CCL2 (also known as monocyte chemoattractant protein-1) were examined.

Additionally, anti-inflammatory cytokine IL-10 mRNA was assessed. With respect to saline injected rats, as expected, LPS injections significantly increased IL-1β mRNA levels in the DG of both control ($p$ = 0.002, $n$ = 10, by one sample $t$-test vs. one) and PAE ($p$ = 0.0039, $n$ = 10, by Wilcoxon Signed Rank Test vs. one) male rats (Fig 3.1A). Similarly, LPS injections significantly increased IL-1β mRNA levels in the DG of control ($p$ = 0.0003, $n$ = 10, by one sample $t$-test vs. one) and PAE ($p$ = 0.0071, $n$ = 9, by one sample $t$-test vs. one) female rats (Fig 3.1B). However, there was not a significant difference between the air control and PAE groups in the LPS-induced increase of IL-1β mRNA levels in either males (Fig 3.1A, $p$ = 0.2443, by Mann-Whitney test) or females (Fig 3.1B, $p$ =0.6828, by unpaired $t$-test).

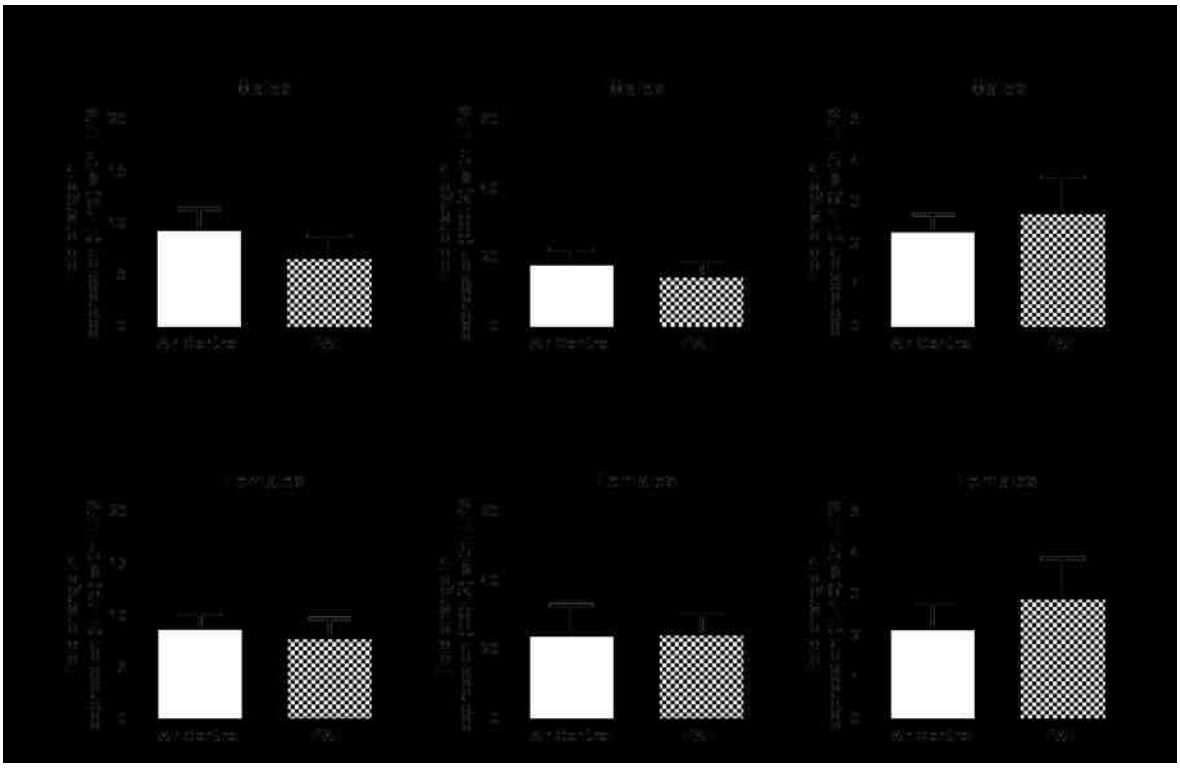

**Figure 3.1: 3rd Trimester-Equivalent Alcohol Exposure did not alter mRNA Expression of IL-1β, CCL2, or IL-10 in the Dentate Gyrus of Male or Female Pups after LPS Injection.**

Levels of IL-1β (A), CCL2 (C), and IL-10 (E) were determined in male pups after LPS injection compared to saline-injected littermates. Levels of IL-1β (B), CCL2 (D), and IL-10 (F) were determined in females after LPS injection compared to saline-injected littermates. The fold change in mRNA expression represents the ratio of levels in LPS-injected pups over saline-injected littermates. (*N.S.* = not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ vs. a theoretical mean of 1).

LPS injections significantly increased CCL2 mRNA levels in the DG of both control ($p = 0.0024$, $n = 10$, by one sample *t*-test vs. one) and PAE ($p = 0.0069$, $n = 10$, by one sample *t*-test vs. one) male rats (Fig 3.1C). LPS injections also significantly increased CCL2 mRNA levels in the DG of both control ($p = 0.002$, $n = 10$, by Wilcoxon Signed Rank Test vs. one) and PAE ($p = 0.0025$, $n = 9$, by one sample *t*-test vs. one) female rats (Fig 3.1D). However, there was not a significant difference between the air control and PAE groups in the LPS-induced

increase of CCL2 mRNA levels in the DG of either males (Fig 3.1C, $p = 0.5339$, by unpaired *t*-test) or females (Fig 3.1D, $p = 0.7065$, by Mann-Whitney test).

LPS injections significantly increased IL-10 mRNA levels in the DG of control ($p = 0.0135$, $n = 8$, by one sample *t*-test vs. one) but not PAE ($p = 0.0805$, $n = 8$, by one sample *t*-test vs. one) male rats (Fig 3.1E). In female rats LPS injections did not significantly affect IL-10 mRNA levels in controls ($p = 0.0915$, $n = 10$, by one sample *t*-test vs one), but did significantly increase levels in the PAE group ($p = 0.0078$, $n = 8$, by Wilcoxon Signed Rank Test vs. one) (Fig 3.1F). However, the fold change in IL-10 expression after LPS injection was not significantly different between control and PAE in male (Fig 3.1E, $p = 0.6472$, by unpaired *t*-test) and female (Fig 3.1F, $p = 0.4038$, by Mann-Whitney test) rats.

### 3.4.2 Alcohol exposure blunts the LPS-induced increase of CCL2 mRNA levels in the frontal cortex of female rats.

With respect to saline injected rats, LPS injections significantly increased IL-1β mRNA levels in the frontal cortex of both control ($p = 0.0466$, $n = 8$, by one sample *t*-test vs. one) and PAE ($p = 0.0156$, $n = 7$, by Wilcoxon Signed Rank Test vs. one) male rats (Fig 3.2A). Similarly, LPS injections significantly increased the IL-1β mRNA levels in the frontal cortex of both control ($p = 0.0156$, $n = 7$, by Wilcoxon Signed Rank test vs. one) and PAE ($p = 0.0106$, $n = 8$, by one sample *t*-test vs. one) female rats (Fig 3.2B). PAE did not significantly alter the LPS-induced increase of IL-1β mRNA in the frontal cortex of male rats (Fig 3.2A, $p = 0.1203$, by Mann-Whitney test). Conversely, PAE significantly blunted the

LPS-induced increase of IL-1β mRNA in female rats (Fig 3.2B, $p$ = 0.0006, by Mann-Whitney test).

LPS injections significantly increased CCL2 mRNA levels in the frontal cortex of control ($p$ = 0.0330, $n$ = 8, by one sample $t$-test vs. one) and PAE ($p$ = 0.0304, $n$ = 7, by one sample $t$-test vs. one) male rats (Fig 3.2C). Similarly, LPS injections significantly increased CCL2 mRNA levels in both control ($p$ = 0.0156, $n$ = 7, by Wilcoxon Signed Rank Test vs. one) and PAE ($p$ = 0.0158, $n$ = 8, by one sample $t$-test vs. one) female rats (Fig 3.2D). However, PAE did not significantly alter the LPS-induced increase of CCL2 mRNA levels in either males (Fig 3.2C, $p$ = 0.1952, by unpaired $t$-test) or females (Fig 3.2D, $p$ = 0.2785, by Mann-Whitney test).

LPS injections did not significantly increase IL-10 mRNA expression in the frontal cortex of control ($p$ = 0.3828, $n$ = 8, by Wilcoxon Signed Rank Test vs. one) or PAE ($p$ = 0.2936, $n$ = 7, by one sample $t$-test vs. one) male rats (Fig 3.2E). Similarly, LPS injections did not significantly increase IL-10 mRNA levels in control ($p$ = 0.1157, $n$ = 7, by one sample $t$-test vs. one) or PAE ($p$ = 0.4119, $n$ = 8, by one sample $t$-test vs. one) female rats (Fig 3.2F). There were no statistically significant differences in IL-10 mRNA levels after LPS in control or PAE samples from either male (Fig 3.2E, $p$ >0.9999, by Mann-Whitney test) or female rats (Fig 3.2F, $p$ = 0.0594, by unpaired $t$-test).

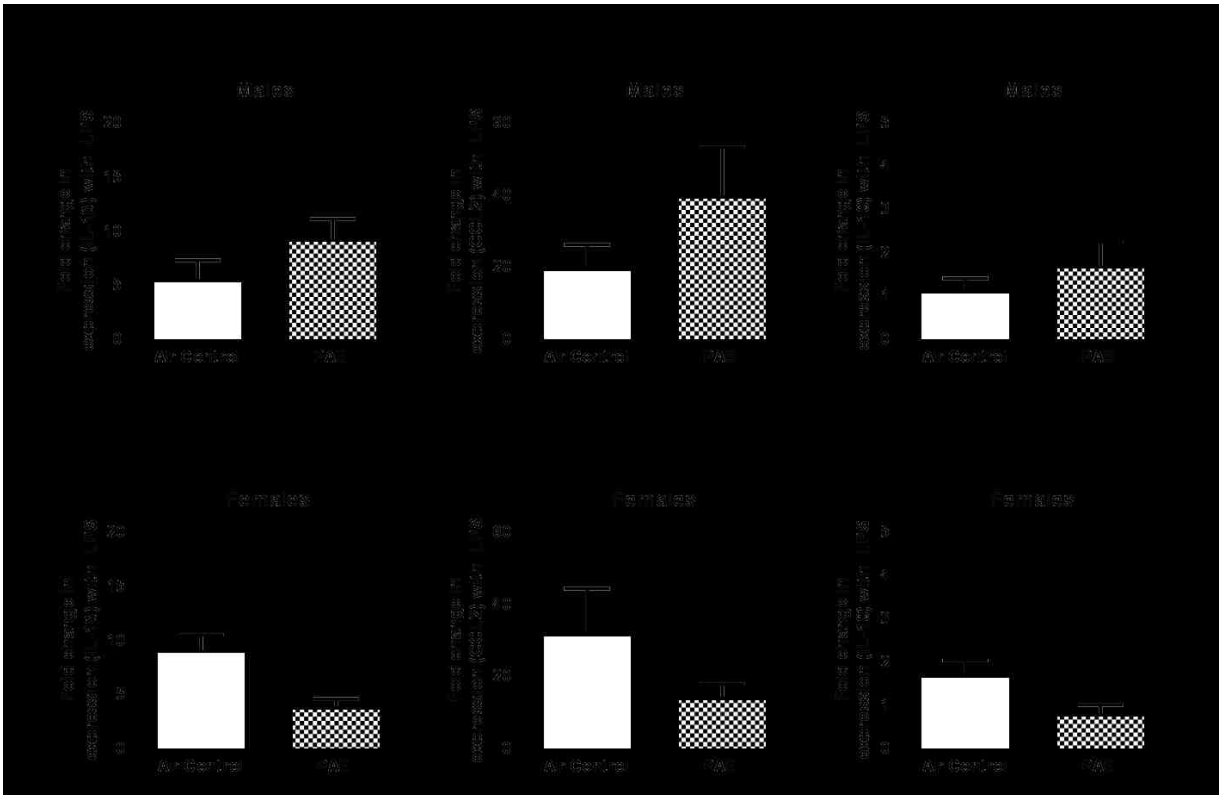

**Figure 3.2: 3rd Trimester-Equivalent Alcohol Exposure blunted the LPS-induced increase in IL-1β mRNA expression in the frontal cortex of female pups but not males.**

The fold change in mRNA expression represents the ratio of levels in LPS-injected pups over saline-injected littermates. Data is displayed as in Figure 3.1. The LPS-induced fold change in IL-1β mRNA expression was significantly attenuated in female rats of the PAE group compared to female rats in the air control group. (*N.S*. = not significant, * $p \leq 0.05$ vs. a theoretical mean of 1, *** $p \leq 0.001$ vs. Air control).

### 3.4.3 Alcohol exposure did not significantly affect IL-1β or CCL2 mRNA levels in the cerebellar vermis of male and female rats

With respect to saline injected rats, LPS injections significantly increased IL-1β mRNA levels in the cerebellar vermis of both control ($p = 0.0160$, $n = 8$, by one sample *t*-test vs. one) and PAE ($p = 0.0200$, $n = 7$, by one sample *t*-test vs. one) male rats (Fig 3.3A). In female rats, LPS significantly increased IL-1β mRNA levels in the cerebellar vermis of the control group ($p = 0.0144$, $n = 7$, by one

sample *t*-test vs. one) but not the PAE ($p$ = 0.1251, $n$ = 6, by one sample *t*-test vs. one) group (Fig 3.3B). PAE did not significantly alter the LPS-induced increase of IL-1β mRNA levels in either males (Fig 3.3A, $p$ = 0.0993, by unpaired *t*-test) or females (Fig 3.3B, $p$ = 0.7949, by unpaired *t*-test).

LPS injections significantly increased CCL2 mRNA levels in the cerebellar vermis of both control ($p$ = 0.0390, $n$ = 8, by one sample *t*-test vs. one) and PAE ($p$ = 0.0169, $n$ = 7, by one sample *t*-test vs. one) male rats (Fig 3.3C). Similarly, LPS injections significantly increased CCL2 mRNA levels in the cerebellar vermis of both control ($p$ = 0.0202, $n$ = 7, by one sample *t*-test vs. one) and PAE ($p$ = 0.0305, $n$ = 6, by one sample *t*-test vs. one) female rats (Fig 3.3D). PAE did not significantly alter the LPS-induced increase of CCL2 mRNA expression in either males (Fig 3.3C, $p$ = 0.2643, by unpaired *t*-test) or females (Fig 3.3D, $p$ = 0.8171, by unpaired *t*-test).

LPS injections did not significantly increase IL-10 mRNA expression in control ($p$ = 0.2791, $n$ = 8, one sample *t*-test vs. one) or PAE ($p$ = 0.0863, $n$ = 6, by one sample *t*-test vs. one) male rats (Fig 3.3E). Similarly, LPS injection did not increase IL-10 mRNA expression in controls ($p$ = 0.2188, $n$ = 7, by Wilcoxon Signed Rank Test vs. one) or in PAE ($p$ = 0.2062, $n$ = 6, by one sample *t*-test vs. one) female rats (Fig 3.3F). There were no statistically significant differences in IL-10 mRNA levels after LPS in control or PAE samples from either male (Fig 3.3E, $p$ = 0.7979, by unpaired *t*-test) or female rats (Fig 3.3F, $p$ > 0.9999, by Mann-Whitney test).

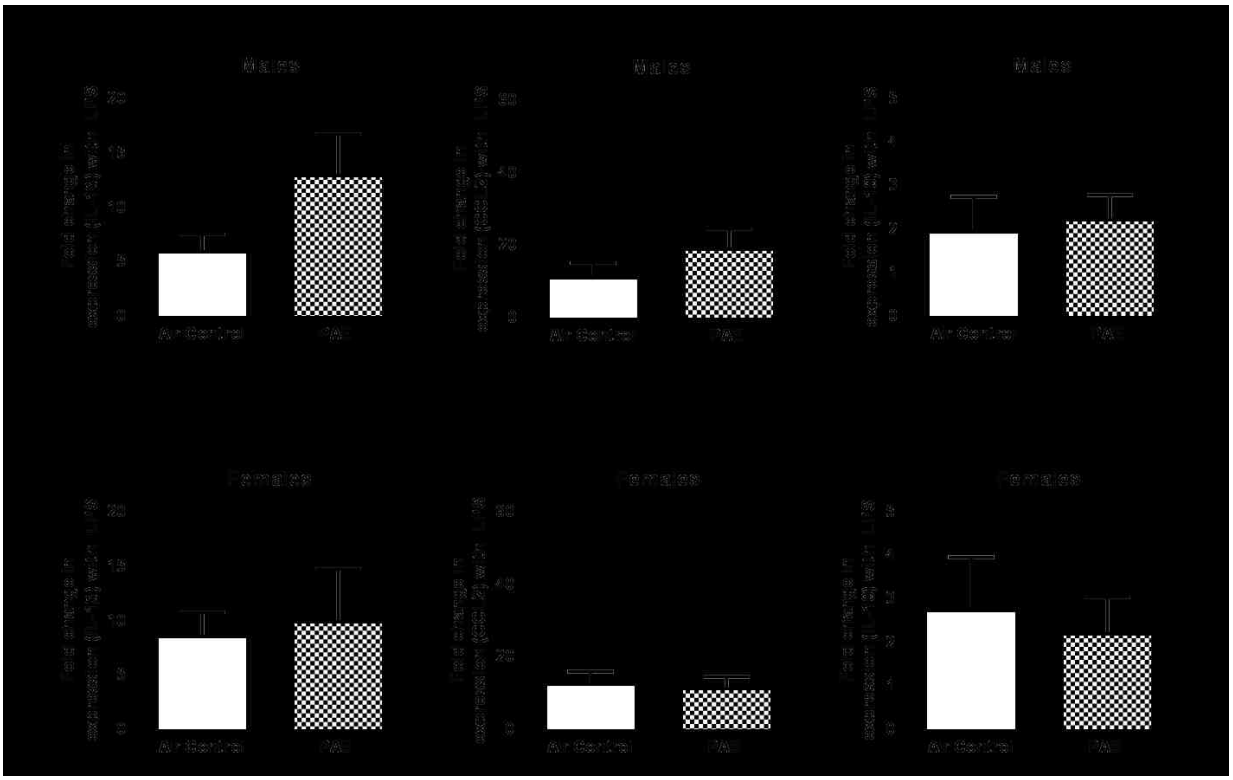

**Figure 3.3: 3rd Trimester-Equivalent Alcohol Exposure did not alter mRNA Expression of IL-1β, CCL2, or IL-10 after LPS Injection in the Cerebellar Vermis of Male or Female pups.**

The fold change in mRNA expression represents the ratio of levels in LPS-injected pups over saline-injected littermates. Data is displayed as in Figure 3.1. (*N.S.* = not significant, * $p \leq 0.05$ vs. a theoretical mean of 1)

## 3.5 Discussion

This study examined the effect of a repeated third-trimester equivalent alcohol exposure on the developing neuroimmune system, concentrating on the interaction of developmental alcohol exposure with a LPS challenge in the rat neonatal period. Many previous studies have demonstrated that neuroimmune activation during the neonatal period can reprogram this system, resulting in long-term deficits that are subtle and essentially undetectable until unmasked by a second insult. The reprogrammed neuroimmune system then over-reacts to later insults with increased pro-inflammatory cytokine production eliciting behavioral deficits[215,216,225,240]. Based on these studies, we hypothesized that ethanol could produce a similar effect; i.e., that exposure to moderate ethanol levels during late stages of pregnancy could program the neuroimmune system to have an exaggerated pro-inflammatory response to an infection, leading to secondary CNS damage. Contrary to our hypothesis, we found that exposure to relatively moderate alcohol levels during the third trimester-equivalent had little effect on neuroimmune function in response to LPS injections. Moreover, PAE had an effect opposite what we expected in the frontal cortex of female rats, where it actually blunted the LPS-induced production of IL-1β mRNA.

Using a previously characterized vapor chamber exposure paradigm[235], we exposed rats to alcohol through vapor inhalation for 2 weeks, from PD2 to 16, which encompasses several of the developmental events that occur in the human brain during the third trimester of human pregnancy[241]. Unlike humans, rodents undergo the brain growth spurt associated with the human third trimester

of gestation during the early postnatal period. This period includes key brain developmental processes, such as synapse formation and elimination[11]. While our paradigm only models alcohol consumption during the third trimester equivalent without any exposure in the first or second trimesters, this has been identified as the third most common drinking pattern during pregnancy in the US[5]. Our laboratory has previously found that this alcohol model, using a slightly higher BAC, induces alterations in GABAergic subunit expression in the cerebellum[242], GABAergic transmission[243] and LTP induction in the hippocampus[35]. However, using lower doses, as in this study, ethanol did not significantly affect either LTP in the hippocampus[35] or complex spikes and long-term depression in cerebellar Purkinje neurons[235]. Additionally, apoptotic neuronal cell death has not been shown to occur at the BACs used in this study[42]. Therefore, we did not expect that the more moderate level of alcohol exposure in this model would cause large changes, but instead sought to investigate whether it might cause more subtle changes in the neuroimmune system that could be unmasked with a second insult (LPS).

Using this paradigm, we attained peak BACs that were approximately twice the legal limit of intoxication (80 mg/dL) and could be achieved by a pregnant woman consuming around 5-6 drinks[244]. Therefore, these levels of alcohol exposure are considered high from the human perspective. However, previous studies have shown that rodents are more resistant to alcohol than humans and higher BACs are needed to elicit similar effects in the former[16]. Using a similar paradigm, our laboratory has previously shown that after this type

of exposure, BACs gradually return to zero over the course of approximately 8 hours [242]. The current study uses pointedly lower BACs, which likely return to baseline in less time. While we did not observe an increase in mortality, there was a significant decrease in body weight in PAE rat pups on PD17. Therefore, this paradigm models some of the characteristics of fetal alcohol syndrome which is, in part, characterized by decreased body weight.

### 3.5.1 Alcohol exposure did not affect the LPS-induced increase in IL-1β, CCL2, and IL-10 mRNA in the DG.

We investigated the effect of PAE followed by immune challenge with a low dose of LPS in order to mimic an infant prenatally exposed to ethanol contracting an infection soon after birth. We gauged the neuroimmune pro-inflammatory response to LPS by measuring the expression of IL-1β and CCL2 mRNA, both of which have shown to be significantly elevated after LPS injection, and are regularly used as markers of neuroinflammation[234]. Additionally, we measured the anti-inflammatory cytokine IL-10, which has been shown to be elevated in the hippocampus of male pups after LPS[234].We therefore expected that IL-10 mRNA would be elevated in other brain regions as well. Two hours after LPS injection, we observed a significant increase in both pro-inflammatory genes in the DG, with a larger increase in CCL2 expression (~20 fold) than in IL-1β (5-10 fold), consistent with previous findings[234]. Conversely, we did not detect robust changes in IL-10 mRNA expression in rats injected with LPS.

Previous studies investigating the role of the neuroimmune response after PAE have focused primarily on male rats. Given the sexually dimorphic effects of

PAE[245-247], as well as the striking gender differences in microglia colonization during development [164], we included both males and females in this study. In the DG, we detected no difference in the LPS-induced pro-inflammatory response between males and females. However, there was a highly variable response to LPS across rats within the same treatment groups, with some animals having very little increase in pro-inflammatory gene production and others having a much more pronounced effect. This may be explained by non-overt differences in maternal care or environmental factors, such as prenatal exposure to stress, as these have been shown to affect the neuroimmune system[248,249]. Future studies should assess this possibility.

A peripheral injection of a low dose of LPS is expected to induce neuroinflammation indirectly given that it does not penetrate the CNS at significant levels[250]. LPS is known to increase both cytokine levels in the periphery and blood brain barrier permeability, potentially allowing cytokines from the periphery to enter the CNS[251,252]. Therefore, changes in CNS cytokine levels could be due in part to invasion of peripheral immune components, such as direct permeation by IL-1β or chemotactic macrophages. Additionally, peripheral immune system infiltration would likely activate neuroimmune cells and induce a local response. Importantly, with the exception of the female frontal cortex, our PAE paradigm did not alter the inflammatory response created by peripheral LPS injections, meaning that the pathway of activation is generally still intact.

Alcohol exposure during the third-trimester equivalent has been shown to cause neuroimmune activation in the hippocampus, including an increase in

several pro-inflammatory markers such as IL-1β[212]; however, BACs were more than twice as high as those used in the current study. Additionally, the route (i.e. intragastic gavage vs. vapor inhalation) and duration (acute vs. chronic) of alcohol administration could contribute to the observed discrepancies between studies.

With regard to the possibility of an anti-inflammatory response, we found that IL-10 expression was increased after LPS in the DG of male controls, but in the females this increase was only significant in the PAE group. Futhermore, there were no significant differences in IL-10 expression after LPS between the PAE and air control rats in either males or females. Thus it is likely that no difference in anti-inflammatory IL-10 expression exists between the two groups in this exposure paradigm. Our findings in the males are consistent with a previous study showing a similar fold increase in IL-10 two hours after LPS injection in the hippocampus of male rat pups[234]. It is unclear why IL-10 production after LPS injection did not also increase in the female rats. While the ability of IL-10 to block the production and function of many pro-inflammatory cytokines is well-established[253,254], it has also been suggested that pro-inflammatory cytokines may increase the expression of IL-10[255-257] acting as an inflammatory brake system. Additionally, IL-10 production has been shown to occur as a result of the activation of specific pathways, such as the p38 MAPK pathway, that are also associated with the production of pro-inflammatory cytokines, including IL-1β[257,258]. Therefore, we expected the increased pro-inflammatory cytokine expression to be accompanied by an increase in IL-10. Surprisingly this was not

typically the case, suggesting that our paradigm does not engage a strong anti-inflammatory response, at least at the time we performed our assays. This could be due, in part to the timing of our assay. We collected mRNA samples 2 hours after LPS expression, at which time pro-inflammatory cytokine expression is reliably increased. Conversely, IL-10 has been suggested to occur on a comparatively delayed timescale[254,257] and consequently it is possible that we missed the peak elevation in IL-10.

