ETHANOL AND MICROGLIA: FROM MOLECULES TO BEHAVIOR

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ABSTRACT

Thomas Jordan Walter: Ethanol and Microglia: From Molecules to Behavior
(Under the direction of Fulton Crews)

Alcohol use disorders (AUDs) are prevalent mental health conditions involving problematic alcohol use despite negative consequences. Available treatments for AUDs are often unhelpful, prompting a search for more effective medications. Interestingly, recent research has identified a role for the neuroimmune system in AUDs, suggesting therapies targeting immune signaling may be helpful. Microglia are the most prominent cell type of the neuroimmune system and are implicated in AUDs. Indeed, alcohol activates microglia. Stress – believed to play a critical role in AUDs – also activates microglia. Alcohol and stress are thought to activate microglia by increasing leakage of bacterial products such as endotoxins from the gut. This causes peripheral inflammation that impacts the neuroimmune system and activates microglia. However, the significance of this microglial activation is unclear. Indeed, microglial activation has multiple functional outcomes ranging from pro-inflammatory and destructive to anti-inflammatory and healing. Microglia can also affect neuronal activity and influence behavior. Understanding how alcohol and stress impact microglia and how this affects the brain and behavior may provide critical insights into the mechanisms of AUDs.

In this dissertation, we investigate the relationship between microglia and AUDs. We used microglial-cell culture models and animal models to investigate the effects of ethanol on microglia. We also used the microglial-depleting compound PLX5622 to study the role of microglia in the

effects of ethanol. We find that acute ethanol withdrawal increased microglial pro-inflammatory cytokine expression (TNFα, Ccl2) *in vitro* and *in vivo*, and that microglial depletion blunted the brain pro-inflammatory response (TNFα, Ccl2) and enhanced the brain anti-inflammatory response (IL-1ra, IL-4) to acute ethanol withdrawal. Furthermore, simultaneous exposure to acute ethanol and acute stress enhanced plasma endotoxin and increased the microglial marker CD11b in multiple brain regions. Chronic intermittent ethanol persistently enhanced stress-induced plasma endotoxin and microglial CD11b across the brain. Finally, microglial depletion blunted the neuronal c-Fos response to acute ethanol withdrawal in some brain regions and decreased voluntary ethanol consumption over time. Overall, these results implicate microglia in AUDs, suggesting therapies that target immune signaling may aid treatment.

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LIST OF ABBREVIATIONS

AA – Alcoholics Anonymous

ACTH – Adrenocorticotropic hormone

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AUD – Alcohol Use Disorder

BAC – blood alcohol concentration

BDNF – brain derived neurotrophic factor

BEC – blood ethanol concentration

BNST – bed nucleus of the stria terminalis

CBT – cognitive behavioral therapy

Ccl2 – chemokine C-C motif ligand 2

CE – chronic ethanol

CeA – central amygdala

CNS – central nervous system

CORT – corticosterone

CRF – corticotropin-releasing factor

CSF1R – colony stimulating factor 1 receptor

DSM – Diagnostic and Statistical Manual of Mental Disorders

FDA – Food and Drug Administration

HMGB1 – high mobility group box 1 protein

HPA – hypothalamic-pituitary-adrenal

IA – intermittent access

IEG – immediate early gene

IL-1ra – interleukin 1 receptor antagonist

IL-4 – interleukin 4

IR – immunoreactivity

LTP – long term potentiation

MEC – media ethanol concentration

MI – motivational interviewing

NAc – nucleus Accumbens

NIAAA – National Institute on Alcohol Abuse and Alcoholism

PFC – prefrontal cortex

PVN – paraventricular nucleus of the hypothalamus

SNS – sympathetic nervous system

TLR – Toll-like receptor

TNFα – tumor necrosis factor α

TREM2 – triggering receptor expressed on myeloid cells 2

TSPO – translocator protein

VTA – ventral tegmental area

CHAPTER 1: INTRODUCTION

1.1 Alcohol Use in Humans: An Overview

Ethanol is a small, organic molecule (C_2H_5OH) that has been used by humans for thousands of years as both a food and a drug. Historically used because fermentation helped protect water from contamination, ethanol is still widely used today in various beverages/foods and as a recreational substance. Indeed, ethanol is one of the most widely used psychoactive substances in the world [1], commonly consumed for its ability to reduce anxiety and induce euphoria. However, ethanol is also very widely abused. The National Institute on Alcohol Abuse and Alcoholism (NIAAA) has described various patterns of alcohol abuse, including heavy drinking and binge drinking [2]. Heavy drinking is defined as ≥8 drinks per week for a woman or ≥15 drinks per week for a man, and binge drinking is defined as alcohol consumption leading to a blood alcohol concentration (BAC) of ≥80 mg/dL [3], or the legal driving limit in the United States. BACs of ≥80 mg/dL are usually achieved by a woman consuming ≥4 drinks in less than 2 hours, or a man consuming ≥5 drinks in less than 2 hours. In the United States, one standard drink contains 14 grams of ethanol, which is equivalent to 12 oz of 5% beer, 5 oz of 12% wine or 1.5 oz of 40% liquor. Individuals who engage in excessive drinking patterns are at higher risk for developing an alcohol use disorder (AUD). The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM 5) defines AUDs as problematic patterns of alcohol use leading to clinically significant impairment or distress, as manifested by at least two of the following criteria occurring within a 12-month period:

- ending up drinking more or longer than intended
- wanting to cut back or stop drinking but being unable

- spending a lot of time drinking and/or getting sick because of alcohol
- craving alcohol
- drinking that interferes with family, job or school
- continuing to drink despite negative consequences
- giving up on important or interesting activities because of drinking
- getting into dangerous situations because of drinking
- continuing to drink despite it worsening other mental or health problems
- developing tolerance to alcohol
- developing withdrawal symptoms

Epidemiological data show that AUDs are highly prevalent, with 13.9% of the U.S. population meeting criteria for an AUD within the past year, and 29.1% meeting criteria over a lifetime [4]. AUDs contribute to substantial morbidity and mortality and are associated with increased risk of several diseases, including alcoholic liver disease, pancreatitis, various cancers, gastrointestinal problems, immune dysfunction, neuropsychiatric conditions and heart problems [5, 6]. Furthermore, substantial economic costs are attributable to AUDs [7]. There is therefore significant need for treatment.

Current treatment options for AUDs include therapies such as motivational interviewing (MI) and cognitive behavioral therapy (CBT), as well as support groups such as Alcoholics Anonymous (AA). Treatment options also include medication. The Food and Drug Administration (FDA) has approved three pharmacological treatments for AUDs, including disulfiram, acamprosate and naltrexone [8]. Disulfiram produces an aversive response to alcohol, acamprosate is thought to correct neurotransmitter imbalances in the alcoholic brain, and naltrexone works by blocking the rewarding effects of alcohol [8]. Other medications, including topiramate, gabapentin, baclofen and

nalmefene are also used to treat AUDs. However, these treatments are often unhelpful or have modest efficacy due to the complexity and heterogeneity of AUDs [8]. There is therefore a pressing need for more effective medications to treat AUDs. Interestingly, recent work has identified a role for the brain neuroimmune system in the pathogenesis of AUDs [9], suggesting that drugs targeting immune signaling may be viable candidates for treatment. Microglia – the resident macrophages of the brain – are the primary cells of the neuroimmune system. While microglia have been implicated in AUDs [10], much remains unknown regarding the role of microglia in this disease.

This dissertation will describe novel studies that investigate the relationship between microglia and AUDs. Chapter 1 will begin by introducing the neurobiology of alcohol use disorders, the neuroimmune system and microglia, the effects of alcohol on microglia, and what is currently known about microglia and AUDs. We will explain concepts relating to alcohol use disorders, including the cycle of addiction and reinforcement. We will also describe the brain regions involved in the development of AUDs. Throughout Chapter 1, we will note the importance of stress in the development of AUDs. We will also introduce the neuroimmune system and microglia, and explain how microglia can influence neuronal activity and subsequently alter behavior. This will lay the foundation for understanding how microglia may contribute to the neuronal and behavioral dysfunction seen in AUDs. We will then describe how the neuroimmune system and microglia may be involved in the addiction cycle. Finally, we will explain the effects of alcohol and stress on the neuroimmune system and microglia, and how this might contribute to psychopathology such as AUDs. Chapter 1 will end with the overarching hypothesis that microglia contribute to alcohol use disorders. Specifically, we hypothesize that alcohol and stress activate microglia, and that activated microglia cause a brain neuroimmune response that alters neuronal function. This changed neuronal function then contributes to the maladaptive behavior of AUDs. In Chapters 2, 3 and 4, we will

describe studies that test this hypothesis. Chapter 2 will describe studies examining how acute ethanol exposure and withdrawal activates microglia and how microglial depletion alters the brain neuroimmune response to acute ethanol withdrawal. Chapter 3 will explain studies showing how alcohol and stress interact to enhance microglial activation. Lastly, Chapter 4 will describe studies showing that microglia contribute to the neuronal response to acute ethanol withdrawal and contribute to ethanol consumption. Finally, Chapter 5 will conclude with a discussion of the significance of this work and ideas for future studies.

1.2 Introduction to Alcohol Use Disorders

1.2.1 The Addiction Cycle and Reinforcement

In this section, we will first introduce concepts relating to alcohol use disorders, including the addiction cycle and reinforcement. We will then describe the neurobiology of alcohol use disorders, including the effects of different BACs, the different brain regions involved in AUDs and how stress contributes to alcohol use disorders. Current theories of addiction conceptualize addiction disorders as encompassing a three-stage cycle that includes (1) binge/intoxication, (2) withdrawal/negative affect, and (3) preoccupation/craving [11] (Fig1.1). The first stage – binge-intoxication – involves heavy use of the substance. The second stage – withdrawal/negative affect – involves the emergence of negative emotional states such as anxiety, irritability and dysphoria following withdrawal from the substance. Finally, the third stage – preoccupation/craving – involves intense thinking about and desiring of the substance, leading to another phase of the binge/intoxication cycle. This cycle of binge/intoxication, withdrawal/negative affect and preoccupation/craving contributes to the development and maintenance of addiction behavior.

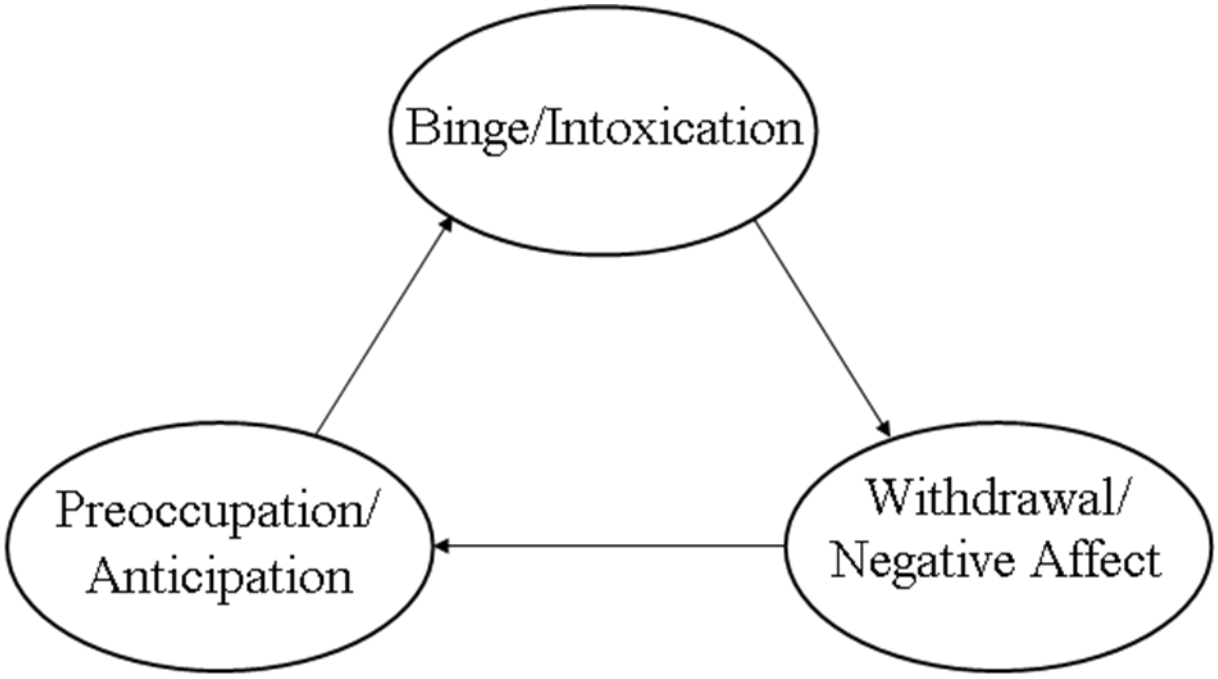


Figure 1-1 Stages of the Addiction Cycle. Current views theorize addiction to be a cycle that involves three stages: binge/intoxication, withdrawal/negative affect and preoccupation/ anticipation. These stages contribute to the development and maintenance of addiction behavior.

The stages of the addiction cycle are involved in the reinforcement of alcohol drinking behavior, especially the binge/intoxication and withdrawal/negative affect stages. Reinforcement is defined as a consequence that increases the likelihood a behavior will be repeated. There are two types of reinforcement: positive and negative. Positive reinforcement occurs when presentation of a rewarding stimulus increases the probability of a behavior. Negative reinforcement occurs when removal of an aversive stimulus increases the probability of a behavior. Both positive and negative reinforcement play a role in driving alcohol consumption [12] (Fig 1.2). Indeed, positive reinforcement is associated with the binge/intoxication stage of addiction. Negative reinforcement is associated with the withdrawal/negative affect stage of addiction. The development of alcohol use disorders is thought to involve a gradual transition from positive to negative reinforcement over time [12, 13]. Alcohol is initially rewarding, and drinking is driven primarily by positive reinforcement of the binge/intoxication stage. However, as drinking continues and increases, changes occur in the

brain that reduce the positive reinforcement caused by binging/intoxication and increase the negative reinforcement caused by withdrawal. As withdrawal becomes increasingly unpleasant, additional alcohol is consumed to alleviate these aversive states. At this point, drinking is driven by negative reinforcement. Therefore, an evolving combination of positive and negative reinforcement contribute to the development and maintenance of alcohol use disorders.

The development of alcohol use disorders often involves an escalation of alcohol intake over

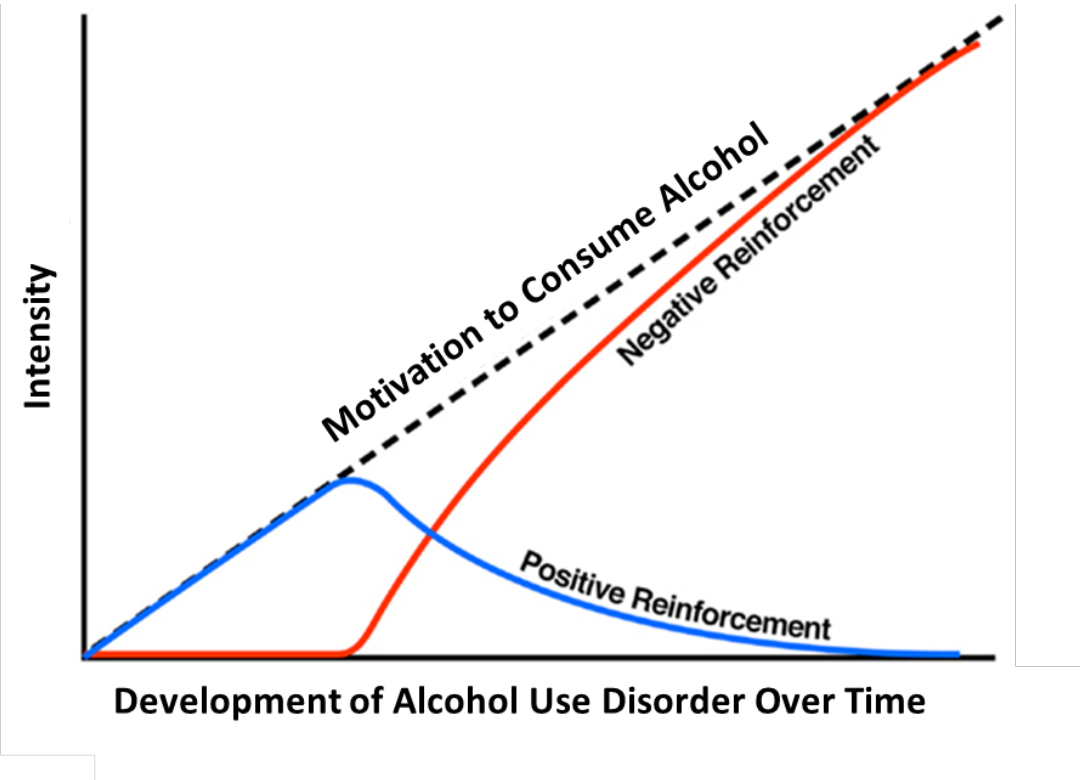


Figure 1-2 Role of Positive and Negative Reinforcement in Driving Alcohol Consumption as Alcohol Use Disorders Develop. In the development of alcohol use disorders, alcohol is initially rewarding, and drinking is driven primarily by positive reinforcement. As excessive alcohol use changes the brain, functioning of reward systems decreases, leading to decreased positive reinforcement. Withdrawal also becomes increasingly aversive and drinking is driven increasingly by negative reinforcement. Adapted from Koob, Frontiers in Psychiatry, 2013.

time. Increasing alcohol intake leads to increasing BACs that have different effects. BACs of 50-100 mg/dL cause desirable effects such as relaxation, disinhibition and euphoria, as well as motor impairment. Higher BACs of 200 mg/dL result in nausea, vomiting, emotional lability and cognitive

impairment. BACs of 300 mg/dL produce stupor and BACs of 400 mg/dL can result in coma. Finally, BACs of 500 mg/dL are lethal for most naïve drinkers [14, 15]. However, individuals with high alcohol tolerances can attain even higher BACs. Multiple studies in humans document individuals attaining a wide range BACs, including levels up to 400 mg/dL [16-18]. Escalation of alcohol intake and BACs impact the brain differently as alcohol use disorders develop, making it important to understand the effects of different doses of alcohol on the brain.

1.2.2 Brain Regions Involved in AUDs

Dysfunction in several brain regions contributes to alcohol use disorders. A basic understanding of the brain regions implicated in AUDs will provide a foundation for understanding work in later chapters. Regions such as the ventral tegmental area (VTA) and nucleus accumbens (NAc) are involved in positive reinforcement, and alcohol is known to interact with these regions [12] (Fig1.3). The VTA is located on the floor of the midbrain and the NAc is located in the basal forebrain. Dopamine neurons in the VTA project to the NAc, and the release of dopamine into the NAc increases reinforcement [19]. This neural pathway is thought to be the common pathway upon which all substances of abuse, including alcohol, act to increase reinforcement. Indeed, acute alcohol increases neuronal activation in the VTA and NAc [20]. One common method for assessing neuronal activity is measuring the expression of immediate early genes (IEGs). IEGs are induced shortly after neuronal activation, and include genes such as c-Fos, EGR1, and Arc [21]. Acute alcohol increases c-Fos levels in the VTA and NAc [20]. Alcohol also increases dopamine release in the NAc in both animals [22] and humans [23]. These studies show that alcohol acts on brain regions involved in positive reinforcement to contribute to alcohol use disorders.

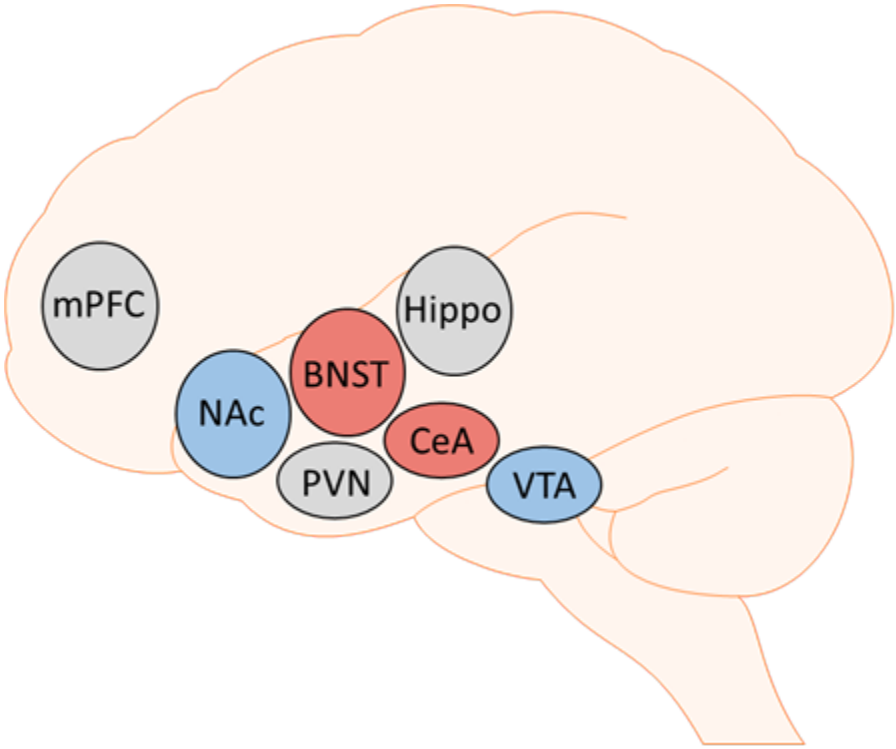


Figure 1-3 Brain Regions Involved in Alcohol Use Disorders. Several brain regions are involved in the development and maintenance of alcohol use disorders. Some regions, such as the ventral tegmental area (VTA) and nucleus accumbens (NAc) are involved in positive reinforcement and the binge/intoxication stage of addiction (blue). Other regions, such as the bed nucleus of the stria terminalis (BNST) and central amygdala (CeA) are involved in negative reinforcement and the withdrawal/negative affect stage of addiction (red). Finally, regions such as the medial prefrontal cortex (mPFC), paraventricular nucleus of the hypothalamus (PVN) and hippocampus also contribute alcohol use disorders (grey), playing various roles in each of the stages of addiction, including the preoccupation/craving stage.

Brain regions such as the bed nucleus of the stria terminalis (BNST) and the central amygdala (CeA) are involved in negative reinforcement, and alcohol is known to interact these regions (Fig1.3). The CeA is located in the medial temporal lobe, and the BNST is located in the forebrain. Both acute alcohol and alcohol withdrawal increase neuronal activation in the CeA and BNST [20]. These brain regions are also activated by acute stress [24, 25] and play a critical role in the stress response [26]. Stress, often defined as a real or perceived threat to homeostasis or well-being, is believed to play an important role in the development of AUDs. Indeed, alcohol use disorders are

considered “stress surfeit disorders” [27], in which over-activity of brain stress systems contributes to alcohol consumption, and excessive alcohol consumption further enhances activity of brain stress systems. Animal studies find that chronic stress can indeed increase alcohol consumption [28]. Studies in human beings also find increased alcohol consumption with stress [29, 30]. Overall, these studies suggest that alcohol and stress act on brain regions involved in negative reinforcement to contribute to alcohol use disorders.