### 3.5.2 Alcohol exposure blunts the LPS-induced increase of IL-1β mRNA in the cortex of female rats.

In the cortex of control animals, LPS injections produced a significant increase in production of IL-1β and CCL2 mRNA in both males and females. This response was very similar to that seen in the DG, with a slightly larger increase in CCL2 mRNA than for IL-1β. However, in PAE female rats the LPS-induced production of IL-1β mRNA was significantly blunted. These findings are consistent with previous studies showing that alcohol exposure has region-specific, selective effects on particular cytokines[192]. While the IL-1β and CCL2 pathways are highly interconnected, the two have different functions. We consistently observed a more pronounced response in CCL2 than IL-1β, indicating that perhaps the subtle impact of PAE on this system only significantly affected the less robust IL-1β response to LPS.

In addition to blunting the response in a cytokine-selective manner, PAE also induced a gender-specific effect. As mentioned previously, the effects of PAE are sexually dimorphic and the development of the neuroimmune system is

different between males and females. Specifically, microglia in juvenile female rats exhibit a more activated morphology than juvenile male rats, are slower to colonize the postnatal brain, and males and females exhibit different gene expression profiles of inflammatory cytokines and chemokines[164]. A combination of these factors could contribute to sex-dependent differences in the effect of PAE observed in the current study. Finally, the effects being region-specific to the cortex may result in PAE offspring having problems with executive, higher functioning, as has been observed in patients with FASD[259]. These findings are also consistent with the literature on the effect of ethanol in the adult brain where region specific changes in neuroinflammation have been identified[192]. Finally, while no significant increase in IL-10 was observed after LPS injection in the frontal cortex of either male or female rats pups, in the female pups there was a strong trend toward decreased IL-10 expression, suggesting that the decrease in IL-1β is not due to an increased anti-inflammatory response, but may instead be due to a diminished overall neuroimmune response, which may be related to toxic effects of PAE on developing microglia[209].

Based on the literature and the idea of neuroimmune system “priming”, we hypothesized that PAE would increase the response to a second insult, eliciting an over-reaction. However, we observed instead that PAE had the opposite effect, blunting the neuroimmune response in the female frontal cortex. Therefore the PAE-induced weakening of the neuroimmune response, could leave the CNS more susceptible to damage by infection, as activation of this system plays an important role in the destruction of pathogenic agents, removal of damaged cells,

and stimulation of repair processes[84]. Further studies are needed to address these possibilities.

### 3.5.3 Alcohol exposure does not affect the LPS-induced increase in IL-1β and CCL2 mRNA in the cerebellar vermis.

In the cerebellar vermis, LPS injections elicited similar effects in control animals as seen in both the DG and frontal cortex. As expected, LPS induced a significant increase in mRNA expression of CCL2 and IL-1β in both males and females. This effect was not significantly altered by PAE for either sex. Additionally, IL-10 was not found to be significantly altered by LPS injections, suggesting that this anti-inflammatory pathway is not engaged in the cerebellar vermis in response to LPS injection, further highlighting the brain regional differences in neuroinflammation at this age. Previous studies utilizing a third trimester binge-like alcohol exposure paradigm have identified both increases in basal levels of pro-inflammatory markers[212] and microglial activation[209] in the cerebellum. However, these studies employed higher doses of alcohol yielding BACs of more than twice the peak BACs found here, and used different methods of administration than the one used in our study (gavage versus inhalation). Differences in paradigm likely play a role in the discrepancies observed in the current report.

### 3.5.4 Overall Conclusion.

To our knowledge, this is the first study of the impact of PAE on neuroimmune activation induced by LPS injection during the neonatal period. We found that, in most cases, PAE had very little effect on neuroimmune function as

in response to a second insult. Importantly, PAE selectively blunted the LPS-induced production of IL-1β mRNA in the cortex of female rats. Taken together with the collective findings regarding third trimester alcohol exposure and the neuroimmune system[220], our findings suggest that chronic exposure to lower levels of alcohol is less disruptive to the neuroimmune system than binge-like exposure to high doses of alcohol.

## 3.6 Acknowledgements

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# 4. Exposure of Neonatal Rats to Alcohol has Differential Effects on Cytokine Levels in the Cerebellum and Hippocampus: Potential Role of Microglia and Astrocytes

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## 4.1 Abstract

**Background:** Fetal alcohol exposure is a leading cause of preventable birth defects, yet drinking during pregnancy remains prevalent worldwide. Studies suggest that activation of the neuroimmune system plays a role in the effects of alcohol exposure during the rodent equivalent to the third trimester of human pregnancy (i.e., first week of neonatal life), particularly by contributing to neuronal loss. Here, we performed a comprehensive study investigating differences in the neuroimmune response in the cerebellum and hippocampus, which are important targets of third trimester-equivalent alcohol exposure.

**Methods:** To model heavy, binge-like alcohol exposure during this period, we exposed rats to alcohol vapor inhalation during postnatal days (P)3-5 (blood alcohol concentration = 0.5 g/dL). The cerebellar vermis and hippocampus of rat pups were analyzed for signs of glial cell activation and neuronal loss by immunohistochemistry at different developmental stages. Cytokine production was measured by reverse transcriptase polymerase chain reaction during peak blood alcohol concentration and withdrawal periods. Additionally, adolescent offspring were assessed for alterations in gait and spatial memory.

**Results:** We found that this paradigm causes Purkinje cell degeneration in the cerebellar vermis at P6 and P45; however, no signs of neuronal loss were found in the hippocampus. Significant increases in pro-inflammatory cytokines were observed in both brain regions during alcohol withdrawal periods. Although astrocyte activation occurred in both the hippocampus and cerebellar vermis, microglial activation was observed only in the latter.

**Conclusions:** These findings suggest that neuroinflammation following third trimester alcohol exposure is time- and brain region- dependent, and that astrocytes may play an important role in this response.

## 4.2 Background

Drinking during pregnancy can result in fetal alcohol spectrum disorders (FASDs), an umbrella term used to describe a wide array of teratogenic effects that occurs in an estimated 2-5% of live births in the United States[3]. FASD range from the most severe form, fetal alcohol syndrome, to less obvious neurocognitive deficits and behavioral abnormalities. Although the mechanisms underlying FASD are not fully understood, one of the most detrimental effects of alcohol exposure in the developing brain is neuronal loss[42,72,260]. Recently, activation of the neuroimmune system has been observed in parallel with neurodegeneration and this effect has been suggested to play a central role in the pathophysiology of FASD.

The neuroimmune system is comprised primarily of microglia and astrocytes, although microglia are much better characterized with respect to this system. Along with their critical functions in defending the CNS from damage, astrocytes and microglia have important roles during development, particularly in synaptic refinement[170,261]. Both cell types undergo maturation concurrent with the brain growth spurt[160,169], which occurs during the third trimester of human pregnancy and is equivalent to the early postnatal period in rodents, suggesting that these cells may be particularly vulnerable to insult during this time. While considerable research has been conducted into alcohol's neuroinflammatory effects in adolescents and adults[175,179,181-183,188,190,192,262,263], the unique neuroimmune response to alcohol during development has only begun to be investigated.

Research focused on prenatal alcohol exposure (i.e., first- and second-trimester-equivalent exposure) have indicated an increased number of microglia/macrophage throughout brain white matter[195] and in the cerebellum[196], and a reduced number of Bergmann glial cells[199]. Models utilizing cell culture or *in-vivo* prenatal exposure have shown that astrocytes can contribute to repair processes engaged in response to alcohol exposure, including promoting neuronal survival, dendritic outgrowth, and plasticity[203].

Additionally, studies in models of third trimester-equivalent alcohol exposure have demonstrated elevations in pro-inflammatory cytokines in multiple brain regions[208,210], which were long-lasting in some cases[212], and widespread signs of neuronal loss[42]. Moreover, blunting neuroinflammation correlated with decreased neuronal loss[209,210] and improved performance in hippocampal-dependent tasks[212]. Based on these results, it was concluded that neuroimmune activation contributes to neuronal loss across several brain regions. Additionally, while postnatal alcohol exposure (PAE) has been shown to activate microglia[209,211], a potential role for astrocytes in the neuroimmune response to PAE received little attention, despite the fact that astrocytes commonly secrete cytokines in response to CNS insults[264] and regulate microglial activation[139]. Furthermore, a more comprehensive analysis of the neuroimmune response to multiple alcohol exposures during development, including the individual effect of withdrawal periods, has yet to be undertaken.

In this study, we further characterized the effect of alcohol exposure during the third trimester-equivalent on the neuroimmune system. To investigate

the relationship between neuronal loss and neuroimmune activation, we utilized a paradigm designed to induce neurodegeneration and compared the respective neuroimmune responses in the cerebellum and hippocampus. We included end points collected during periods of both peak blood alcohol levels and withdrawal. These end points incorporate an investigation into both astrocyte and microglial activation with an in-depth analysis of the specific layers in which activation of these cells occurs, with respect to neuronal loss. In addition, we measured mRNA levels for both pro- and anti-inflammatory cytokines.

## 4.3 Materials and Methods

### 4.3.1 Animal treatments

Animal procedures were approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center. Time-pregnant Sprague-Dawley rats were obtained from Harlan Laboratories Inc. (Indianapolis, IN) and allowed to acclimate for one week before giving birth. Only male offspring were used for experiments, as developmental alcohol exposure has been shown to have sexually dimorphic effects[245-247] and microglial colonization of the brain is different in males and females[164]. Previous studies have demonstrated that binge-like exposure to high levels of alcohol is effective at inducing neuronal cell loss in the rodent brain[42]. Additionally, binge-like, heavy alcohol-exposure paradigms in the early postnatal period have been shown to be more effective in generating a robust neuroimmune response[209,210,212] when compared to more moderate, but long-term exposures[265]. Importantly, heavy alcohol exposure during late pregnancy has been documented in humans [4,244]. We chose to expose pups to higher levels of alcohol than those typically achieved in humans because the developing rodent brain is comparatively more resistant to alcohol than the human brain[16,17]. We used a vapor inhalation paradigm because it is less invasive than other methods, requires minimum pup handling, and allows pups to remain with their mothers throughout exposure.

Three days after litter birth, dams and their respective offspring were housed together in the vapor chamber apparatus. Litters were randomly assigned to either alcohol treatment or control. Starting at 10:00 am, litters were

exposed to alcohol via vapor inhalation for 4 hours daily during their light cycle, from postnatal day (P)3 through P5, a period of exposure that has previously been shown to activate the neuroimmune system[209]. Alcohol vapor levels were 8.03 g/dL ± 0.21 at 4 hours (*n* = 9 rounds of exposure). Control litters were housed in identical chambers that had only air flowing through them. Pups were handled daily only for weighing purposes. Blood alcohol concentrations (BACs) were determined using an alcohol dehydrogenase-based assay, as previously described[236]. On P4, BACs were measured at 0, 2, 4, 8, 12 and 21 hours after exposure began, with 0 hours being the time immediately preceding exposure. Maternal BACs were also measured in a separate group of dams subjected to the 4 hour exposure paradigm after pups had been weaned.

Nursing ability was assessed by counting the number of pups in each litter who had a visible presence of milk in their stomachs on the mornings after each exposure day. Finally, maternal care was assessed as previously described[266]. Briefly, on P4, starting at exposure hour 0, litters were filmed for 15 minutes out of every hour during the 4 hour exposure, plus 4 additional hours following exposure (8 total hours). Maternal behavior was scored by a blinded observer every 3 minutes during each 15 minute clip for the following actions: no contact with the pups, arched-back nursing, blanket nursing, passive nursing, licking pups, and licking pups during arched-back nursing.

### 4.3.2 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Samples were collected on P3 and P5 within 30 minutes following the 4 hour alcohol exposure, and during periods of alcohol withdrawal on P4 and P6. Additionally, to address the duration of effects, samples were collected at P11-13. Eight pups (from a total of 8 litters) were used per treatment group. Animals were anesthetized with ketamine (250 mg/kg). Whole hippocampi and cerebellar vermises were collected. Tissue was homogenized by sonication in ice cold RNeasy Lysis Buffer and RNA was extracted using an RNeasy Mini Kit according to the manufacturer's instructions (SABiosciences/Qiagen, Valencia, CA). The RNA was stored at -20 °C until use. cDNA was synthesized from 1 µg of RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, cat# 4368813, Foster City, CA) according to the manufacturer's instructions. The following primers from Qiagen were used: interleukin-1β (IL-1β, cat# PPR06480B), tumor necrosis factor α (TNFα, cat# PPR06411F), interleukin-10 (IL10, cat# PPR06479A), transforming growth factor β (TGFβ, cat# PPR06430B) and hypoxanthine phosphoribosyltransferase 1(HPRT1, cat# PPR42247F). Only samples with threshold cycle values (CT) over 35 were used for analysis. All values were normalized to HPRT1 via the ΔΔCT method. HPRT1 was not affected by PAE at any age in either the cerebellar vermis (interaction $F_{(4,70)}$ = 0.22, $p$ = 0.929; alcohol $F_{(1,70)}$ = 0.20; $p$ = 0.657; age $F_{(4,70)}$ = 2.50, $p$ = 0.050, $n$ = 8 pups from 8 litters) or the hippocampus (interaction $F_{(4,69)}$ = 0.36, $p$ = 0.836; alcohol $F_{(1,69)}$ = 0.003, $p$ = 0.956; age $F_{(4,69)}$ = 13.76, $p$ < 0.0001, $n$ = 7-8 pups from 7-8 litters). Then, for each cytokine the average value of the P3 air control

was computed and each individual data set was divided by this average, including the P3 air control that was used to compute the average.

### 4.3.3 Immunohistochemistry

On P4, P6, and P45, 4 pups (from a total of 4 litters) per treatment group were anesthetized by injection of ketamine (250 mg/kg). Animals were transcardially perfused first with phosphate buffered saline (PBS), pH 7.4, containing procaine hydrochloride (1 g/L) and heparin (1 USP unit/L) for 4 minutes, followed by ice cold 4% paraformaldehyde in PBS. Brains were removed by decapitation and placed in 4% paraformaldehyde for 48 hours at $4^{o}$C, then 30% sucrose for 24-48 hours at $4^{o}$C. Brains were embedded in Optimal Cutting Temperature compound (Fisher Healthcare, Houston, TX) and frozen before sectioning on a cryostat (Microm, model# HM 505E, Walldorf, Germany) at 16 µm. Sections for staining were incubated with PBS containing 1% bovine serum albumin, 0.2% Triton X-100, and 5% of either donkey or goat serum to match the host species of the secondary antibodies described below. Primary antibodies were applied overnight at 4 °C. Astrocytes were stained with rabbit anti-glial fibrillary acidic protein (GFAP, 1:500, Dako, ref# Z0334, Carpinteria, CA), microglia with rabbit anti-IBA-1 (1:500, Wako, ref#Z0334, Richmond, VA), Purkinje cells with mouse anti-Calbindin (1:500, Santa Cruz, cat# sc-70478, Dallas, TX), hippocampal neurons with mouse anti-NeuN (1:500, Millipore, cat# MAB377A5, Bedford, MA), neurofilament with mouse anti-neurofilament (1:500, Abcam, cat# ab24574, Cambridge, MA) and cell nuclei with 4',6-diamidino-2-phenylindole (DAPI, 1:1000, cat# D3571, Life Technologies, Carlsbad, CA).

Secondary antibodies were either donkey anti-rabbit or goat anti-mouse conjugated to Alexa 555 (1:1000, Invitrogen, Waltham, MA) and were applied at room temperature (20-22°C) for 2 hours.

All immunohistochemical analyses were performed by a blinded researcher using ImageJ (NIH, Bethesda, MD). At least two sections per animal, at least 5 sections apart, were averaged for each antibody. Sections were imaged on a Nikon TE2000 microscope (Nikon, Melville, NY) with a nuance spectral camera (Quorum, model# N-MSI-FX, Guelph, Ontario), which allows for elimination of background and low-intensity fluorescence as previously described [267]. Images were taken at 20X and 60X for all but GFAP intensity, which was imaged at 10X and 60X. Lobules of the cerebellar vermis have been shown to have different sensitivities to PAE [53] likely based on their rates of maturation. Therefore, lobules were grouped into three regions for quantification; lobules I-III, IV-VII, and IX-X. The hippocampus was divided by regions CA1, CA3, and the dentate gyrus (DG).

For measurements of intensity, the region of interest was traced and fluorescent intensity was quantified within. GFAP intensity was used to assess astrocytes[268] and staining for neurofilament was used to track Purkinje cell maturation during the postnatal period[269].

Microglia were stained with ionized calcium-binding adapter molecule 1 (IBA-1) and classified into one of four morphological stages, similar to as previously described[270,271]. In brief, cell types were categorized as either

resting/ramified (characterized by small cell body and long dendritic processes), transitional 1 (slightly swollen cell body with thicker, shorter processes), transitional 2 (large cell body with slight protrusion of processes) or amoeboid (large, round cell body with no processes and more intensely stained with IBA-1).

To quantify neuronal numbers, the thickness of the cell layer and/or the cell density was assessed. In the case of cell layer thickness, measurements were made randomly in three separate locations and averaged per image. The Purkinje cell layer (PCL) was traced in each image and the total area was quantified. Purkinje cells within the layer were then counted and displayed as cells per area, similar to as previously described[272]. In the hippocampus, because of the large number of cells, density of the granule cell layer (GCL) in the DG and the *stratum pyramidale* (SP) of the CA1 and CA3 were measured by placing three 30 $\mu m^2$ boxes at random in each region. The neurons within each box were counted and averaged.

### 4.3.4 Gait Analysis

Alterations in gait are associated with cerebellar impairment and particularly with Purkinje cell dysfunction[273-275]. Gait was assessed using the Catwalk XT system (Noldus, Wageningen, Netherlands) located at Animal Behavioral Core of the Biomedical Research and Integrative Neuroimaging Center (BRaIN), UNM-HSC; 2-3 animals per litter were used from a total of 4 litters per treatment group. Rats aged P45-50 were placed on an enclosed glass walkway illuminated from above by a red fluorescent light and along the walkway

by green LED lights. Disruption of the green light allowed for tracking of paw prints, while disruption of red light allowed for visualization of silhouettes. Rats were allowed to walk freely across the runway to a dark box, containing bedding from their home cage, located at the opposite end. A digital high-speed camera recorded each trial until three compliant trials were captured in which the animal did not stop or turn around and crossed the walkway within 0.5 - 10s with a maximum body speed variation of less than 60%. PAE significantly increased the total number of trials required to achieve 3 compliant trials, and these were 3.44 ± 0.41 and 6.44 ± 1.28 trials for the control and alcohol groups, respectively ($t_{(16)}$ = 2.23, $p$ = 0.041, by unpaired t-test). After each trial, the walkway was wiped down with 70% ethanol and deionized water. Catwalk XT 8.1 software (Noldus) was used to analyze the data. A detailed description of the measured parameters can be found in Table 4.2 (Additional File 3). For each animal, print area, average print intensity, base of support, support %, print position, phase dispersions, swing speed, cadence, and average speed were calculated, similar to as previously described[276]. Parameters were analyzed for the right forepaw (RF), right hind paw (RH), left forepaw (LF) and left hind paw (LH).

### 4.3.5 Contextual Fear Conditioning

Multiple pre-exposure contextual fear conditioning was used to test hippocampal-dependent behavioral differences. Pre-exposing animals to the context one day prior to shock conditioning, which has been shown to increase freezing, is impaired in a model of neonatal alcohol exposure[277]. Animals aged P36-48 were individually housed 1 day prior to testing. One animal per litter from

5-7 litters per treatment group was used. Between animals, the chamber was cleaned with ~5% ammonium chloride and left to dry for 2 minutes before the next animal was placed into the chamber. Testing took place over 3 days. On day 1, the animals were allowed to explore the chamber for 5 minutes. Then, the animals were removed from the chamber for 1 minute and then returned to the chamber for the next minute. This was repeated a total of 5 times before returning the animal to the home cage. On the second day, animals were placed into the chamber and received a 2s foot shock (1.5 mA) ~45-60 seconds later, after which they were placed back into their home cage. On the final day of testing, the animal was placed in the chamber for 5 minutes and total freezing time, defined as the body becoming immobile without any licking or grooming activity, was video-recorded and then scored by a blinded observer.

### 4.3.6 Statistical Analysis

All statistics were analyzed with GraphPad Prism Version 6.05 (GraphPad, San Diego, CA). Data were analyzed by student's unpaired *t*-test or two-way ANOVA followed by Bonferroni post hoc test. Significance was determined as $p < 0.05$. Data are shown as mean ± SEM.

## 4.4 Results

### 4.4.1 Characterization of the Exposure Paradigm

PAE significantly decreased pup body weight on P17 (Fig. 4.1a, two-way ANOVA, interaction $F_{(5,98)} = 4.00$, $p = 0.002$; age $F_{(5, 98)} = 153.2$, $p < 0.0001$; alcohol $F_{(1,98)} = 12.01$, $p = 0.0008$); Bonferroni post hoc test: $p = 0.0004$ on P17). Nursing, as assessed by the proportion of pups with milk in their stomachs, was not significantly affected by PAE (Fig. 4.1b, two-way ANOVA, interaction $F_{(3,36)} = 2.44$, $p = 0.080$; age $F_{(3,36)} = 2.15$, $p = 0.111$; alcohol $F_{(1,36)} = 0.73$, $p = 0.400$). Litter sizes were comparable between PAE and air control groups (Fig. 4.1c, $t_{(32)} = 1.19$, $p = 0.242$, unpaired t-test). In pups, BACs reached a peak of 121.1 ± 11.75 mM (0.56 ± 0.05 g/dL) at the end of exposure and returned to baseline by the following morning (Fig. 4.1d). Conversely, peak maternal BACs were much lower at 32.22 ± 8.49 mM (Fig. 4.1d). Measurements of maternal care were unaffected by alcohol exposure (Table 4.1).

**Table 4.1. Assessment of Maternal Care**

| Characterization | Air (% of total observation time | PAE (% of total observation time) | t | *p* value |
|---|---|---|---|---|
| No Contact with Pups | 23.98 ± 4.05 | 12.50 ± 8.41 | 1.23 | 0.242 |
| Licking Pups and Arched-Back Nursing | 7.10 ± 2.16 | 3.95 ± 2.54 | 0.94 | 0.365 |
| Arched-Back Nursing | 29.86 ± 7.00 | 26.02 ± 9.51 | 0.33 | 0.751 |
| Blanket Nursing | 23.90 ± 7.76 | 46.68 ± 11.18 | 1.67 | 0.120 |
| Passive Nursing | 7.80 ± 2.67 | 5.66 ± 2.91 | 0.54 | 0.597 |
| Licking Pups | 7.37 ± 1.24 | 5.19 ± 1.48 | 1.13 | 0.281 |

Maternal Care was assessed hourly throughout exposure and for several hours following exposure (as described in the methods). Key maternal behaviors were scored and displayed above. DF = 12, *n* = 7.

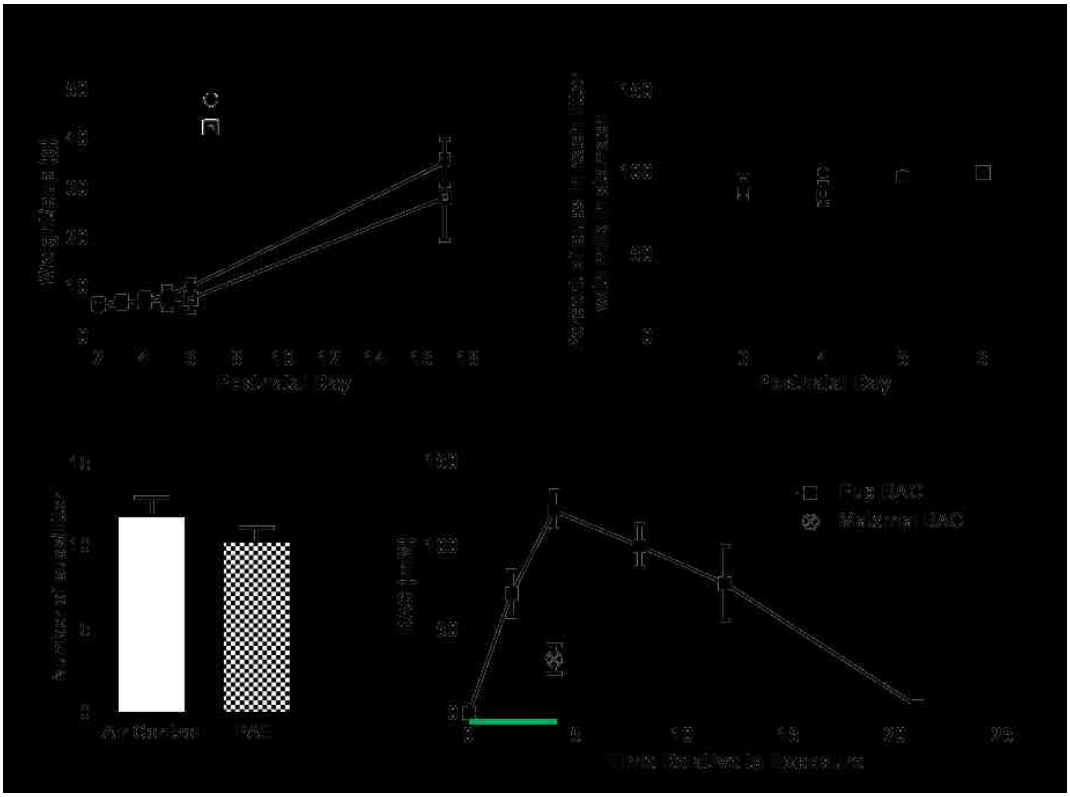

**Figure 4.1: Characterization of the Exposure Paradigm**

Pups were exposed to alcohol for 4 hours/day via vapor inhalation from P3 to P5. (A) Pups were weighed daily and the average weight per pup was recorded (*n* = 3-12 litters). (B) Nursing ability was quantified as the percentage of pups in each litter with milk visible in their stomachs each morning prior to exposure (*n* = 5-6 litters). (C) Similar litter sizes were assigned to each treatment group on P3 (*n* = 17 litters). (D) Pup blood alcohol concentrations (*n* = 4 pups from 4 litters) were each measured at the beginning (0 hours), middle (2 hours), and end (4 hours) of the exposure, as well as at several time points after. The green line represents the four hour exposure period. Peak maternal BAC was assessed at 4 hours (*n* = 3 dams exposed after pups had been weaned). (*** $p < 0.001$).