In addition to those described above, there are many other brain regions are involved in regulating alcohol consumption (Fig1.3). The prefrontal cortex (PFC) is the foremost region of the frontal lobe, and is involved in executive control. Executive control includes functions such as reasoning, planning, decision-making, impulse inhibition, etc. Various studies show that alcohol impacts prefrontal cortical functioning. For example, heavy drinking reduces several executive functions in humans [31]. Prefrontal dysfunction and decreased executive control are thought to contribute to decreased impulse control, increased compulsive behavior, and decreased planning/goal-setting, all of which contribute to escalation and loss of control over alcohol consumption [32]. Another brain region involved in alcohol use disorders is the paraventricular nucleus of the hypothalamus (PVN). The PVN plays a critical role in the stress response. Neurons in the PVN release corticotropin-releasing factor (CRF), which stimulates the anterior pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH then stimulates the adrenal glands to produce glucocorticoid stress hormones such as cortisol. This pathway is referred to as the hypothalamic-pituitary-adrenal (HPA) axis. Acute alcohol activates neurons of the PVN [20] and increases plasma glucocorticoid levels [33]. Dysregulation of the HPA axis by chronic alcohol is thought to contribute to alcohol use disorders [33]. Indeed, stress is thought to play an important role in AUDs. Also, glucocorticoids play important roles in reward, learning and memory [33], each of

which are involved in alcohol use disorders. Lastly, the hippocampus is another important brain region involved in alcohol use disorders. The hippocampus is located in the medial temporal lobe and is involved in learning and memory. Acute alcohol and chronic alcohol activates neurons in the hippocampus [20], and chronic alcohol is associated with learning and memory deficits. Indeed, dysregulation of learning and memory can contribute to alcohol drinking behavior [34]. For example, external cues (i.e. – environments associated with drinking) or internal cues (i.e. – memories of drinking) can elicit craving that leads to alcohol consumption. Therefore, maladaptive memory formation and reactivation can promote excessive alcohol use. Overall, these studies implicate multiple brain regions, including the mPFC, PVN and hippocampus, in the development of alcohol use disorders. However, much remains unknown regarding the mechanisms by which AUDs develop. Indeed, how alcohol and stress impact the brain to contribute to AUDs is still unclear.

1.3 Introduction to the Neuroimmune System and Microglia

1.3.1 Introduction to the Neuroimmune System

Interestingly, accumulating research finds alcohol and stress both activate the neuroimmune system, suggesting this system may contribute to AUDs [9, 35]. In this section, we will introduce the neuroimmune system, including the molecules and cell types that constitute it. We will also introduce microglia – the primary cell type of the neuroimmune system. We also describe how the neuroimmune system and microglia can alter neuronal function and subsequently behavior. This will lay the foundation for understanding how microglia may contribute to the neuronal and behavioral dysfunction of AUDs. The neuroimmune system is composed of the immune molecules and cells that interact with the nervous system. (Fig1.4). There are many types of immune

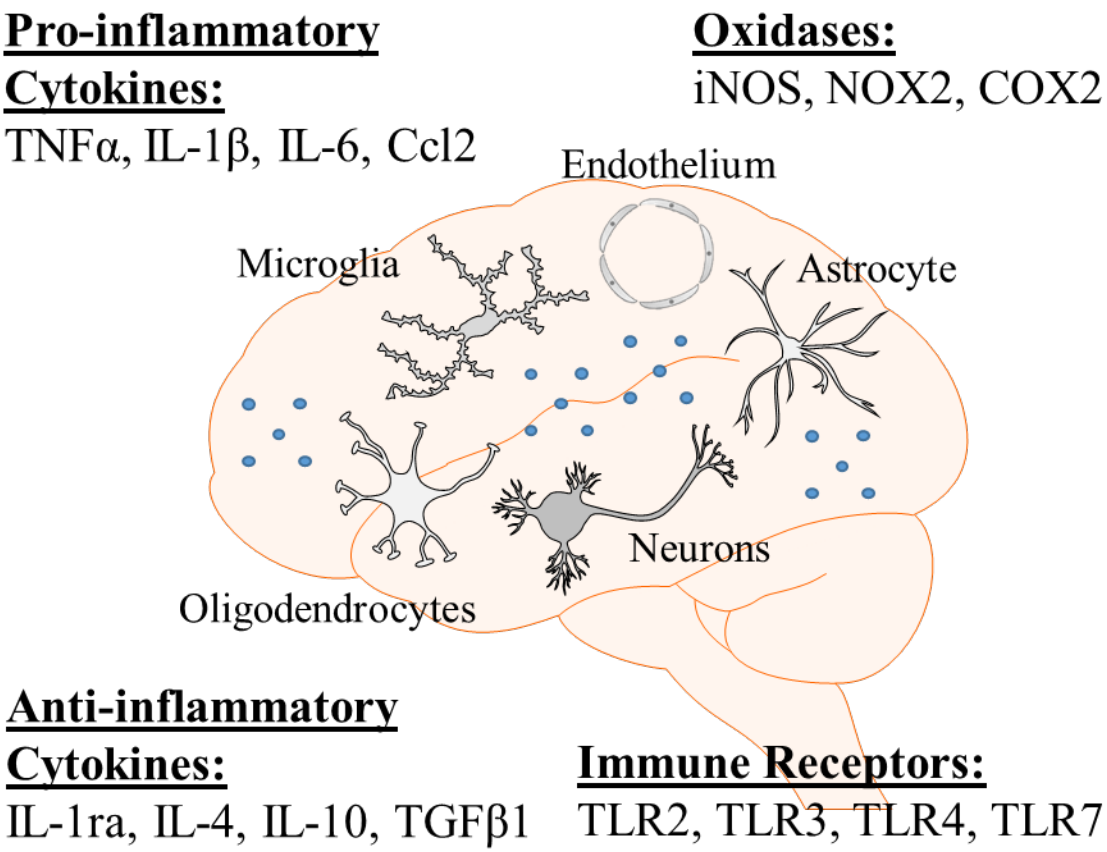


Figure 1-4 The Neuroimmune System. The neuroimmune system consists of the immune molecules and cells that interact with the nervous system. Many types of immune molecules, such as cytokines, oxidases, immune receptors and others are expressed in the brain. These molecules are expressed on a variety of different brain cell types, including microglia, astrocytes, oligodendrocytes, endothelium and even neurons. In addition to having classic immune functions, the neuroimmune system also impacts the function of neurons. This is significant, because neurons are the final effectors of behavior. Therefore, the neuroimmune system has the capacity to impact behavior.

molecules present in the brain. Classic examples include cytokines: small, secreted proteins that play important roles in immune signaling. There are various types of cytokines, including pro-inflammatory cytokines that promote inflammation, and anti-inflammatory cytokines that inhibit inflammation. Tumor-necrosis factor α (TNFα), chemokine ligand 2 (Ccl2), interleukin-1β (IL-1β) and interleukin-6 (IL-6) are well-known pro-inflammatory cytokines, while interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-1 receptor antagonist (IL-1ra) and tissue growth factor β1 (TGFβ1) are examples of anti-inflammatory cytokines. The neuroimmune system also includes

chemokines - a specific type of cytokine that promotes migration of immune cells toward the source of the chemokine. Other examples of neuroimmune molecules include the complement proteins, a system of proteases involved in phagocytosis and microbial clearance. There are also various kinds of immune receptors, such as the Toll-like receptors (TLRs). TLRs interact with exogenous microbial-derived molecules as well as endogenous danger signaling molecules such as HMGB1 to impact immune function. Indeed, activation of Toll-like receptors contributes to inflammation. Furthermore, transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) play a key role in regulating transcription of immune genes. Overall, a variety of neuroimmune molecules are expressed in the brain, and these can impact brain function in several important ways.

There are also many cell types that constitute the neuroimmune system, including microglia, astrocytes, oligodendrocytes, endothelial cells and even neurons. Microglia are the resident macrophages of the brain and the primary cells of the neuroimmune system. They will be discussed in more detail in later sections. Astrocytes are also an important component of the neuroimmune system [36]. Astrocytes are involved in numerous physiological processes, such as metabolic support of neurons and modulation of synaptic transmission [37]. Astrocytes express multiple neuroimmune molecules that allow them to participate in immune processes [38], including pro-inflammatory cytokines such as IL-1β [39] and IL-6 [40], and Toll-like receptors such as TLR3 [41]. Oligodendrocytes are another cell type that provides support to neurons by generating myelin sheaths to insulate axons. Interestingly, these cells express a range of immune molecules, including cytokines such as IL-6 and TLRs such as TLR2 and TLR3 [42]. Cytokine expression by oligodendrocytes appears to increase during disease conditions [42]. Endothelial cells are another important cell type of the brain neuroimmune system. These cells line the blood vessels of the brain

and constitute a primary component of the blood-brain-barrier. Endothelial cells produce a variety of cytokines such as IL-1β and IL-6, as well as other immune mediators such as nitric oxide and prostaglandins [43]. Furthermore, immune signals from the periphery are detected by endothelial cells, which then trigger an immune response in the CNS. Endothelial cells are therefore critical to immune signaling between the periphery and the central nervous system. Lastly, even neurons contribute to the neuroimmune system. While not usually thought of as having an immune function, neurons express immune receptors, such as TL4 [44-46], and release chemokines, such as Cxcl10, in response to brain injury [47]. Neurons also express several molecules such as fractalkine and CD200 that inhibit the activation of microglia [48]. Finally, neurons release neurotransmitters, which can impact the activity of other neuroimmune cells such as microglia [48]. Therefore, all the cell types of the brain, including neurons, contribute to the neuroimmune system.

In addition to performing classic immune functions (e.g. – defense against pathogens), the neuroimmune system interacts with neurons to impact neuronal activity. For example, brain cytokines such as IL-1β and TNFα influence synaptic strength. Indeed, moderate levels of IL-1β are required for hippocampal long-term potentiation (LTP), a process by which synapses are strengthened [49]. Moderate levels of TNFα are also required for hippocampal LTP to occur efficiently [50, 51]. TNFα also increases the surface expression of the excitatory glutamatergic AMPA receptors on neurons, leading to increased frequency and amplitude of excitatory currents in neurons [52]. Other neuroimmune molecules such as the oxidase COX2 can influence synaptic plasticity. Increased COX2 activity is seen during the induction of LTP [53], and inhibition of COX2 results in impaired hippocampal LTP [54], showing that COX2 plays a role in synaptic plasticity. Complement proteins also impact neuronal function. The complement system includes multiple proteins, such as C1q and C3, that can “tag” synapses, marking them for elimination by microglial

phagocytosis. This is an example of how microglia can impact neuronal function. Overall, these studies show that the neuroimmune system impacts neuronal function. This is important because neurons are the final effectors of behavior, and suggests that the neuroimmune system can ultimately affect behavior (Fig1.5).

Interestingly, multiple studies find that the neuroimmune system plays an important role in behavior. Mice that overexpress TNFα specifically in the central nervous system show decreased performance on spatial learning and memory tasks [55]. These studies suggest that TNFα in the brain is important for cognitive functioning, even in the absence of an immune stimulus. Other studies find that IL-1β also plays a role in behavior. Mice over-expressing IL-1β in the hippocampus show delayed acquisition and decreased retention of a spatial learning task [56]. Interestingly, even immune receptors such as the TLRs have been implicated in behavior. Indeed, TLR2 knockout mice show behavioral deficits such as hyper-locomotion, social withdrawal and cognitive impairments

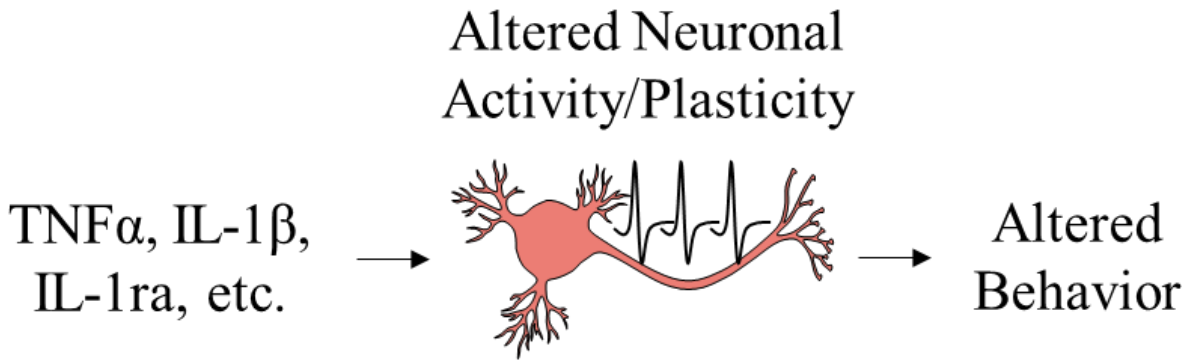


Figure 1-5 The Neuroimmune System Affects Neuronal Activity to Impact Behavior. Neuroimmune mediators present in the brain, such as TNFα, IL-1β, IL-1ra and others, can alter neuronal activity and plasticity. This is significant because neurons are the final effectors of behavior, implying the neuroimmune system can impact behavior. Indeed, various studies find that the neuroimmune system plays a role in both normal and pathological behaviors.

[57], and TLR4 knock-out mice show reduced novelty-seeking and social interaction [58]. Overall, these studies show the neuroimmune system impacts behavior, suggesting the neuroimmune system may contribute to pathological behaviors such those seen in alcohol use disorders.

1.3.2 Introduction to Microglia

Among the cell types of the neuroimmune system, microglia are the most prominent. Microglia play many important roles in the healthy brain. Some of these functions are immune functions, such as surveillance, defense and debris clearance [59]. Indeed, microglia express a wide range of cytokines, such as TNFα and IL-1β, oxidases, such as NOX2, complement proteins, TLRs, and many other immune molecules. They are critical effectors of several neuroimmune processes. However, recent studies find that microglia play additional roles. Indeed, microglia are unique among macrophage-like cells [60] and are specifically adapted to the CNS environment [61]. They selectively express many receptors such as TREM2 that may serve as ideal candidates for therapeutic targeting [60]. Microglia engage in functions such as synaptic pruning, synaptic formation, modulation of synaptic plasticity, regulation of neurogenesis and much more [59, 62]. Microglia can prune synapses via phagocytosis, release neurotrophins such as brain-derived neurotrophic factor (BDNF) that contribute to synapse formation [63] and modulate synaptic plasticity through the release of cytokines such as TNFα [64]. Furthermore, microglia can promote or inhibit neurogenesis depending on their activation state [65, 66]. Therefore, microglia are actively involved in normal brain function [63].

Microglia normally exist in a "resting" state. Despite the title "resting", microglia in this state are highly active [67], as their processes constantly scan the environment for threats or damage. In response to insults or disruptions of homeostasis, resting microglia undergo a process called activation. Microglial activation is a complex phenomenon and there are a variety of different activation states. Functional microglial activation is traditionally understood as occurring along a spectrum between two extremes, ranging from the pro-inflammatory, destructive M1 state to the anti-inflammatory, reparative M2 state [68], although recent studies suggest an even more

complicated picture [69, 70]. M1 microglia secrete pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 and produce reactive oxygen species through molecules such as iNOS and NADPH oxidase. M2 microglia secrete anti-inflammatory cytokines such as IL-10 and synthesize extracellular matrix products to assist with healing [71]. Overall, microglial activation is a complex phenomenon that can have a variety of different functional outcomes (Fig1.6). Indeed, how microglia respond to various stimuli will play a critical role in how they impact brain function.

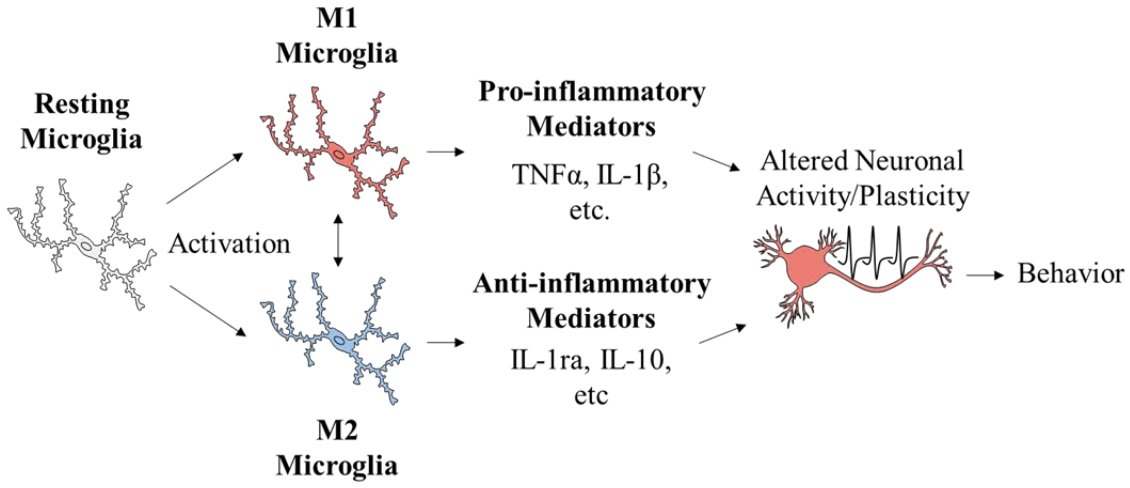


Figure 1-6 Microglial Activation and its Effects on the Brain. Microglia usually exist in a resting state in the brain. In response to various stimuli, microglia undergo a process known as activation. Microglial activation is a complex phenomenon involving multiple different activation states with different functional outcomes. The type of microglial activation that occurs is critically important to how the rest of the brain is impacted. Indeed, the M1 state is inflammatory and destructive while the M2 state is anti-inflammatory and reparative. The cytokines and other neuroimmune molecules produced by activated microglia will impact neuronal function, potentially changing behavior.

Indeed, microglial activation has important consequences for neurodegeneration and neuronal activity. Activated microglia can protect against [72] or contribute to neuronal cell death [73, 74] depending on their activation state. A variety of studies show that microglia activated to a pro-inflammatory state contribute to neurodegeneration through a variety of mechanisms (for review, see [75]), including production of toxic reactive-oxygen species and pro-apoptotic cytokines such as TNFα. Microglia also impact neuronal function in several other ways. Indeed, microglia strip

synapses from neurons, thereby altering neuronal activity [72]. Microglia also release cytokines such as TNFα that drive the internalization of AMPA receptors on neurons, leading to decreased glutamatergic synaptic strength [64]. In addition to cytokines, microglia release other compounds such as neurotrophins to impact neuronal function. Deletion of BDNF specifically from microglia decreased levels of glutamatergic NMDA receptor subunits and NMDA-mediated mini-excitatory post-synaptic currents (mEPSCs) in cortical pyramidal neurons [63]. This was associated with decreased formation and elimination of neuronal spines. Overall, these studies show microglial BDNF impacts neuronal function. Finally, mice lacking CX_3CR1, a microglial protein involved in neuronal-microglial communication, show weakened synaptic strength and brain regional connectivity compared to wild-type controls [76]. Overall, these studies show microglia impact neuronal function, suggesting that microglial activity may be able to influence behavior.

Indeed, various studies suggest that microglia can impact behavior (Fig1.6). Cocaine increases microglial TNFα in the nucleus accumbens, thereby altering NAc neuronal activity. This contributes to increased locomotor activity. Deletion of TNFα specifically from microglia blocks changes in NAc neurons and enhances the locomotor activity response to cocaine. These studies suggest microglial TNFα contributes to the behavioral effects of cocaine. Other studies find that deletion of BDNF specifically from microglia resulted in learning deficits. Mice lacking microglial BDNF showed decreased learning improvement over multiple rotarod training sessions. These studies find that microglial BDNF affects learning. Overall, this research suggests microglia play a role in behavior. It is therefore possible microglia contribute to the pathological behaviors associated with AUDs. However, much remains unknown regarding the role of microglia in the pathogenesis of AUDs.

1.4 Role of the Neuroimmune System and Microglia in Addiction

1.4.1 The Binge Intoxication Stage

Interestingly, accumulating evidence suggests that the neuroimmune system plays an important role in each stage of the addiction cycle. As the primary cells of the neuroimmune system, microglia may be critically involved in these processes. In this section, we will describe the role of the neuroimmune system in each stage of the addiction cycle. We will note how stress also activates the neuroimmune system, thereby potentially contributing to the progression of addiction. Regarding the binge/intoxication stage, blockade of IL-1β in the VTA with naloxone, a TLR4 antagonist, prevented cocaine-induced dopamine release in the NAc and decreased cocaine self-administration [77]. This effect may be mediated in part by microglia, as treatment with minocycline, a microglial inhibitor, blocked cocaine-induced conditioned place preference. This suggests a role for microglia in the rewarding effects of drugs of abuse. Several studies have also investigated the effects of the neuroimmune system on alcohol consumption. Experiments in knock-out mice show that immune genes influence alcohol intake. For example, deletion of *Ccr2*, the gene encoding the receptor for the pro-inflammatory cytokine Ccl2, reduced voluntary alcohol consumption in mice without reducing overall fluid intake [78]. Deletion of the *IL1rn* and *IL-6* genes also decreased voluntary alcohol consumption without changing total fluid intake [79]. This suggests the immune system may impact the desire to consume alcohol or the rewarding effects of alcohol. Additional studies show that while knocking out either the IL-1 receptor (IL-1R) or TNF receptor (TNFR) alone does not change alcohol consumption, knocking out both receptors simultaneously decreases voluntary alcohol consumption in mice. Furthermore, double IL-1R and TNFR knockouts blocked stress-induced alcohol intake [80]. This suggests the neuroimmune system may influence alcohol

consumption driven by negative reinforcement. Other studies show that injection of the inflammogen lipopolysaccharide increases alcohol consumption in mice [81]. This suggests increased inflammatory signaling promotes alcohol intake. As the primary immune cells of the brain, microglia may play a key role in mediating these effects. Overall, these studies show that the neuroimmune system influences alcohol intake and contributes to the binge/intoxication stage of the

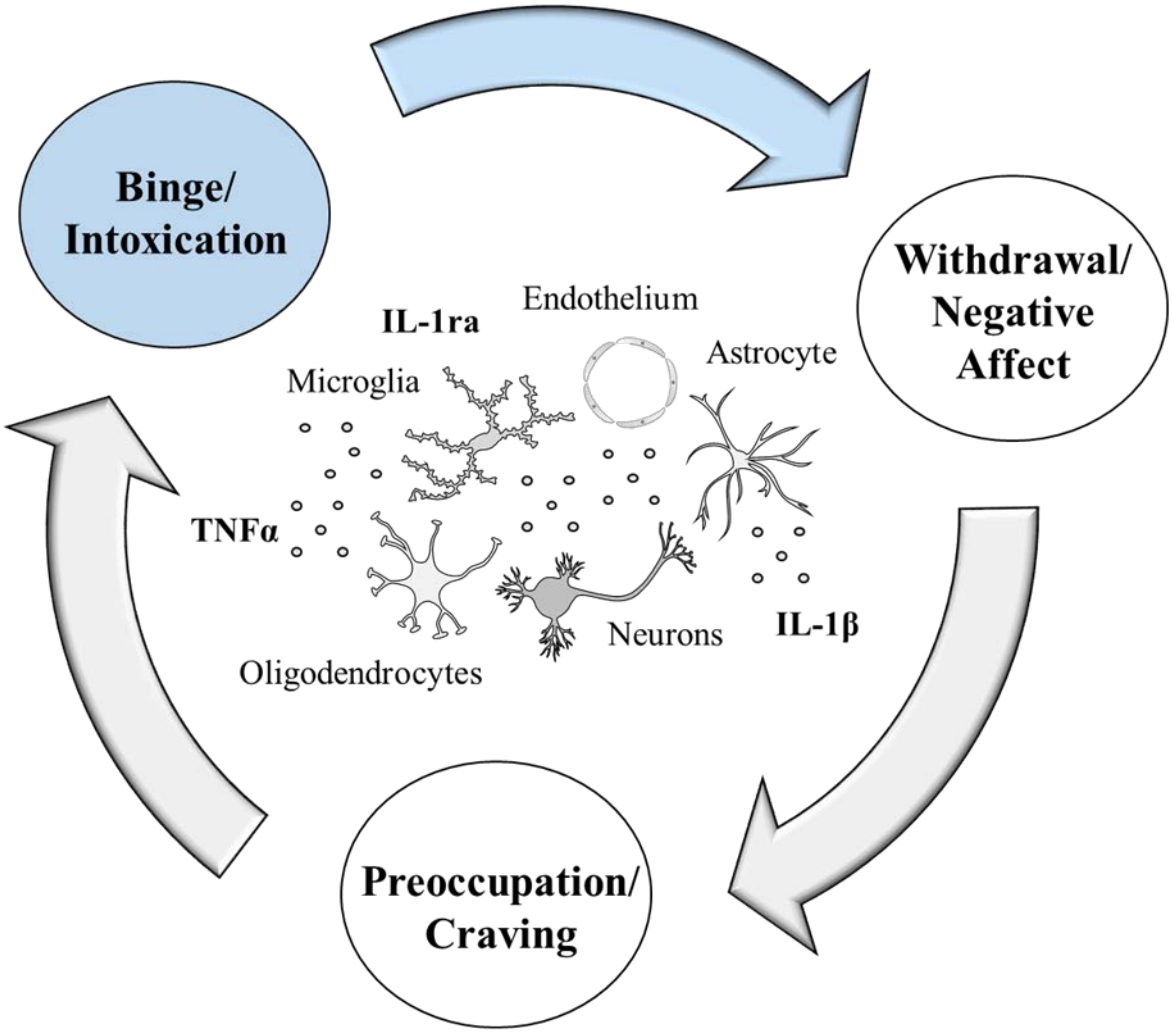


Figure 1-7 The Neuroimmune System Contributes to the Binge/Intoxication Stage of the Addiction Cycle. Several studies find that neuroimmune molecules such as IL-1β and IL-1ra impact ethanol consumption, suggesting the neuroimmune system plays a role in the binge/intoxication stage of the addiction cycle.

addiction cycle.