To determine if this PAE paradigm induced deficits in cerebellar-dependent behavior, rats were tested on a Catwalk apparatus and several parameters for gait were analyzed as described in Table 4.2/Additional file 3. Alterations in gait are associated with cerebellar impairment and particularly with Purkinje cell dysfunction [273-275]. PAE had significant effects on many aspects of gait (Table 4.3/Additional File 4, Fig. 4.2a-f), including phase dispersions

between the LF→ LH paws (Fig. 4.2b, $t_{(16)} = 2.67$, $p = 0.017$, unpaired t-test), the RF→ RH paws (Fig. 4.2f, $t_{(16)} = 4.35$, $p = 0.0005$, by unpaired t-test) and the LF→ RF paws (Fig. 4.2e, $t_{(16)} = 2.21$, $p = 0.042$, unpaired t-test), which are determinants of inter-limb coordination. Of note, print positions, on both the right (Fig. 4.2c, $t_{(16)} = 3.26$, $p = 0.005$, by unpaired t-test) and left (Fig. 4.2d, $t_{(16)} = 2.66$, $p = 0.017$, by unpaired t-test) sides of the body were affected after PAE. In a normal gait, the hind paw makes contact with the walkway in nearly the same location as the previous step of the forepaw on the same side of the body. In PAE animals, the distance between placement of these paws was noticeably increased (Fig. 4.2a). Spatial parameters related to individual paws such as print area and intensity were unaffected by PAE (Table 4.3/Additional File 4). Additionally, while PAE decreased the average speed of the animal, and the swing(s) of the LF, it had no other effects on temporal parameters (Table 4.3/Additional File 4).

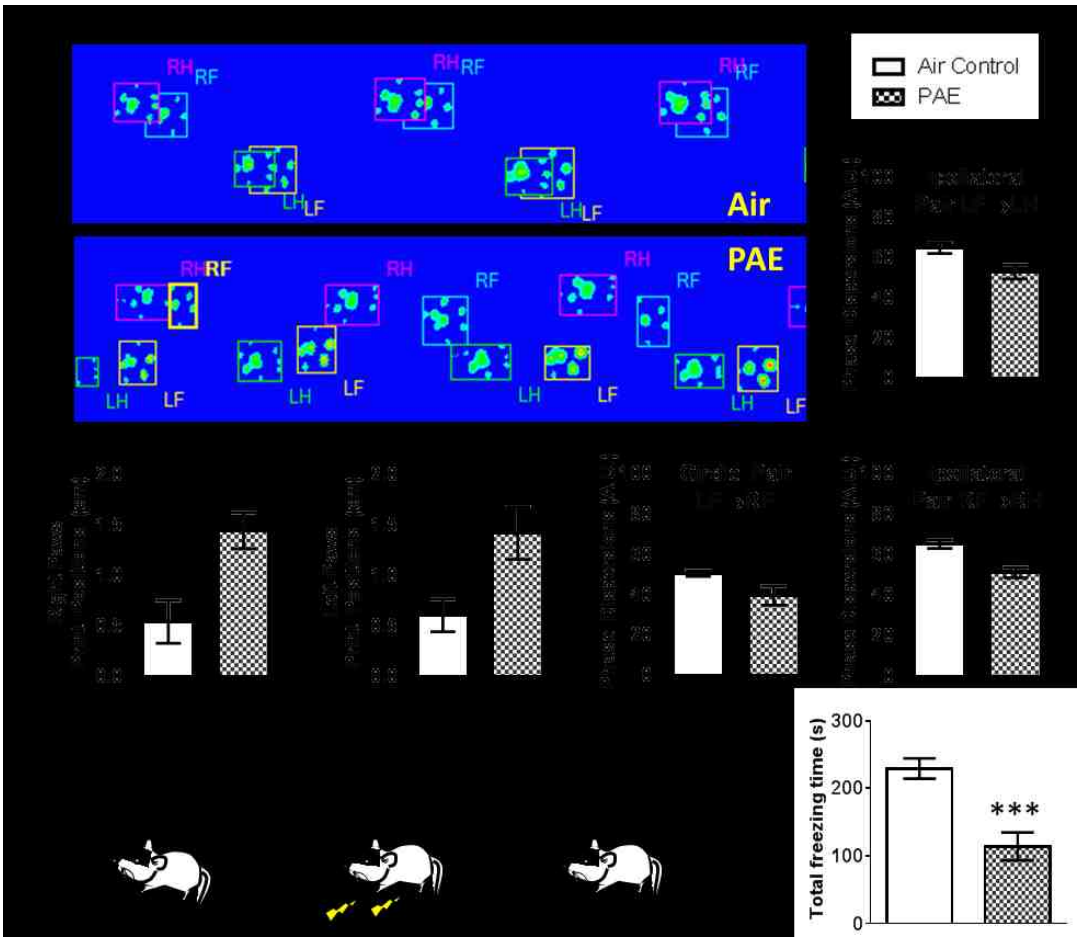


**Figure 4.2: Postnatal Alcohol Exposure (PAE) Induced Alterations in Gait and Deficits in Fear Conditioning**

(A-F) Animals aged P45-50 were placed on a Catwalk apparatus and differences in gait were assessed (*n* = 9 animals from 4 litters). (A) Representative Catwalk images are shown illustrating a PAE-induced reduction in overlap between the position of front and hind paws (LF =Left Forepaw, LH = Left Hindpaw, RF = Right Forepaw, RH = Right Hindpaw). Summary graphs illustrating the overall effect of PAE on the distance between placement of fore and hind paws on the right (B) and left (C) side of the body were measured. (D-F) Phase dispersions, which are indicators of inter-limb coordination, were measured between paws. (G-H) Animals aged P36-48 (*n* = 5-7 animals from 5-7 litters) were tested for performance on a variation of the fear conditioning test in which they were pre-exposed to the context the day before being re-exposed to the context and then shocked. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

To determine if PAE elicited any deficits in hippocampal-dependent behavior, performance in the multiple pre-exposure contextual fear conditioning test was assessed (Fig. 4.2g). PAE animals spent significantly less time freezing

in response to the context in which they had previously received a foot shock than control animals (Fig. 4.2h, $t_{(10)} = 4.64$, $p = 0.0009$, unpaired *t*-test).

### 4.4.2 PAE Decreases Neuronal Number in the Cerebellar Vermis

On the morning after the first exposure (P4), no effect of PAE was observed on Purkinje cell density across any of the lobule regions (Fig. 4.3a-b, two-way ANOVA, interaction $F_{(2,18)} = 0.15$, $p = 0.859$; lobule $F_{(2,18)} = 5.78$, $p = 0.012$; alcohol $F_{(1,18)} = 0.28$, $p = 0.602$). Similarly, thickness of the external granule layer (EGL) was unaffected by PAE (Figure 4.11a/Additional file 1a, two-way ANOVA, interaction $F_{(2,18)} = 0.21$, $p = 0.816$; lobule $F_{(2,18)} = 0.21$, $p = 0.031$; alcohol $F_{(1,18)} = 0.39$, $p = 0.538$) as well as the internal granule layer (IGL, Figure 4.11b/Additional file 1b, two-way ANOVA, interaction $F_{(2,18)} = 0.20$, $p = 0.820$; lobule $F_{(2,18)} = 2.32$, $p = 0.127$; alcohol $F_{(1,18)} = 1.93$, $p = 0.182$). However, at the end of exposure on P6, the alcohol exposed pups showed a dramatic reduction in the number of Purkinje neurons present in the cerebellar vermis in all lobule regions (Fig. 4.3c-d, two-way ANOVA, interaction $F_{(2,18)} = 0.83$, $p = 0.451$; lobule $F_{(2,18)} = 1.04$, $p = 0.375$; alcohol $F_{(1,18)} = 68.81$, $p < 0.0001$; Bonferroni post hoc test: I-III, $p < 0.0001$; IV-VIII, $p = 0.002$; IX-X, $p = 0.001$). There was also an overall decrease in thickness of the EGL (Figure 4.11c/Additional file 1c, two-way ANOVA, interaction $F_{(2,18)} = 0.26$, $p = 0.776$; lobule $F_{(2,18)} = 0.66$, $p = 0.527$; alcohol $F_{(1,18)} = 7.87$, $p = 0.012$), although there was no significant effect in individual lobule regions as measured by post-hoc analysis. Furthermore, IGL thickness was not affected (Figure 4.11d/Additional file 1d, two-way ANOVA,

interaction $F_{(2,18)} = 0.37$, $p = 0.694$; lobule $F_{(2,18)} = 1.101$, $p = 0.354$; alcohol $F_{(1,18)} = 0.01$, $p = 0.912$).

On P45, the number of Purkinje cells continued to be decreased in lobule regions I-III and IX-X, but not in regions IV-VII (Fig. 4.3e-f, two-way ANOVA, interaction $F_{(2,18)} = 9.24$, $p = 0.002$; lobule $F_{(2,18)} = 0.55$, $p = 0.586$; alcohol $F_{(1,18)} = 28.00$, $p < 0.0001$; Bonferroni post hoc test: I-III $p = 0.001$; IX-X $p = 0.0002$). These findings are consistent with previous studies showing lobules IV-VIII to be less sensitive to PAE [53]. Interestingly, granule cell layer thickness was no longer affected at P45 (Figure 4.11e/Additional file 1e, two-way ANOVA, interaction $F_{(2,18)} = 1.16$, $p = 0.337$; lobule $F_{(2,18)} = 1.664$, $p = 0.217$; alcohol $F_{(1,18)} = 0.08$, $p = 0.774$), indicating that PAE induces long-term selective loss of Purkinje neurons.

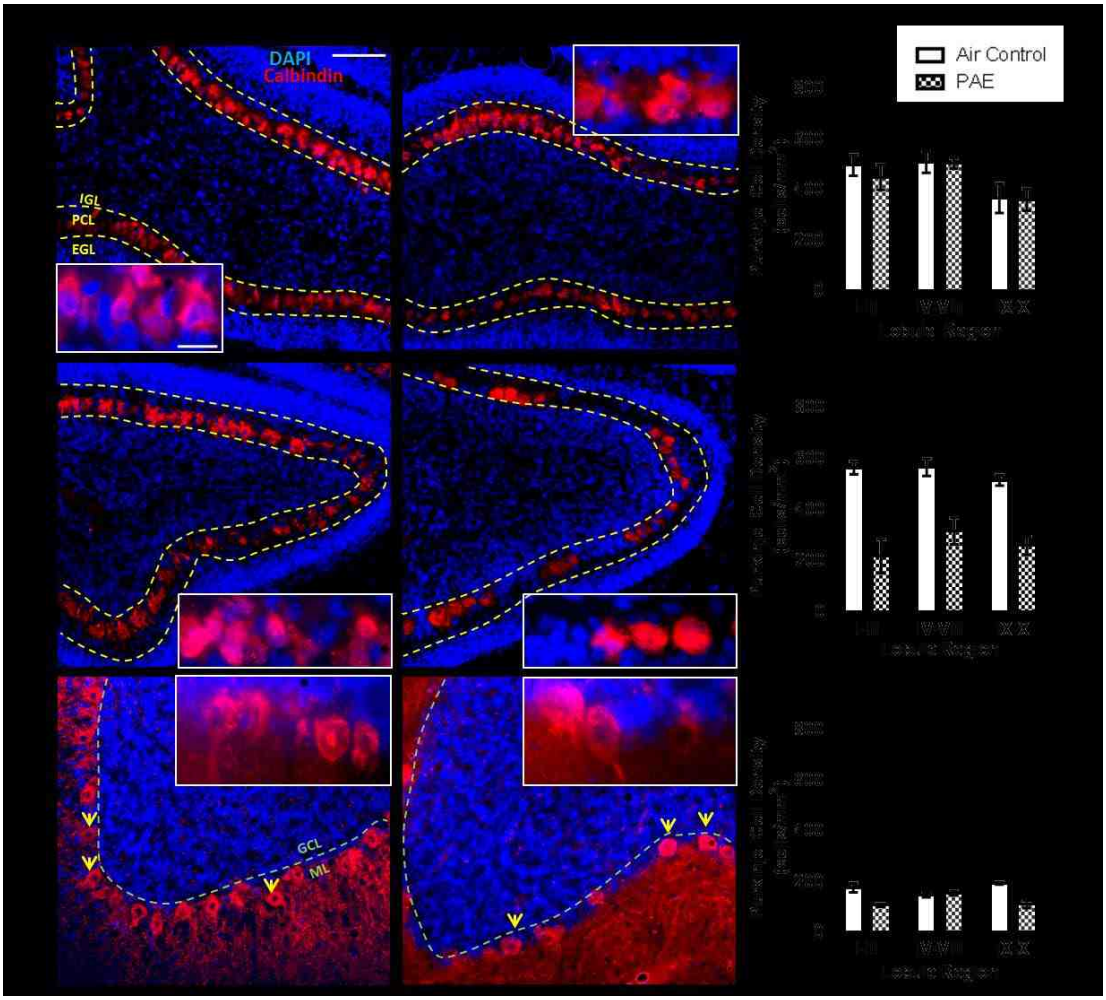


**Figure 4.3: Postnatal Alcohol Exposure (PAE) Reduced the Number of Purkinje Neurons in the Cerebellar Vermis**

Representative images of the cerebellar vermis stained for calbindin (red) to label Purkinje neurons and 4',6-diamidino-2-phenylindole (DAPI, blue) to label cell nuclei. Purkinje cell density was quantified during the first withdrawal period on P4 (A-B), the third withdrawal period on P6 (C-D) (see top panel in Fig 4.4) and also on P45 (E-F). The cerebellar vermis was divided into three lobule regions and quantified separately. The Purkinje cell layer (PCL) was traced as shown and the number of Purkinje cells within were counted. (** $p < 0.01$, **** $p < 0.0001$). $n = 4$ animals from 4 litters. The thickness of the external and internal granule cell layers (EGL and IGL, respectively) was also quantified and results are shown in Fig 4.11). Scale bars are 40 µm and 10 µm for low and high magnification images, respectively.

### 4.4.3 PAE Increases Cytokine Expression in the Cerebellar Vermis during Periods of Withdrawal

To determine how the neuroimmune response evolves as a function of multiple exposures, pro- and anti-inflammatory cytokine responses were

measured by RT-PCR throughout the exposure. PAE increased the expression of pro-inflammatory IL-1β during withdrawal periods on P4 and P6 (Fig. 4.4a, two-way ANOVA, interaction $F_{(4,70)} = 1.34$, $p = 0.265$; age $F_{(4,70)} = 4.72$, $p = 0.002$; alcohol $F_{(1,70)} = 20.65$, $p < 0.0001$; Bonferroni post hoc test for PAE: P4 $p = 0.045$; P6 $p = 0.002$). Similarly, TNFα expression was increased on P4 (Fig. 4.4b, two-way ANOVA, interaction $F_{(4,70)} = 5.70$, $p = 0.0005$; age $F_{(4,70)} = 5.66$, $p = 0.0005$; alcohol $F_{(1,70)} = 14.06$, $p = 0.0004$; Bonferroni post hoc test for PAE: P4 $p < 0.0001$). Additionally, PAE significantly increased overall expression of anti-inflammatory cytokines TGFβ (Fig. 4.4c, two way ANOVA, interaction $F_{(4,69)} = 0.95$, $p = 0.442$; age $F_{(4,69)} = 0.73$, $p = 0.577$; alcohol $F_{(1,69)} = 12.24$, $p = 0.0008$) and IL10 (Fig. 4.4d, two way ANOVA, interaction $F_{(4,70)} = 0.88$, $p = 0.481$; age $F_{(4,70)} = 2.71$, $p = 0.037$; alcohol $F_{(1,70)} = 4.17$, $p = 0.045$). However, expression was not found to be significantly elevated by post-hoc test on any individual postnatal day.

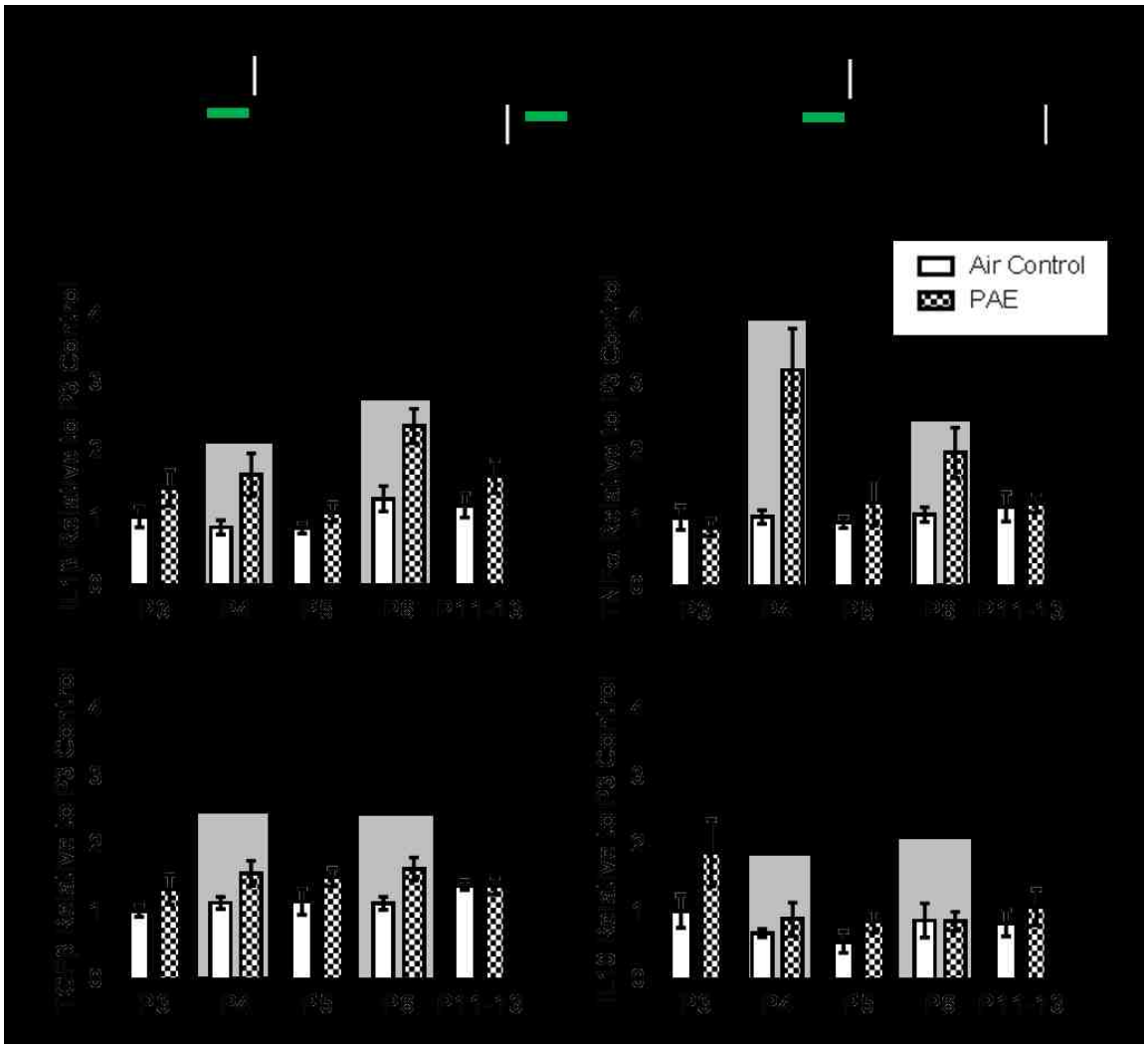


**Figure 4.4: Postnatal Alcohol Exposure (PAE) Increases Cytokine Expression in the Cerebellar Vermis**

Effect of PAE on interleukin-1β (IL-1β, A), tumor necrosis factor α (TNFα, B), transforming growth factor β (TGFβ, C) and interleukin-10 (IL10, D) in the cerebellar vermis. Animals were exposed to alcohol from P3-5 for 4 hours daily (10 AM to 2 PM, represented by green lines in top panel). Expression of cytokines was measured by RT-PCR throughout the exposure. Time points during peak blood alcohol levels were collected on P3 (I) and P5 (III). Time points during withdrawal periods were collected on P4 (II) and P6 (IV) and are also indicated by grey shading in panels A-D. For more details on the timecourse of the blood alcohol concentrations, please see Fig 4.1D. To address if effects were persistent, P11-13 was also included. Threshold cycle values (CT) were normalized to hypoxanthine phosphoribosyltransferase 1(HPRT1) and each individual value was normalized with respect to the average of the P3 air controls (see Materials and Methods). (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$). $n = 8$ animals from 8 litters.

### 4.4.4 PAE Induces Morphological Changes in Microglia and Increases Astrocytic GFAP Expression in the Cerebellar Vermis

To determine if the increase in cytokine levels is associated with alterations in glial cells, we stained for the microglia marker IBA-1 and the astrocyte marker GFAP. During the first withdrawal period (P4), the majority of microglia existed in a transitional state, with none exhibiting a ramified/resting morphology. However, microglia in PAE animals appeared very similar to those in controls (Fig. 4.5a-c). The proportion of microglia in each morphological state was not altered by PAE (Fig. 4.5b, two-way ANOVA, interaction $F_{(3,24)} = 2.0$, $p = 0.141$; morphology $F_{(3,24)} = 124.2$, $p < 0.0001$; alcohol $F_{(1,24)} = 9.17e\text{-}12$, $p > 0.9999$) and there was no increase in amoeboid morphology across the lobule regions (Fig. 4.5c, two-way ANOVA, interaction $F_{(2,18)} = 0.09$, $p = 0.911$; lobule $F_{(2,18)} = 0.57$, $p = 0.574$; alcohol $F_{(1,18)} = 1.53$, $p = 0.232$).

By the end of the exposure paradigm, during the third withdrawal period (P6), while most microglia in control animals continued to exhibit a transitional morphology, PAE animals had a significantly elevated presence of amoeboid microglia in the cerebellar vermis and a subsequent decrease in the number of microglia in the transitional 1 (T1) morphology (Fig. 4.5d, e, two-way ANOVA, interaction $F_{(3,24)} = 11.69$, $p < 0.0001$; morphology $F_{(3,24)} = 19.69$, $p < 0.0001$; alcohol $F_{(1,24)} = 1.81e\text{-}13$, $p > 0.999$; Bonferroni post hoc test: amoeboid cells $p = 0.0004$; T1 cells $p = 0.008$). Similarly, the proportion of microglia in the amoeboid morphology was significantly increased in lobule regions IV-VIII and IX-X, but not in lobule region I-III (Fig. 4.5f, two-way ANOVA, interaction $F_{(2,18)} = 0.73$, $p = 0.496$; lobule $F_{(2,18)} = 0.51$, $p = 0.609$; alcohol $F_{(1,18)} = 30.24$, $p < 0.0001$;

Bonferroni post hoc test: IV-VIII $p = 0.002$; IX-X $p = 0.024$). Since the majority of microglia were located within the center of the lobules, rather than in the PCL where neurodegeneration occurred, we used a neurofilament stain to investigate the possibility that amoeboid microglia were directly damaging neuronal axons which exist in this area in adult animals. However, neurofilament staining showed little presence of neuronal axons extending into this region by P6. Additionally, no difference was seen in the intensity of neurofilament staining between PAE and control animals in any lobule region (Figure 4.12/Additional file 2, two-way ANOVA, interaction $F_{(2,18)} = 0.34$, $p = 0.72$; lobule $F_{(2,18)} = 0.94$, $p = 0.410$; alcohol $F_{(1,18)} = 3.83$, $p = 0.066$)

To investigate if changes in microglial morphology persist, staining was also assessed on P45. By this age, nearly all microglia in both PAE and control animals existed in a resting state and there was no interaction between treatment and morphology (Fig. 4.5g, h, two-way ANOVA, interaction $F_{(3,24)} = 1.33$, $p = 0.289$; morphology $F_{(3,24)} = 10612$, $p < 0.0001$; alcohol $F_{(1,24)} = 1.87e\text{-}10$, $p > 0.999$). Similarly, there was no effect of PAE on the proportion of microglia in a resting morphology across lobule regions (Fig. 4.5i, two-way ANOVA, interaction $F_{(2,18)} = 0.47$, $p = 0.633$; lobule $F_{(2,18)} = 0.36$, $p = 0.706$; alcohol $F_{(1,18)} = 0.79$, $p = 0.385$).