Other studies show that administration of neuroimmune molecules directly into the brain changes alcohol consumption. Indeed, injection of TLR4 siRNA into the central amygdala reduces alcohol self-administration [82]. Other studies find that intracerebroventricular infusion of the pro-inflammatory cytokine Ccl2 increases self-administration of sweetened alcohol, but not sucrose [83]. Furthermore, injections of IL-1ra, the IL-1 receptor antagonist, into the basolateral amygdala (BLA) reduced alcohol self-administration in mice [84], but did not alter sucrose self-administration or locomotor activity. Another study found that injection of the anti-inflammatory cytokine IL-10 into the BLA also reduced alcohol consumption without changing locomotor activity. These studies are important for demonstrating the role of the neuroimmune system in alcohol consumption behavior, as they show neuroimmune signaling solely within the brain can change alcohol intake. Overall, these data suggest the neuroimmune system plays a role in the binge/intoxication phase of addiction and alcohol use disorders (Fig1.7). As the primary immune cells of the brain, microglia may play a critical role in these processes; however, much remains regarding the role of microglia in the binge intoxication stage of AUDs.

1.4.2 The Withdrawal/Negative Affect Stage

There is also evidence implicating the neuroimmune system and microglia in the withdrawal/negative affect stage of addiction. Substance abuse is associated with the development of negative affective states, such as anxiety, irritability, depression and dysphoria during withdrawal [85]. These negative affective states are a driving force behind substance use. Multiple studies show that pro-inflammatory cytokines such as TNFα and Ccl2 increase in the brain during alcohol withdrawal [86, 87]. Another study finds brain IL-1β and TNFα are increased during morphine

withdrawal [88]. Increased pro-inflammatory cytokines during withdrawal contribute to the negative affective states. Indeed, alcohol withdrawal-induced anxiety and depression is increased following administration of the inflammogen lipopolysaccharide or the pro-inflammatory cytokine TNFα [89]. Also, knockout of TLR4 protects against withdrawal-induced anxiety-like behavior and memory

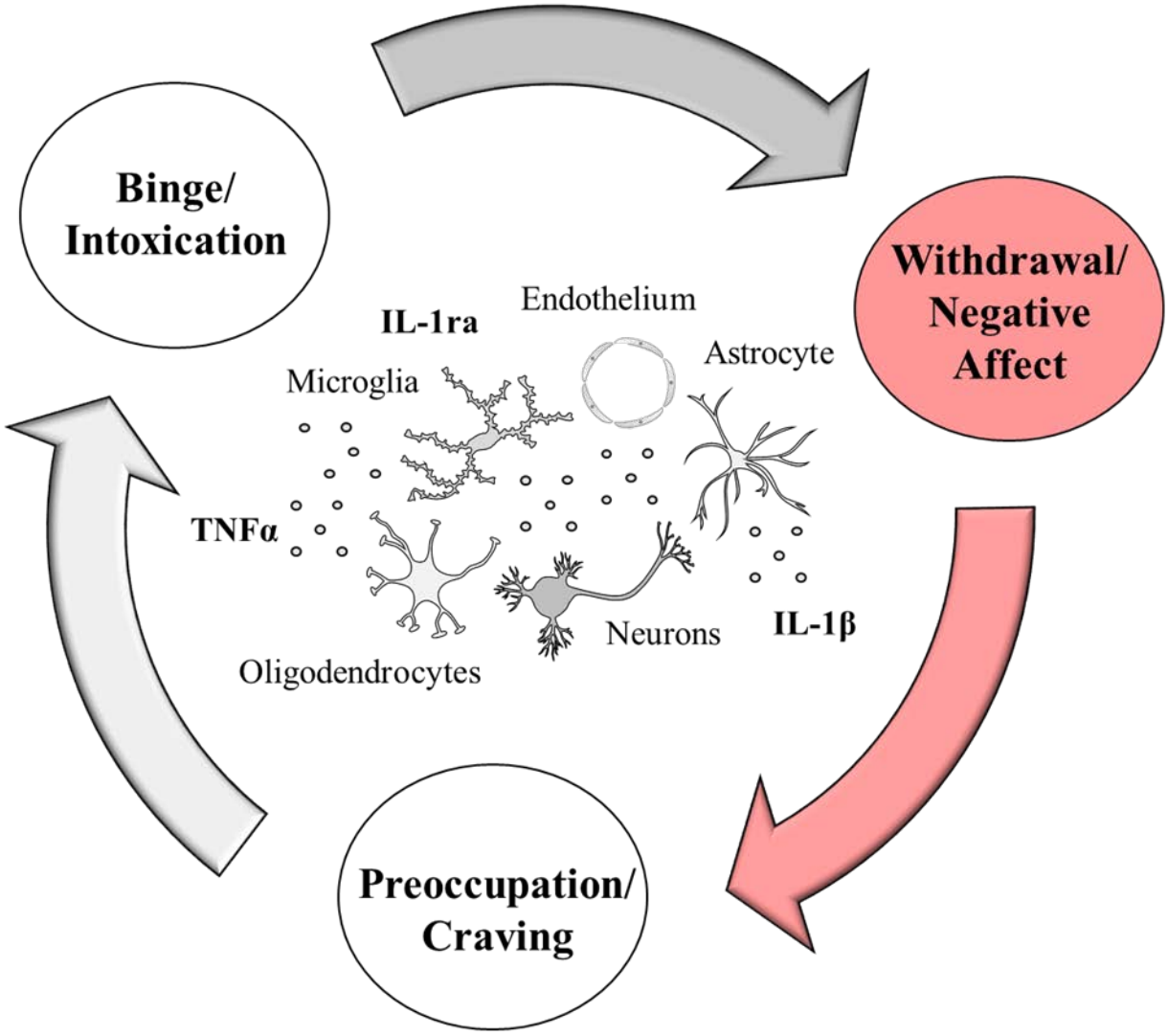


Figure 1-8 The Neuroimmune System Contributes to the Withdrawal/Negative Affect Stage of the Addiction Cycle. Various studies find that neuroimmune molecules increase in the brain during withdrawal, and that this contributes to aversive emotional states such as anxiety and depression. This suggests the neuroimmune system plays a role in the withdrawal/ negative affect stage of the addiction cycle.

impairment [90]. These data are consistent with the neuroimmune system contributing to negative

affect during withdrawal. A possible mechanism for this is the influence of the neuroimmune system on hippocampal neurogenesis. Hippocampal neurogenesis is involved in regulation of mood and affective states [91], and inflammation reduces neurogenesis and contributes to depression-like behavior [92]. Indeed, reductions in hippocampal neurogenesis are observed during alcohol withdrawal and are associated with increased depression-like behavior [93]. As the primary cell of the neuroimmune system, microglia may play a key role in these effects. Overall, these studies suggest a role for the neuroimmune system in the withdrawal/negative affect stage of addiction (Fig 1.8).

Stress is also critically involved in the development of alcohol use disorders and like the withdrawal/negative affect stage of addiction, contributes to drinking through negative reinforcement. Drinking studies in mice suggest stress drives alcohol consumption through activation of the neuroimmune system [80]. Indeed, stress increases expression of genes such as TNFα, IL-1β and Ccl2 in the brain following acute stress [94-96]. Stress-induced neuroimmune activation contributes to negative affect, similar to the negative affect seen during withdrawal. Indeed, stress induces IL-1β in the hippocampus to decrease neurogenesis and contributes to depression-like behavior [97], while inhibition of IL-1β blocks stress-induced decreases in neurogenesis and depression-like behavior [98]. Overall, this suggests that the neuroimmune system contributes to the effects of stress and the withdrawal/negative affect stage; however, much remains unknown concerning the role of microglia in the withdrawal/negative affect stage.

1.4.3. The Preoccupation/Craving Stage

Various studies also implicate the neuroimmune system in the third stage of addiction – the preoccupation/craving stage. This stage involves thinking about and desiring the substance of abuse,

and is associated with increased risk of relapse. Interestingly, plasma levels of the pro-inflammatory cytokines IL-1β and IL-8 were positively correlated with alcohol craving in human alcohol-dependent subjects [99]. Treatment of abstinent human alcoholics with naltrexone, a TLR4 antagonist, reduced subjective reporting of craving [100]. Similarly, treatment with the (+)-isomer

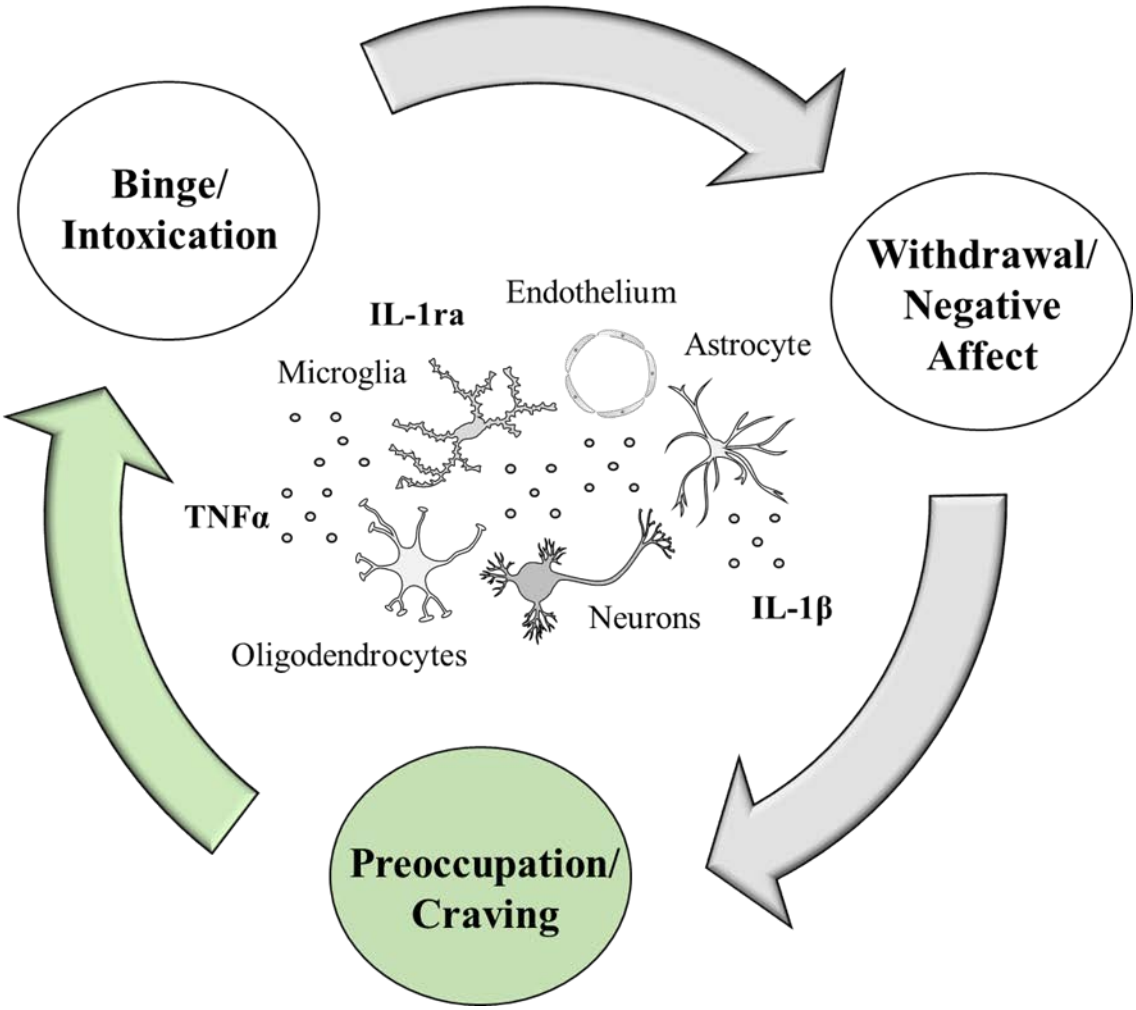


Figure 1-9 The Neuroimmune System Contributes to the Preoccupation/Craving Stage of the Addiction Cycle. Studies find that plasma levels of inflammatory cytokines positively correlate with craving in human alcoholic subjects and that treatment with TLR4 antagonists reduce reports of craving. These studies suggest that the neuroimmune system plays a role in the preoccupation/craving stage of addiction.

of naltrexone, which is inactive at the opioid receptor, blocked heroin-seeking behavior in dependent

rats [101]. Furthermore, triggers associated with relapse, such as stress [102, 103] activate the neuroimmune system, suggesting the neuroimmune system may play a role in craving and relapse. Overall, these studies suggest that the neuroimmune system contributes to the preoccupation/craving stage of addiction (Fig1.9). While this research suggests the neuroimmune system and microglia are involved the stages of addiction, much remains unknown. Indeed, the effects of alcohol and stress on microglia are not well defined, and the role that microglia play in the pathogenesis of AUDs is unclear. In the following sections, we will describe what is currently known about the effects of alcohol and stress on the neuroimmune system and microglia. This will lay the foundation for the hypotheses tested in later chapters.

1.5 Effects of Alcohol & Stress on the Neuroimmune System, Microglia & Psychopathology

1.5.1 Effects of Alcohol and Stress on the Peripheral Immune System

Repeated exposure to alcohol and stress is thought to contribute to alcohol use disorders [104]. How this occurs remains poorly understood. However, several studies find alcohol and stress both activate the neuroimmune system and microglia, suggesting they may play a role in alcohol use disorders. The effects of alcohol and stress on the neuroimmune system and microglia occur at least partly through peripheral inflammation (Fig 1.10). Physical stressors, such as injury, increase inflammation through tissue damage, causing release of intracellular compounds such as ATP, HMGB1, heat-shock proteins, etc. These compounds activate immune cells and increase inflammatory signaling. Interestingly, even psychological stressors (e.g. – social stress) can cause inflammation [105] through activation of the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis. Activation of the SNS releases norepinephrine, which increases pro-inflammatory TNFα production in macrophages [106]. Activation of the HPA axis releases glucocorticoids, which are acutely anti-inflammatory at high doses, but are pro-

inflammatory under certain circumstances [107]. Indeed, glucocorticoids prime the inflammatory response, meaning that glucocorticoids enhance the immune response to subsequent stimuli once glucocorticoid levels return to normal [108]. Furthermore, alcohol and stress both enhance intestinal permeability and bacterial translocation from the gut lumen [109]. Leaked bacterial products such as endotoxins cause peripheral inflammation and increase pro-inflammatory cytokines [110] [9]. Inflammation in the periphery can then increase neuroimmune signaling in the brain [111]. For example, pro-inflammatory cytokines can diffuse into the brain from the periphery through leaky regions of the blood brain barrier. They can also be transported across the blood brain barrier by transport molecules. Indeed, TNFα transporters on the blood brain barrier are essential for systemic inflammation to cause brain inflammation [112]. Activated immune cells such

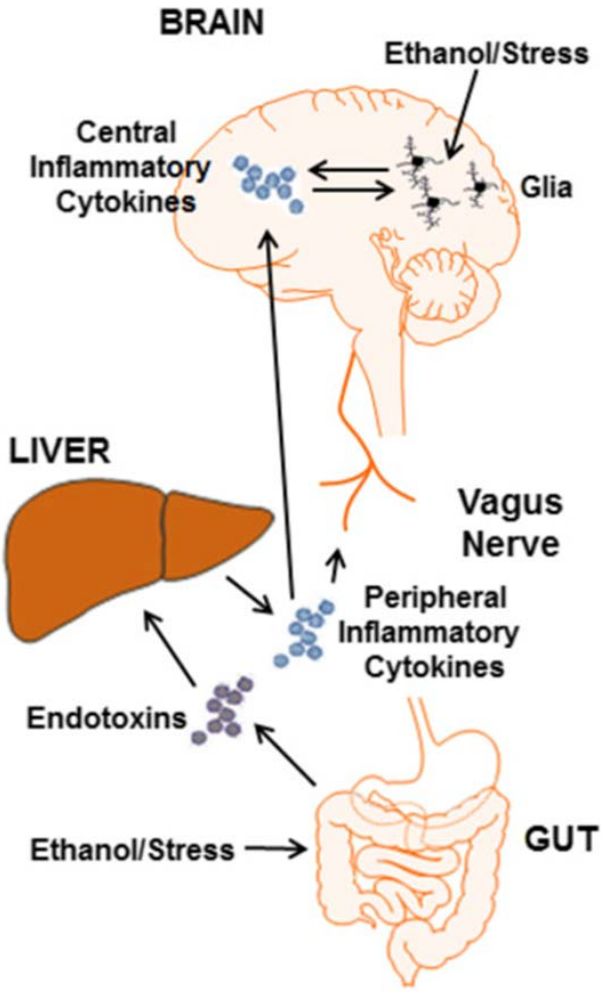


Figure 1-10 Mechanisms of Stress- and Ethanol-induced Immune Activation. Ethanol and stress both activate the peripheral and neuroimmune system in multiple ways. Both can enhance gut leakiness. This causes increased translocation of bacterial products such as endotoxins from the intestinal lumen to the periphery. Leaked bacterial products induce an inflammatory response in the liver from resident macrophages. Increased peripheral inflammatory cytokines such as TNFα, IL-1β and IL-6 impact the brain and behavior through multiple mechanisms. One way is the neural route. The vagus nerve expresses cytokines receptors and is activated by peripheral inflammation. The signal of peripheral inflammation is transmitted to central brain regions involved in the regulation of sickness behavior. Another route is the humoral route. Peripheral cytokines can cross the blood brain barrier either by transport proteins or by diffusion in regions where the barrier is leaky. This can lead to a central immune response. Stress and ethanol also activate glia through more direct mechanisms. Indeed, stress-induced glucocorticoids prime microglia, and ethanol exposure can directly activate glia.

as monocytes can also traffic into the brain. [113] where they can increase inflammation. This peripheral inflammation also triggers adaptive behavioral changes known as “sickness behavior.” Sickness behavior includes social withdrawal, decreased activity, somnolence/sleepiness, anhedonia, etc. that help to conserve energy and facilitate recovery [114]. While stress can lead to inflammation and the adaptive response of sickness behavior, intense or chronic stress and inflammation can become maladaptive and lead to neuropsychiatric disease such as depression. Indeed, the similarities between sickness behavior and depression have been noted [115]. The inflammation caused by excessive alcohol consumption may have similar maladaptive consequences. Overall, these studies suggest both alcohol and stress induce peripheral inflammation, and this peripheral inflammation can activate the neuroimmune system and microglia.

1.5.2 Effects of Stress on the Neuroimmune System, Microglia & Psychopathology

Stress is thought to be critical to the development of multiple psychopathologies, including AUDs. Indeed, stress and the resulting peripheral inflammation impact the neuroimmune system. Acute stress sensitizes microglia to inflammation in an HMGB1-dependent manner [116]. Chronic stress activates microglia in multiple brain regions [117] and causes depression-like behavior. Administration of microglial-modulating agents can block the development of depression-like behavior, suggesting that microglia may play a causal role in the development of depression [97]. Human studies also support a role for chronic stress and inflammation in affective disorders. Administration of inflammatory agents (such as interferon-α for the treatment of hepatitis C) can cause depression in previously non-depressed patients [118]. Individuals with increased plasma levels of CRP, an acute phase marker of inflammation, have an increased risk for depression [119]. Interestingly, increased CRP in adolescents has also been found to predict addiction later in life [120]. Other studies find peripheral inflammation can increase brain expression of innate immune

signals and contribute to depression and negative affect [121]. Imaging studies in humans show that a marker of microglial activation, TSPO, is elevated in patients with depression [122]. Depressed patients who committed suicide showed increased expression of microglial markers Iba1 and CD45 and increased levels of the inflammatory cytokine Ccl2 in the cingulate cortex [123]. Studies also find changes in astrocyte markers in the brains of depressed patients [124]. These findings suggest that stress contributes to increased neuroimmune signaling and psychopathology.

Stress-induced CNS inflammation can contribute to affective disorders through many mechanisms. Acute inflammation alters the activity of neural circuitry implicated in anxiety and depression. For example, acute inflammation changes activity in the cingulate cortex, medial prefrontal cortex and amygdala [125]. Acute inflammation can also impair spatial memory in humans [121], potentially contributing to the cognitive changes seen in affective disorders. Furthermore, studies find that a variety of cytokine receptors, such as those for TNFα, IL-1β, IL-6 and the interferons, are expressed on neurons [126], suggesting that cytokines act directly on neurons to influence their activity. The trafficking of peripheral inflammatory monocytes into the CNS has also been implicated in stress-induced behaviors. Stress causes trafficking of peripheral monocytes into the brain that, in turn, promotes anxiety-like behavior [127]. Thus, stress-induced neuroimmune and microglial activation contributes to increased negative affect.

Inflammation can also impact neurogenesis, which is implicated in affective disorders. Neurogenesis occurs throughout adulthood in discrete brain regions, including the forebrain subventricular zone and the subgranular zone of the hippocampal dentate gyrus. Hippocampal neurogenesis contributes not only to learning and memory [128], but also mood and affective state [91]. Decreased neurogenesis contributes both to anxiety and depression [129]. The ability of anti-depressants to alleviate depression-like behavior is dependent on hippocampal neurogenesis [130].

Both inflammation [92] and chronic stress [97] reduce neurogenesis and cause depression-like behavior. Indeed, stress induces IL-1β in the hippocampus which decreases neurogenesis and contributes to depression. Inhibition of IL-1β blocks stress-induced decreases in neurogenesis and depression-like behavior [98]. Furthermore, enhanced neurogenesis is sufficient to reverse the anxiety-like and depression-like behavior caused by chronic corticosterone treatment [129]. Therefore, stress can increase CNS immune signaling, which contributes to affective disorders.

1.5.3 Effects of Alcohol on the Neuroimmune System, Microglia & Psychopathology

Like stress, alcohol and the resulting peripheral inflammation impacts the neuroimmune system and contributes to psychopathology. Some studies suggest alcohol can directly activate the neuroimmune system and microglia. *In vitro* studies find exposure to high concentrations of alcohol increases expression of pro-inflammatory cytokines such as TNFα and Ccl2 in hippocampal slices beginning at 8 hours, while expression of IL-1β is increased at 24 hours [131]. Expression of these pro-inflammatory cytokines remains elevated with continued alcohol exposure. Some studies have also examined the effects of alcohol on microglia *in vitro*. Exposure to constant levels of ethanol induced expression of pro-inflammatory cytokines such as TNFα and IL-1β [132], as well as oxidases such as iNOS and COX2. Ethanol also induced the phosphorylation of transcription factors that mediate inflammation, including ERK, JNK and p38 [132]. Overall, these studies suggest ethanol can directly activate microglia; however, much remains unknown.

In vivo studies also find that alcohol affects the neuroimmune system. Indeed, a single, heavy dose of acute alcohol increases expression of pro-inflammatory cytokines TNFα and Ccl2 in the brain 24 hours after treatment [133]. Ten daily doses of heavy binge alcohol also increased brain TNFα and Ccl2 expression, as well as NOX2 and reactive oxygen species, 24 hours after the final treatment [134]. Alcohol also increases anti-inflammatory cytokine expression. Indeed, IL-10 and

TGFβ1 are increased in the hippocampus seven days following a prolonged heavy alcohol treatment [135]. Other studies have examined the effects of chronic experimenter-administered alcohol on the neuroimmune system. Chronic intermittent alcohol administration increased levels of TLR3 and TLR4 in the prefrontal cortex persistently for 25 days [136]. This treatment protocol also persistently increased TNFα, Ccl2, COX2 and NOX2 expression in the frontal cortex. Other studies find that self-administered alcohol also impacts the neuroimmune system. Chronic alcohol consumption for five months increased TNFα, IL-1β and IL-6 in the cortex of mice [137]. This treatment also increased brain oxidases iNOS and COX2. Overall, these studies show that alcohol activates the neuroimmune system *in vivo*.

Studies also find that alcohol increases microglial markers *in vivo*. The microglial marker CD11b is increased in the cerebral cortex of mice treated with alcohol for 3 consecutive days [132]. Intermittent alcohol treatment over 3 weeks increased staining for the microglial marker MHC II in the hippocampus of rats. Also, five months of continuous ethanol consumption increased staining for the microglial marker CD11b in the frontal cortex [137]. This continuous ethanol consumption was associated with increased expression of pro-inflammatory cytokines TNFα, IL-1β and IL-6, as well as inflammatory mediators iNOS and COX2 in cortical homogenates [137]. Furthermore, after ten days of alcohol treatment, microglia showed increased NF-κB activation and reactive oxygen species production. One heavy, 4-day binge alcohol exposure increased CD11b staining in the hippocampus that persisted for four weeks [135]. Interestingly, a second 4-day binge treatment further enhanced CD11b expression in the hippocampus [138]. This suggests cycles of alcohol exposure sensitize the microglial response to alcohol. Overall, these studies show that alcohol increases microglial markers *in vivo*, consistent with microglial activation.

Increased microglial markers following alcohol exposure suggests alcohol activates

microglia. However, there are multiple types of microglial activation, each with different functional outcomes and consequences. Microglia can become pro-inflammatory and destructive, as well as anti-inflammatory and reparative. Understanding the type of functional activation produced by alcohol is critical to understanding how alcohol-activated microglia impact the brain. A few studies have attempted to define the type of activation alcohol induces in microglia. A heavy, 4-day binge alcohol model produces morphological changes in hippocampal microglia, suggesting activation; however, there was no detectable change in CD68 or MHCII [139]. Since CD68 and MHCII are indicators of full microglial activation, these experiments suggest heavy, four-day binge alcohol produces a mild, homeostatic microglial activation. Furthermore, this treatment did not change hippocampal IL-6 or TNFα, but increased hippocampal TGFβ and IL-10 seven days post-treatment, suggesting an anti-inflammatory microglial activation state. However, other studies frequently find increased pro-inflammatory mediators with alcohol treatment, implying pro-inflammatory microglial activation. Therefore, while some studies have begun to investigate the type of microglial activation produced by alcohol, much remains unknown.

Multiple studies have also examined the effects of alcohol on the neuroimmune system and microglia in the human brain. Indeed, the brains of post-mortem human alcoholics show increased Ccl2 in several different regions [140]. Levels of TLR2, TLR3 and TLR4 and NOX2 are also increased in the alcoholic brain [134, 141]. Transcriptome analyses of post-mortem human alcoholic brain also find changes in neuroimmune gene expression [142, 143]. Furthermore, phosphorylation of the immune transcription factors p38 and ERK are increased in the post-mortem alcoholic brain. Phosphorylation of these proteins indicates activation, suggesting these transcription factors are more active in the brains of human alcoholics. Staining for microglial markers Iba1 and GluT5 are also increased in the cingulate cortex of post-mortem alcoholic brains. Staining for GluT5 was

increased in other brain regions as well, including the midbrain and ventral tegmental area [140]. Other studies have also noted increased Iba1 in the prefrontal cortex of post-mortem human alcoholic subjects [144]. Overall, these studies show that alcohol activates microglia in human alcoholics, implicating microglia in the pathogenesis of AUDs.

These studies find that alcohol activates microglia, and is associated with increased expression of pro-inflammatory cytokines such as TNFα and IL-1β in the brain. Like stress, alcohol-induced neuroimmune signaling may contribute to psychopathology through many different mechanisms. As described previously, inflammation can also impact neurogenesis, which is implicated in affective disorders. Indeed, decreased neurogenesis contributes to anxiety and depression [129]. Induction of pro-inflammatory cytokines such as IL-1β in the hippocampus decreases neurogenesis and contributes to depression, while inhibition of IL-1β blocks decreases in neurogenesis and depression-like behavior [98]. Furthermore, inflammatory cytokines such as TNFα and IL-1β can also impact neuronal plasticity. Excessive TNFα can inhibit LTP in hippocampal slices [51], and TNFα overexpressing mice show decreased performance on spatial learning and memory tasks [55]. Excessive IL-1β also inhibits hippocampal LTP and impairs memory [145]. Overall, these studies show alcohol increases brain immune signaling, which may contribute to psychopathology. As the primary cells of the neuroimmune system, microglia may be important effectors of these processes; however, much remains unknown regarding the role of microglia in the development of AUDs.

1.6 Summary and Hypotheses

In the previous sections, we introduced alcohol use disorders in humans and how there is a pressing need for more effective medications. We explained how recent research has implicated microglia in AUDs and how they may represent a novel target for treatment. To explain this idea

further, we described the neurobiology of alcohol use disorders, noting the role stress plays in the development of these conditions. We then introduced the neuroimmune system and microglia, describing how they impact neuronal activity and subsequently behavior. We also explained the role of the neuroimmune system in the addiction cycle, and how microglia may potentially contribute to this. Finally, we described how alcohol and stress impact the neuroimmune system and microglia and how this may contribute to psychopathologies such as AUDs. Overall, these studies implicate microglia in AUDs; however, much remains unknown. For example, how alcohol affects microglia is not clearly defined, and how alcohol-activated microglia impact the rest of the brain is not fully understood. Indeed, microglia are highly dynamic cell types and how they respond to even a single cycle of binge ethanol and withdrawal is unknown. Furthermore, while alcohol and stress are thought to contribute to AUDs, it is unclear how alcohol and stress interact to impact microglia and whether this impacts neuronal activity. Finally, no studies have examined whether microglia impact the neuronal response to alcohol exposure or withdrawal or whether microglia contribute to alcohol consumption. Overall, we hypothesize that alcohol and/or stress activate microglia and that activated microglia contribute to a neuroimmune response that alters neuronal function. These changes in neuronal function then lead to behavioral changes that may contribute to AUDs (Fig1.11).

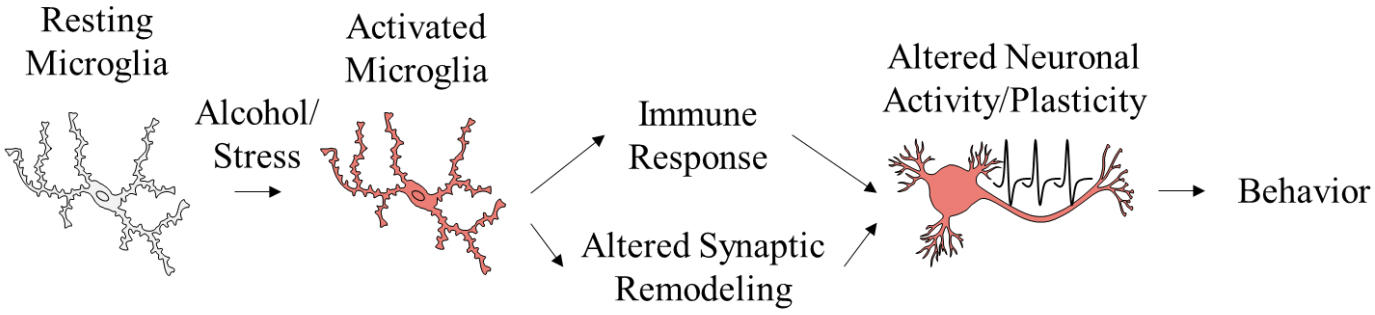


Figure 1-11 Proposed Role of Alcohol and Stress in Alcohol Use Disorders. Resting microglia are activated by stimuli such as alcohol and stress. Activated microglia then produce immune mediators, such as cytokines and oxidases, or alter synaptic remodeling to impact neuronal activity/plasticity. This can result in behavioral changes related to psychopathologies such as AUDs.

Specifically, our first hypothesis is that acute binge ethanol exposure and withdrawal activate

microglia to a pro-inflammatory state and that microglia contribute to the brain neuroimmune response to alcohol withdrawal. In Chapter 2, we test this hypothesis by examining the effects of acute binge ethanol on microglia over time *in vitro* and *in vivo*. We also examine the effects of microglial depletion on the brain neuroimmune response to acute ethanol withdrawal. We find that acute ethanol withdrawal increases microglial pro-inflammatory cytokine expression (TNFα, Ccl2) *in vitro* and *in vivo*. We also find that microglial depletion blunts the brain pro-inflammatory response (TNFα, Ccl2) and enhances the brain anti-inflammatory response (IL-1ra, IL-4) to acute ethanol withdrawal. Our second hypothesis is that alcohol and stress interact to enhance microglial activation and alter neuronal activity. In Chapter 3, we test this hypothesis by examining the effects of ethanol and stress on microglial and neuronal activation. We find that acute ethanol and acute stress interact to increase plasma endotoxin and microglial markers across the brain, but decrease the neuronal response in some brain regions. Furthermore, chronic ethanol persistently sensitized the stress-induced plasma endotoxin response and microglial CD11b response without impacting the neuronal response. Finally, our third hypothesis is that microglia contribute to the neuronal response to acute ethanol withdrawal and that microglia play a role in ethanol consumption. In Chapter 4, we test this hypothesis by examining the effects of microglial depletion on neuronal activity during acute ethanol withdrawal, as well as the effects of microglial depletion on ethanol consumption. We find that microglial depletion blocks acute ethanol withdrawal-induced neuronal activation in the nucleus accumbens and hippocampus. Furthermore, microglial depletion decreases voluntary ethanol consumption. Finally, Chapter 5 concludes with a discussion of the significance of this work and ideas for future directions. Overall, this work provides insight into the relationships between microglia and alcohol use disorders, and may help inform strategies to treat alcohol use disorders in humans.

CHAPTER 2: MICROGLIAL DEPLETION ALTERS THE BRAIN NEUROIMMUNE RESPONSE TO ACUTE BINGE ETHANOL WITHDRAWAL

2.1 Introduction

Alcohol use disorders (AUDs) are common mental health diseases in which individuals drink excessive amounts of alcohol despite negative consequences. Many individuals with AUDs engage in heavy patterns of alcohol consumption known as binge drinking, defined as drinking to a blood alcohol concentration (BAC) greater than 80 mg/dL [3]. However, many studies in humans have documented even more extreme binge drinking leading to BACs around 400 mg/dL [16-18]. Excessive alcohol consumption has several detrimental effects on the brain, contributing to neurodegeneration [146], neuronal dysfunction [147] and various other pathologies. Research even suggests excessive alcohol changes the brain to promote further alcohol consumption [13]. However, much remains unknown regarding the mechanisms by which excessive alcohol use negatively impacts the brain.

Interestingly, recent studies have implicated the innate immune system in the pathophysiology of alcohol use disorders. Deletion of innate immune genes such as *IL1rn* or *IL6* decreases voluntary alcohol consumption [79, 80]. Furthermore, alcohol treatment increases innate immune gene expression in the brain [137, 148]. Even a single, heavy dose of alcohol increases expression of innate immune genes such as TNFα and Ccl2 in the brain [133]. Microglia are the resident innate immune cells of the brain and thought to be the primary mediators of the brain immune response to alcohol. Indeed, microglial markers such as Iba1 are increased in the brains of post-mortem alcoholics [140, 144], consistent with microglial activation. However, there are

multiple types of microglial activation [70], with functional effects ranging from pro-inflammatory and destructive to anti-inflammatory and healing, and increased markers such as Iba1 do not indicate activation type [68, 149]. While a few studies have begun to investigate the effects of alcohol on microglial function *in vivo* [135], much remains unknown. *In vitro* studies in human monocytes suggest ethanol may have complex, dynamic effects, as short-term ethanol exposure decreases inflammatory signaling, while long-term ethanol exposure increases inflammatory signaling [150]. Even the microglial response to a single episode of ethanol exposure and withdrawal *in vivo* is not fully understood. Therefore, in this study, we sought to investigate the dynamic effects of acute binge ethanol on microglia *in vivo*.

Understanding the effects of ethanol on microglia is critical, as microglia interact extensively with other cell types of the brain to impact brain function. Microglia can alter synapse formation through brain-derived neurotrophic factor (BDNF) [63], synapse elimination through complement-dependent phagocytosis [151], and synaptic plasticity through release of cytokines such as TNFα [64]. Furthermore, microglia can either promote or inhibit neurogenesis depending on their activation state [65, 66]. Activated microglia can also protect against neuronal cell death [72] or contribute to neuronal cell death [73, 74] depending on whether they are activated to an anti-inflammatory or pro-inflammatory activation state. Indeed, ethanol-induced changes in microglia may have an important impact on brain functioning, and ultimately behavior. Interestingly, recent research has identified a new pharmacological tool for studying the role of microglia *in vivo*. Inhibitors of the colony stimulating factor 1 receptor (CSF1R) deplete microglia from the brain without known detrimental effects or behavioral changes [152]. This allows for an opportunity to examine the function of microglia *in vivo* in a novel way. We sought to utilize this method to investigate the effects of microglial depletion on both the normal brain and the ethanol-treated brain.

Furthermore, microglial depletion allows for examination of the contributions of non-microglial cells to the brain response to ethanol. We examined the effect of acute binge ethanol and microglial depletion on brain expression of microglial genes, pro-inflammatory genes, anti-inflammatory genes, and various other genes including Toll-like receptors and death receptors. We hypothesized that acute binge ethanol would cause time and dose-dependent changes in microglial and neuroimmune gene expression, and that microglial depletion would alter brain neuroimmune gene expression. We further hypothesized that microglial depletion would alter the brain neuroimmune response to acute binge ethanol withdrawal.

2.2 Results

2.2.1 Acute binge ethanol biphasically and dose-dependently alters microglial gene expression *in vivo*

Previous studies have found that chronic ethanol increases microglial markers *in vivo* [153] and that acute ethanol has varying effects over time on human monocytes *in vitro* [150]. However, few studies have investigated the effects of acute ethanol on microglia *in vivo*. Furthermore, it is unclear what dose of ethanol is required to impact microglia acutely *in vivo*. We therefore performed time course and dose response experiments with acute binge ethanol *in vivo*. Mice were treated intragastrically with various doses of ethanol (3, 4.5 and 6 g/kg) and a time course was done with the highest dose (6 g/kg). Brain mRNA levels of commonly studied microglial markers, Iba1 and

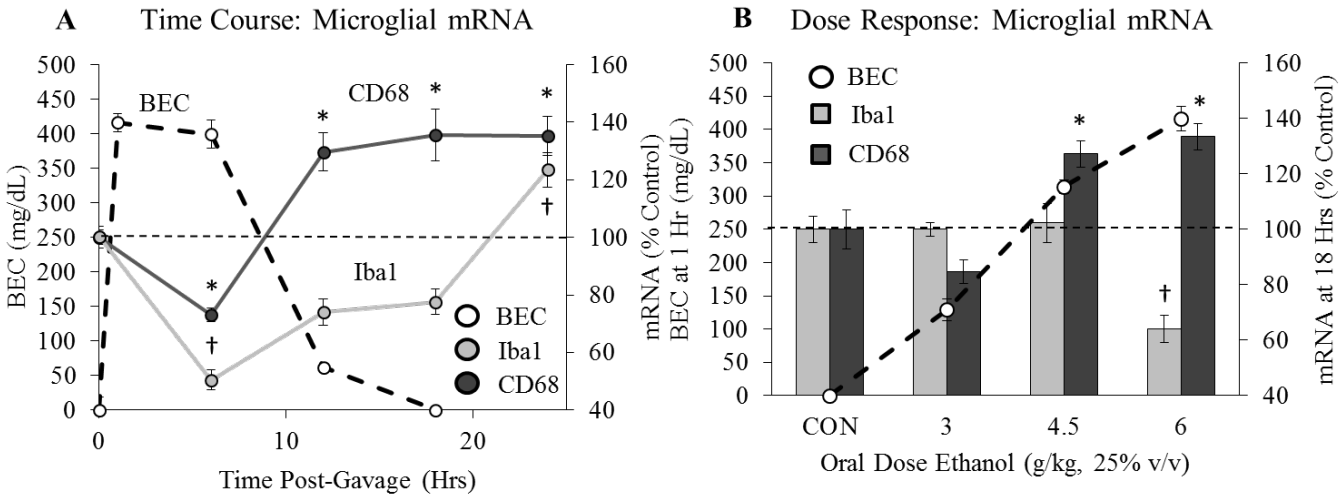


Figure 2-1 Acute binge ethanol biphasically and dose-dependently changes microglial gene expression. A) Time course of blood alcohol concentrations and brain microglial mRNA following acute binge ethanol: Mice were gavaged with ethanol (6 g/kg, 25% v/v) and tail blood was collected at 1, 6, 12 and 18 hours to determine BACs (dashed line – left axis). Brain mRNA was collected at 0, 6, 12, 18 or 24 hours and microglial Iba1 and CD68 mRNA levels were measured (solid lines – right axis). Note that Iba1 and CD68 mRNA decreased when BACs were high and increased when BACs dropped. **B)** Ethanol dose-response curve: Mice were gavaged with ethanol (3, 4.5 or 6 g/kg, 25% v/v) or water. Tail blood was collected 1 hour post gavage to assess BACs (dashed line – right axis). At 18 hours post gavage, brain mRNA was collected and microglial Iba1 and CD68 mRNA levels were measured (light and dark grey bars, left axis). Ethanol dose-dependently altered Iba1 and CD68 mRNA. Data are presented as mean±s.e.m. *$p<0.05$ for CD68, $^{\dagger}p<0.05$ for Iba1. n=5-7/group.

CD68, were assessed. Only high doses of ethanol (i.e. – 4.5 and 6 g/kg) altered Iba1 and CD68 mRNA (Fig1). Although these doses yielded high BACs (≥ 300 mg/dL), no mice died (FigS1). In order to determine the behavioral effects of these doses of alcohol over time in mice, movement and pain response were assessed. There was a transient decrease in movement and pain response while BACs were high, with normal behavior returning as BACs approached zero (FigS1). For the time course, tail blood was collected at 1, 6, 12 and 18 hours post gavage. BACs were approximately 400 mg/dL at 1 hour and decreased to 0 mg/dL by 18 hours (Fig1A). Interestingly, a biphasic effect on Iba1 mRNA levels was observed, with a 50% decrease ($p<0.05$) at 6 hours, followed by a 23% increase at 24 hours ($p<0.05$) (Fig1A). CD68 mRNA showed a similar biphasic response, with a 27% decrease ($p<0.05$) at 6 hours and a 30% ($p<0.05$), 36% ($p<0.05$) and 35% ($p<0.05$) increase at

12, 18 and 24 hours, respectively (Fig1A). Gavage with water did not change brain CD68 or Iba1 mRNA over time (FigS2). For the dose response studies, tail blood was collected at 1 hour post-gavage. Increasing doses of ethanol caused a proportional increase in 1 hour BACs (Fig1B). Mice were sacrificed 18 hours post gavage, a point at which time course data showed elevated microglial CD68 mRNA. The 4.5 g/kg dose increased CD68 mRNA 27% ($p<0.05$), but did not change Iba1 mRNA (Fig1B). The 6 g/kg dose increased CD68 mRNA 33% ($p<0.05$), while Iba1 mRNA was still decreased by 36% ($p<0.05$) at this 18 hour time point (Fig1B). These results find that acute binge ethanol biphasically changes microglial gene expression *in vivo*, with initial decreases during intoxication and later increases during withdrawal. Furthermore, these changes only happen at high doses of acute ethanol.