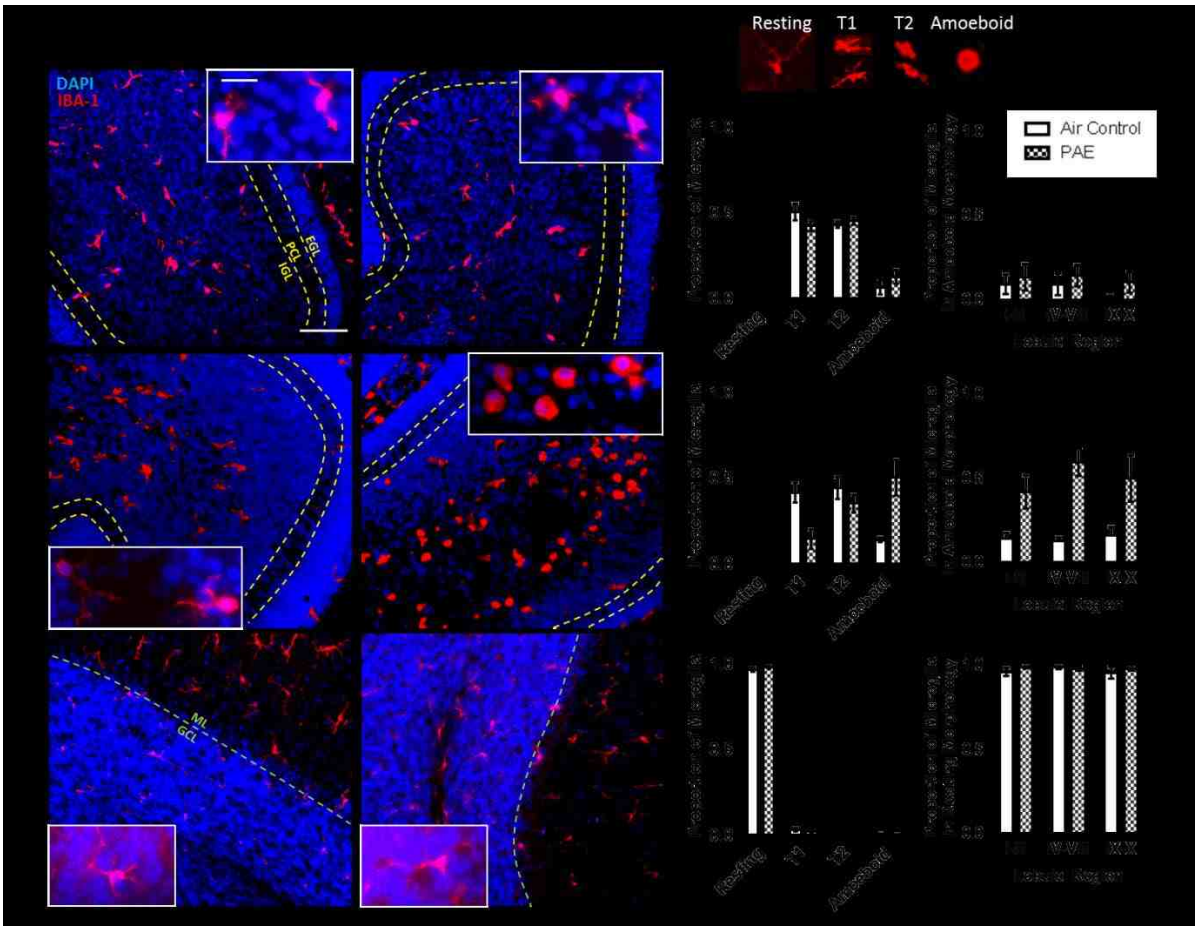


**Figure 4.5: Postnatal Alcohol Exposure (PAE) Activates Microglia in the Cerebellar Vermis**

Representative images of the cerebellar vermis are stained for ionized calcium-binding adapter protein molecule 1 (IBA-1, red) to label microglia and 4',6-diamidino-2-phenylindole (DAPI, blue) to label cell nuclei. Microglial morphology was quantified as resting, transitional 1 (T1), transitional 2 (T2), or amoeboid as shown in the top right panel. The proportion of microglia exhibiting each morphology was quantified during the first withdrawal period on P4 (A-B), the third withdrawal period on P6 (D-E) (see top panel in Fig. 4.4), and also on P45 (G-H). To investigate regional differences, the cerebellar vermis was divided into three lobule regions and the proportion of microglia in an amoeboid morphology was quantified in each region on P4 (C) and P6 (F). On P45 (I) the proportion of resting microglia was quantified by region as there were no amoeboid microglia. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). $n = 4$ animals from 4 litters. Scale bars are 40 µm and 10 µm for representative low and high magnification images, respectively. The yellow labels represent the external granule cell (EGL), Purkinje cell (PCL) and Internal Granule Cell (IGL) layers.

To examine astrocytes, changes in GFAP intensity were measured in the EGL, PCL, and IGL of the lobules. During the first withdrawal period (P4), PAE caused an overall significant increase in GFAP expression in the EGL (Fig. 4.6a,

b, two-way ANOVA, interaction $F_{(2,18)} = 0.05$, $p = 0.954$; lobule $F_{(2, 18)} = 3.70$, $p = 0.045$; alcohol $F_{(1,18)} = 15.59$, $p = 0.001$) and the PCL (Fig. 6a, c, two-way ANOVA, interaction $F_{(2,18)} = 0.21$, $p = 0.817$; lobule $F_{(2,18)} = 4.34$, $p = 0.029$; alcohol $F_{(1,18)} = 8.55$, $p = 0.009$) of the cerebellar vermis, but not in any individual lobule region by post hoc test. In the IGL, GFAP expression was significantly increased in lobule regions I-III (Fig. 4.6a, d, two-way ANOVA, interaction $F_{(2,18)} = 0.44$, $p = 0.649$; lobule $F_{(2,18)} = 1.87$, $p = 0.182$; alcohol $F_{(1,18)} = 14.21$, $p = 0.001$; Bonferroni post hoc test: $p = 0.039$).

By the end of the exposure paradigm, during the third withdrawal period (P6), post-hoc analysis showed that PAE increased astrocytic GFAP expression across all three lobule regions in the EGL (Fig. 4.6e, f, two-way ANOVA, interaction $F_{(2,18)} = 0.01$, $p = 0.987$; lobule $F_{(2,18)} = 0.06$, $p = 0.938$; alcohol $F_{(1,18)} = 25.61$, $p < 0.0001$; Bonferroni post hoc test: I-III $p = 0.022$; IV-VIII $p = 0.027$; IX-X $p = 0.035$), the PCL (Fig. 4.6e, g, two-way ANOVA, interaction $F_{(2,18)} = 0.21$, $p = 0.812$; lobule $F_{(2,18)} = 0.49$, $p = 0.620$; alcohol $F_{(1,18)} = 27.97$, $p < 0.0001$; Bonferroni post hoc test: I-III $p = 0.007$; IV-VIII $p = 0.025$; IX-X $p = 0.049$), and in the IGL (Fig. 4.6e, h, two-way ANOVA, interaction $F_{(2,18)} = 0.00$, $p = 0.998$; lobule $F_{(2,18)} = 0.22$, $p = 0.803$; alcohol $F_{(1,18)} = 37.33$, $p < 0.0001$; Bonferroni post hoc test: I-III $p = 0.008$; IV-VIII $p = 0.007$; IX-X $p = 0.007$). On P45, PAE no longer increased GFAP expression in either the ML (Fig. 4.6i, j, two-way ANOVA, interaction $F_{(2,18)} = 0.01$, $p = 0.988$; lobule $F_{(2,18)} = 0.12$, $p = 0.884$; alcohol $F_{(1,18)} = 2.83$, $p = 0.110$) or the GCL (Fig. 4.6i, k, two-way ANOVA, interaction $F_{(2,18)} = 0.16$, $p = 0.852$; lobule $F_{(2,18)} = 0.07$, $p = 0.934$; alcohol $F_{(1,18)} = 4.19$, $p = 0.056$).

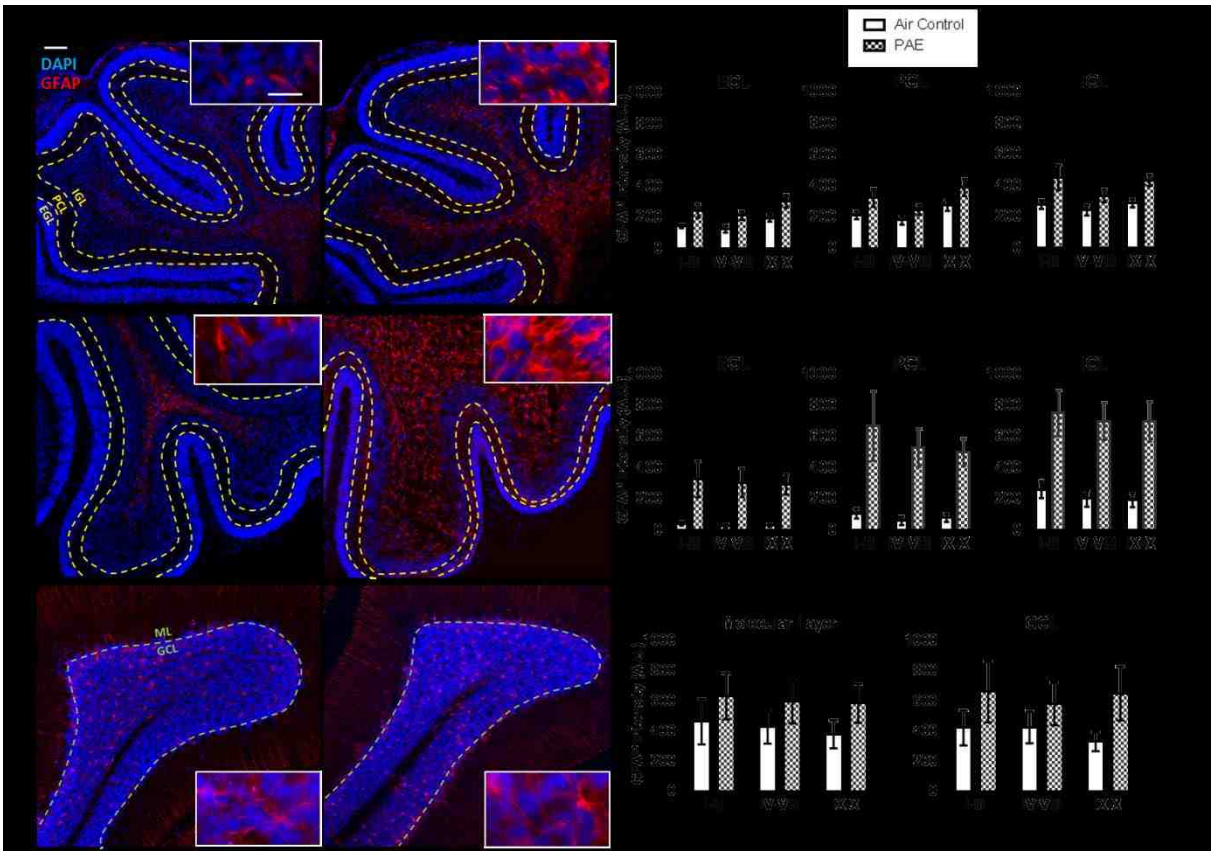


**Figure 4.6: Postnatal Alcohol Exposure (PAE) Increases Astrocytic Glial Fibrillary Acidic Protein (GFAP) Expression in the Cerebellar Vermis**

Representative images of the cerebellar vermis are stained for GFAP (red) to label astrocytes and 4',6-diamidino-2-phenylindole (DAPI, blue) to label cell nuclei. The cerebellar vermis was divided into three lobule regions and quantified separately during the first withdrawal period on P4 (A-D), the third withdrawal period on P6 (E-H) (see top panel in Fig. 4.4) and also on P45 (I-K). On P4 and P6 the external granule layer (EGL), Purkinje cell layer (PCL), and internal granule layer (IGL) of each lobule were traced as shown. On P45 the molecular layer (ML) and granule cell layer (GCL) of each lobule were traced (the EGL is no longer present at this age). GFAP intensity levels were measured within each layer. (* $p < 0.05$, ** $p < 0.01$). $n = 4$ animals from 4 litters. Scale bars are 40 µm and 10 µm for low and high magnification images, respectively.

### 4.4.5 PAE Does Not Reduce the Number of Granule Cells or Pyramidal Neurons in the Hippocampal Formation

To investigate potential regional differences in the effects of PAE, the hippocampal formation was also analyzed for neuronal loss in the GCL of the DG and in the pyramidal cell layer of the CA1 and CA3 subregions. During the first withdrawal period (P4), PAE animals exhibited no difference in neuronal

densities within the respective subregions (Fig. 4.7a, b, two-way ANOVA, interaction $F_{(2,18)} = 0.44$, $p = 0.650$; region $F_{(2,18)} = 28.28$, $p < 0.0001$; alcohol $F_{(1,18)} = 2.13$, $p = 0.162$) as well as similar cell layer thicknesses (Fig. 4.7a, c, two-way ANOVA, interaction $F_{(2,18)} = 1.21$, $p = 0.322$; region $F_{(2,18)} = 47.25$, $p < 0.0001$; alcohol $F_{(1,18)} = 1.66$, $p = 0.214$). Additionally, at the end of the exposure paradigm, during the third withdrawal period (P6), PAE animals showed no change in neuronal density in any subregion (Fig. 4.7d, e, two-way ANOVA, interaction $F_{(2,18)} = 0.00$, $p = 0.999$; region $F_{(2,18)} = 6.39$, $p = 0.008$; alcohol $F_{(1,18)} = 1.47$, $p = 0.240$) nor were there any differences in cell layer thicknesses (Fig. 4.7d, f, two-way ANOVA, interaction $F_{(2,18)} = 1.30$, $p = 0.297$; region $F_{(2,18)} = 68.30$, $p < 0.0001$; alcohol $F_{(1,18)} = 0.17$, $p = 0.685$).

Similarly, on P45 there was no significant change in either neuronal density (Fig. 4.7g, h, two-way ANOVA, interaction $F_{(2,18)} = 0.13$, $p = 0.878$; region $F_{(2,18)} = 12.73$, $p = 0.0004$; alcohol $F_{(1,18)} = 3.38$, $p = 0.082$) or cell layer thicknesses (Fig. 4.7g, i, two-way ANOVA, interaction $F_{(2,18)} = 2.48$, $p = 0.112$; region $F_{(2,18)} = 109.2$, $p < 0.0001$; alcohol $F_{(1,18)} = 0.32$, $p = 0.579$).

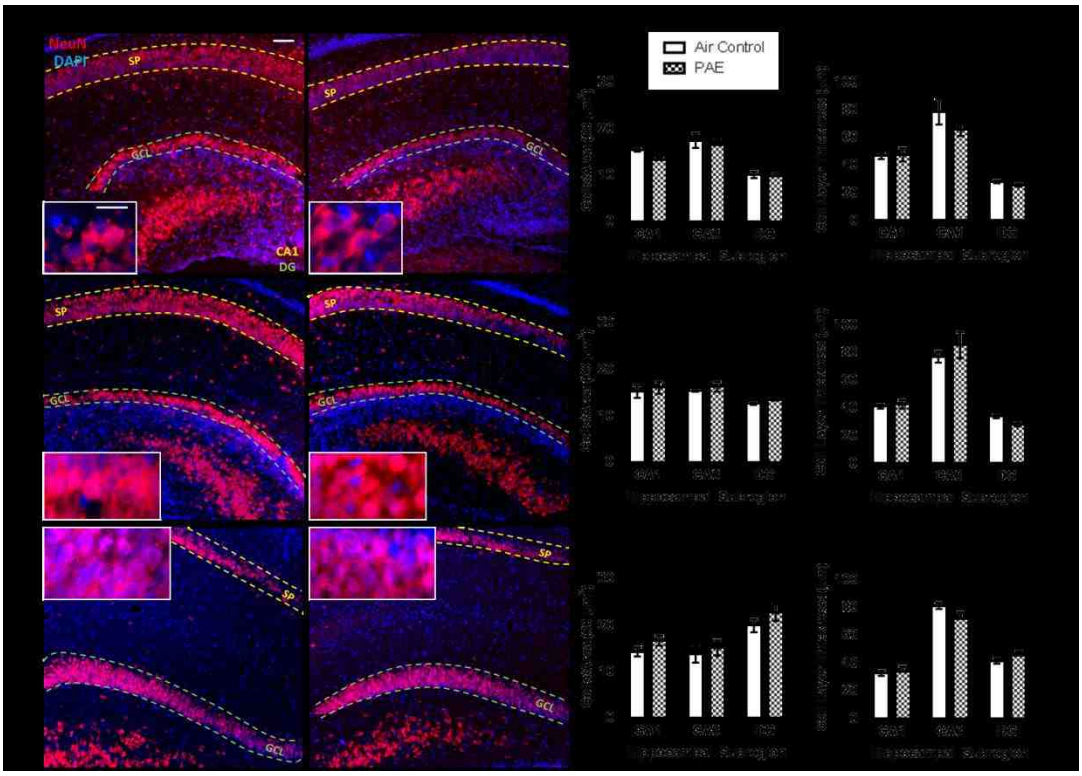


**Figure 4.7: Postnatal Alcohol Exposure (PAE) Does not Alter the Number of Pyramidal Neurons in the Hippocampus**

Representative images of the hippocampus are stained for NeuN (red) to label neurons and 4',6-diamidino-2-phenylindole (DAPI, blue) to label cell nuclei during the first withdrawal period on P4 (A-C), the third withdrawal period on P6 (D-F) (see top panel in Fig. 4) and also on P45 (G-I). Cells were counted within 30 $um^2$ areas in the *stratum pyramidale* (SP*)* layer of the CA3 and CA1, and in the granule cell layer (GCL) of the dentate gyrus (DG) subregion. Thickness of the cell layers was also measured. *n* = 4 animals from 4 litters. Scale bars are 40 µm and 10 µm for representative images and high magnification pictures, respectively.

### 4.4.6 PAE Increases Cytokine Expression in the Hippocampus

To compare the neuroimmune response in the hippocampus with the cerebellar vermis, pro- and anti-inflammatory cytokines were measured by RT-PCR throughout the exposure. PAE had no effect on the expression of pro-inflammatory IL-1β (Fig. 4.8a, two-way ANOVA, interaction $F_{(4,69)} = 1.55$, $p = 0.197$; age $F_{(4,69)} = 1.61$, $p = 0.183$; alcohol $F_{(1,69)} = 2.21$, $p = 0.142$).

Conversely, there was a significant interaction between PAE and TNFα expression, and post-hoc analysis revealed an increase during the first withdrawal period on P4 (Fig. 4.8b, two-way ANOVA, interaction $F_{(4,69)} = 3.30$, $p = 0.016$; age $F_{(4,69)} = 1.81$, $p = 0.137$; alcohol $F_{(1,69)} = 2.45$, $p = 0.122$; Bonferroni post hoc test: P4 $p = 0.005$). Consistent with findings in the cerebellum, TNFα expression was not significantly elevated by post-hoc analysis the following day during peak BAC (P5). PAE did not affect expression of anti-inflammatory TGFβ (Fig. 4.8c, two-way ANOVA, interaction $F_{(4,69)} = 1.78$, $p = 0.142$; age $F_{(4,69)} = 0.97$, $p = 0.431$; alcohol $F_{(1,69)} = 0.95$, $p = 0.334$). In contrast, PAE increased overall expression of anti-inflammatory IL10 (Fig. 4.8d, two-way ANOVA, interaction $F_{(4,69)} = 1.76$, $p = 0.147$; age $F_{(4,69)} = 2.03$, $p = 0.100$; alcohol $F_{(1,69)} = 6.00$, $p = 0.017$), but not on any individual day as assessed by post-hoc analysis.

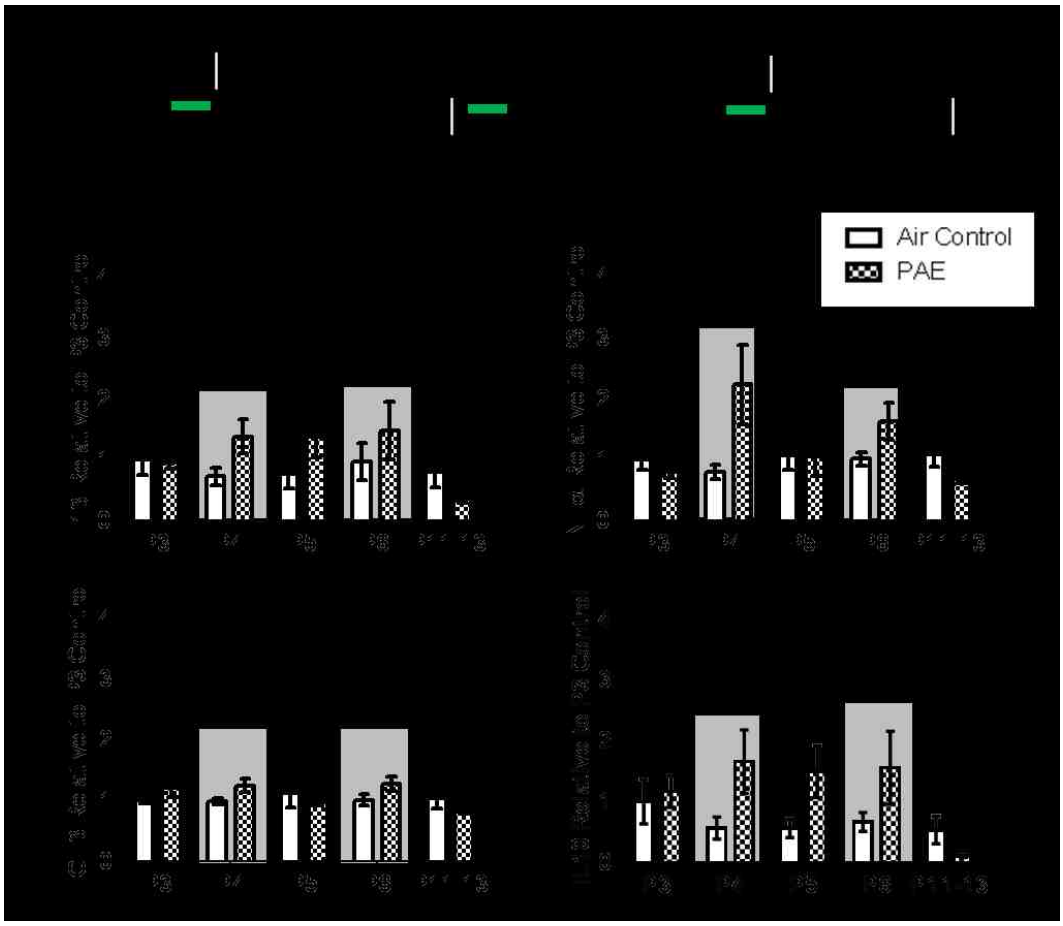


**Figure 4.8: Postnatal Alcohol Exposure (PAE) Increases Cytokine Expression in the Hippocampus**

Effect of PAE on interleukin-1β (IL-1β, A), tumor necrosis factor α (TNFα, B), transforming growth factor β (TGFβ, C) and interleukin-10 (IL10, D) in the hippocampus. Animals were exposed to alcohol from P3-5 for 4 hours daily (10 AM to 2 PM, represented by green lines). Expression of cytokines was measured by RT-PCR throughout the exposure. Time points during peak blood alcohol levels were collected on P3 (I) and P5 (III). Time points during withdrawal periods were collected on P4 (II) and P6 (IV) and are also indicated by grey shading in panels A-D. For more details on the timecourse of the blood alcohol concentrations, please see Fig. 4.1D. To address if effects were persistent, P11-13 was also included. Threshold cycle values (CT) were normalized to hypoxanthine phosphoribosyltransferase 1(HPRT1) and each individual value was normalized with respect to the average of the P3 air controls (see Materials and Methods). . (** $p < 0.01$). $n$ = 8 animals from 8 litters.

### 4.4.7 PAE Increases Astrocytic GFAP Expression in the Hippocampus

To assess morphological changes in glial cells in the hippocampus, astrocytes and microglia were stained with GFAP and IBA-1, respectively. During

the first withdrawal period (P4), the majority of microglia exhibited a T1 morphology and there was no interaction between PAE and morphology (Fig. 4.9a, two-way ANOVA, interaction $F_{(3,24)} = 0.16$, $p = 0.924$; morphology $F_{(3,24)} = 59.46$, $p < 0.0001$; alcohol $F_{(1,24)} = 0.0$, $p > 0.999$). By the third withdrawal period (P6), the majority of microglia in both groups exhibited a more mature, resting/ramified morphology, but there was a significant interaction between PAE and morphology, and post-hoc analysis revealed a decrease in the proportion of microglia in a resting morphology (Fig. 4.9b, two-way ANOVA, interaction $F_{(3,24)} = 4.21$, $p = 0.016$; morphology $F_{(3,24)} = 145.7$, $p < 0.0001$; alcohol $F_{(1,24)} = 2.87e-11$, $p > 0.999$; Bonferroni post hoc test: resting cells $p = 0.021$). However, PAE did not significantly increase the number of microglia in T1, T2, or amoeboid forms. Finally, on P45 no differences in microglial morphology were evident in the hippocampus of PAE animals (Fig. 4.9c, two-way ANOVA, interaction $F_{(3,24)} = 1.31$, $p = 0.295$; morphology $F_{(3,24)} = 35$, $p < 0.0001$; alcohol $F_{(1,24)} = 0.0$, $p > 0.999$) and nearly all microglia in both groups were in a resting morphological state.