2.2.2 Acute binge ethanol withdrawal dose-dependently increases brain pro-inflammatory cytokine expression *in vivo*

Increased microglial markers suggest microglial activation, but do not indicate the functional changes occurring in microglia. Microglia can adopt both pro- and anti-inflammatory activation states [68, 149]. We therefore sought to assess changes in pro-inflammatory cytokine expression following acute binge ethanol. Time course experiments were performed as described above. We measured the brain expression of TNFα and Ccl2, key pro-inflammatory cytokines. TNFα expression decreased by 54% at 6 hours post treatment and increased dramatically during withdrawal, peaking at 600% ($p<0.05$) at 18 hours (Fig2A). Ccl2 showed a similar pattern of expression and increased during ethanol withdrawal, peaking at 1002% ($p<0.05$) at 18 hours

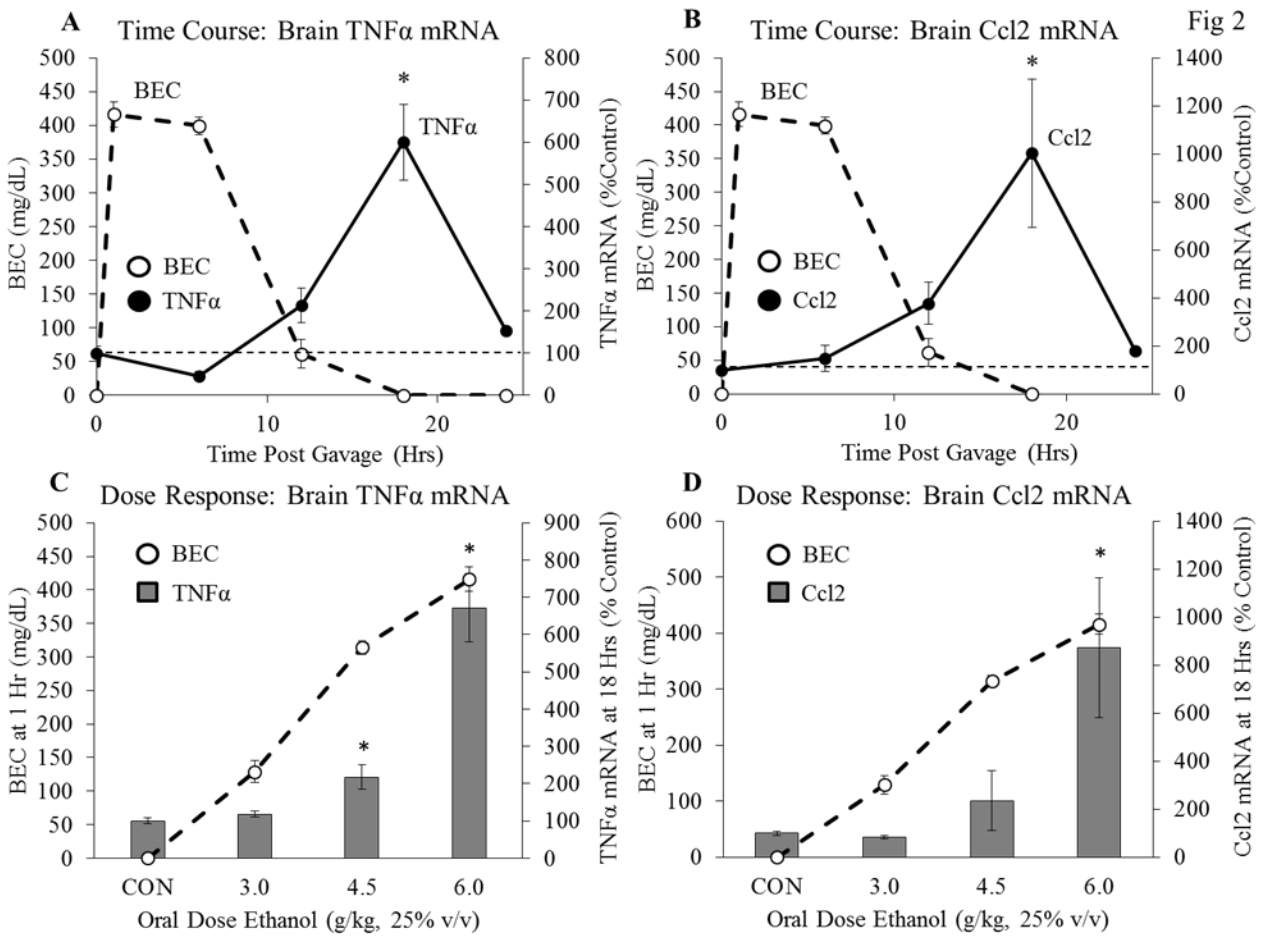


Fig 2

Figure 2-2 Acute binge ethanol withdrawal increases brain pro-inflammatory gene expression. A, B) Time course of blood alcohol concentrations and brain TNFα and Ccl2 mRNA following acute binge ethanol: Mice were gavaged with ethanol (6 g/kg, 25% v/v) and tail blood was collected at 1, 6, 12 and 18 hours to determine BACs (dashed line – left axis). Brain mRNA was collected at 0, 6, 12, 18 or 24 hours and TNFα mRNA and Ccl2 mRNA was assessed (solid line – right axis). Note that TNFα and Ccl2 mRNA increased during withdrawal when BACs dropped. **C, D)** Ethanol dose-response curve: Mice were gavaged with ethanol (3 g/kg, 4.5 g/kg or 6 g/kg, 25% v/v) or water. Tail blood was collected 1 hour post gavage to assess BACs (dashed line – right axis). At 18 hours post gavage, brain mRNA was collected and TNFα and Ccl2 transcript levels were assessed (grey bars, left axis). Data are presented as mean±s.e.m. *p<0.05. n=5-8/group.

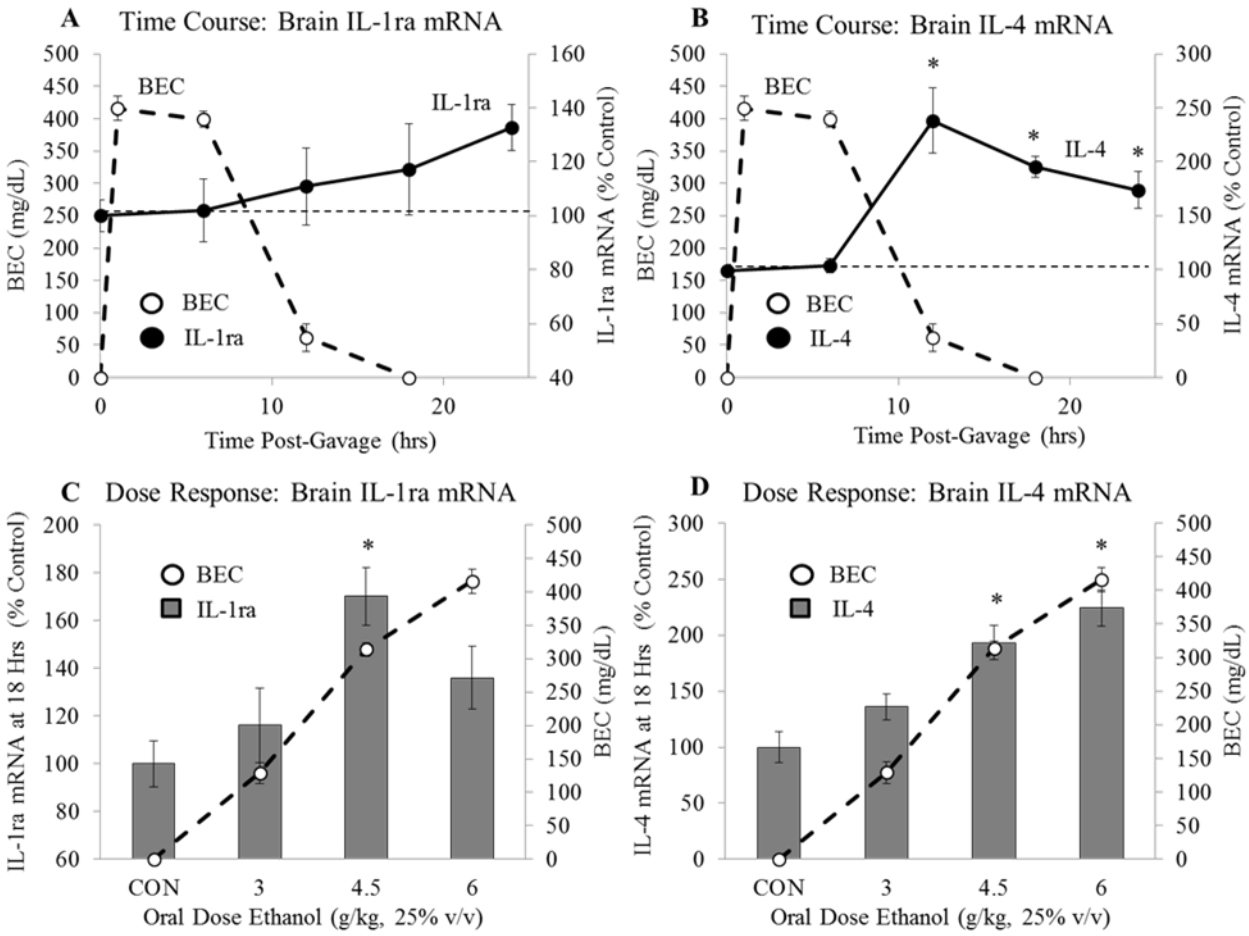
(Fig2B). Gavage with water did not significantly increase TNFα or Ccl2 mRNA at any time point (FigS3A,B). These marked changes in mRNA levels were accompanied by increased brain TNFα and Ccl2 protein levels at 18 hours post gavage. TNFα protein increased 345%, while Ccl2 increased from less than 1 pg/mL to approximately 17 pg/mL as determined by ELISAs (FigS4A,B). These

results find that acute binge ethanol withdrawal increases brain TNFα and Ccl2 mRNA and protein during withdrawal, similar to the microglial markers.

In order to determine the dose of acute binge ethanol required to impact pro-inflammatory cytokine expression, we performed *in vivo* dose-response curves as described above. The 3 g/kg dose of ethanol did not change TNFα or Ccl2 at the 18 hour time point. The 4.5 g/kg dose of ethanol increased TNFα 218% ($p<0.05$) and Ccl2 236% at 18 hours post gavage (Fig2C,D). TNFα and Ccl2 expression increased exponentially to 672% ($p<0.05$) and 873% ($p<0.05$), respectively, with the 6 g/kg dose (Fig2C,D). These results find that acute binge ethanol dose-dependently increases brain pro-inflammatory cytokine expression during withdrawal beginning at high doses.

2.2.3 Acute binge ethanol withdrawal dose-dependently increases brain anti-inflammatory cytokine expression *in vivo*

Microglia often react to insults in a complex and dynamic manner consisting of both pro-inflammatory and anti-inflammatory responses. We therefore assessed changes in brain anti-inflammatory gene expression following acute binge ethanol. Mice were treated with acute binge ethanol as described above. We measured expression of IL-1ra and IL-4, key anti-inflammatory cytokines. IL-1ra expression increased modestly to 132% at 24 hours after ethanol treatment (Fig3A). Expression of IL-4 peaked at 238% ($p<0.05$) at 12 hours and decreased to 174% ($p<0.05$) by 24 hours (Fig3B). Gavage with water did not significantly increase IL-1ra or IL-4 mRNA at these time points (FigS3C,D). Similar changes in brain IL-1ra and IL-4 protein were observed 18 hours post gavage. While IL-1ra protein was unchanged, IL-4 protein increased approximately 9-



fold as determined by ELISAs (FigS4C,D). These results find that acute binge ethanol increases **Figure 2-3 Acute binge ethanol withdrawal increases brain anti-inflammatory gene expression. A, B)** Time course of blood alcohol concentrations and brain IL-1ra and IL-4 mRNA following acute binge ethanol: Mice were gavaged with ethanol (6 g/kg, 25% v/v) and tail blood was collected at 1, 6, 12 and 18 hours to determine BACs (dashed line – left axis). Brain mRNA was collected at 0, 6, 12, 18 or 24 hours and IL-1ra and IL-4 mRNA was assessed (solid line – right axis). **C, D)** Ethanol dose-response curve: Mice were gavaged with ethanol (3, 4.5 or 6 g/kg, 25% v/v) or water. Tail blood was collected 1 hour post gavage to assess BACs (dashed line – right axis). At 18 hours post gavage, brain mRNA was collected and IL-1ra and IL-4 mRNA was assessed (grey bars, left axis). Data are presented as mean±s.e.m. *p<0.05. n=5-8/group.

brain IL-4 mRNA and protein during withdrawal.

In order to determine the dose of acute binge ethanol required to impact anti-inflammatory cytokine expression, we performed *in vivo* dose-response curves as described above. The 3 g/kg dose of ethanol did not change IL-1ra or IL-4 at the 18 hour time point. The 4.5 g/kg dose of ethanol increased IL-1ra 170% ($p<0.05$) and IL-4 193% ($p<0.05$) at 18 hours post gavage (Fig3C,D). IL-1ra

expression decreased to 136% with the 6 g/kg dose, while IL-4 expression increased to 224% ($p<0.05$) (Fig3C,D). These data find that acute binge ethanol dose-dependently alters brain anti-inflammatory cytokine expression during withdrawal.

2.2.4 Acute ethanol exposure alters microglial cytokine expression *in vitro*

Ethanol-induced changes in neuroimmune gene expression *in vivo* could be due to direct

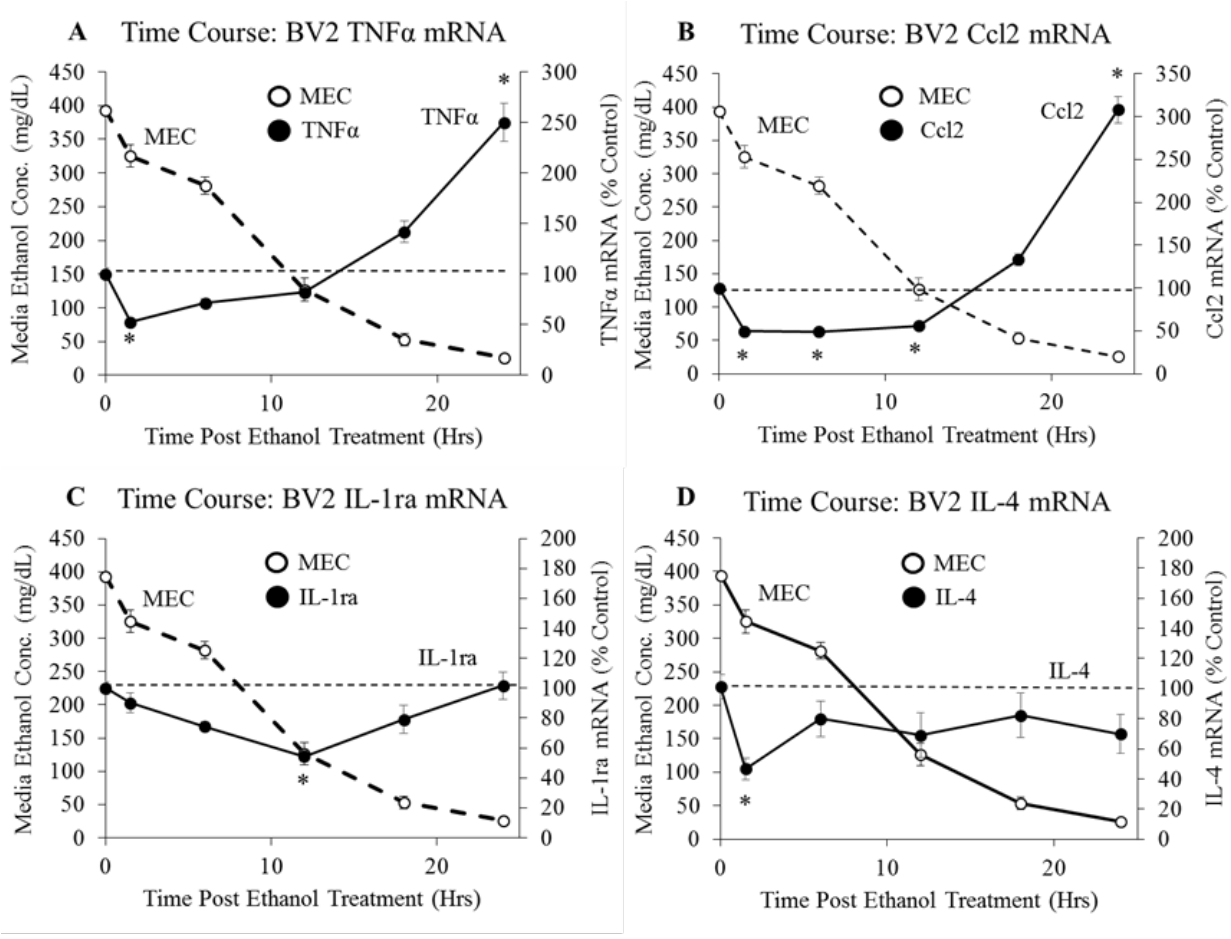


Figure 2-4 Acute ethanol changes BV2 microglial cytokine gene expression *in vitro*. Time course of cytokine expression in ethanol-treated BV2 cells: Microglia-like BV2 cells were treated with ethanol (85 mM) and the ethanol evaporated over time. Media ethanol concentrations (MECs) were measured (dashed line – left axis) and mRNA was collected from the BV2 cells. mRNA levels of **A)** TNFα **B)** Ccl2 **C)** IL-1ra and **D)** IL-4 in ethanol-treated cells were normalized to mRNA levels in PBS-treated control cells (solid line – right axis). Data are presented as mean±s.e.m. *$p<0.05$. n=4-6/group.

actions of ethanol on microglia or due to immune signaling across other brain cell types, as

endothelial cells, astrocytes and even neurons express immune genes [36, 136, 154]. To determine whether ethanol exposure and withdrawal can directly alter microglial gene expression, cultured BV2 microglia-like cells were treated with ethanol *in vitro*. Cells were treated with ethanol, but without vaporized ethanol in the incubation chamber, allowing the ethanol to evaporate from the media and simulate the ethanol clearance that occurs *in vivo*. Media ethanol concentrations were measured and BV2 mRNA was collected over time. Ethanol concentrations decreased from an initial concentration of approximately 400 mg/dL to nearly 0 mg/dL over 24 hours (Fig4). Gene expression in ethanol-treated BV2 cells was normalized to gene expression in PBS-treated BV2 cells at each time point. This was done to normalize gene expression changes over time (Fig. S5). Ethanol treatment initially decreased TNFα mRNA 47% ($p<0.05$) at 1.5 hours post-treatment, but later increased TNFα mRNA to 250% ($p<0.05$) of controls 24 hours post-treatment (Fig. 4A). Ccl2 mRNA showed a similar pattern, with an initial decrease in expression through 12 hours of treatment followed by a 312% ($p<0.05$) increase at 24 hours post-treatment (Fig4B). Ethanol also decreased IL-1ra expression 45% ($p<0.05$) at 12 hours and decreased IL-4 expression 53% ($p<0.05$) at 1.5 hours (Fig4C,D). To determine whether decreases in BV2 gene expression were due to cell death, we used the vital stain Trypan blue to determine cell viability. Results showed no changes in BV2 viability at multiple time points post ethanol treatment (FigS6). To determine whether increased pro-inflammatory gene expression was dependent on ethanol evaporation, identical experiments were performed, except ethanol was vaporized into the incubator chamber to keep MECs constant (FigS7A). After 24 hours of continuous ethanol exposure, BV2 TNFα mRNA was unchanged, while Ccl2 mRNA was decreased 17% ($p<0.05$) (FigS7B,C), consistent with ethanol evaporation being required for increased pro-inflammatory gene expression. These results find that ethanol treatment biphasically alters BV2 microglial-like gene expression *in vitro*, with increased pro-inflammatory

gene expression occurring after ethanol clearance, and consistent with ethanol having direct effects on microglia.

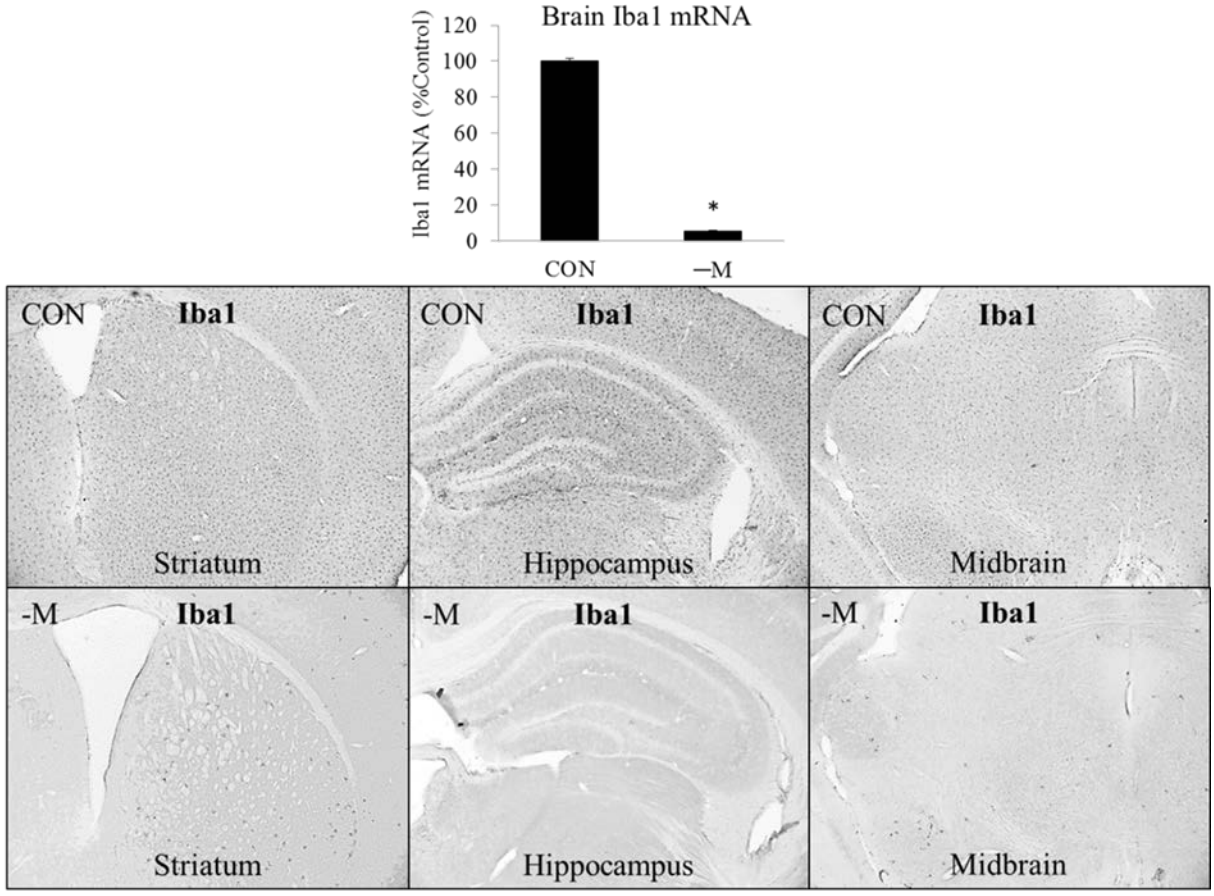


Figure 2-5 Treatment with CSF1R inhibitor PLX5622 depletes microglia from the brains of mice. C57BL/6J mice received chow containing 1200 mg/kg PLX5622 for three weeks. **A)** RT-PCR on whole brain mRNA showed a 94% reduction in Iba1 mRNA, indicating substantial depletion of microglia. Data are represented as mean ± s.e.m. *$p<0.05$, student's t-test, n=6/group. **B)** Immunohistochemical staining for Iba1 confirmed depletion of microglia across the brain. Illustrations and insets show a section of hippocampus.