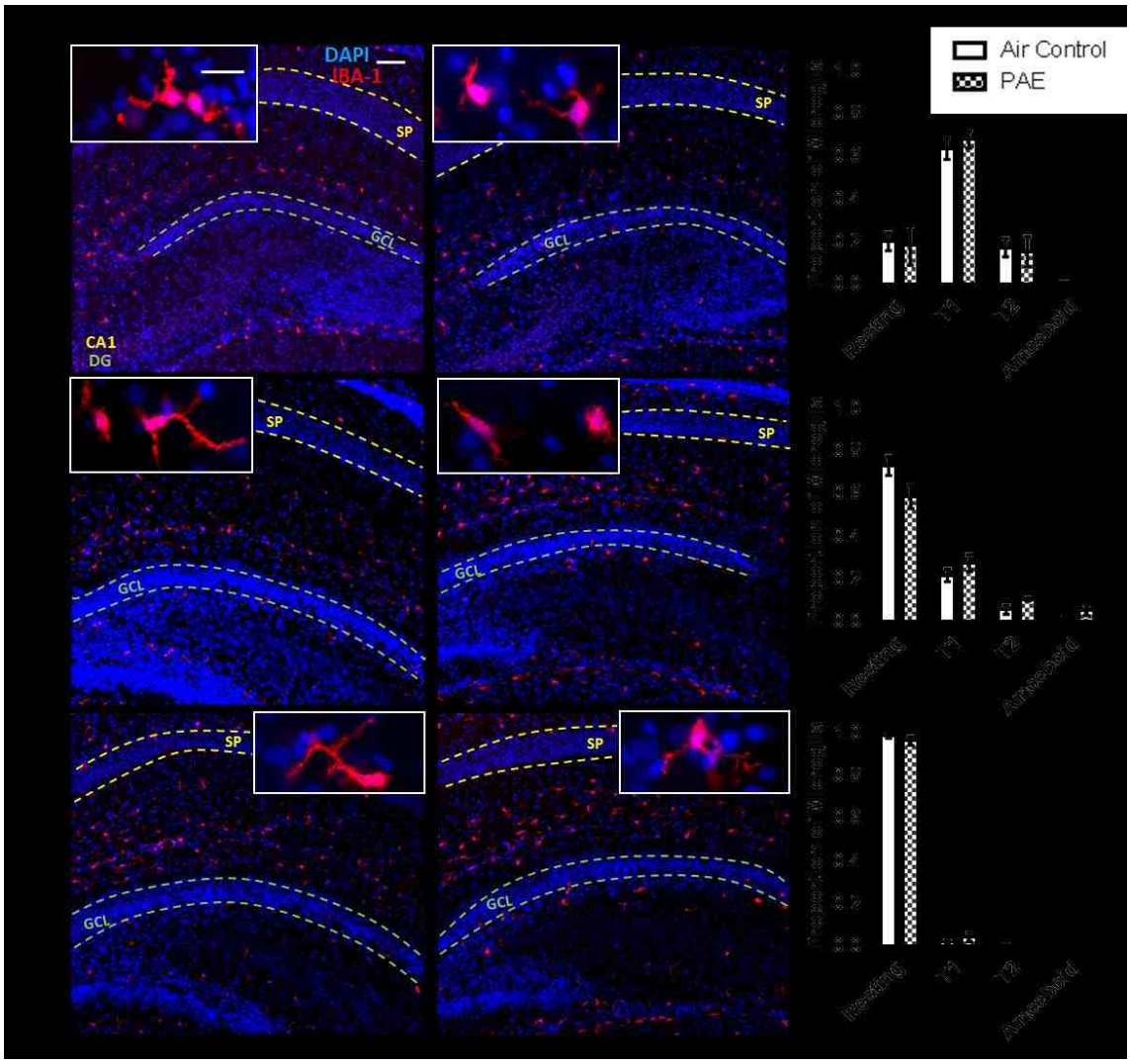


**Figure 4.9: Postnatal Alcohol Exposure (PAE) Decreases Resting Microglia on P6**

The effect of PAE on microglial morphology in the hippocampus during the first withdrawal period on P4 (A), the third withdrawal period on P6 (B) (see top panel in Fig. 4.4) and also on P45 (C). Representative images of the hippocampus are stained for ionized calcium-binding adapter protein molecule 1(IBA-1, red) to label microglia and 4',6-diamidino-2-phenylindole (DAPI, blue) to label cell nuclei. Microglial morphology was quantified as resting, transitional 1 (T1), transitional 2 (T2), or amoeboid as shown in Fig. 4.5. The proportion of microglia exhibiting each morphology was quantified. Since no significant differences were found in activation, morphology by region was not analyzed. Asterisks represent significance by post-hoc test. (* $p < 0.05$). $n = 4$ animals from 4 litters. Scale bars are 40 µm and 10 µm for low and high magnification images, respectively. *Stratum pyramidale* (SP*)* of the CA1 region and the granule cell layer (GCL) of the dentate gyrus (DG) are labeled in the images.

In the CA1 and CA3 subregions, changes in GFAP intensity were quantified within the *stratum oriens* (SO), *SP,* and either within the *stratum*

*radiatum* (SR), and *stratum lacunosum moleculare* (SLM) or the *stratum lucidum* (SL) and SR/SLM, respectively.. Similarly, the molecular layer (ML), GCL, and hilus were traced in the DG subregion and GFAP intensity was quantified. During the first withdrawal period (P4), PAE did not increase GFAP expression in the DG (Fig. 4.10a,b, two-way ANOVA, interaction $F_{(2,18)} = 0.28$, $p = 0.760$; layer $F_{(2,18)} = 1.17$, $p = 0.334$; alcohol $F_{(1,18)} = 1.16$, $p = 0.295$), CA3 (Fig. 4.10a,c, two-way ANOVA, interaction $F_{(3,24)} = 0.06$, $p = 0.979$; layer $F_{(3,24)} = 2.99$, $p = 0.051$; alcohol $F_{(1,24)} = 0.83$, $p = 0.371$), or the CA1 subregion (Fig. 4.10a, d, two-way ANOVA, interaction $F_{(3,24)} = 0.05$, $p = 0.986$; layer $F_{(3,24)} = 10.51$, $p = 0.0001$; alcohol $F_{(1,24)} = 0.81$, $p = 0.378$).

By the third withdrawal period on P6, astrocyte activation was evident in the DG subregion in the ML and the hilus (Fig. 4.10e, f, two-way ANOVA, interaction $F_{(2,18)} = 1.42$, $p = 0.267$; layer $F_{(2,18)} = 9.03$, $p = 0.002$; alcohol $F_{(1,18)} = 28.42$, $p < 0.0001$; Bonferroni post hoc test: ML $p = 0.002$; hilus $p = 0.014$). Similarly, the CA3 subregion exhibited increased GFAP expression in the SO, SL, and SR/SLM layers (Fig. 4.10e, g, two-way ANOVA, interaction $F_{(3,24)} = 0.89$, $p = 0.460$; layer $F_{(3,24)} = 17.76$, $p < 0.0001$; alcohol $F_{(1,24)} = 49.81$, $p < 0.0001$; Bonferroni post hoc test: SO $p = 0.006$; SL $p = 0.011$; SR/SLM $p = 0.0003$). Additionally, in the CA1, the SO, SR, and SLM exhibited increased GFAP expression (Fig. 4.10e, h, two-way ANOVA, interaction $F_{(3,24)} = 1.47$, $p = 0.247$; layer $F_{(3,24)} = 17.38$, $p < 0.0001$; alcohol $F_{(1,24)} = 44.65$, $p < 0.0001$; Bonferroni post hoc test: SO $p = 0.001$; SR $p = 0.048$; SLM $p = 0.0007$). Of note, the SP in

the CA3 and CA1 and the GCL in the DG did not show significant increases in GFAP expression.

On P45, there was a significant overall increase in GFAP expression in the DG (Fig. 4.10i, j, two-way ANOVA, interaction $F_{(2,18)} = 0.54$, $p = 0.591$; layer $F_{(2,18)} = 6.92$, $p = 0.006$; alcohol $F_{(1,18)} = 5.70$, $p = 0.028$) and CA3 (Fig. 4.10 i,k, two-way ANOVA, interaction $F_{(3,24)} = 0.11$, $p = 0.953$; layer $F_{(3,24)} = 0.84$, $p = 0.487$; alcohol $F_{(1,24)} = 11.35$, $p = 0.003$) subregions, although post-hoc analysis indicated that no individual layers exhibited a significant increase. Additionally, no increase was found in the CA1 subregion (Fig. 4.10i, l, two-way ANOVA, interaction $F_{(3,24)} = 0.26$, $p = 0.854$; layer $F_{(3,24)} = 3.10$, $p = 0.046$; alcohol $F_{(1,24)} = 1.73$, $p = 0.201$).

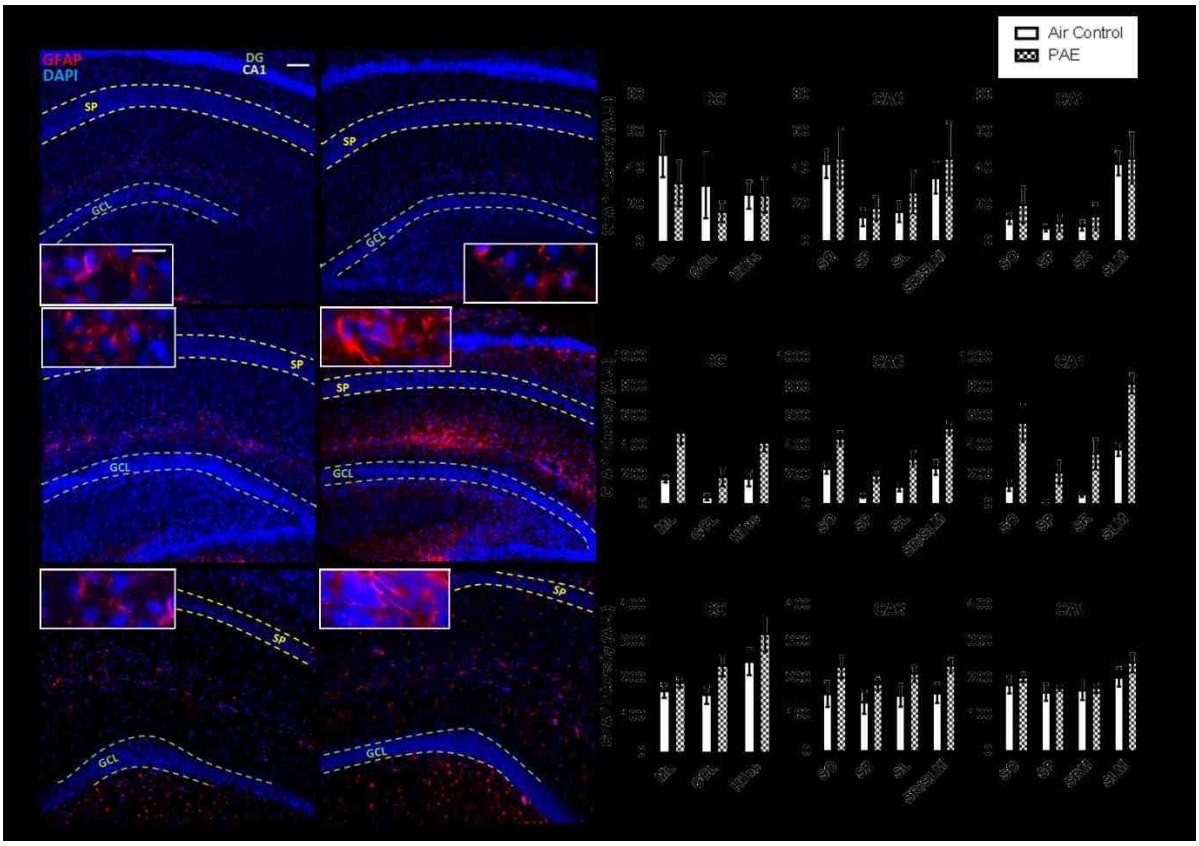


**Figure 4.10: Postnatal Alcohol Exposure (PAE) Increases Astrocytic Glial Fibrillary Acidic Protein (GFAP) Expression in the Hippocampus**

Representative images of the hippocampus are stained for GFAP (red) to label astrocytes and 4',6-diamidino-2-phenylindole (DAPI, blue) to label cell nuclei during the first withdrawal period on P4 (A-D), the third withdrawal period on P6 (E-H) (see top panel in Fig. 4.4), and also on P45 (I-L). The CA1 and CA3 subregions the *stratum oriens* (SO), *stratum pyramidale* (SP) and either the *stratum radiatum* (SR), and *stratum lacunosum moleculare* (SLM) layers or the *stratum lucidum* (SL), respectively, were traced and GFAP intensity was quantified within. In the dentate gyrus (DG), the molecular layer (ML), granule cell layer (GCL), and hilus were quantified. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). $n = 4$ animals from 4 litters. Scale bars are 40 µm and 10 µm for low and high magnification images, respectively. *SP* of the CA1 region and the GCL of the DG are labeled in the images.

## 4.5 Discussion

This study is the first to perform a comprehensive investigation of neuroinflammation throughout multiple alcohol exposure and withdrawal episodes during the third trimester-equivalent period of development. We report findings that complement earlier studies on the effects of PAE on the neuroimmune system. Specifically, we find vast differences in the hippocampus and cerebellar vermis with respect to cytokine production, neuronal loss, and glial cell activation. We report that signs of both microglial and astrocyte activation occurred in parallel with neurodegeneration in the cerebellar vermis and with gait disturbances, while in the hippocampus indications of astrocyte activation was evident in conjunction with spatial memory alterations. Intriguingly, at no measured point in the cerebellar vermis was there significant microglial presence within the Purkinje cell layer, where the majority of cell death transpired. Additionally, PAE increased cytokine expression in both brain regions, albeit to a lesser extent in the hippocampus. Interestingly, we find that, rather than building with subsequent exposures, cytokine production is transiently increased during withdrawal periods and a different response is elicited after one binge episode compared to three. The cytokines produced in this model were primarily pro-inflammatory, with only minor elevations in anti-inflammatory cytokines, and increases in either pathway were not long-lasting.

### 4.5.1 Increased mRNA Expression of Pro-inflammatory Cytokines Occurs in the Hippocampus and Cerebellar Vermis, but Only during Periods of Withdrawal

In the hippocampus and cerebellar vermis, we confirm that PAE stimulates production of pro-inflammatory cytokines. Surprisingly, cytokine production did not escalate as a function of exposure, but rather spiked during periods of withdrawal and dropped off when BACs were at peak levels at the end of 4 hour exposure periods. Withdrawal from alcohol is associated with processes known to stimulate neuroinflammation, such as excitotoxicity[278], prevention of which has been shown to alleviate behavioral deficits in a PAE model [279]. Furthermore, withdrawal from binge exposure increases the number of microglia in adult rat brains[280]. As such, it is likely that withdrawal, rather than alcohol itself, directly contributes to cytokine production in the developing brain, although we cannot discount the possibility that this is in part explained by a delayed onset of cytokine production induced by alcohol exposure.

Additionally, it has been suggested that anti-inflammatory cytokines contribute to the alcohol-induced neuroimmune response in adults[181]. To this end, we included anti-inflammatory cytokines in our analysis. While small increases were seen in TGFβ and IL10 in the cerebellar vermis and hippocampus, respectively, the majority of cytokine production was pro-inflammatory. However, anti-inflammatory cytokines could be responsible for the diminished pro-inflammatory response over the course of the paradigm as both TGFβ and IL10 can blunt the production of pro-inflammatory cytokines[116,281,282].

Our findings in the cerebellar vermis are consistent with previous observations that just one day of heavy PAE can increase levels of IL-1β in this region, and that multiple days of exposure amplifies this effect[210]. Interestingly, while we also found TNFα expression to be elevated during the first withdrawal period, levels were not significantly above those in control animals after three withdrawal periods. This suggests that, in this case, the IL-1β and TNFα activation pathways are differentially affected. It is also possible that if anti-inflammatory cytokines are influencing pro-inflammatory cytokine expression as described above, they could elicit a greater effect on TNFα expression.

While the exact mechanism by which alcohol activates the neuroimmune system is not fully understood, particularly in the context of developmental exposure, studies in adults have shown that alcohol likely activates microglia through toll-like receptor 4[185]. Moreover, alcohol exposure can increase secretion of the endogenous cytokine high mobility box 1 (HMGB1) by neurons and other cells[189]. HMGB1 then activates toll-like receptor 4, expressed on microglia, and induces cytokine secretion in a positive feedback loop. It is possible that the mechanism for microglial activation by alcohol in the developing brain is similar.

### 4.5.2 Regional Differences between the Hippocampus and Cerebellar Vermis may be a Result of the Window of Exposure

Specific brain regions are susceptible to alcohol-induced damage, particularly neuronal loss, only during limited developmental windows. In the cerebellum, Purkinje cell loss is associated with exposure on P5 or before, but not later[70,72,73], while the hippocampus is more resistant to cell loss during this

period[44,45]. Therefore, our exposure paradigm (P3-5) was expected to more heavily affect cells in the cerebellar vermis. In support of this, we saw no signs of neuronal loss in the hippocampus, but massive Purkinje cell loss in the cerebellar vermis on P6. This loss was still evident on P45, although to a lesser extent. There was also a decrease in Purkinje cell density on P45 compared to P4 and P6, likely due to the period of programmed cell death that occurs in the early postnatal period[283], as well as the expanding overall area of the cerebellum during development.

In the hippocampus, pro-inflammatory cytokine production was not as robust as in the cerebellar vermis. Of the cytokines measured, TNFα was the only one significantly increased on a specific day of the paradigm, and only in the first withdrawal period. These observations challenge previous findings showing dramatically escalated levels of both pro- and anti-inflammatory cytokines in the hippocampus several weeks after exposure[212]. They also contrast with studies finding elevated levels of both TNFα and IL-1β in the hippocampus during withdrawal, 24 hours after a P4-9 PAE paradigm[208]. However, in both cases these discrepancies could be explained by differences in the method of administration (oral gavage versus vapor inhalation), rodent strain or type, the kind of sample collected (mRNA versus protein), and, in particular, the longer duration of the exposure paradigm. While our study specifically included a time window during which alcohol exposure more highly affects the cerebellum, the hippocampus has been shown to be more vulnerable to alcohol at a slightly later

postnatal time point[42,277,284,285], which overlap with the exposure paradigms used in the studies described above.

Another important aspect of the timing of alcohol exposure, as it pertains to regional susceptibility, is the maturation rate of the neuroimmune system. During the early postnatal period, microglia mature faster from an amoeboid (activated) state to a branched (resting) morphological state in the hippocampus than in the cerebellum[166]. Indeed, we found that microglia in the hippocampus exhibited a more mature phenotype on P4 and P6 than those in the cerebellar vermis. Microglia that are amoeboid or in the early stages of transition could be primed to overreact to insult[163], explaining the alcohol-induced increase in cytokine production in the cerebellar vermis, when compared to the hippocampus. Additionally, in agreement with previous studies[208,209], by the end of our PAE paradigm, we observed a dramatic increase in the proportion of microglia in an amoeboid morphology in the cerebellar vermis, consistent with microglia activation. Conversely, we saw little sign that microglia were transitioning to a morphology associated with activation within the hippocampus. This is in contrast with observations in other models of PAE utilizing a longer exposure window that overlaps with hippocampal vulnerability[208]. Again, this is likely due to temporal differences in regional susceptibility and the maturation rate of microglia. These findings highlight the importance of considering the window of exposure when anticipating the effects of PAE on the neuroimmune system.

### 4.5.3 PAE-Induced Increases in Astrocytic GFAP Expression Occur with or without Microglial Morphological Transitions: Possible Relationships to Neurodegeneration

As stated above, neuronal loss was seen with this paradigm in the cerebellar vermis, but not in the hippocampus. Interestingly, an increase in amoeboid microglia occurred only in the cerebellar vermis, while robust elevations in astrocytic GFAP expression was observed in both brain regions. While microglial morphological transitions and increased astrocytic GFAP expression do not always equate to activation, they provide considerable evidence of neuroinflammation and are highly associated with reactive glial cell functions[78,286-288]. Based on this, our findings support the idea that microglial activation is strongly associated with PAE-induced neuronal loss; however, whether microglial activation contributes to or is caused by it remains unclear. In support of the latter, there was little presence of microglia, amoeboid or otherwise, in or near the cell layer in which neurodegeneration occurred. However, it is possible that secreted factors, such as cytokines, could diffuse from a distance to directly affect Purkinje cell soma or damage the axons of these neurons, ultimately leading to neurodegeneration. Cytokine secretion did precede neuronal loss in the cerebellar vermis, although there were no signs of neuronal loss in the hippocampus, which also had significantly elevated levels of TNFα on P4.

Similarly reactive astrocytes were observed alongside neuronal loss and microglial morphological changes in the cerebellar vermis. However, unlike microglia, there was a robust increase in astrocytic GFAP expression in the PCL,

where neurodegeneration occurred. Although this increase was not markedly higher than in the other cell layers. Importantly, signs of astrocyte activation were also evident in the hippocampus, where there was no neuronal loss and little indication of microglial activation. In this brain region, the increase in astrocytic GFAP expression was lowest surrounding neuron somata. Taken together, these findings indicate that astrocytes contribute to the effects of PAE in a brain region-specific manner.

Astrocyte activation varies heavily based on the type of insult and neighboring environmental cues[137]. As such, it is likely that astrocytes in the hippocampus and cerebellar vermis have distinct functions. In some cases, astrocytes can be heavily anti-inflammatory, can restrict migration of local and peripheral inflammatory cells, and can secrete growth factors. Conversely, astrocytes can also have pro-inflammatory functions including secretion of IL-1β and TNFα[137]. Moreover, astrocytes have been shown to regulate microglial activation[139]. It is possible that astrocyte activation in the hippocampus prevents microglial activation and subsequent neuronal loss. Indeed, astrocytes have been shown to have many positive effects on neurons after alcohol exposure in cell culture and *in-vivo* prenatal models[289-291]. For instance, as astrocytes are often strongly associated with increased neuronal survival[292,293], they could also be directly preventing neuronal loss.

Finally, we cannot discount the possibility that there is some contribution by invading peripheral immune cells, such as macrophages. Astrocyte activation, as seen in this model, can be indicative of astrocytic scars, which can block

migration of peripheral immune cells[137]. However, the blood brain barrier is not fully formed during the early postnatal period[294] making it easier for peripheral cells to infiltrate the brain. Additionally, increased levels of pro-inflammatory cytokines, such as those seen in this model, can recruit peripheral immune cells[295] and future studies should address this possibility.

### 4.5.4 PAE Results in Deficits in both Hippocampal and Cerebellar-Dependent Behavior

PAE animals exhibited deficits in contextual fear conditioning, which is hippocampal dependent[296,297]. Although we did not see signs of neuronal loss in the hippocampus, previous studies have identified several other mechanisms of PAE-induced damage in this brain region, including alterations in synaptic plasticity and transmission[35,243], which could contribute to the observed deficit. Interestingly, both of these processes have been shown to be affected by TNFα[298-300] which we found to be up-regulated in the hippocampus of PAE rats. Therefore, neuroinflammation could be causing hippocampal-dependent deficits through more subtle alterations, rather than through cell loss.

Additionally, PAE animals had significant alterations in gait. While gait has been shown to be highly dependent on the cerebellum and particularly on Purkinje cells[274,275], it is also affected in other types of neurodegenerative diseases that have more widespread effects in the brain[276,301,302]. Therefore, while it is likely that PAE-induced Purkinje cell loss heavily impacts gait in these animals, this paradigm may also cause damage to other structures, such as descending spinal pathways or motor cortex, which could further contribute to

alterations in gait. Whether or not neuroinflammation is directly contributing to these deficits remains unclear as the nature of the relationship between neuroimmune activation and neuronal damage/loss requires further research.

### 4.5.5 Overall Conclusions

In summary, this study provides information that fills the following gaps in knowledge within this field: 1) that PAE induces neurodegeneration, neurobehavioral deficits, and neuroimmune activation in a region- and time-specific manner rather than globally; 2) that cytokine secretion in response to PAE is primarily pro-inflammatory and transient, mainly occurring during alcohol withdrawal periods; and 3) that not only microglia, but also astrocytes, are important participants in the neuroinflammatory response to developmental alcohol exposure. Future work should investigate the mechanisms underlying these region- and time-specific effects of PAE, as well as the potential utility of anti-inflammatory agents in the treatment of FASDs.