2.2.5 PLX5622 decreases expression of microglial genes and alters expression of some neuroimmune genes *in vivo*

To further understand which gene expression changes are due to microglia, we sought to deplete microglia from the brain and then administer acute binge ethanol. This would allow us to assess the contribution of microglial and non-microglial cells to the brain response to ethanol. Microglial depletion was performed by administering the CSF1R inhibitor PLX5622 to C57BL/6J

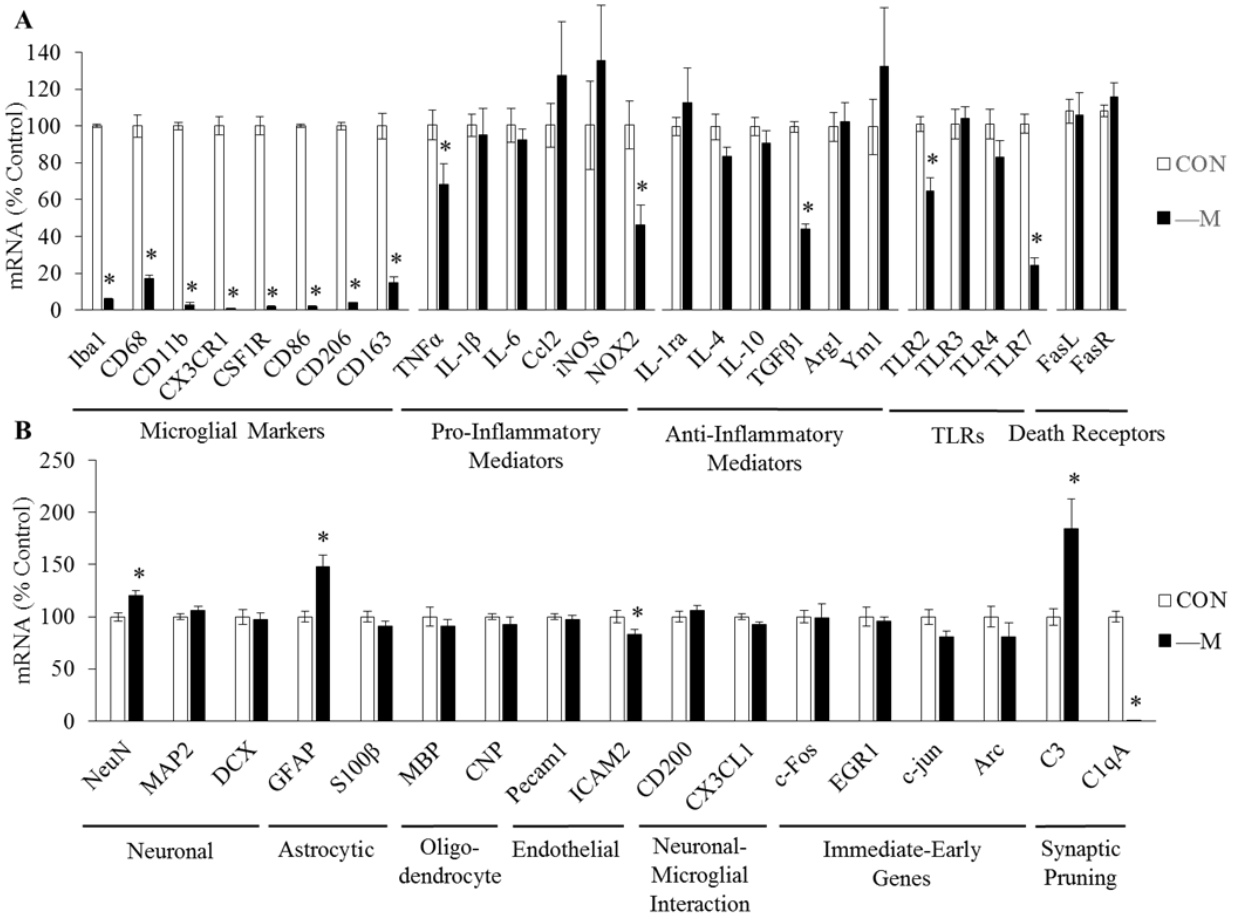


Figure 2-6 Treatment with CSF1R inhibitor PLX5622 alters brain gene expression. Mice received chow containing PLX5622 for three weeks and mRNA was isolated from whole brain **A.)** RT-PCR showed marked reduction in several microglial transcripts, indicating substantial microglial depletion. Other neuroimmune transcripts were also assessed. **B.)** The effects of PLX5622 on non-microglial transcripts were also assessed. Note that PLX5622 had little to no effect on neuronal, oligodendrocyte or endothelial transcripts, but increased the astrocyte marker GFAP. Also note the marked changes in synaptic pruning genes, C3 and C1qA. Data are represented as mean ± s.e.m. *p<0.05, student's t-test, n=6/group.

mice for three weeks, a duration previously shown to result in maximal microglial depletion [152]. We first characterized the effects of PLX5622 treatment on microglial and neuroimmune gene expression. RT-PCR and immunohistochemistry for the microglial marker Iba1 found that PLX5622 reduced Iba1 mRNA 94% ($p<0.05$) (Fig5A) and almost completely eliminated Iba1+ cells (Fig5B), consistent with PLX5622 depleting microglia from the brain. Expression of several other microglial markers (CD68, CD11b, CX_3CR1, etc.) was reduced by 80% or more by PLX5622 ($p<0.05$) (Fig6A), further suggesting substantial microglial depletion. Expression of pro-inflammatory genes, anti-inflammatory genes, and many other genes was also assessed. Interestingly, microglial depletion decreased basal expression of TNFα 32% ($p<0.05$) and NOX2 54% ($p<0.05$) (Fig6A), but did not decrease basal expression of IL-1β, IL-6 or iNOS, suggesting these genes may be expressed in other cell types. PLX5622 decreased expression of TGFβ1 56% ($p<0.05$), but did not decrease expression of other examined anti-inflammatory genes. We also examined expression of multiple Toll-like receptors (TLRs) – a group of innate immune receptors. PLX5622 decreased brain expression of TLR2 36% ($p<0.05$) and TLR7 76% ($p<0.05$), but surprisingly did not decrease expression of TLR3 and TLR4 (Fig6A). This suggests these neuroimmune genes may be expressed in other CNS cell types. Furthermore, expression of genes encoding death receptors – receptors that contribute to apoptosis – and death receptor ligands, such as FasL and FasR, were unchanged with microglial depletion. These results suggest that PLX5622 depletes microglia, but does not alter expression of many genes associated with neuroimmune signaling.

In order to investigate the specificity of PLX5622 for microglia and potential effects on other brain cell types, we assessed the expression of several other genes. Microglial depletion caused no change in expression of the neuronal marker MAP2 or neuroprogenitor marker DCX. However,

there was a small, but statistically significant increase in expression of neuronal marker NeuN (Fig6B) and a 48% increase in astrocyte marker GFAP ($p<0.05$). There was no effect on astrocytic S100β expression (Fig6B). Oligodendrocyte markers myelin basic protein (MBP) and 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP) were not changed by microglial depletion (Fig6B). There was a small, but statistically significant 17% decrease in endothelial marker ICAM2, but no change in endothelial marker Pecam1 (Fig6B). We also examined expression of the neuronal-microglial interaction genes, CD200 and CX_3CL1 (also known as fractalkine). CD200 and CX_3CL1 are expressed on neurons and interact with the CD200 receptor and CX_3CR1 located on microglia to regulate their activity [48]. Interestingly, microglial depletion did not change neuronally expressed CD200 and CX_3CL1 (Fig6B), despite microglial CX_3CR1 being markedly decreased. Since microglia play a role in synaptic elimination and synaptic plasticity [62], we also assessed the expression of C3 and C1qA, complement factors involved in synaptic pruning, as well as four immediate-early genes (IEGs), genes that are induced following neuronal activation. Microglial depletion increased C3 expression 84% ($p<0.05$) and decreased C1qA expression to 1% ($p<0.05$). No significant changes in either c-Fos, EGR1, c-jun or Arc were found (Fig6B). These results find that microglial depletion has little effect on basal expression of several markers of other CNS cell types.

2.2.6 Microglial depletion alters the brain immune response to acute binge ethanol withdrawal

To determine the role of microglia in the brain response to acute binge ethanol withdrawal, microglia were depleted from the brains of mice as described above.

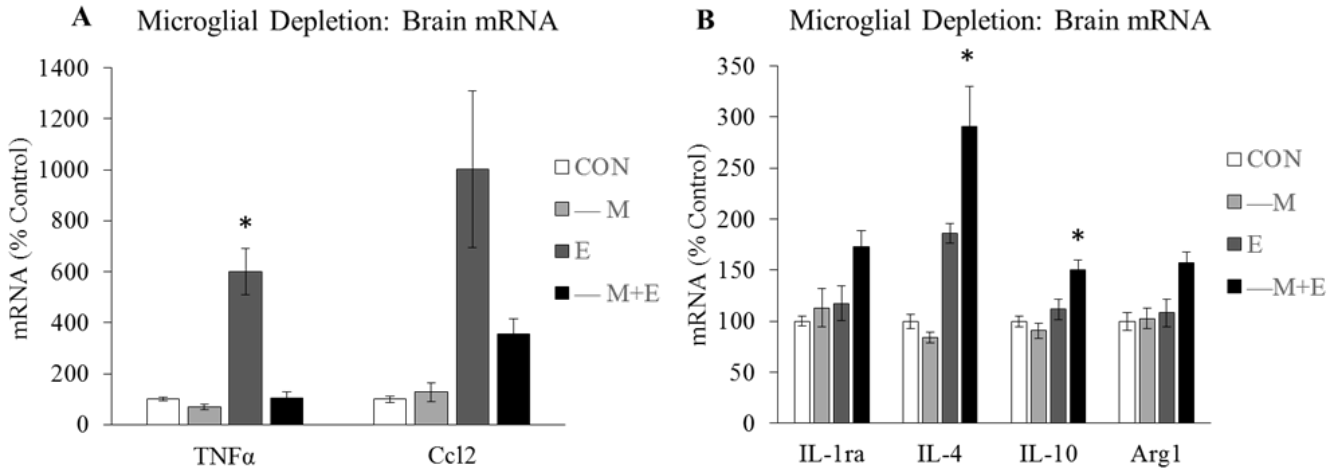


Figure 2-7 Microglial depletion alters the neuroimmune response to acute binge ethanol withdrawal. Microglia-depleted mice were gavaged with either ethanol (6 g/kg, 25% v/v) or water and sacrificed 18 hours later. **A)** Levels of pro-inflammatory TNFα and Ccl2 transcripts were determined in whole brain mRNA. **B)** Levels of anti-inflammatory IL-1ra, IL-10, IL-4 and Arg1 were determined in whole brain mRNA. Data are represented as mean ± s.e.m. * = $p<0.05$ Tukey's *post-hoc* test compared to all other groups. n=6-8/group.

Microglial-depleted mice were gavaged with ethanol and brain mRNA was isolated 18 hours post-treatment, a time when expression of microglial and cytokine genes was maximal. Expression of several genes was assessed via RT-PCR (Table 2.1). Microglial depletion completely blocked ethanol withdrawal-induced TNFα expression (Fig7A), reducing TNFα mRNA from about 600% to 104% ($p<0.05$). Microglial depletion partially blocked ethanol withdrawal-induced Ccl2 expression, decreasing Ccl2 mRNA from approximately 1000% to 355% (Fig7A). Interestingly, microglial depletion enhanced the ethanol withdrawal response of several anti-inflammatory genes. Withdrawal-induced IL-1ra was increased from 117% to 173% with microglial depletion (Fig7B). Microglial depletion also enhanced the ethanol withdrawal response of anti-inflammatory cytokines IL-4 and IL-10 to 291% ($p<0.05$) and 151% ($p<0.05$), respectively (Fig7B). These results find that microglial depletion blunts the acute binge ethanol withdrawal pro-inflammatory gene response and enhances the anti-inflammatory gene response.

Previous studies have implicated microglia in ethanol-induced neurodegeneration [153]. We

therefore sought to examine whether microglial depletion changed ethanol induction of cell death processes. One mechanism by which ethanol can induce cell death is through death receptors – a group of cytokine receptors that play a role in apoptosis. We therefore examined the role of microglia in ethanol-induced expression of death receptor genes. Microglial depletion enhanced the ethanol response of brain LTα, TL1A and FasL to 233% ($p<0.05$), 157% and 186% ($p<0.05$) of control levels, respectively (Table 1). Microglial depletion also enhanced the ethanol response of FasR to 210% of control levels (Table 1). These results find that microglia depletion enhances ethanol withdrawal-induced expression of multiple death receptor genes.

Since microglia can impact neuronal function, we also sought to investigate the role of microglia in ethanol-induced behavioral changes, specifically motor impairment. Previous studies suggest that microglia are involved in some of the behavioral effects of acute ethanol [155]. After one week of PLX5622 treatment, a time when microglia are mostly depleted [152], mice were treated with binge ethanol and tested for motor impairment on the rotarod as previously described [155]. There was no effect of one week of microglial depletion on motor coordination at any time point up to 110 minutes after ethanol treatment (FigS8A). To further test this hypothesis, we also examined the effects of the recombinant IL-1ra antagonist, Kineret, as done previously [155]. Mice were injected with two different doses of Kineret, 100 and 300 mg/kg, 30 minutes before ethanol treatment. We did not observe an effect of either dose of Kineret on ethanol-induced motor impairment (FigS8B,C). These results do not support the hypothesis that microglia play a role in the effects of acute ethanol-induced motor impairment.

2.3 Discussion

In this present study, we examined the effects of acute binge ethanol on microglia and how microglial depletion changes the brain neuroimmune response to acute binge ethanol withdrawal.

We report that acute binge ethanol biphasically changes microglial marker gene expression, with initial decreases during intoxication, followed by later increases during withdrawal when ethanol was gone (Fig8A). Acute binge ethanol withdrawal dose-dependently increased neuroimmune gene expression, starting at high doses. Cultured microglial-like cells showed biphasic changes in pro-inflammatory gene expression with ethanol treatment and evaporation *in vitro*, consistent with direct effects on microglia. Also, microglial depletion reduced expression of some neuroimmune genes, while many others were unchanged, suggesting that neuroimmune genes are expressed across many brain cell types (Fig8B). Finally, microglial depletion blunted withdrawal-induced pro-inflammatory gene expression and enhanced withdrawal-induced anti-inflammatory gene expression (Fig8C). These findings suggest microglia contribute to the impact of heavy binge alcohol on the brain, particularly during withdrawal.

These studies found that acute ethanol had dose-dependent effects on microglial and neuroimmune gene expression *in vivo*. Acute binge ethanol increased expression of microglial and

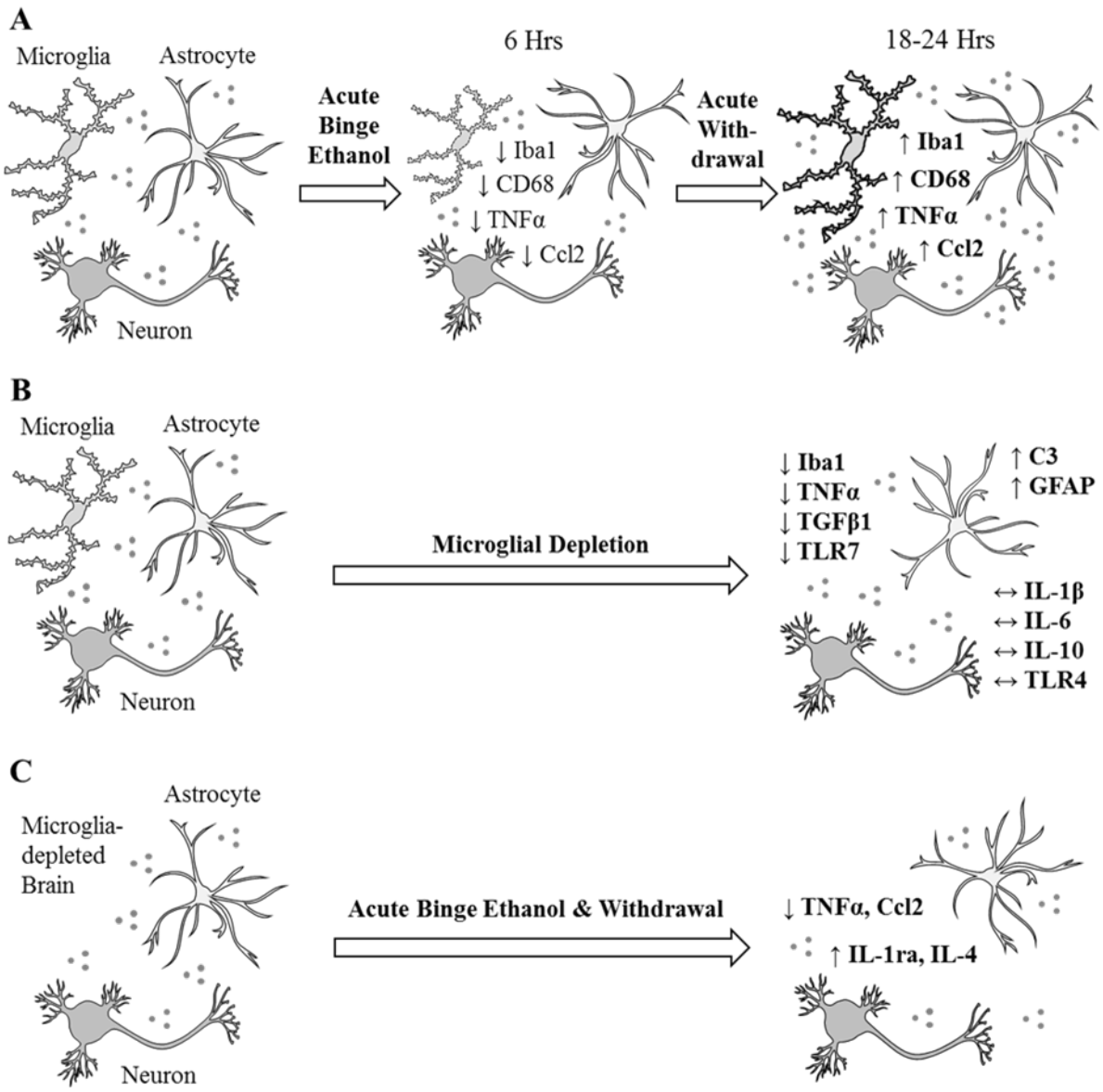


Figure 2-8 Effects of acute binge ethanol and microglial depletion on brain neuroimmune gene expression. A) Acute binge ethanol causes biphasic changes in microglial gene expression. Various microglial genes such as Iba1 and CD68 decrease during acute intoxication. Later, during withdrawal, expression of these genes increases. These data suggest the effects of ethanol on microglia depend on time after exposure. **B)** Microglial depletion alters brain gene expression. Various microglial genes, such as Iba1, decrease with microglial depletion. Other neuroimmune genes, such as TNFα or TGFβ1, also decrease. However, some neuroimmune genes, such as IL-1β, IL-6 and TLR4, do not decrease, suggesting these may be predominantly expressed in other cell types. Other genes, such as C3 and GFAP, increase with microglial depletion. **C)** Microglia mediate a balance between pro- and anti-inflammatory gene expression during acute binge ethanol withdrawal. Microglial depletion blocks withdrawal-induced pro-inflammatory gene induction (TNFα and Ccl2). However, microglial depletion enhances withdrawal-induced anti-inflammatory gene induction (IL-1ra and IL-4).

neuroimmune genes beginning at BACs of ~300 mg/dL. To understand the behavioral effects of

these BACs in mice over time, we assessed movement, pain response and mortality. Behaviorally, these BACs were associated with transient reductions in movement and pain response, but no mortality. BACs of this level have also been noted in humans [16, 18, 156, 157], suggesting these studies model brain pathology relevant to some human alcoholics. Increasing the ethanol dose further increased the expression of brain microglial and neuroimmune genes. Also, this acute binge ethanol treatment consistently models microglial and neuroimmune changes seen with moderate-dose, chronic ethanol treatment [133, 137]. Indeed, it seems that either high-dose, acute ethanol or moderate-dose, chronic ethanol is required to impact microglia and neuroimmune gene expression. These results are also consistent with studies in post-mortem human alcoholic brains that find increased microglial Iba1 [140, 144]. Overall, these results find that acute binge ethanol withdrawal dose-dependently increases microglial and neuroimmune gene expression, with high binge doses required to increase expression acutely.

We also report that acute binge ethanol has biphasic effects on microglial gene expression *in vivo*. Iba1 and CD68 expression initially decreased during intoxication and later increased during withdrawal. Increased Iba1 during ethanol withdrawal is consistent with previous chronic ethanol studies [134]. However, increased CD68 protein was not observed in previous *in vivo* studies [135]. Since those studies report comparably high BACs, differences may be due to examining protein versus mRNA, or due to different patterns of ethanol treatment. Indeed, the microglial response to stimuli can be dynamic and complex [97], and the response to ethanol likely depends on several factors, including the dose, pattern of administration and timing after ethanol exposure. The use of different species may also account for the differences between these studies. Overall, these results show acute binge ethanol has biphasic effects on microglial gene expression *in vivo* (Fig8A).

These studies also find acute binge ethanol withdrawal increases both pro-inflammatory and

anti-inflammatory gene expression in the brain. This was accompanied by increased TNFα, Ccl2 and IL-4 protein. Our results are consistent with previous studies showing acute ethanol increased brain TNFα and Ccl2 gene expression in mice [133]. Our results add to these previous observations by defining the time course of acute ethanol-induced pro-inflammatory gene expression. Specifically, it is during withdrawal that acute binge ethanol increases brain pro-inflammatory cytokines. These results are also consistent with studies in rats showing that withdrawal from both acute and chronic ethanol increase TNFα gene expression in the brain [87, 158]. While many studies have focused on the brain pro-inflammatory response to ethanol [133, 137, 158], only a few studies have examined the brain anti-inflammatory response to ethanol [135]. These studies show that IL-4, a key anti-inflammatory cytokine, is also increased in the brain during withdrawal from acute binge ethanol. Overall, these results show induction of both pro- and anti-inflammatory gene expression in the brain during acute binge ethanol withdrawal.

Results also showed ethanol had biphasic effects on pro-inflammatory cytokine expression in microglial-like cells *in vitro*, with expression decreased shortly after ethanol exposure and increased when the ethanol evaporated. However, expression of anti-inflammatory cytokines only decreased when ethanol was present and did not increase at any time point. Changes in gene expression were not due to BV2 cell death. Furthermore, increased pro-inflammatory gene expression was dependently on ethanol evaporation, as continuous ethanol exposure did not increase TNFα or Ccl2 gene expression. These results are similar to those of other studies finding biphasic effects of ethanol on human monocytes *in vitro*. While acute ethanol exposure decreases inflammatory signaling [159], chronic ethanol exposure enhances inflammatory signaling [150]. These data also suggest increased TNFα and Ccl2 gene expression *in vivo* reflect direct effects of ethanol withdrawal on microglia. Indeed, previous studies find that constant ethanol exposure can

act directly on microglia to change inflammatory signaling [160]. Since ethanol did not increase BV2 anti-inflammatory gene expression *in vitro,* this suggests the anti-inflammatory response observed *in vivo* may be mediated by other cell types. Indeed, previous studies have noted that astrocytes provide an anti-inflammatory balance to the pro-inflammatory activity of microglia [161]. Overall, these data show that ethanol has biphasic effects on microglial pro-inflammatory gene expression *in vitro,* with no increases in anti-inflammatory gene expression.

Treating mice with the CSF1R inhibitor PLX5622 for 3 weeks led to substantial microglial depletion, consistent with other studies [152, 162]. Indeed, mRNA levels of several microglial markers were reduced by more than 90% (Fig6A). It is worth noting that mRNA levels of CSF1R itself were markedly reduced following PLX5622 treatment, suggesting CSF1R is located predominantly on microglia. Microglial depletion decreased TNFα expression, consistent with previous studies [152]. However, mRNA levels of IL-1β, IL-6, Ccl2 and several other immune genes were surprisingly unchanged in PLX5622 treated mice (Fig6A). As the main immune cells of the CNS, one might expect microglia to be prominent expressers of these inflammatory mediators. While immunohistochemical studies revealed small, scattered populations of microglia remaining in the brain (Fig5), the extent of depletion was striking. It is possible these genes are expressed in other brain cells types. Indeed, astrocytes have been observed to produce IL-1β both *in vitro* [39] and *in vivo* . Other studies suggest IL-6 is produced by many other brain cell types, including neurons [163, 164], astrocytes [40], and endothelial cells [165]. Ccl2 expression has also been observed in neurons [166] and astrocytes [167]. We also examined expression of multiple TLRs. TLRs are believed to be prominently expressed on microglia [168]. PLX5622 decreased expression of TLR2 and TLR7, but surprisingly did not significantly decrease TLR3 or TLR4 expression. Indeed, microglial TLR4 is thought to play an important role in the detrimental effects of alcohol on the

brain [132]. Data from these studies suggest that TLR3 and TLR4 are primarily expressed in other cell types or that expression on other cell types is increased in response to microglial depletion. Overall, these results find that PLX5622 treatment decreased expression of many microglial genes and some, but not all, neuroimmune genes (Fig8B).