## 4.6 Acknowledgements

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## 4.7 Additional Files

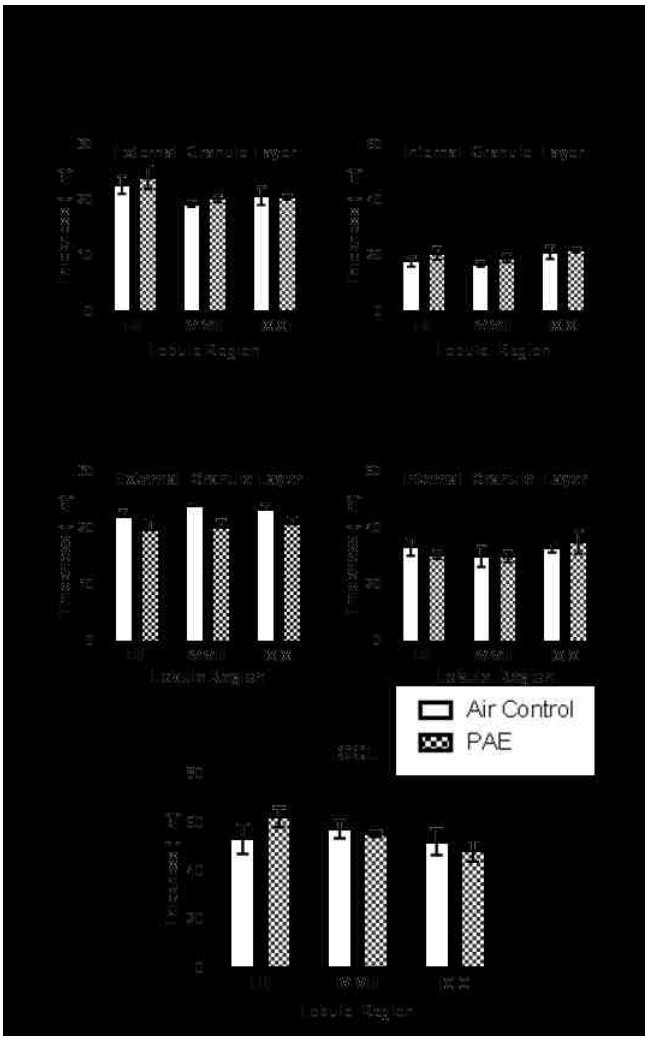


**Figure 4.11: (Additional File 1) Postnatal Alcohol Exposure (PAE) does not Affect Granule Cell Layer Thickness in the Cerebellar Vermis**

Effect of PAE on external granule layer (EGL) and internal granule layer (IGL) thickness in the cerebellar vermis during the first withdrawal period on P4 (a-b), the third withdrawal period on P6 (c-d) (see top panel in Fig. 4.4) and also on P45 (e). Animals were treated as described in Fig. 4.1. To investigate regional difference, lobules I-X of the cerebellar vermis were grouped into three lobule regions and quantified separately. For sample images see Fig. 4.3. *n* = 4 animals from 4 litters.

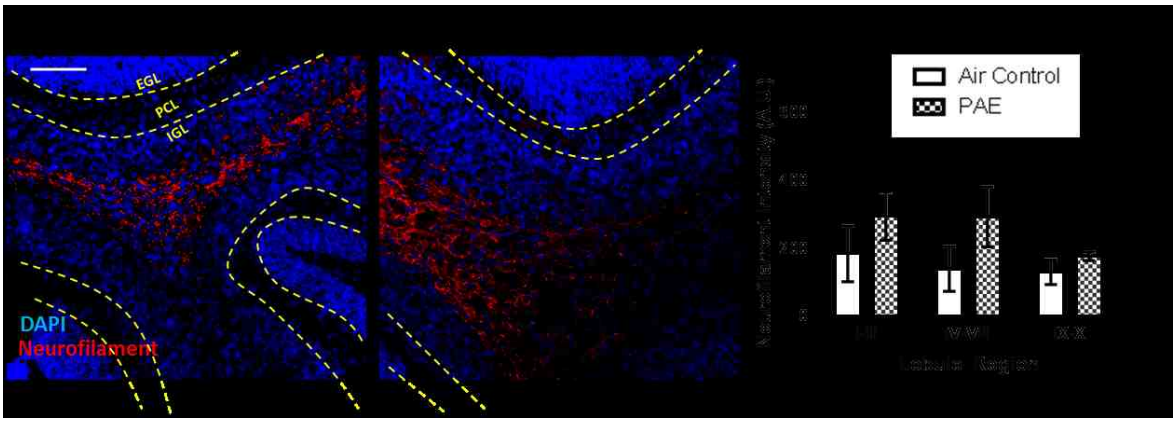


**Figure 4.12: (Additional File 2) Postnatal Alcohol Exposure (PAE) does not Affect Neurofilament Levels in the Cerebellar Vermis**

Effect of PAE on neurofilament in the cerebellar vermis during the third withdrawal period on P6 (see top panel in Fig. 4.4). Animals were treated as described in Fig. 4.1. Representative images of the cerebellar vermis are stained for neurofilament (red) to label axons and 4',6-diamidino-2-phenylindole (DAPI, blue) to label cell nuclei. To investigate regional difference, lobules I-X of the cerebellar vermis were grouped into three lobule regions and neurofilament intensity was quantified separately. *n* = 4 animals from 4 litters. Scale bar = 40 µm.

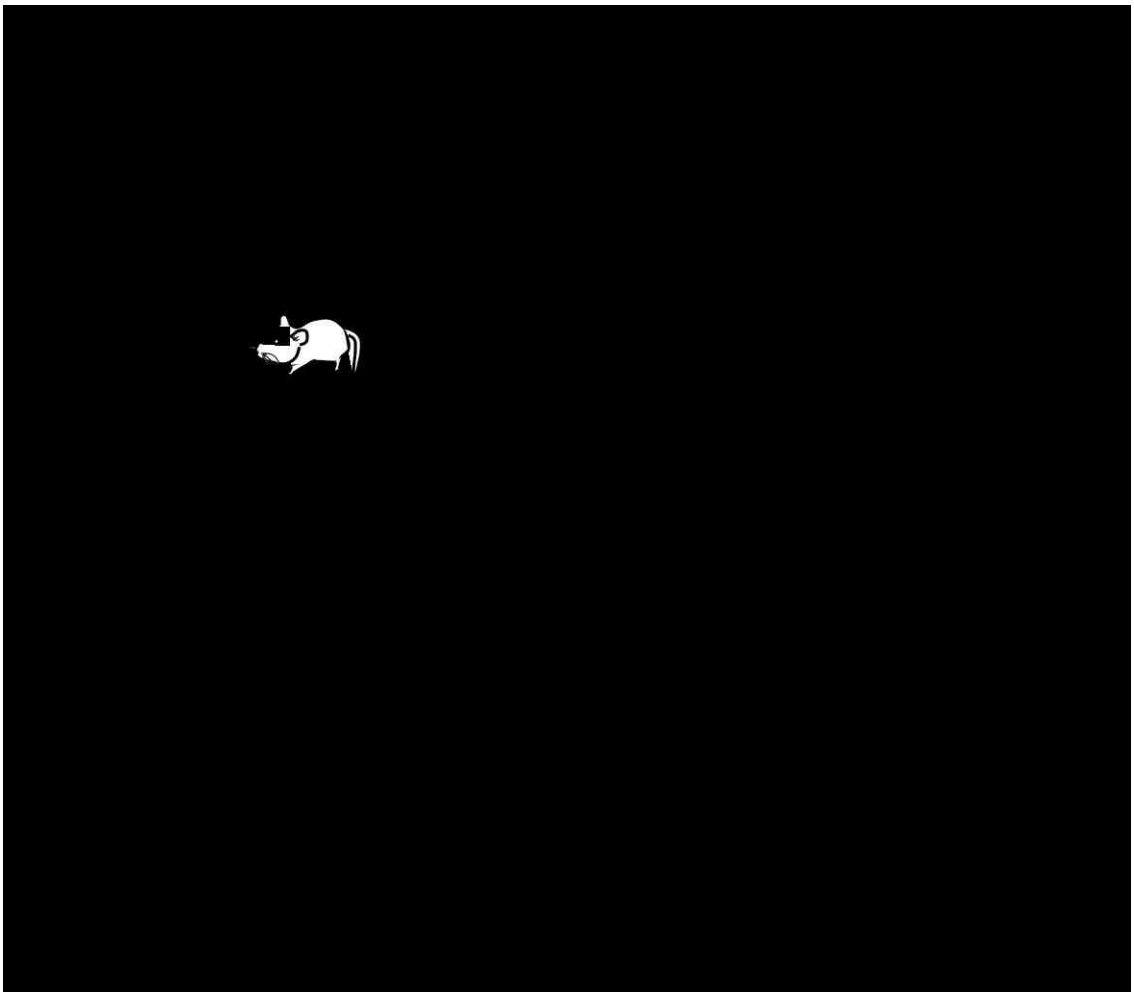

**Table 4.2: (Additional File 3) Table providing a description of the parameters used in gait assessment.**

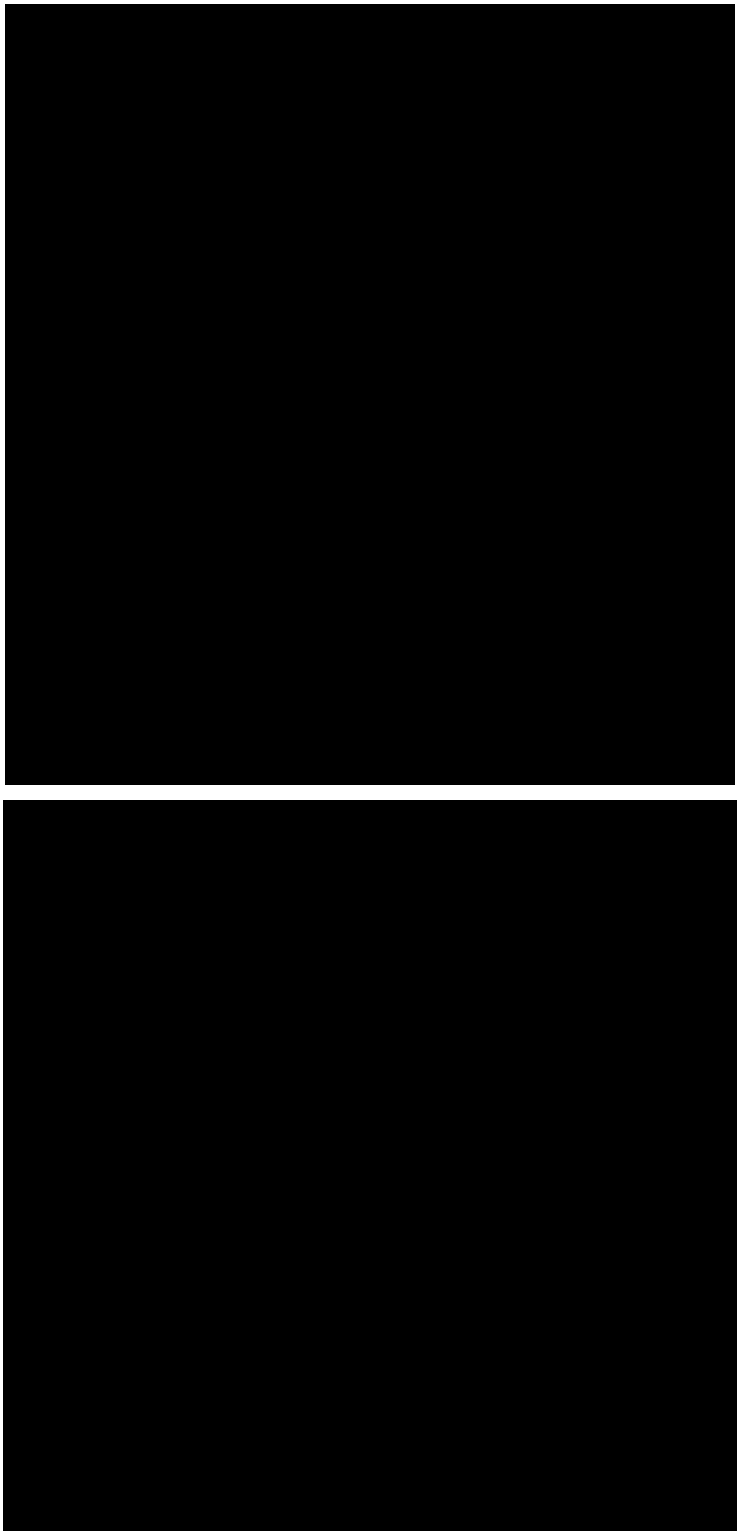

**Table 4.3: (Additional File 4) Table providing additional measurements of gait in PAE and control animals.**

# 5. Discussion

## 5.1 Summary of Major Findings

The research described in this dissertation investigated several aspects of neuroimmune system activation following PAE. Many of the results were unexpected, either contrasting with the originally stated hypotheses, or revealing surprising discoveries that we had not originally sought to investigate.

Comparing the results of study #1 with those of study #2, provides conclusive evidence that alcohol dose is the major determinant of neuroimmune system activation. We confirmed that binge-like alcohol exposure results in robust neuroinflammation. Importantly, we showed that just one day of exposure to a high dose of alcohol results in dramatic increases in pro-inflammatory cytokine production. Conversely, exposure to more moderate levels, over a longer period of time, had little effect on neuroimmune system function or activation.

We identified several other factors that can heavily influence the type of neuroimmune response generated. Results from the study #1 showed differential outcomes between males and females, indicating that neuroinflammation is dependent on gender. Additionally, we found the neuroimmune response to be highly brain-region specific, as findings from both study #1 and study #2 show differences across brain regions. Most dramatic were the contrasting findings between the hippocampus and cerebellum in study #2 after the binge exposure paradigm (Figure 5.1). While the cerebellum showed robust neuronal loss and

microglial activation, the hippocampus exhibited no signs of neuronal loss and little change in microglial morphology. Furthermore, cytokine production was not as pronounced in the hippocampus. Finally, comparing the results from study #2 with the literature indicates that the window of exposure is a major effector of neuroinflammatiory outcomes. Our contrasting findings in the hippocampus and cerebellum are likely explained by the limited window of exposure (P3-5), as neuronal loss and neuroinflammation in the hippocampus have been observed after windows of exposure encompassing slightly later time points (~P7-9) [42,208,213]. These findings further highlight the complex and dynamic nature of the neuroimmune system.

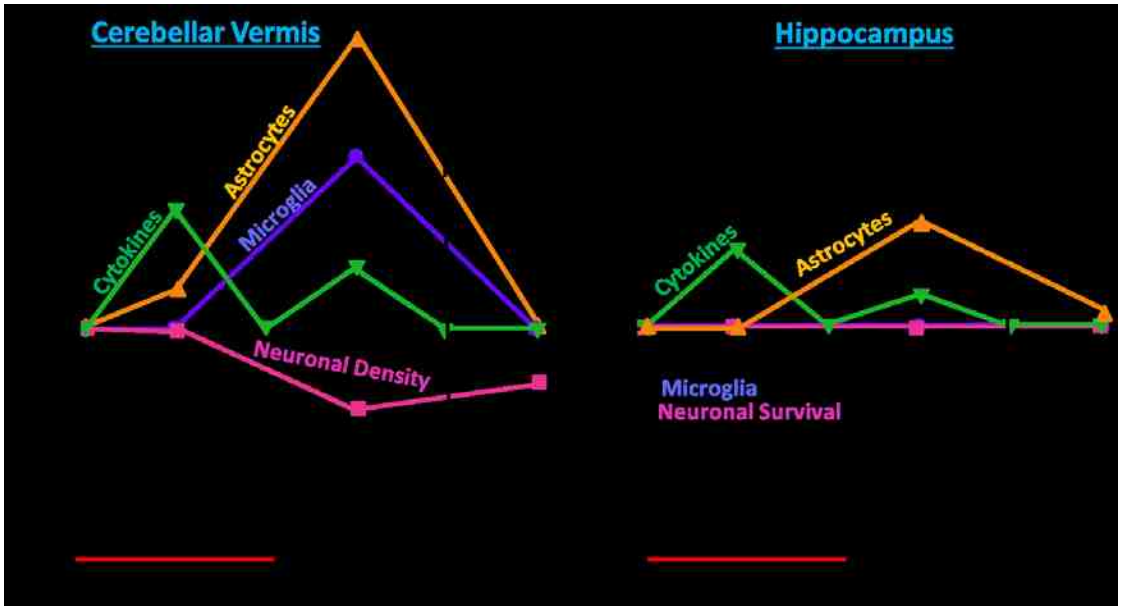


**Figure 5.1 Summary of Findings for Study #2.**

The time course for production of pro-inflammatory cytokines, microglia and astrocyte activation, and neuronal loss is shown relative to the exposure paradigm. All measurements are shown as fold changes from air controls. Neonates were exposed to alcohol for 4 hrs daily on P3, P4, and P5. Samples collected on P3 and P5 were at times of peak BAC (labeled BAC). Samples collected on P4 and P6 were taken during withdrawal (labeled W.D.)

As previously stated, we originally designed study #2 around the hypothesis that there would be a polarization switch in neuroimmune activation from M1 to M2 over the course of the binge-like exposure paradigm. While we did not see evidence of this, there was a subtle, yet definitive increase in anti-inflammatory cytokines that persisted throughout the exposure paradigm. This indicates that there is a component of this response that is anti-inflammatory and it likely influences the course of neuroinflammation and neuronal survival.

One of the most surprising findings was with respect to the impact of withdrawal periods in study #2. We expected cytokine production to increase with each day of alcohol exposure, however mRNA expression was most pronounced during withdrawal periods and dropped off during peak BACs. Additionally, we found that astrocyte activation was evident in both brain regions, occurring independently of microglial activation and neuronal loss. Little has been done previously to address astrocyte activation in a model of PAE, and astrocytes have often been designated to play a more secondary role with respect to microglia. However, these findings indicate that astrocytes may have an important role in mediating PAE that is distinct from microglia. Specifically, the fact that robust astrocyte activation occurred without corresponding neuronal loss supports that idea that astrocytes likely do not play a neurotoxic role, and may instead contribute to neuronal survival.

## 5.2 Potential Mechanisms

### 5.2.1 Study #1

While in study #1 we did not expect to see the type of robust neuroinflammation associated with acute binge exposures, it is surprising that there was so little effect on neuroimmune function. The exposure paradigm used in this study encompassed the full two postnatal weeks, a period in which the majority of gliogenesis and glial cell maturation is thought to occur[163,169]. The evidence for microglial maturation during the first two weeks of life is based primarily on the well-characterized changes in microglial morphology. This structural transition consists of amoeboid microglia adopting the small cell body and long dendritic processes associated with a ramified morphology, and take place during the first two weeks of postnatal life[163]. A similar transition occurs in the human brain, in which fully ramified microglia are not evident until close to term (35 weeks)[303]. However, the phenotypic changes that occur during microglial maturation, including expression of the multitude of receptors exhibited by microglia in the adult brain, is not yet fully characterized. Several recent studies have begun to investigate this process.

In an elegant study by Butovsky and colleagues, microglia were shown to have a distinct genetic and proteomic profile. This signature consists of hundreds of genes and proteins that distinguish them from other neural and/or immune cells, including macrophage, astrocytes, and oligodendrocytes. The authors then demonstrated that this profile does not exist in microglia on P1 or P4. Moreover, microglia at this young age had very little expression of any of the investigated

microglial genes, including many for receptors (such as purinergic receptors), transcription factors, and ion channels. Instead, the full genetic signature of a mature microglia was adopted between P21 and 2 months of life[304]. These findings indicate that microglia are not fully mature until ~ 2 months of age, despite the fact that they adopt a mature morphology by P14 (Figure 5.2). Similarly, a study by Crain and colleagues showed that the expression of purinergic receptors in microglia is highly dependent on age. Assessing multiple ages, they showed evidence that also suggests microglia do not display a mature receptor profile until several months post birth. Interestingly, certain purinergic receptor types were expressed preferentially at a very young age (P3), compared to more mature microglia[305].

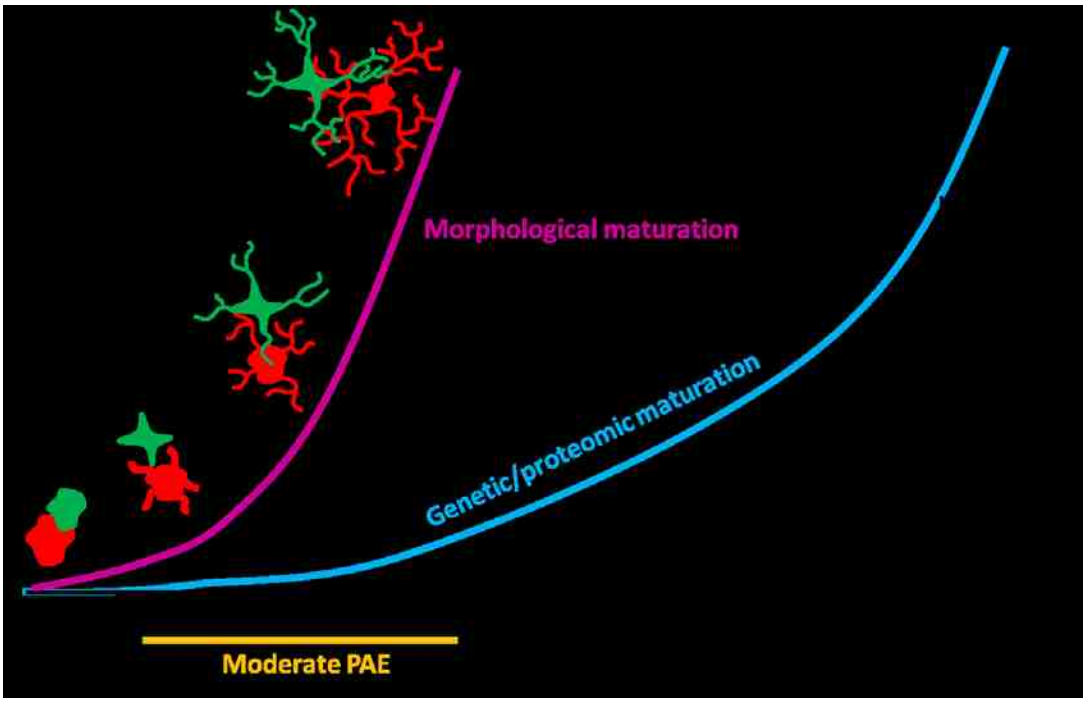


**Figure 5.2: Glial Cell Maturation**

The approximate time course for morphological and molecules maturation rates of astrocytes and microglia are shown relative to the moderate PAE paradigm used in study #1.

Considering these two studies, it is possible that moderate alcohol exposure during the postnatal period does not readily activate microglia, as they are still too immature. If the expression of the receptors and other proteins necessary for microglial activation is very low at this age, then perhaps microglia are much less sensitive to insult and a larger dose of alcohol is necessary to evoke a response. For example, ATP is a strong regulator of microglial activity, capable of causing rapid migration and cytokine production, and is released by glial cells and neurons[306]. ATP binds to purinergic receptors; many of the purinergic receptor types that are expressed in mature microglia were among those shown to be minimally expressed in young microglia[304,305]. Conversely, there was a higher expression at this age of other purinergic receptor types not seen heavily in adults[305]. It is possible that different receptors are expressed in the postnatal period to help with developmental processes, such as proper migration. Therefore, it is likely that neuroimmune signaling is impaired to some extent in the postnatal period, or at least different from in a mature microglia.

The process of microglial maturation has been shown to be dependent on a number of factors. In the Butovsky study, adoption of the mature microglial genetic profile was dependent on TGFβ signaling. Conversely, research by Zusso and colleagues showed that microglial morphological maturation requires the transcription factor Runx1. Runx1 is highly expressed in microglia during the embryonic and early postnatal stages, however it is down-regulated as microglia progress to a ramified form. The authors demonstrated that Runx1 blocks the

proliferation of amoeboid microglia and promotes progression to ramification. Furthermore, in the adult brain Runx1 was found to inhibit microglial activation, and its expression increases with activation, potentially acting as an automatic brake system. The authors speculate that Runx1 may act similarly in immature amoeboid microglia, preventing activation[307]. If this is the case, then the increased Runx1 expression seen in the early postnatal stages may further inhibit microglial activation by alcohol exposure at this age.

In addition to Runx1 and TGFβ, the transcription factors IRF-8 and PU.1, as well as the microRNA miR124 and the tyrosine kinase transmembrane receptor CSF1R, have all been implicated in the regulation of microglia or monocyte maturation[224]. In humans, some of these same factors have been shown to control important processes in human microglia, such as proliferation, suggesting human microglial development may be similarly regulated[308,309]. Of the factors listed above, to the extent of my knowledge, only PU.1 and miR124 have been demonstrated to be affected by alcohol, and only in adult models[310,311]. While the affects seen in an adult model are often not reflective of changes in a neonate, it is still somewhat surprising that neonatal alcohol exposure did not alter neuroimmune programming in study #1. However, changes in the expression of PU.1 in response to alcohol was investigated only in the periphery, not the CNS, and the exposure model was chronic, as compared to the acute exposure in study #2[311]. Similarly, the changes in mir124 were seen after a 10-day alcohol exposure model and only in the limbic forebrain[310]. Therefore, findings from both of these studies are likely highly

influenced by the longer duration of the exposure paradigms, the fact that these were adults, and the specific regions in which expression was affected. Furthermore, both study #2 and previous studies in the literature[212,213] have shown that PAE can elevate TGFβ expression, which the Butovsky study showed regulates the adoption of a mature genetic and proteomic profile in microglia. However, in that study, the authors only showed that this process was impaired in TGFβ deficient animals, and it is unclear what the effect would be, if any, from elevated expression.

As for astrocytes, they too undergo pronounced morphological changes and gliogenesis during the postnatal period. Again, this is reflective of what has been reported to occur in the human brain[312]. Similar to microglia, the molecular maturation of astrocytes occurs after their morphological development[169] (Figure 5.2). Astrocytes begin expressing several genes important to their mature function, including the glutamate transporter GLT1, proteins involved in astrocytic gap junctions, and potassium channels, at around 3-4 weeks of age. Therefore, there is a low expression of these genes during the postnatal period, which further suggests that neuroimmune signaling is impaired during this time. Particularly important is the loss of gap junction proteins, as much of astrocytic signaling is thought to occur across this network. On the other hand, GLT1 is critical for the proper clearance of glutamate from neuronal synapses, suggesting that this function may be blunted in young astrocytes, and increasing the risk for neuronal excitotoxicity[169].