Expression of certain other genes was also impacted by microglial depletion. Neuronal markers MAP2 and DCX were unchanged, while expression of NeuN was slightly increased, consistent with other studies showing little to no effect of CSF1R inhibitors on neuronal markers [152]. PLX5622 increased the astrocyte marker GFAP, and had no effect on oligodendrocyte markers MBP and CNP, consistent with previous studies [152]. PLX5622 had no effect on the endothelial cell markers Pecam1 and ICAM2. Since microglia interact extensively with the other cells types of the CNS, we also examined expression of some genes that mediate these interactions. Neurons express CD200 and CX3CL1 (also known as fractalkine). These ligands interact with their cognate receptors on microglia and function to keep the microglia in a quiescent state [48]. Interestingly, microglial depletion did not alter expression of neuronal CD200 or CX3CL1. Microglia have also been found to play an important role in synaptic pruning via components of the complement cascade [169]. Components such as C3 and C1qA “tag” neural synapses for elimination by microglia. Interestingly, PLX5622 increased expression of C3 and dramatically reduced expression of C1qA. It is possible that compensatory upregulation of C3 may be occurring due to a lack of microglial synaptic elimination. These data also suggest that C1qA is predominantly expressed in microglia. It is possible that in the absence of microglia, there may be altered synaptic pruning. Finally, since depleting microglia may change neuronal functioning, we measured the expression of four immediate early genes, genes that increase with neuronal activation. There was no significant difference in expression of either c-Fos, EGR1, c-jun or Arc following PLX5622

treatment. Overall, these data suggest that microglial depletion has little effect on markers of other CNS cell types, but markedly alters expression of synaptic pruning genes.

These studies also examined the role of microglia in the brain response to acute binge ethanol withdrawal. These studies are, to our knowledge, the first to do so by depleting microglia *in vivo*. Multiple cell types of the CNS express immune genes [170], leaving it unclear which ethanol responses are due to microglial. As expected, microglial depletion reduced expression of multiple microglial genes, including Iba1, CD68, CD11b, etc. Furthermore, ethanol-induced TNFα was completely blocked by microglial depletion, suggesting ethanol-induced TNFα is microglial-derived. These results are similar to those of other studies finding microglial depletion completely blocked LPS-induced TNFα in the brain [152]. Ccl2 mRNA was partially, but not completely blocked by microglial depletion, suggesting multiple CNS cell types contribute to ethanol-induced Ccl2 expression. Surprisingly, microglial depletion did not reduce ethanol-induced IL-1β or IL-6. This finding is unexpected, as one might expect microglia to be the primary sources of these inflammatory mediators, especially following an insult. Indeed, microglial depletion blunted LPS-induced IL-1β in the brain [152]. However, microglial depletion did not decrease LPS-induced IL-6, suggesting that other CNS cell types are the primary sources of IL-6. Microglial depletion also enhanced withdrawal-induced anti-inflammatory gene expression, including IL-1ra, IL-4, IL-10 and Arg1. It is possible these genes are expressed by other brain cell types, such as astrocytes, during ethanol withdrawal, and microglia normally suppress expression of these genes. These results suggest that microglia play a role mediating the balance of pro- and anti-inflammatory forces during ethanol withdrawal. Overall, these results find that microglia play a role in the neuroimmune effects of ethanol on the brain (Fig8C).

This study also examined the effects of microglial depletion on death receptor gene

expression following acute binge ethanol. Interestingly, microglial depletion enhanced the ethanol response of multiple death receptor/ligand genes. Previous studies find that ethanol causes FasR-FasL induced cell death in the hepatocytes [171]. Furthermore, ethanol was found to increase FasL mRNA and cell death in cortical slices *in vitro* [172]. Since death receptors/ligands play important roles in apoptosis, these results indicate that microglial depletion may increase cell death following acute binge ethanol. While microglia are often thought to contribute to neurodegeneration [74], there is literature supporting a role for microglia in neuroprotection [73]. Indeed, it is likely that microglia can be either neurotoxic or neuroprotective depending on the circumstances. Future studies could further examine the role of microglia in ethanol-induced cell death. Overall, these results find that microglial depletion enhances the ethanol response of death receptor/ligand expression.

Furthermore, results of this study support acute ethanol withdrawal inducing a pro-inflammatory microglial phenotype. This is supported by the fact that microglia-like cells treated with ethanol *in vitro* show a pro-inflammatory response, but not an anti-inflammatory response, and that depleting microglia *in vivo* blunts the ethanol-induced pro-inflammatory response. Microglial activation phenotypes are usually described as occurring along an M1-M2 spectrum, with M1 representing the pro-inflammatory, destructive phenotype and M2 representing the anti-inflammatory, reparative phenotype. Previous studies have found varying results regarding the microglial phenotype induced by ethanol, with some studies suggesting an M1 phenotype [134] and others suggesting an M2 phenotype [135]. Microglia have been shown to exhibit complex and dynamic responses to stimuli [97]. Therefore, it is likely that the microglial response to ethanol is complex, and depends on the species being studied, as well as the dose, duration and pattern of alcohol administration. Indeed, previous studies suggest a single ethanol binge causes a milder, homeostatic microglial activation phenotype, while a second binge causes a more robust, pro-

inflammatory phenotype [138]. A complete understanding of the effects of ethanol on microglia may require examining entire microglial transcriptomes. Indeed, recent studies have called into question the M1-M2 activation schema, describing instead a complex constellation of several microglial activation states [69, 70]. Our results also question various aspects of the M1-M2 scheme, as various M1-M2 markers, such as iNOS and Arg1, were not reduced in microglial-depleted animals. Indeed, further studies will be necessary to define thoroughly the complex effects of ethanol on microglial function. Such studies will be critical, as microglia have been found to alter neuronal functioning and even behavior [63, 64], and may contribute to the development or consequences of alcoholism. Overall, these studies suggest acute ethanol withdrawal induces pro-inflammatory gene expression in microglia.

Table 2-1 Effects Acute Binge Ethanol and/or Microglial Depletion on Brain Gene Expression.

	CON	− M	E	− M + E
Microglial Markers				
Iba1 [*, †, ‡]	100 ± 1[A]	6 ± 0.4[B]	68 ± 3[C]	6 ± 1[B]
CD11b [*, †, ‡]	100 ± 2[A]	3 ± 1[B]	57 ± 2[C]	5 ± 1[B]
CD45 [*]	100 ± 7	52 ± 7	121 ± 15	69 ± 5
MHCII	100 ± 8	68 ± 22	67 ± 4	48 ± 15
M1 Microglial Markers				
CD68 [*, †]	100 ± 6	17 ± 2	136 ± 9	42 ± 4
CD86 [*, †, ‡]	100 ± 1[A]	2 ± 0.3[B]	127 ± 6[C]	5 ± 2[B]
iNOS [*, †, ‡]	100 ± 24[A]	135 ± 37[A]	170 ± 37[A]	812 ± 145[B]
NOX2 [*]	100 ± 13	46 ± 11	137 ± 14	51 ± 10
M2 Microglial Markers				
CD206 [*]	100 ± 2	4 ± 0.2	91 ± 5	5 ± 0.3
CD163 [*, †]	100 ± 7	15 ± 3	127 ± 11	30 ± 3
Arg1 [*, †]	100 ± 8	103 ± 10	108 ± 13	157 ± 11
Ym1	100 ± 15	133 ± 32	194 ± 36	197 ± 57
Pro-inflammatory Cytokines				
IL-1β [†]	100 ± 6	95 ± 14	143 ± 17	174 ± 16
TNFα [*, †, ‡]	100 ± 8[A]	68 ± 11[A]	600 ± 90[B]	104 ± 26[A]
IL-6 [†]	100 ± 9	92 ± 6	131 ± 13	164 ± 9
Ccl2 [†]	100 ± 12	127 ± 36	1002 ± 308	355 ± 60

Anti-inflammatory Cytokines

IL-10 †, ‡	100 ± 5[A]	91 ± 7[A]	112 ± 11[A]	152 ±9[B]
IL-4 †, ‡	100 ± 7[A,B]	84 ± 5[A]	186 ± 10[B]	291 ± 40[C]
IL-1ra *, †	100 ± 5	113 ± 19	117 ± 17	173 ± 16
TGF-β1 *	100 ± 3	44 ± 3	107 ± 11	58 ± 8

CNS Cell Types

NeuN *	100 ± 4	120 ± 5	108 ± 5	125 ± 10
MAP2	100 ± 3	106 ± 4	108 ± 4	102 ± 5
DCX †	100 ± 7	97 ± 7	84 ± 3	83 ± 6
GFAP *	100 ± 5	148 ± 11	111 ± 11	145 ± 10
S100β	100 ± 5	91 ± 5	91 ± 4	95 ± 6
CNP	100 ± 3	93 ± 7	86 ± 2	92 ± 7
MBP	100 ± 9	91 ± 6	84 ± 7	85 ± 5
ICAM2 †, ‡	100 ± 6[A]	83 ± 5[A]	108 ± 11[A]	144 ± 8[B]
Pecam1	100 ± 3	97 ± 4	103 ± 9	110 ± 7

Toll-like Receptors

TLR2 *, †	100 ± 4	64 ± 7	133 ± 8	94 ± 7
TLR3	100 ± 8	103 ± 6	99 ± 3	100 ± 5
TLR4 †	100 ± 8	82 ± 9	137 ± 11	125 ± 11
TLR7 *, †, ‡	100 ± 5[A]	24 ± 4[B]	152 ± 6[C]	38 ± 3[B]

Neuron-Microglia Signaling

CX3CL1 $^{*, \dagger, \ddagger}$	100 ± 3^{A}	93 ± 2^{A}	96 ± 4^{A}	75 ± 3^{B}
CX3CR1 *	100 ± 5	1 ± 0.1	112 ± 15	4 ± 1
CD200	100 ± 5	106 ± 5	102 ± 3	104 ± 3
CD200R1	100 ± 16	70 ± 9	105 ± 16	91 ± 8
CSF1	100 ± 2	120 ± 5	113 ± 5	104 ± 5
CSF1R *	100 ± 5	2 ± 0.3	91 ± 4	4 ± 1
Neurotrophins				
BDNF $^{*, \ddagger}$	$100 \pm 4^{A,B}$	$101 \pm 4^{A,B}$	113 ± 6^{A}	86 ± 5^{B}
NGF	100 ± 6	103 ± 3	96 ± 4	107 ± 4
NT3	100 ± 7	111 ± 9	96 ± 6	117 ± 10
NT4/5 $^{*, \dagger}$	100 ± 6	115 ± 15	131 ± 10	176 ± 17
Cytokine Receptors				
IL-1R1 $^{*, \dagger}$	100 ± 4	113 ± 8	130 ± 6	189 ± 20
TNFR1 †	100 ± 2	101 ± 5	131 ± 4	120 ± 6
TNFR2 $^{*, \dagger}$	100 ± 5	55 ± 2	124 ± 5	70 ± 6
CCR2	100 ± 11	110 ± 21	131 ± 22	144 ± 13
Immediate Early Genes				
c-Fos	100 ± 6	99 ± 13	155 ± 26	123 ± 20
EGR1	100 ± 9	96 ± 4	121 ± 8	92 ± 13
Arc	100 ± 10	81 ± 13	105 ± 17	75 ± 19
c-Jun $^{\dagger, \ddagger}$	$100 \pm 7^{A,B}$	81 ± 5^{A}	$105 \pm 5^{A,B}$	116 ± 8^{B}

Apoptosis Pathways				
FasL [*, †, ‡]	100 ± 6[A]	98 ± 11[A]	121 ± 8[A]	186 ± 20[B]
FasR [†]	100 ± 3	107 ± 7	146 ± 17	210 ± 25
TL1A [†]	100 ± 9	88 ± 8	127 ± 25	157 ± 12
DR3 [*]	100 ± 5	71 ± 7	91 ± 9	55 ± 9
TRAIL [†]	100 ± 5	108 ± 5	88 ± 7	67 ± 13
DR5 [*]	100 ± 6	106 ± 5	91 ± 6	139 ± 17
LTα [*, †, ‡]	100 ± 5[A]	95 ± 10[A]	120 ±19[A]	233 ± 25[B]
FADD	100 ± 3	98 ± 5	99 ± 4	114 ± 14
Caspase-3	100 ± 4	112 ± 4	112 ± 4	130 ± 12
Clock Genes				
Per1 [†]	100 ± 3	111 ± 8	155 ± 6	185 ± 41
Per2 [†, ‡]	100 ± 8[A]	123 ± 5[A,B]	202 ± 7[C]	147 ± 12[B]
BMAL1	100 ± 5	95 ± 5	104 ± 4	110 ± 6
Neurotransmitter Systems				
CRF [*]	100 ± 8	87 ± 7	93 ± 6	73 ± 7
DAT	100 ± 5	98 ± 7	92 ± 4	100 ± 4
GluR1 [†]	100 ± 2	101 ± 4	123 ± 3	121 ± 10
NPY	100 ± 2	95 ± 4	96 ± 4	96 ± 3
Other				
Glucocorticoid Receptor	100 ± 5	99 ± 3	96 ± 3	90 ± 6

HMGB1	100 ± 4	103 ± 7	101 ± 4	109 ± 4
COX2 †	100 ± 7	106 ± 5	140 ± 7	151 ± 9
MMP9 †	100 ± 2	106 ± 7	125 ± 6	133 ± 8
IL-12	100 ± 5	112 ± 7	99 ± 8	101 ± 6
IL-34	100 ± 7	101 ± 6	96 ± 7	262 ± 80
NLRP3 *, †	100 ± 3	27 ± 4	159 ± 22	57 ± 6
Nogo	100 ± 4	114 ± 6	124 ± 5	116 ± 3

Columns represent water-treated control mice (CON), microglial depleted mice (—M), ethanol-treated mice (E) and microglial-depleted ethanol-treated (—M+E) mice**.** Values are for mRNA levels set to 100% of controls. Control or microglia-depleted mice were gavaged with either water or ethanol (6 g/kg, 25% v/v) and sacrificed 18 hours post-treatment. * = Main effect of microglial depletion, † = Main effect of ethanol treatment, ‡ = Interaction. A, B, C, D = Means with different letters are significantly different ($p<0.05$, Tukey's *post-hoc*) from each other. Means with the same letters are not significantly different from each other. Only means with significant interactions are labeled with letters. Data represent mean ± s.e.m.

Table 2-2 Effects of Acute Binge Ethanol on Brain Gene Expression over Time.

	0 Hr	6 Hrs	12 Hrs	24 Hrs	48 Hrs
Microglial Markers					
Iba1	100 ± 3	51 ± 4*	84 ± 5	123 ± 6*	103 ± 4
CD11b	100 ± 8	74 ± 5*	65 ± 3*	84 ± 7	102 ± 4
M1 Microglial Markers					
CD68	100 ± 4	73 ± 2*	130 ± 7*	135 ± 7*	111 ± 4
CD86	100 ± 5	115 ± 5	129 ± 4*	118 ± 7	107 ± 8
iNOS	100 ± 6	31 ± 5*	92 ± 7	106 ± 6	90 ± 7
NOX2	100 ± 24	113 ± 18	148 ± 31	135 ± 23	116 ± 31
M2 Microglial Markers					
CD206	100 ± 8	88 ± 3	94 ± 8	106 ± 6	84 ± 4
CD163	100 ± 7	224 ± 9*	116 ± 13	104 ± 7	118 ± 9
Arg1	100 ± 7	95 ± 9	95 ± 16	98 ± 7	86 ± 10
Ym1	100 ± 8	162 ± 25	212 ± 47	195 ± 44	116 ± 12
Pro-inflammatory Cytokines					
IL-1β	100 ± 3	94 ± 7	112 ± 12	117 ± 6	95 ± 7
TNFα	100 ± 17	46 ± 10	214 ± 41*	154 ± 18	119 ± 14
IL-6	100 ± 6	83 ± 8	105 ± 10	125 ± 7	109 ± 13
Ccl2	100 ± 9	148 ± 54	376 ± 89*	178 ± 18	147 ± 24
Anti-inflammatory Cytokines					

IL-10	100 ± 5	99 ± 9	106 ± 13	126 ± 3	108 ± 14
IL-4	100 ± 6	104 ± 7	238 ± 30*	174 ± 17*	105 ± 8
IL-1ra	100 ± 6	102 ± 12	111 ± 14	132 ± 8	112 ± 13
TGF-β1	100 ± 7	68 ± 2*	121 ± 14	96 ± 5	95 ± 6
CNS Cell Types					
NeuN	100 ± 7	104 ± 7	108 ± 4	100 ± 4	117 ± 2
MAP2	100 ± 3	84 ± 2*	106 ± 6	95 ± 3	97 ± 3
GFAP	100 ± 10	81 ± 7	128 ± 19	125 ± 12	102 ± 7
MBP	100 ± 10	101 ± 7	104 ± 6	99 ± 4	100 ± 5
Inflammation					
HMGB1	100 ± 9	96 ± 9	89 ± 5	118 ± 4	109 ± 7
Neuron-Microglia Signaling					
CX3CL1	100 ± 4	91 ± 4	108 ± 5	118 ± 8	84 ± 5
CX3CR1	100 ± 3	43 ± 5*	86 ± 6	97 ± 5	92 ± 4
CD200	100 ± 6	107 ± 5	94 ± 3	99 ± 4	90 ± 3
CD200R1	100 ± 18	90 ± 7	80 ± 7	94 ± 12	91 ± 10
Neurotrophins					
BDNF	100 ± 7	66 ± 2*	124 ± 8	129 ± 8*	89 ± 4
NGF	100 ± 3	90 ± 2	115 ± 8	111 ± 4	102 ± 4
NT3	100 ± 11	114 ± 10	93 ± 16	88 ± 7	86 ± 8
NT4/5	100 ± 10	77 ± 3	103 ± 6	119 ± 9	115 ± 10

Neurogenesis

DCX	100 ± 5	85 ± 2	106 ± 8	97 ± 10	102 ± 4

Mice were gavaged with acute binge ethanol (6 g/kg, 25% v/v) and sacrificed 6, 12, 24 or 48 hours post-treatment. A non-gavaged "0" hour group was included as a control. * = $p<0.05$, ANOVA followed by Dunnett's post-hoc test compared to 0 hr control.

Table 2-3 Effects of Acute Binge Ethanol Dose on Brain Gene Expression During Withdrawal.

	CON	3 g/kg	4.5 g/kg	6 g/kg
M1 Microglial Markers				
CD68	100 ± 5	85 ± 2	127 ± 7*	133 ± 5*
CD86	100 ± 8	118 ± 4	116 ± 7	127 ± 9
M2 Microglial Markers				
CD206	100 ± 7	102 ± 8	100 ± 6	84 ± 6
CD163	100 ± 11	85 ± 4	97 ± 4	118 ± 9
Pro-inflammatory Cytokines				
IL-1β	100 ± 10	100 ± 10	151 ± 10*	130 ± 13
TNFα	100 ± 8	118 ± 8	218 ± 33	672 ± 93*
IL-6	100 ± 9	125 ± 15	186 ± 11*	140 ± 10
Ccl2	100 ± 8	84 ± 7	236 ± 126	873±290*
Anti-inflammatory Cytokines				
IL-10	100 ± 10	101 ± 13	141 ± 7*	122 ± 9
IL-4	100 ± 14	136 ± 12	193 ± 16*	224 ± 16*
IL-1ra	100 ± 10	116 ± 16	170 ± 12*	136 ± 14
Clock Genes				
Per1	100 ± 6	102 ± 4	185 ± 13*	204 ± 9*

Mice were gavaged with water or acute binge ethanol (6 g/kg, 25% v/v) and sacrificed 18 hours post-treatment. * = $p<0.05$, ANOVA followed by Dunnett's *post-hoc* test compared to water gavaged control.

Table 2-4 Effects of Ethanol Treatment on BV2 Gene Expression Over Time.

	0 Hr	1.5 Hrs	6 Hrs	12 Hrs	18 Hrs	24 Hrs
Microglial Markers						
Iba1	100 ± 5	99 ± 3	91 ± 7	81 ± 4	90 ± 4	111 ± 9
CD11b	100 ± 4	94 ± 5	114 ± 5	133 ± 9*	204 ± 2*	285±12*
M1 Microglial Markers						
CD68	100 ± 5	105 ± 4	113 ± 6	114 ± 6	130 ± 5*	155 ± 8*
CD86	100 ± 4	109 ± 4	141 ± 5*	156 ± 5*	161 ± 6*	172 ± 7*
iNOS	100 ± 28	48 ± 4	59 ± 8	34 ± 4	28 ± 4	31 ± 7
NOX2	100 ± 4	90 ± 4	89 ± 3	102 ± 5	137 ± 6*	174 ± 8*
M2 Microglial Markers						
CD206	100 ± 2	99 ± 3	104 ± 4	117 ± 6	144 ± 12*	152 ± 7*
CD163	100 ± 11	79 ± 19	106 ± 18	83 ± 6	111 ± 26	126 ± 8
Arg1	100 ± 10	109 ± 8	106 ± 13	90 ± 9	95 ± 9	105 ± 7
Ym1	100 ± 11	77 ± 6	73 ± 6	90 ± 11	60 ± 7	46 ± 10*
Pro-inflammatory Cytokines						
IL-1β	100 ± 2	113 ± 4	92 ± 4	107 ± 4	135 ± 12	226 ± 20
TNFα	100 ± 6	66 ± 3*	105 ± 6	104 ± 5	156 ± 12*	265±11*
IL-6	100 ± 10	94 ±5	121 ± 10	123 ± 7	133 ± 10	213±27*
Ccl2	100 ± 5	65 ± 4*	52 ± 2*	58 ± 2*	89 ± 5	148 ± 7*
Anti-inflammatory Cytokines						

IL-10	100 ± 8	139 ± 9	155 ± 9*	146 ± 9*	200 ± 7*	209±18*
TGF-β1	100 ± 6	97 ± 3	97 ± 4	104 ± 7	117 ± 11	121±10
Inflammation						
HMGB1	100 ± 5	103 ± 5	97 ± 4	70 ± 5	60 ± 5	61 ± 4

BV2 microglia-like cells were treated with ethanol (85 mM) *in vitro*. mRNA was collected at 0, 1.5, 6, 12, 18 and 24 hours and the expression of various genes was assessed. * = $p<0.05$, ANOVA followed by Dunnett's *post-hoc* test compared to 0 hr control.

2.4 Materials and Methods

2.4.1 Animals

Male C57BL/6J mice were ordered from the Jackson Laboratory and housed in an animal facility at the University of North Carolina at Chapel Hill. All mice were group housed (n=3-4) in a temperature- (20°C) and humidity-controlled vivarium on a 12 hr/12 hr light/dark cycle (light onset at 0700 hr), and provided *ad libitum* access to food and water. Experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill, and conducted in accordance with National Institutes of Health regulations for the care and use of animals.

2.4.2 Mouse Time Course and Dose Response Experiments

For the time course experiment, 12 week old mice were gavaged with either ethanol (6 g/kg, 25% v/v) or a comparable volume of water and sacrificed 6, 12, 18, 24 or 48 hours later. A group of non-gavaged "0 hour" mice was included as a control. All mice were sacrificed mid-morning (9-10 AM). A parallel group of mice was gavaged with ethanol (6 g/kg, 25% v/v) and tail blood was collected at 1, 6, 12, and 18 hours for the determination of blood alcohol concentrations (BACs). For the dose response experiment, 12 week old mice were gavaged with ethanol (3 g/kg, 4.5 g/kg or 6 g/kg, 25% v/v) or comparable volume of water and sacrificed 18 hours later. Tail blood was collected at 1 hour for the determination of BACs. All mice were sacrificed mid-morning (9-10 AM). BACs were determined using a GL5 Analyzer (Analox; London, UK).