Little is known about the process of priming and in what way a primed neuroimmune cell differs from normal. However, up-regulation in microglial surface receptors and an increase in number are associated with this change. Additionally, particularly in cases of neurodegeneration, there can be a loss of inhibitory signaling from neurons, making the microglia more easily activated[313]. In a previous study showing neuroimmune system priming after a neonatal insult, the initial infection induced a neuroinflammatory response, characterized by increased cytokine production[227]. While expression of most cytokines was no longer elevated in adulthood, there was an increase in the microglial activation marker, CD11b[227]. This increase in CD11b could be a lingering result of microglial activation by the neonatal infection. Therefore, it is possible that initial neuroimmune activation is necessary to induce priming of these cells to future insults. With the moderate PAE model used in study #1, we did not find any signs of neuroimmune activation by alcohol exposure (Section A.1), and this may explain why there was no exaggerated response to a subsequent injection of LPS.

Compared to microglia, the role of astrocytes in neuroimmune system programming has been much less explored. However, astrocytes have been shown to become primed in neurodegenerative diseases, producing markedly exaggerated increase in chemokines after stimulation with inflammatory cytokines, which resulted in infiltration of peripheral immune cells[314]. Therefore, this suggests that astrocytes may be an important component of neuroimmune priming. Furthermore, astrocytes can exert powerful control over microglial

activation, often times preventing it[139]. As a result, astrocytes could be responding to moderate PAE by secreting factors that prevent microglial activation. This represents an additional mechanism by which neuroimmune system activation is blunted following moderate PAE.

Taken together, the possibility described above that the immature neuroimmune system may be more difficult to activate than in adult and adolescent rats, combined with the idea that neuroimmune activation is necessary to induce priming, could explain why there are no changes in neuroimmune system function following moderate PAE. Figure 5.3 summarizes the potential mechanisms for study #1 described above.

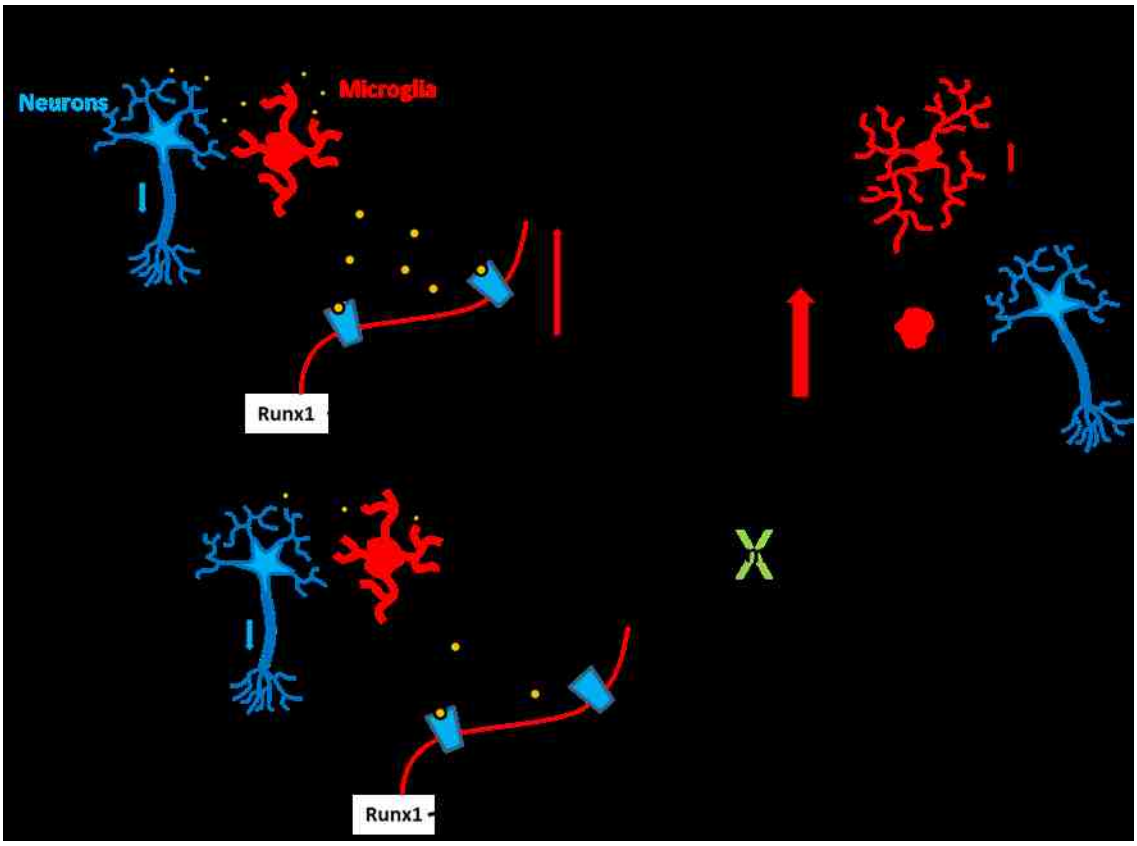


**Figure 5.3 Summary of Potential Mechanisms for Study #1**

Normal priming likely involves activation of the neuroimmune response during the postnatal period (A). Peripheral insults, such as infections, cause inflammation throughout the body. These increases in cytokines and other pro-inflammatory molecules can cross the BBB and distress neurons, causing them to remove signaling that inhibits microglial activation, such as fractalkine, and release other molecules that can activate microglia (ATP, glutamate). Immature microglia likely express lower levels of certain receptors, however high concentrations of peripheral cytokines or neurotransmitters can bind to the limited number of receptors and induce transcription of genes involved in microglial activation. Activated microglia then produce cytokines and up-regulate cell-surface receptors. In adulthood, CD11b remains up-regulated in primed microglia and a subsequent insult by LPS leads to an over-production of inflammatory molecules which can damage neurons.

In the moderate PAE model used in study #1 (B), the relatively low dose of alcohol may cause some peripheral cytokine infiltration and minimal neuronal distress. However, the lower concentration of signaling molecules, combined with the limited number of receptors are not enough to overcome the inhibitory actions of Runx1 at this age. Microglial activation does not occur, and therefore does not influence neuroimmune programming to subsequent insults.

### 5.2.2 Study #2

One of the most puzzling findings of study #2 is the differential susceptibility of the cerebellum and hippocampus to PAE. It is well-described that different brain regions, and even the cells within these regions, have different vulnerabilities and periods of increased susceptibility to developmental alcohol exposure[31,233]. Likely this is due to the fact that the individual brain regions, and cells within them, do not share the same maturation rates and timelines for developmental processes.

In models of PAE, the cerebellum has continuously been shown to be the most susceptible to damage, particularly neuronal loss, on P4-P5[70,72], while the vulnerability of this region drops off later in the postnatal week. In an effort to explain this phenomenon, a study by Heaton and colleagues investigated the production of reactive oxygen species (ROS) in the cerebellum, after exposure to alcohol at different developmental time points. Using vapor inhalation, the authors exposed pups to alcohol on P4, P7, or P14. Exposure on P4 resulted in rapid and dramatic increases in ROS, while exposure on P7 or P14 decreased ROS expression[315]. ROS are extremely reactive molecules that are well-established as contributing to tissue damage and neuronal death[316]. In the CNS, they can be secreted by activated microglia and astrocytes[317,318], and under conditions of excitotoxicity neurons have a markedly elevated production of ROS[319]. Changes in ROS expression likely contributes to, or is a result of, the increased neuronal death seen after alcohol exposure during the P4-5 time window. Additionally, in another similarly designed study, Heaton and colleagues

showed that exposure to alcohol on P4-5 was the only temporal window that caused a decrease in nerve growth factor[320]. Nerve growth factor supports Purkinje cells and increases neuronal survival[321], and a decreased expression could further explain this temporal susceptibility to neuronal loss.

In the hippocampus, alcohol exposure on P7 has repeatedly been shown to cause neuronal damage/loss and affect neurogenesis[42,322-324]. However, alcohol exposure on P4 does not cause neuronal loss in the hippocampus [44] and causes the most pronounced neuronal loss in the cerebellum, compared to other brain regions[71]. To the extent of my knowledge, little has been done to investigate the mechanisms responsible for this developmentally-dependent susceptibility of the hippocampus. However, one interesting study by Ikonomidou and colleagues looked at the vulnerability of the developing brain to damage by excitotoxicity. The authors injected the animals with N-methyl aspartate, which activates glutamate receptors, on P4, P6, or P10 and performed analyses within 4 hours of injection. They found that the hippocampus was resistant to excitotoxic damage after injection on P4 or P10, but that there was a significant increase in neuronal death after injection on P6[325]. Indeed, excitotoxicity and alterations in glutamate receptors have long been hypothesized as a potential mechanism for mediating alcohol-induced damage in the developing brain[326]. Models of PAE have shown increases in NMDA receptor subunits[327,328] and decreased GABAergic inhibition of some neurons[329]. Therefore, it is likely that the temporal susceptibility to excitotoxicity in the hippocampus contributes to its similar window of vulnerability to PAE-induced neuronal loss.

In study #2, we found that it was during the periods of withdrawal that cytokine expression was increased, and not during peak BACs. Interestingly, withdrawal following PAE can cause excitotoxicity in the hippocampus and cerebellum[330,331], and in the cerebellum has been suggested to cause more damage than the alcohol exposure itself [331]. Furthermore, mitigation of alcohol withdrawal periods in neonates rescues behavioral deficits associated with impaired functioning of the hippocampus and the cerebellum[279,332,333]. Finally, alcohol withdrawal has been implicated in neuroimmune activation, as it increases the number of microglia[280] and the density of astrocytes[334] in the adult brain. Taken together, it is possible that in study #2 alcohol withdrawal leads to excitotoxic neurons and neuroimmune activation.

In support of this, as mentioned above, the hippocampus is resistant to excitotoxic insult during the period of the exposure paradigm utilized in study #2, which would explain why this region is much less affected. Additionally, in the studies by Heaton and colleagues discussed above, PAE on P4 increased ROS production in the cerebellum, which is strongly associated with excitotoxic neurons. Therefore, the ROS production they observed could be due, in part, to PAE-induced excitotoxicity. Furthermore, as previously discussed, astrocytes at this age are still molecularly immature and do not fully express GLT1. GLT1 is important for astrocyte removal of glutamate from neuronal synapses, thereby preventing excitotoxicity. Therefore, neurons may be particularly at risk for excitotoxic insult during this time. Under this theory, withdrawal periods could

directly cause neuronal damage and/or loss in the cerebellum through excitotoxicity.

Neuronal distress can activate microglia, causing increased cytokine production. A potential mechanism for neuronal activation of microglia, following alcohol exposure, was shown in a recent study by Zou and Crews. The authors demonstrated that, in adults, neurons release HMGB1 in response to alcohol, and that HMGB1 then activates microglia by binding to TLR4. Importantly, HMGB1 can be released from dying neurons[189]. Therefore, HMGB1 release by damaged neurons represents an additional mechanism for microglial activation during withdrawal periods of PAE. Microglial activation could then further contribute to neuronal loss through increased pro-inflammatory cytokine production.

Given the massive neuronal death seen in study #2, it is odd that there is such little presence of microglia within the Purkinje cell layer. In both the air control and PAE cerebellar sections, microglia are located centrally within the lobules, in the places that contain the deep white matter in an adult vermis. Based on this, we hypothesized that microglia may be centered on Purkinje cell axonal tracts, however staining for PanNeuronal and myelin binding protein showed no evidence of this type of patterning (Section A.4). Additionally, the white matter seems to be only beginning to be formed by P6 (Section A.4, Figure 4.12). Instead, it seems that this PAE paradigm does not induce chemotaxis in microglia. This contrasts with the idea of excitotoxic neurons, as microglia should be phagocytosing dying neurons, and excess glutamate has been shown to

induce chemotaxis in microglia[335]. However, it is possible that microglia are slow to respond given their immaturity and the fact that they do not fully express some of the receptors necessary for neuroinflammatory chemotaxis. As discussed above, the Butovsky and Crain studies show that microglia in the postnatal period have different receptor expression than in mature microglia, including receptors such as purinergic receptors, which are critical for mediating chemotaxis toward insults[304,305]. Additionally, TGFβ has been shown to inhibit microglial chemotaxis[336], and in study #2 we found that alcohol caused a significant, prolonged increase in TGFβ mRNA expression.

Another puzzling result of study #2, pertains to the unique neuroimmune response of astrocytes. Astrocytes are closely organized around neuronal synapses where they normally remove excess glutamate and also help to combat the production of ROS by releasing the antioxidant glutathione (GSH)[337]. In times of excitotoxicity, astrocytes can respond by increasing GLT1 expression to promote glutamate up-take[338], and in neurodegenerative diseases they can become a structural replacement for dying neurons and promote neuronal survival[339]. Therefore, these important roles of astrocytes may mean that astrocytes are quicker to respond to neuronal insult following PAE, and may provide insight into their unique role.

A study by Douhou and colleagues found strikingly similar results to study #2, concerning the relationship between astrocytes, microglia, and neuronal loss during a developmental time period. In this study, the authors used a mouse model of Parkinson's disease, in which Dopaminergic cell loss begins to occur

around P15. They observed a rapid increase in GFAP expression at this time, including in regions in which neuronal loss was not found. Moreover, they found that areas with the highest increases in GFAP expression correlated with the best neuronal survival. Furthermore, the authors described an increase in microglial activation that was delayed compared to astrocytes and coincided only with the peak of neuronal loss[340]. This is very similar to the results of study #2, in which astrocyte activation occurs in both the hippocampus and cerebellum, regardless of neuronal loss, and astrocyte activation seems to precede microglial activation. Furthermore, in study #2, microglial activation was only observed in the brain region in which neuronal loss occurred.

Douhou and colleagues speculated that, in their model, astrocyte activation served as a support mechanism for distressed neurons, and that astrocyte activation in areas without neuronal loss was a preventative measure. Additionally, microglial activation seemed to be secondary to neuronal loss, likely to remove neuronal debris[340]. Therefore, it is possible that neurons in the hippocampus could still be experiencing distress from this high dose PAE paradigm that, while much less in comparison to the cerebellum, still activates astrocytes to provide neuronal support. In the hippocampus astrocyte activation could be enough to rescue any neuronal damage, and if microglia are activated secondarily only to neuronal death, then this would explain why little morphological activation is seen in the hippocampus.

The potential mechanisms described in this section are summarized in Figure 5.4. Importantly, in this mechanism, neuronal death is initially caused by

excitotoxicity, with only a partial contribution by microglia. Conversely, astrocyte activation is critical for neuronal support and survival, and there is a distinct possibility that there is some M2 microglial activation that contributes to the anti-inflammatory cytokine response. Therefore, according to this model, neuroimmune activation has opposing roles in neuronal damage.

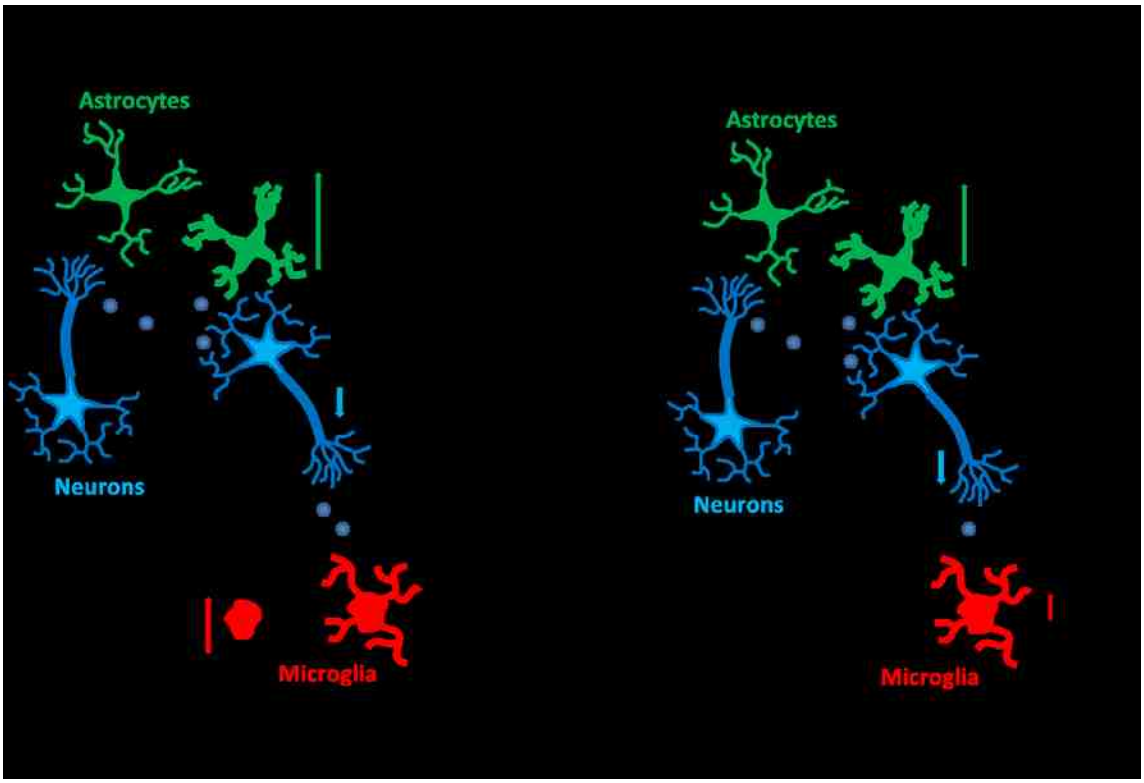


**Figure 5.4 Summary of Possible Mechanisms for Study #2.**

In the cerebellum (A) exposure to alcohol and particularly alcohol withdrawal periods induces excitotoxicity in Purkinje neurons, potentially through alterations in NMDA receptors (1). Excitotoxicity results in rapid response by astrocytes (2) as they become reactive and up-regulate glutamate transporter GLT1 (to remove excess glutamate), the antioxidant GSH (to combat ROS production), and anti-inflammatory/neuroprotective cytokines. Excitotoxic neurons reduce signaling of inhibitory molecules, such as fractalkine, which normally block microglial activation. Excitotoxic neurons also release molecules such as HMGB1, ATP, and ROS, which can activate microglia (3). Importantly, microglial chemotaxis is not induced by ATP as the correct purinergic receptors are not yet expressed, and production of TGFβ also blunts chemotaxis. Activated microglia produce pro-inflammatory IL1β, TNFα, and ROS which further contribute to neuronal damage (4).

In the hippocampus (B), neurons are resistant to excitotoxic insult (1). However, withdrawal following binge alcohol exposure still causes some neuronal distress, activating astrocytes (2). Alternatively, astrocytes are activated by signaling from cerebellar astrocytes. The distressed neurons reduce signaling of inhibitory fractalkine, leading to slight microglial activation and production of TNFα (3). The immature microglia of the hippocampus do not become fully activated without secretion of additional molecules, and therefore do not further damage neurons.

## 5.3 Clinical Implications

Blunting of neuroimmune system activation has been proposed as a potential therapeutic option for the treatment of FASDs. Several previous studies have shown improved outcomes in neuronal survival and behavioral tasks after PAE if neuroinflammation is prevented[209,210,212,213]. However, the observations in this dissertation indicate that treatment designed around this concept will need to strongly consider each patient case individually, given the enormous number of factors identified herein that can influence neuroinflammatory outcomes. Importantly, therapeutics aimed at blocking neuroimmune system activation will likely be ineffective in women who do not binge drink, although they may be helpful in women who drink heavily on even one day of pregnancy.

While this work does not provide a conclusion on the relationship between neuroinflammation and neuronal loss, it provides some additional evidence for both sides of the argument. On one hand, microglial activation corresponded heavily with neuronal loss. Conversely, microglia were rarely located within the cell layer in which neurodegeneration occurred. However, if microglial activation does contribute to neurotoxicity it is important to consider that agents designed to block neuroimmune activation will likely also reduce the anti-inflammatory component and astrocyte activity. This may block any beneficial effects that TGFβ, IL10, or astrocytes may have in this case.

To sum, the neuroimmune system is a promising therapeutic target for cases of binge-like exposure, however completely blocking neuroinflammation may have some negative consequences. I suggest that a focus on shifting the

polarization from M1 to M2, or mitigating withdrawal periods should also be considered as a potential treatment option.

## 5.4 Critique

Both study #1 and study #2 utilized an exposure model in which the pups inhaled vaporized alcohol rather than ingesting it. Despite the advantages of this paradigm, it could be a potential confound in these studies, as the alcohol is not metabolized by the stomach and liver upon first entering the body as it would be if the alcohol was ingested orally. Normally, alcohol is broken down by the enzyme alcohol dehydrogenase, producing acetaldehyde, which is a reactive and toxic metabolite[10]. However, alcohol can be metabolized by other tissues, such as the brain[10]. Additionally, the alcohol now enters the bloodstream through the lungs, where the high dose likely causes inflammation and potentially damages the tissue. This peripheral inflammation could contribute to, or influence the neuroimmune response, as these systems are highly communicative and influential with each other[76]. Additionally, the exposure model in study #2 elicited very high BACs. While this was done intentionally, in part because rodents may be less sensitive to alcohol[16], this still represents an additional confound as it may not represent physiologically relevant BACs. Furthermore, the high levels of alcohol vapor within the chambers likely introduce an element of stress to the pups, as the smell is very potent and aversive. There is a strong relationship between stress and the neuroimmune system[341,342], and FAE can exacerbate stress responses[343,344]. Either of these relationships could potentially influence outcomes and should be taken into account when considering the results of this

study. Finally, while nursing and maternal care were not found to be significantly affected in study #2, there were still indications (trends) that these may be slightly altered in the PAE animals. Both maternal care and nutrition can affect the neuroimmune system[345,346], and should continue to be carefully monitored in future studies.

In study #2, glial cell morphology was used as the main determinant of activation. While morphological transition is considered strongly indicative of activation, this is not always the case and, importantly, does not provide a complete picture of phenotype. As a result, it is difficult to determine how the glial cells are responding to PAE and what their actions are. Additionally, while IBA-1 was used to label microglia, this marker labels monocytes and monocyte-derived cells. Since microglia and macrophage are both monocyte-derived, this stain cannot differentiate between the two. By immunohistochemistry, macrophages appear similar to amoeboid microglia, therefore it is possible that the microglia labeled as being in an amoeboid morphology are actually peripheral macrophages. Furthermore, no assessment of peripheral immune cells was included in this study. As a result, we cannot discount the possibility that infiltrating cells are contributing to increased cytokine production and potentially neuronal loss.

In both study #1 and study #2, cytokine production is assessed by measuring mRNA. While perfusions were performed before collecting samples for immunohistochemistry, brains were not perfused before collection of samples for mRNA. As a result, the blood vessel network within the collected tissue

sample likely contains circulating cytokines that are not actually within the CNS parenchyma, potentially biasing the results. Additionally, for study #2 we confirmed that IL1β protein expression mimics what occurs at the level of mRNA on P4 in both the hippocampus and cerebellum (Section A.2, Figure A.2.1). However, protein expression was not measured along the full time course, and no other cytokines were included. Therefore, we cannot definitively say that the changes observed in mRNA are reflective of what occurs at the level of protein. Moreover, even if cytokine mRNA is translated into protein, we do not know that it is being processed into the activated form. Furthermore, while there exist hundreds of different cytokines, we have only included four in this study as it would have been unrealistic to assess all of them. As a result, there could be important actions by, or changes in the levels of, other cytokines that we are unaware of. Finally, we have made assumptions about the pro- and anti-inflammatory actions of the cytokines measured, yet, as detailed in the introduction, activation of these cytokine signaling pathways can have a number of different outcomes. Therefore it is impossible to say for sure what their exact actions are and, because both glial cells and neurons express many of these receptors, upon which cells they are acting.

For study #1 and study #2, we used ketamine to humanely anesthetize the pups before euthanizing them. Since ketamine has been shown to cause neuronal apoptosis, using this drug could confound the results in this study[347,348]. While, this is unlikely as typically prolonged exposure to ketamine is needed to cause neuronal loss, any type of insult to neurons can potentially elicit a

neuroimmune response. Additionally, ketamine has also been shown to have some effects on microglia[349,350]. Therefore, this should be considered when interpreting results.

Finally, while study #2 provided new information critical to understanding neuroimmune system activation following PAE, it does not include an investigation into the mechanism of how alcohol is activating the neuroimmune system at this age. It is still unclear whether or not neuroinflammation contributes to neuronal loss. Future work should expand on these findings and address these lingering questions.

## 5.5 Future Directions

There are a number of different experiments that could be done to follow up on this work. One of the most pertinent would be to investigate more the individual roles of the neuroimmune cells in the binge exposure paradigm. To discover what cytokine expression is due to astrocytes and what is produced by microglia, the cells could be isolated and assessed by flow cytometry. Extracellular markers can be used for GFAP to label astrocytes, and CD11b to label microglia. Then, intracellular markers could be used for expression of cytokines. Additionally, co-staining for CD11b and CD45 will allow for identification of any infiltrating macrophage that could not be distinguished from microglia by immunohistochemistry. Specifically, both microglia and macrophage express CD11b, but microglial expression of CD45 is low, while macrophages more highly express CD45[351]. To more thoroughly investigate contributions by

peripheral immune cells, additional IHC could be performed for lymphocytes (such as T cells and B cells) and leukocytes. Furthermore, it would be interesting to see if this paradigm increases BBB permeability given the dramatic increases in pro-inflammatory cytokines. There are a number of assays that can be used to assess BBB breakdown. One of the simplest assessments involves injecting a fluorescent dye, such as fluorescein, that does not cross the BBB under normal conditions. If the dye is found in the brain, this indicates that the BBB is no longer tightly sealed.

Another future experiment would be to investigate the mechanism for how alcohol activates the neuroimmune system. As described in the introduction section, in adults there is evidence that activation of microglia by alcohol is dependent on toll-like receptors, particularly TLR4[185,189]. Furthermore, activation of TLR4 has been shown to be caused by primarily neuronal secretion of the cytokine HMGB1[189]. It would be interesting to see if this is also the case in FAE. To start, TLR4 knockout animals could be exposed to the binge alcohol exposure paradigm used for study #2, and neuroinflammation could again be assessed. If the TLR4 knockout animals have diminished neuroinflammation, the effect this has on neuronal loss could also be investigated. Additionally, increased levels of HMGB1 could be assessed by measuring protein levels. If the proposed mechanism for alcohol in adult animals is relevant to neonates, then HMGB1 expression should be increased in PAE animals.

To follow-up on the differences seen in the hippocampus and cerebellum in study #2, it would be interesting to perform this study again using a different

window of exposure. Specifically, exposing neonates to the same dose of alcohol used in this study, again over a 3-day period, however this time utilizing a window shown to cause neuronal loss in the hippocampus (P7-9). I would expect that the results would be flipped from what was seen in this study, with the hippocampus having more neuronal loss than the cerebellum. Whether or not neuroimmune activation is also increased in the hippocampus could shed more light on the relationship between neuronal loss and neuroinflammation.

Additionally, while many studies have begun to investigate the use of agents that prevent neuroimmune activation in PAE, the observations in this study point to a potent effect of withdrawal periods. As a result, mitigating the effects of withdrawal and assessing how this changes the neuroimmune activation and neuronal loss seen after PAE could yield important insights. There are a number of clinically approved drugs to treat alcohol withdrawal symptoms, and if my findings are correct, I would expect that these drugs could reduce pro-inflammatory cytokine production, and potentially glial cell activation as a result. In particular, more investigation should be conducted into the potential role excitotoxicity during PAE and during withdrawal. Since NMDA receptors are often implicated in excitotoxic mechanisms, perhaps NMDA receptor antagonists could potentially represent an additional treatment option for prevention of FAS.

Finally, I think it would be important to confirm these findings in both other models of exposure, and in females. As mentioned above, this vapor inhalation has a number of potential confounds, as do most exposure paradigms, and so it is important to confirm findings in the FAE field in multiple models. Alcohol could

be administered by gavage, and some of the most pertinent findings of the binge study could be repeated, such as the elevated cytokine levels on P4 and the drop off on P5, and measurements of glial cell activation and neuronal loss on P6. Additionally, as I found in study #2, gender influences neuroimmune outcomes. Therefore, the experiments of study #2 should be repeated in females, and the results should be compared. I expect that there could be vast differences between the two groups, given the sexually dimorphic effects of alcohol [245-247] and the differences in the neuroimmune system between males and females at this age[132,164].

# Appendix A: Supplemental Data

## A.1 Investigation of basal effects of moderate PAE on neuroimmune activation.

**Purpose:**

Before performing study #1, in which pups received LPS injections following moderate PAE, we sought to test whether or not the moderate PAE paradigm itself caused any neuroimmune changes. Specifically, we focused on the dentate gyrus as this region has been shown to be particularly susceptible to FAE[32,230,231]. This experiment was designed to be a general screening for any changes in neuroinflammatory gene expression following this exposure.

**Methods:**

*Ethanol treatment and collection of samples*

Pups were exposed to alcohol via vapor inhalation for four hours daily, as described in study #1. Moderate BACs were achieved from P2 through P16. On P17, the dentate gyrus was isolated and harvested for mRNA as described in study #1. For protein extraction, dentates were placed into ice cold homogenization buffer (25 mM HEPES, 500 mM NaCl, 2 mM EDTA, 1 mM DTT, 10% Tween 20, 1 mM phenylmethanesulfonyl fluoride, 20 mM NaF, 5 µM Cyclosporin A, 1% v/v phosphatase inhibitor cocktail [Sigma, Cat. No. P2850], and 1 complete Mini Protease tablet [Roche]), homogenized, and stored in aliquots at -80°C. Both males and females were used.

*PCR Arrays and Validation*

Gene expression was initially measured using a quantitative real-time PCR array focusing on rat inflammatory cytokines and receptors (SABiosciences/Qiagen, Cat No. PARN-011Z) in combination with the $RT^2$ SYBR Green qPCR Master Mix (SABiosciences/Qiagen). Additional quantitative real-time PCR was conducted to validate any significant findings of the array. For validation of array findings, primers were identical to those on the array and were purchased from SABiosciences/Qiagen including Cd40lg (Cat. No. PPR49715A), Ccl5 (Cat. No. PPR06854F), Il11 (Cat. No. PPR06463F), and HPRT1 (Cat. No. PPR42247F). RT-PCR was carried out as described in study #1.

*Western blotting*

After homogenization by sonication the dentate gyri from 2 pups in each litter were pooled, tissues were frozen and stored at -80 °C until use. Protein was quantified using a Bio-rad protein assay (Cat. No. 500-0006). Samples were mixed with a 5X SDS-PAGE sample buffer containing 250 mM Tris–HCl (pH 6.8), 10% sodium dodecyl sulfate, 30% glycerol, 5% β-mercaptoethanol, and 0.02% bromophenol blue, and boiled for 5 min. Final concentration of protein homogenates was 1 mg/mL and each sample was loaded as 5, 10, and 15 µg/lane. The proteins were separated in 4-15% Tris-HCl precast gels (BioRad) at 110 V for 70 min, then transferred to a polyvinylidene fluoride (0.45 µm) at 100 V for 70 min. After, blots were incubated in blocking buffer (LI-COR, Lincoln, NE) for 2 hours, and then allowed to sit overnight at 4 °C in 1X phosphate buffered saline with 0.1% Tween 20 containing a rabbit polyclonal IL-11 antibody (1:1000,

Santa Cruz Biotechnology,sc-7924) and a monoclonal mouse β-actin antibody (1:25,000, Sigma). Secondary antibodies were Goat anti-mouse (LI-COR, IRDye 680) and Goat anti-rabbit (LI-COR, IRDYE 800CW). Blots were developed using an Odyssey infrared imager (LI-COR) and quantified with Odyssey V3.0 software (LI-COR) using integrated intensity. All primary antibodies used were found to fall within the linear dynamic range (data not shown) at this dilution and with this quantification method.

*ELISA*

Protein samples were centrifuged at 8 X $10^3$ g for 10 minutes at 4°C. The supernatants were transferred to a fresh tube, aliquoted, and stored at -80°C until use. Protein was quantified using a Bio-rad protein assay (Cat. No. 500-0006). Samples were normalized to the one containing the lowest concentration of protein and an ELISA measuring CCL5 was run according to the manufacturer's instructions (R&D systems, Cat. No. MMR00)

**Results:**

The arrays revealed no significant changes in gene expression in female pups (Table A.1.2), however the array showed three genes were significantly altered in males (Table A.1.1). CD40l (CD40 ligand, important for activation of T cells) and CCL5 (chemokine C-C motif ligand 5, pro-inflammatory chemokine) were significantly up-regulated by moderate PAE, while the anti-inflammatory cytokine IL11 (interleukin-11) was significantly down-regulated. However, PCR validation was unable to confirm the findings of the array for any of these genes

(Figure A.1.1, A-C). Similarly, IL-11 and CCL5 were tested for changes in protein levels, and these were also found to be unaffected by moderate PAE (Figure A.1.1, D-F).

**Table A.1.1 Inflammatory Gene Expression in Males after Postnatal Alcohol Exposure**

mRNA isolated from the dentate gyrus of male pups was run on an inflammatory gene array containing 84 different genes. This table shows the genes most affected by moderate PAE. n = 4 litters

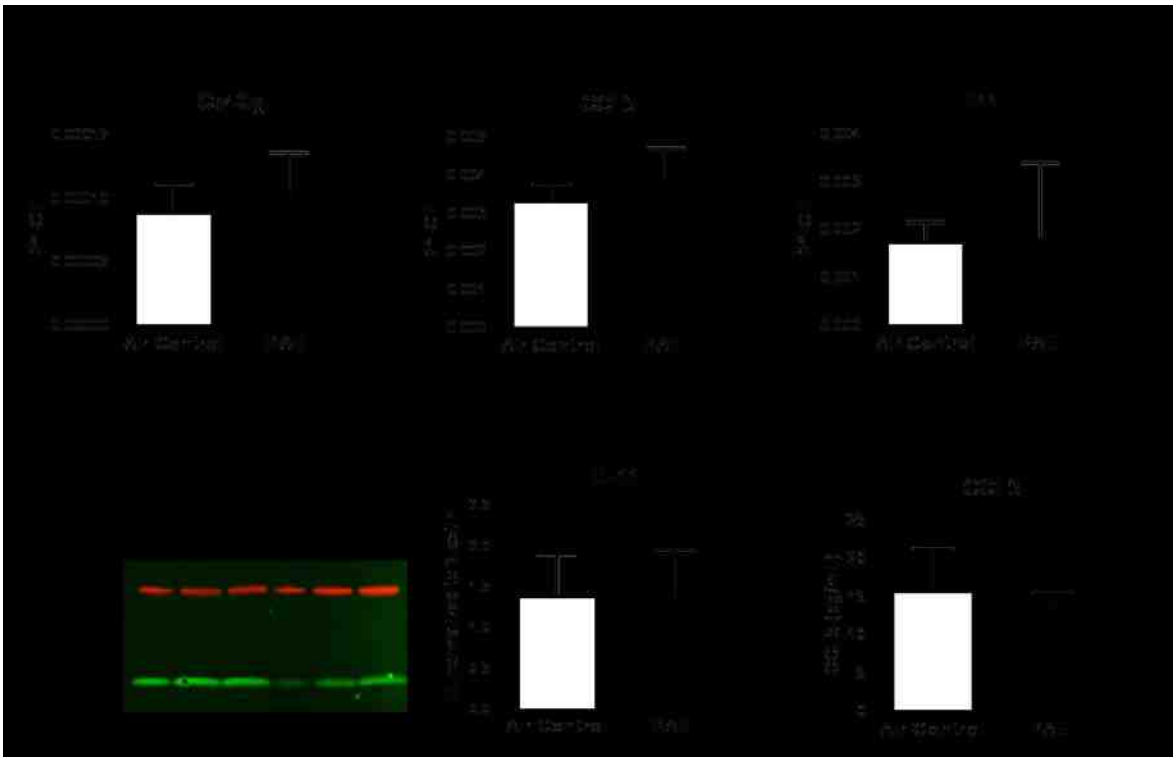

**Figure A.1.1: Effect of moderate PAE on the Dentate Gyrus of Male Pups.**

PCR validation shows that moderate PAE does not affect expression of CD40lg (A, n =4 litters), CCL5 (B, n = 6-7 litters), or Il11 (C, n = 6-7 litters) at the level of mRNA. Protein levels of IL-11 are not affected by moderate PAE, as measured by western blot (D, E, n = 7 litters). An example western blot for IL-11 is shown in (D) in which each sample was loaded in 5, 10, and 15 µg protein concentrations and normalized to β-actin. Protein levels of CCL5 were unaffected by moderate PAE, as measured by ELISA (F, n = 4 litters).

**Table A.1.2 Inflammatory Gene Expression in Females after Postnatal Alcohol Exposure**

mRNA isolated from the dentate gyrus of female pups was run on an inflammatory gene array containing 84 different genes. This table shows the genes most affected by moderate PAE. n = 5-6 litters

## Conclusions:

These data support the idea that moderate PAE has little effect on neuroimmune system activation.

## A.2 Binge alcohol exposure increases IL1β protein expression on P4 in the cerebellar vermis

**Purpose:**

This experiment was designed to test whether or not the changes in IL1β mRNA after binge alcohol exposure translated to the level of protein.

**Methods:**

Pups were exposed to alcohol and sacrificed as described in study #2. On P4, hippocampi and cerebellar vermis were removed and sonicated in ice cold homogenization buffer containing 20 mM Tris, 0.25 M sucrose, 2 mM EDTA, 10 mM EGTA, 1% Triton x-100, and 1 SIGMAFAST Protease Inhibitor tablet. Samples were centrifuged for 40 min at 100,000 X g and the supernatant was collected and kept at -80 until use. Protein was quantified using a Bio-rad protein assay (Cat. No. 500-0006). Levels of IL1β protein expression were quantified using a Rat IL-1β/IL-1F2 Quantikine ELISA (R&D Systems, RLB00) according to the manufacturer's instructions. Statistics were determined by unpaired t-test.

**Results:**

IL1β expression on P4 was significantly increased by PAE in the cerebellum to a similar extent as was seen in mRNA in study #2. In the hippocampus, no increase was seen in IL1β protein on P4, which again replicates the findings in mRNA in study #2.

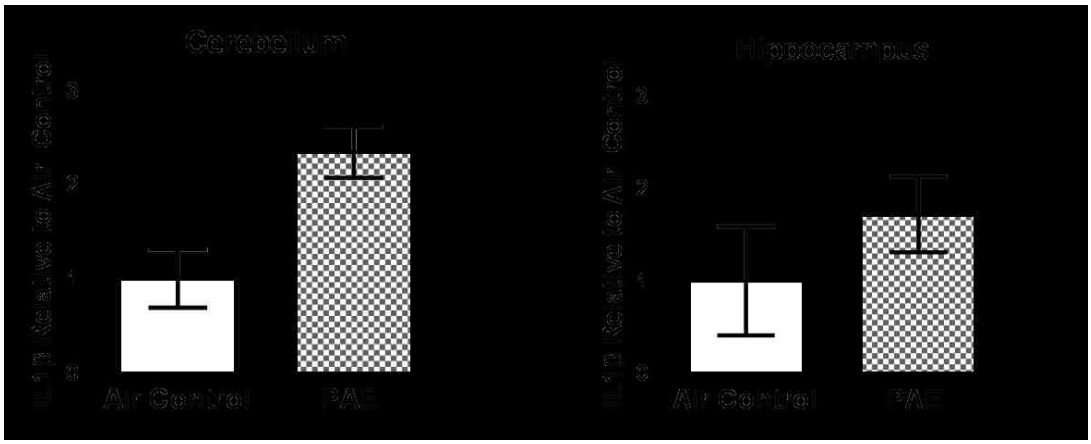


**Figure A.2.1: Protein Expression of IL1β Following Binge Alcohol Exposure was Measured on P4 in the Hippocampus and Cerebellum by ELISA.**

Protein levels for the air control were averaged and then each individual data point for both air control and PAE samples was normalized to this average. n = 4 litters. (* $p < 0.05$).

## Conclusions:

These results show that on P4, the results seen in IL1β mRNA are closely mirrored by expression at the level of protein.

## A.3 Binge alcohol exposure does not increase TUNEL staining in the cerebellum on P4.

**Purpose:**

The purpose of this experiment was to see if signs of neuronal apoptosis were already evident at P4, before neuronal loss occurred but concurrent with increased cytokine production. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) can be used to detect DNA fragmentation associated with late-stage apoptosis in cells.

**Methods:**

Pups were exposed to alcohol, sacrificed, and slices for immunohistochemistry were collected on P4, as described in study #2. To label neurons undergoing apoptosis, TUNEL staining was performed using the TACS 2 TdT-Fluor *In Situ* Apoptosis Detection Kit (Trevigen, cat no. 4812-30-K, Gaithersburg, MD), according to the manufacturer's instructions. Slices were imaged as described in study #2, and an analysis of TUNEL intensity was completed in the external granule layer, the Purkinje layer, and the internal granule layer.

**Results:**

In both air control and PAE animals, there were pockets of intense TUNEL staining within the cerebellar vermis (Figure A.3.1a). No difference was observed between the air control and PAE and there staining intensities were not elevated in the Purkinje cell layer compared to the other layers (Figure A.3.1b).

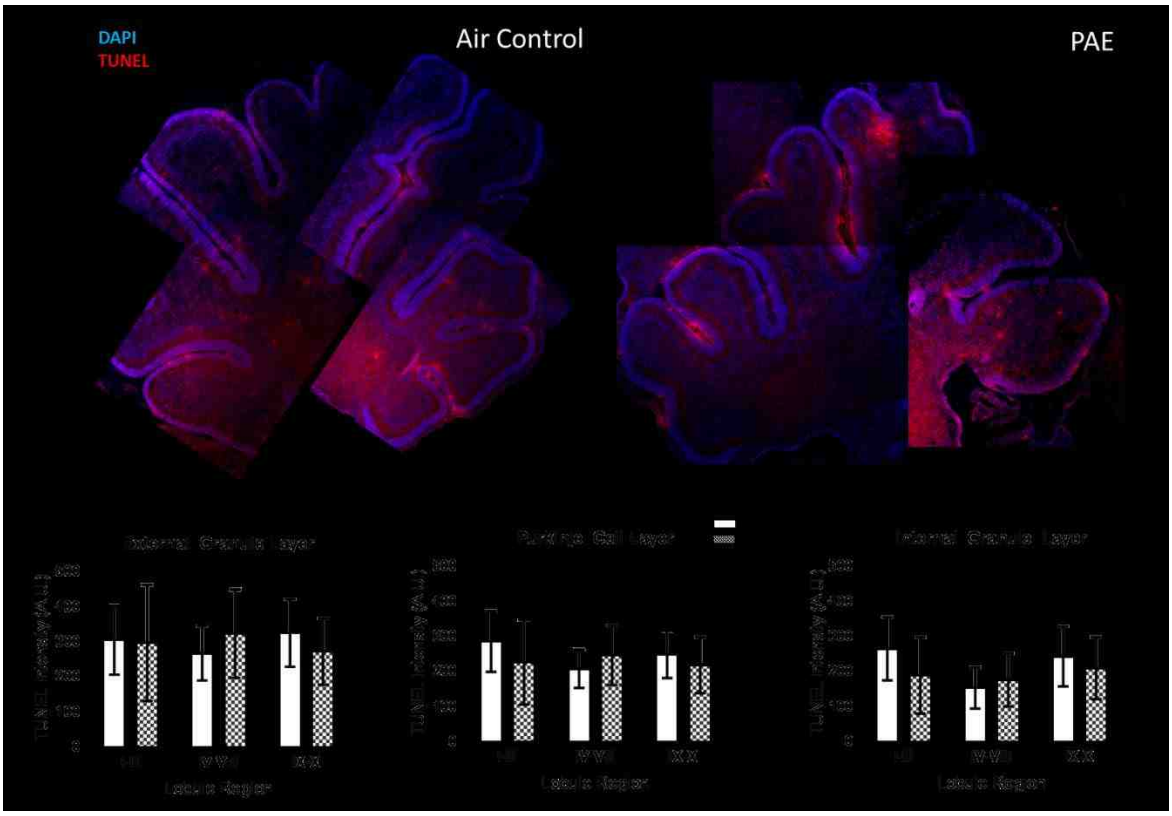


**Figure A.3.1: Binge Alcohol Exposure does not Increase TUNEL Staining Intensity at P4 in the Cerebellum.**

Cerebellar sections were stained for TUNEL (red) to label cells undergoing apoptosis, and DAPI (blue) to label cell nuclei. Example 10X images for PAE and air controls are shown (A). The TUNEL staining intensity is quantified for the external granule layer, purkinje cell layer, and internal granule layer (B). (*n* = 3-4 litters)

## Conclusions:

TUNEL is a late marker for apoptosis, so there may be no increase in the PAE animals at this age because cytokine production is only just spiking and apoptosis is just beginning to occur. It is possible that TUNEL is most elevated around P5, before neuronal loss is evident. Additionally, the pockets of intense TUNEL staining in both groups is likely due to the period of programmed cell death that occurs around this time [352].

## A.4 Microglia are not organized around neuronal axons at P6 in the cerebellum

**Purpose:**

Staining for microglia in the postnatal cerebellum indicated that microglia were not organized in the Purkinje cell layer, where neuronal death occurred. Instead they were located centrally within the cerebellum and cerebellar lobules, in the places that white matter would normally reside in an adult brain. We hypothesized that microglia might be interacting with neuronal axons. To test this, we stained for PanNeuronal, which is an antibody blend for multiple different proteins expressed throughout neurons including in neuronal axons, and myelin binding protein (MBP) which labels myelinated axons.

**Methods:**

Pups were exposed to alcohol, sacrificed, and slices for immunohistochemistry were collected on P6, as described in study #2. The following antibodies were used: rabbit anti-MBP (1:500, abcam, cat# ab40390 Cambridge, MA) and mouse anti-PanNeuronal (1:500, Millipore, cat# MAB2300, Darmstadt, Germany). The immunohistochemistry protocol and secondary antibodies are described in study #2. Images were taken and assembled such that the entire cerebellar vermis section was visible and compared to similar sections (used in study #2) from the same brain in which IBA-1 was used to label microglia.

**Results:**

MBP staining was barely visible within the cerebellar vermis at P6 in either air controls or PAE pups. PanNeuronal staining was evident within the center of the cerebellar vermis and was beginning to intensify within some lobules. No differences were seen between air control and PAE animals. Additionally, comparison of staining for microglia revealed no evidence of patterning in areas in which PanNeuronal was seen.

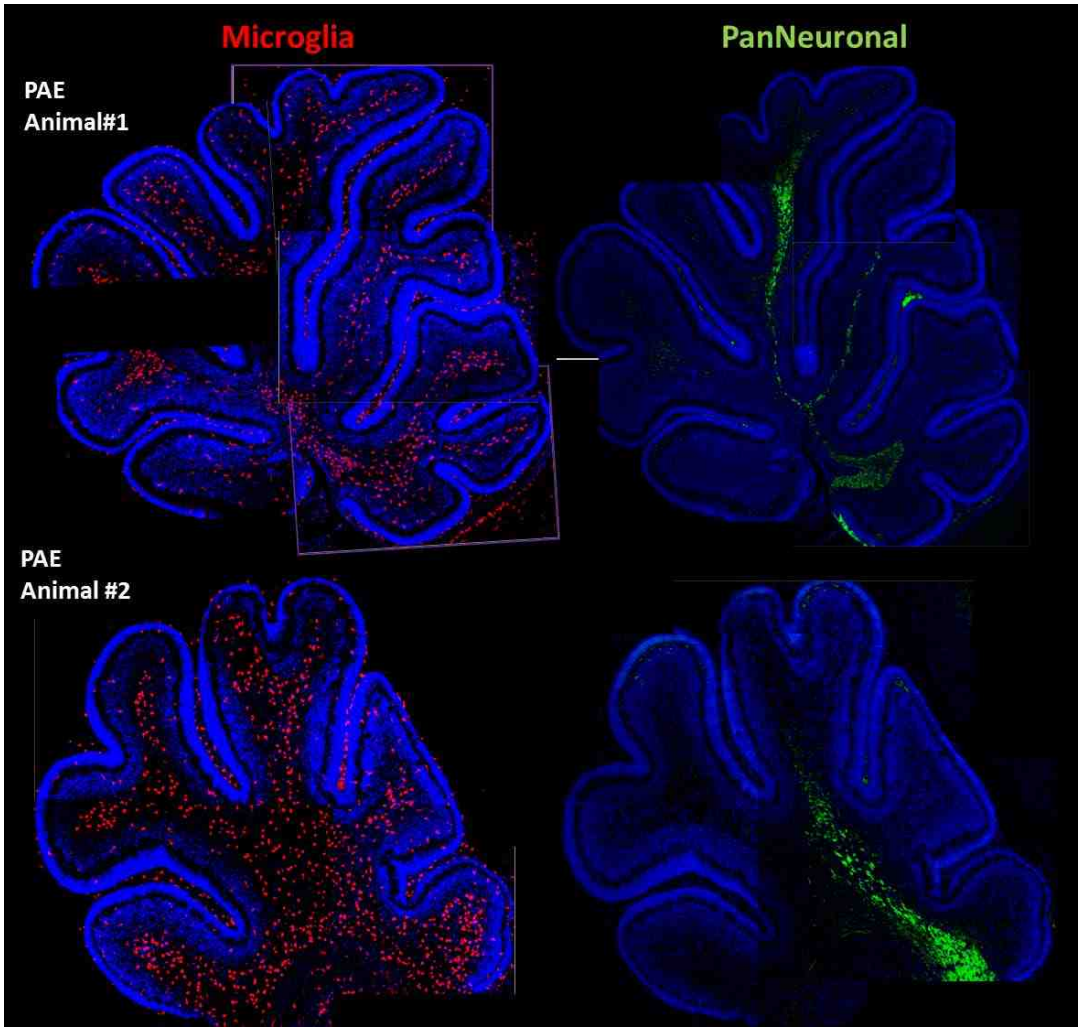


**Figure A.4.1**

Representative images are shown from two PAE pups from separate litters. Staining for IBA-1 (microglia, red) and PanNeuronal (green) are shown in consecutive sections. Air controls had similar PanNeuronal staining to these PAE animals.

**Conclusions:**

Neuronal axons are not myelinated within the cerebellar vermis at P6. Additionally, little PanNeuronal staining is evident within the lobules at P6, indicating that the axons are only beginning to extend outward from their cell layers. The centralized organization of microglia does not seem to be in a response to neuronal axons, however the massive number of amoeboid microglia after PAE is evident throughout the cerebellar vermis.

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