2.4.3 Cell Culture Time Course Experiment

The BV2 immortalized mouse microglial cell line was maintained in DMEM supplemented with 10% FBS, 2 mM L-alanyl-L-glutamine dipeptide (GlutaMAX, ThermoFisher, 35050061), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Antibiotic-

Antimycotic 100X, ThermoFisher, 15240062). Cells were maintained in a humidified incubator with 5% CO_2. For time course experiments, BV2 cells were plated at a density of $1.5x10^5$ cells/well in 6-well plates. After plating, cells were allowed a few hours to adhere, and media was replaced with DMEM supplemented with 2% FBS, 2 mM L-alanyl-L-glutamine dipeptide, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. The cells were then allowed to incubate overnight. The next day, cells were treated with PBS or 85 mM ethanol in PBS at noon (24 hour time point), 6 PM (18 hour time point), midnight (12 hour time point), 6 AM the next day (6 hour time point) or 10:30 AM the next day (1.5 hour time point). At noon the next day, media was collected for analysis of ethanol concentrations and TRI reagent was added to the cells. Total mRNA was extracted as described below. For evaporation experiments, cells were placed in the incubator without vaporized ethanol in the chamber, thereby allowing media ethanol to evaporate over time. For continuous ethanol exposure experiments, cells were placed in the incubator with vaporized ethanol to keep media ethanol concentrations constant. Ethanol was vaporized into the incubator by placing a beaker with 200 mL of 4% ethanol into the chamber.

2.4.4 Microglial Depletion Experiment

The CSF1R inhibitor PLX5622 was provided by Plexxikon Inc. (Berkeley, CA) formulated at a dose of 1200 mg/kg in AIN-76A chow by Research Diets (New Brunswick, NJ). Control chow was also provided. Twelve week old mice received either PLX5622 chow or control chow for 3 weeks. Mice (average weight: 27 g; range: 23 g – 30 g) were then acutely gavaged with ethanol (6 g/kg, 25% v/v, i.g.) or an equivalent volume of water and sacrificed 18 hours later. All mice were sacrificed mid-morning (9-10 AM).

2.4.5 mRNA Isolation, Reverse Transcription, and RT-PCR

Total mRNA was extracted from frozen half-brains by homogenization in TRI reagent

(Sigma-Aldrich, St. Louis, MO, Cat.# T9424) following the single-step method [173]. Total mRNA was reverse transcribed. Primers used for RT-PCR are listed in Table M1. In all experiments, 18S rRNA was used as a reference gene. SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA, Cat.# 4367659) was used for the RT-PCR. The real time RT-PCR was run with an initial activation for 10 min at 95°C, followed by 40 cycles of denaturation (95°C, 15 s), annealing/extension (57-58°C, 1 min) and finally a melt curve. The threshold cycle (C_T) of each target product was determined and the $\Delta\Delta C_T$ method was used to calculate the percent change compared to the control group.

2.4.6 Protein Isolation and ELISAs

Total protein was extracted from frozen half-brains by homogenization in cold lysis buffer (20 mM Tris, 0.25 M sucrose, 2 mM EDTA, 10 mM EGTA, 1% Triton X-100 plus 1 tablet of Complete ULTRA protease inhibitor cocktail [Sigma, St. Louis, MO] per 10 mLs solution). Homogenates were centrifuged at 100,000 × g for 40 min, supernatant was collected, and protein levels determined using the BCA protein assay reagent kit (PIERCE, Milwaukee, WI). Levels of TNFα, Ccl2, IL-1ra and IL-4 were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN), as per the manufacturer's instructions.

2.4.7 Perfusion and Brain Tissue Preparation

Mice were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4). Brains used for RT-PCR or ELISAs were extracted and immediately frozen in liquid nitrogen. Brains used for immunohistochemistry were perfused with 4.0% paraformaldehyde in PBS, extracted, post-fixed in 4.0% paraformaldehyde/PBS solution overnight and then placed in a 30% sucrose solution in PBS for a few days. Brain tissue was sectioned coronally at a thickness of 40 μm on a sliding microtome

(MICROM HM450; ThermoScientific, Austin, TX). Sections were sequentially collected into well plates and stored at −20 °C in a cryoprotectant solution consisting of 30% glycol/30% ethylene glycol in PBS for immunohistochemistry.

2.4.8 Immunohistochemistry

Free-floating sections were washed in 0.1 M PBS, incubated in 0.3% H_2O_2 for 30 minutes, washed again in PBS and blocked for one hour at room temperature in 0.25% Triton-X100/5% normal serum (MP Biomedicals, Solon, OH, Cat.# 19135680). Sections were transferred directly from the block to primary antibody (rabbit anti-Iba1, WAKO, Japan) diluted in blocking solution and were incubated overnight at 4°C. Sections were then washed in PBS, incubated for one hour in biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA), washed and incubated for one hour in avidin–biotin complex solution (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, Cat.# PK6100). The chromogen, nickel-enhanced diaminobenzidine (Sigma–Aldrich, St. Louis, MO, Cat.# D5637), was used to visualize immunoreactivity. Tissue was mounted onto slides, dehydrated, and coverslipped.

2.4.9 Rotarod Behavioral Experiments

Rotarod testing was performed as previously described [155] and consisted of walking on a rotarod apparatus (Ugo Basile, Italy, Mouse Rota-rod, 47600) set at a fixed speed of 16 rotations-per-minute (rpm). Each mouse underwent training one day prior to experimental testing. Training consisted of the mouse remaining on the rod for three consecutive trials of 180 seconds. Mice that did not pass this criterion within 18 trials were not included in subsequent testing. The next day, mice performed a baseline test to ensure they could remain on the rotarod for 180 seconds. Mice were then injected i.p. with either vehicle or the recombinant IL-1ra drug, Kineret (obtained from the pharmacy at UNC Hospitals) at a dose of either 100 or 300 mg/kg. 30 minutes later, mice

underwent another rotarod test to ensure there were no effects of IL-1ra on motor activity. Mice were then injected i.p. with ethanol (2.0 g/kg, 20% v/v) and tested on the rotarod at 2, 5, 8, 14, 20 and every subsequent 10 minutes until 110 minutes had passed. The time the mice remained on the rotarod was recorded.

Rotarod testing was performed on another group of mice, except that these mice received either control chow or PLX5622 chow for 1 week prior to testing, a treatment time previously shown to result in substantial microglial depletion [162]. These mice were otherwise trained and tested as described above.

2.4.10 Movement and Pain Response Behavioral Assessments Following Acute Binge Ethanol

Mice were gavaged with ethanol (4.5 or 6 g/kg, 25% v/v) and movement and pain response were qualitatively assessed each following hour. For movement, a complete absence of movement other than breathing was recorded as “No Activity.” Movement of the head or limbs or impaired ambulation was recorded as “Impaired Activity.” Movement that appeared indistinguishable from a sober control mouse was recorded as “Full Activity.” For pain assessment, each hindpaw was pinched. A complete absence of a response was recorded as “No response.” Slight flinching or movement following any pinch was recorded as “Weak Response.” Full paw withdrawal for any pinch was recorded as “Full Response.”

2.4.11 Statistical Analyses

The Statistical Package for the Social Sciences (SPSS; Chicago, IL) was used for all statistical analyses. Data from time course experiments was analyzed via ANOVA with Tukey’s *post hoc* test for multiple comparisons. The data from dose-response experiments was analyzed via ANOVA with Dunnett’s *post hoc* test compared to controls. Data from microglial depletion experiments was analyzed using a two-by-two ANOVA with significant interactions being further

investigated using Tukey's *post hoc* test for multiple comparisons. Comparison of two means was analyzed using student's t-test. All values are reported as mean ± S.E.M., and significance was defined at a level of $p \leq 0.05$.

2.5 Supplemental Figures

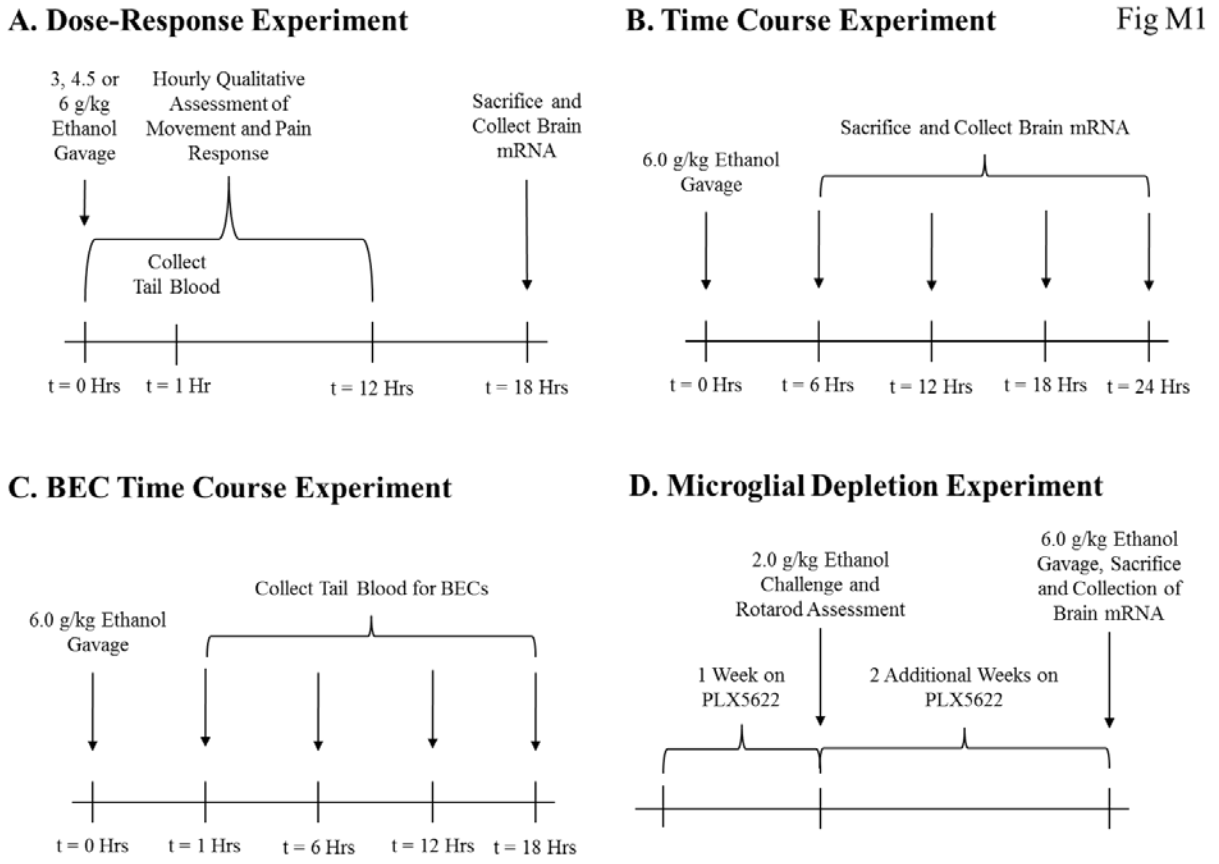


Fig S2.0. Schematic of experimental designs. A) For the Dose-Response Experiment, mice were gavaged with 3.0, 4.5 or 6.0 g/kg ethanol (25% v/v) and pain response and movement were qualitatively assessed each following hour. For each group, BACs were collected 1 hour post-gavage. Mice in these groups were sacrificed at 18 hours and brain mRNA was collected for RT-PCR. Note that mice in the group receiving 3.0 g/kg ethanol were not qualitatively assessed for behavioral changes. **B)** For the Time Course Experiment, mice were gavaged with 6.0 g/kg ethanol (25% v/v) and sacrificed at 6, 12, 18 or 24 hours. A non-gavaged "0 hour" control group was also included. Brain mRNA was collected for RT-PCR. **C)** A separate group of mice was gavaged with 6.0 g/kg ethanol (25% v/v) and tail blood was collected at 1, 6, 12 and 18 hours for assessment of BACs. **D)** Mice were given either Control chow or PLX5622 chow. One week after starting PLX5622 chow to deplete microglia, mice were injected intraperitoneally with 2.0 g/kg ethanol (20% v/v) and tested on the rotarod. After two more weeks of treatment with PLX5622 chow, mice were gavaged with 6.0 g/kg ethanol (25% v/v) and brain mRNA was collected 18 hours later for RT-PCR.

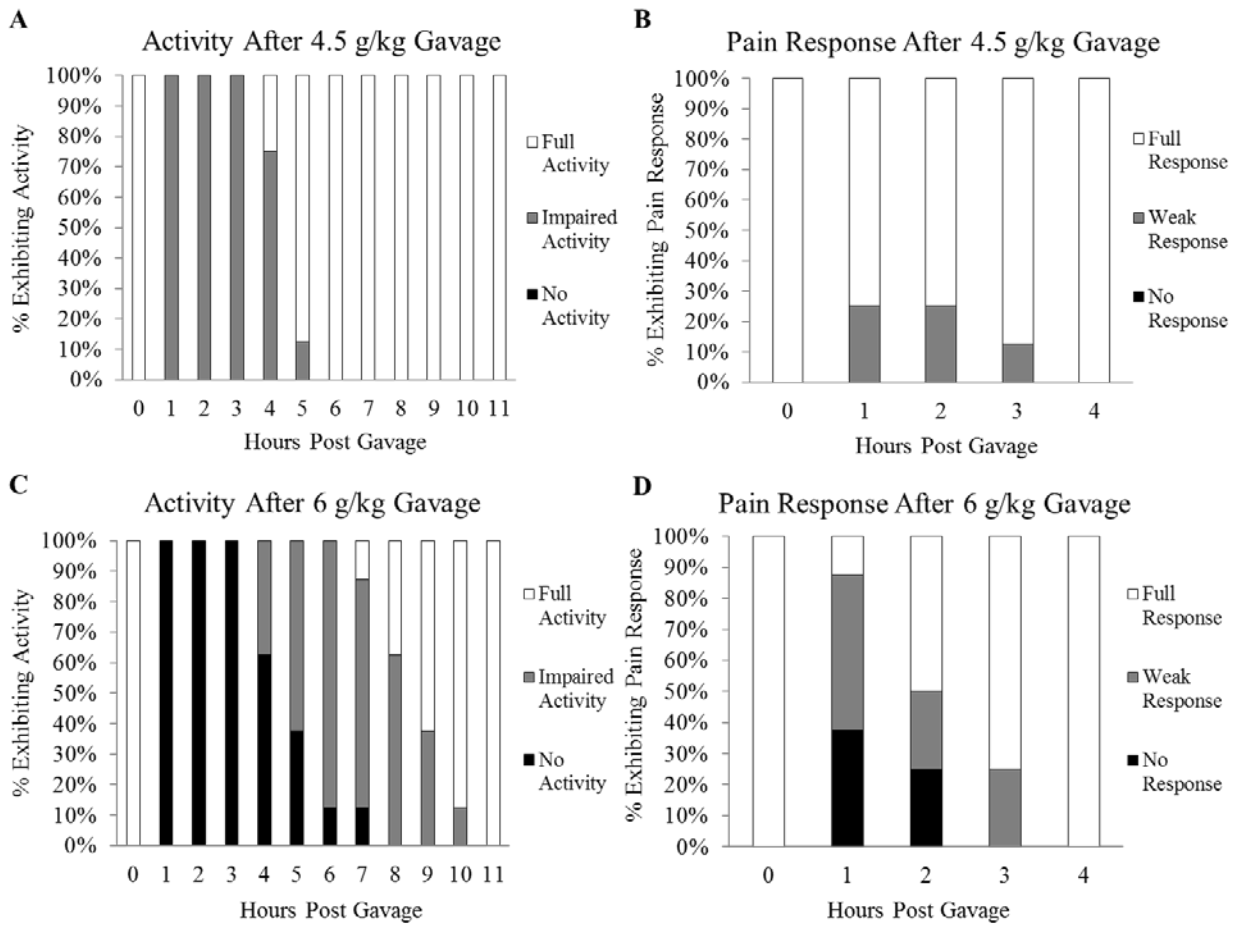


Fig S2.1. Behavioral characterization of the acute binge ethanol model. Mice were gavaged with ethanol (4.5 g/kg or 6 g/kg, 25% v/v) and pain response and movement were qualitatively assessed each following hour. Note that there was 0% mortality, as indicated by behavior recorded for 100% of mice in each group at each time point. **A, C)** For movement, a complete absence of movement other than breathing was recorded as "No Activity." Head movement, limb movement or impaired ambulation was recorded as "Impaired Activity." Movement that was indistinguishable from a control mouse was recorded as "Full Activity." **B, D)** For pain assessment, each hindpaw was pinched. A complete absence of a response was recorded as "No response." Slight flinching or movement following any pinch was recorded as "Weak Response." Full paw withdrawal following any pinch was recorded as "Full Response." n=8/group.

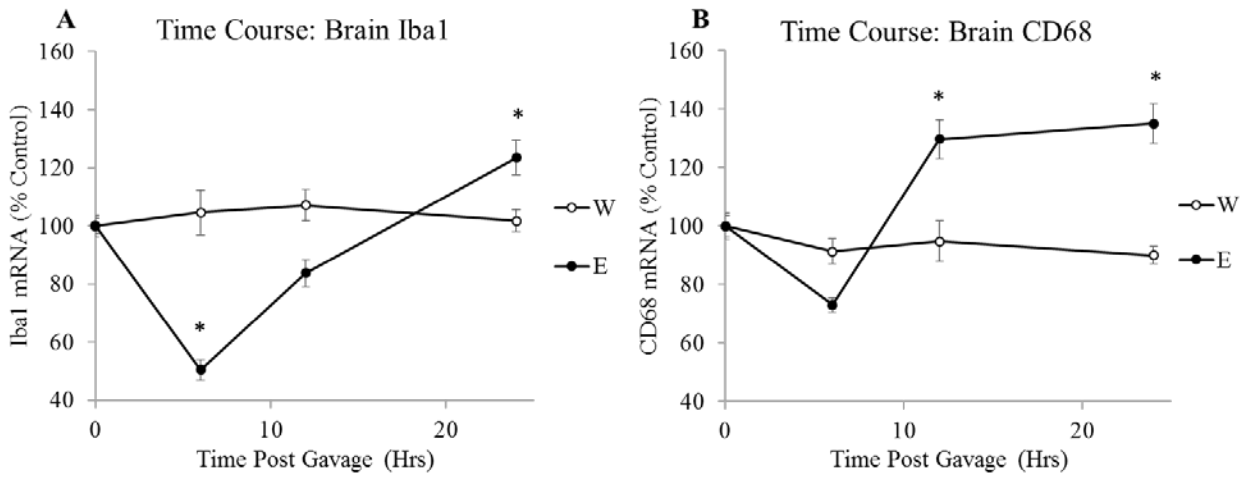


Fig S2.2. Acute binge ethanol causes biphasic changes in brain Iba1 and CD68 mRNA compared to controls. Mice were gavaged with acute binge ethanol (6 g/kg, 25% v/v) or water and sacrificed various times post-treatment. **A)** Brain Iba1 was measured over time by RT-PCR in both water- and ethanol-treated mice. **B)** Brain CD68 was measured over time by RT-PCR in both water- and ethanol-treated mice. Note there is no change in brain Iba1 or CD68 mRNA in water-treated mice over time. Data are represented as mean ± s.e.m. * = $p<0.05$ compared to controls. n=5-6/group.

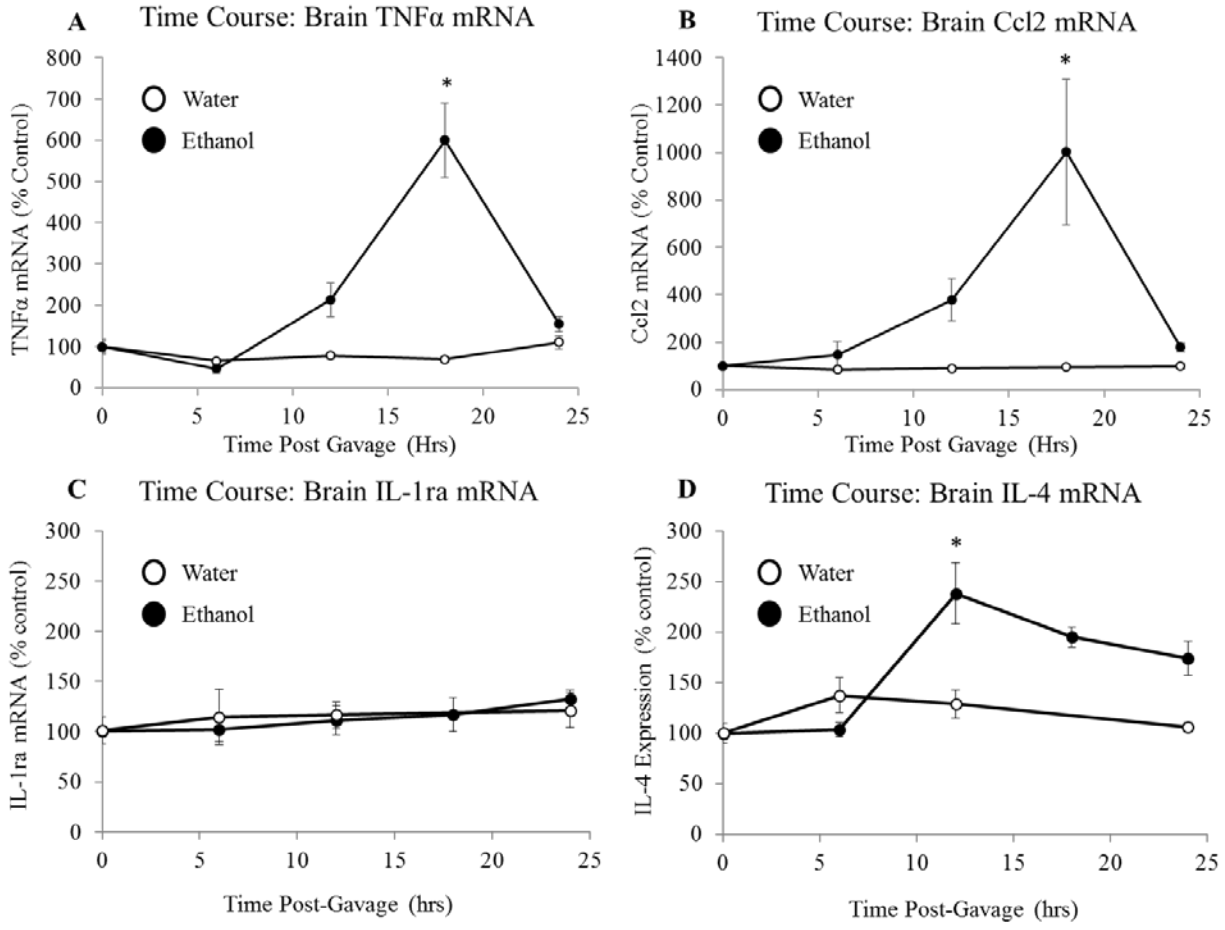


Fig S2.3. Acute binge ethanol withdrawal increases brain TNFα, Ccl2 and IL-4 mRNA compared to controls. Mice were gavaged with acute binge ethanol (6 g/kg, 25% v/v) or water and sacrificed various times post-treatment. **A)** Brain TNFα, **B)** Ccl2, **C)** IL-1ra, and **D)** IL-4 was measured over time by RT-PCR in both water- and ethanol-treated mice. Data are represented as mean ± s.e.m. * = $p<0.05$ compared to controls. n=5-7/group.

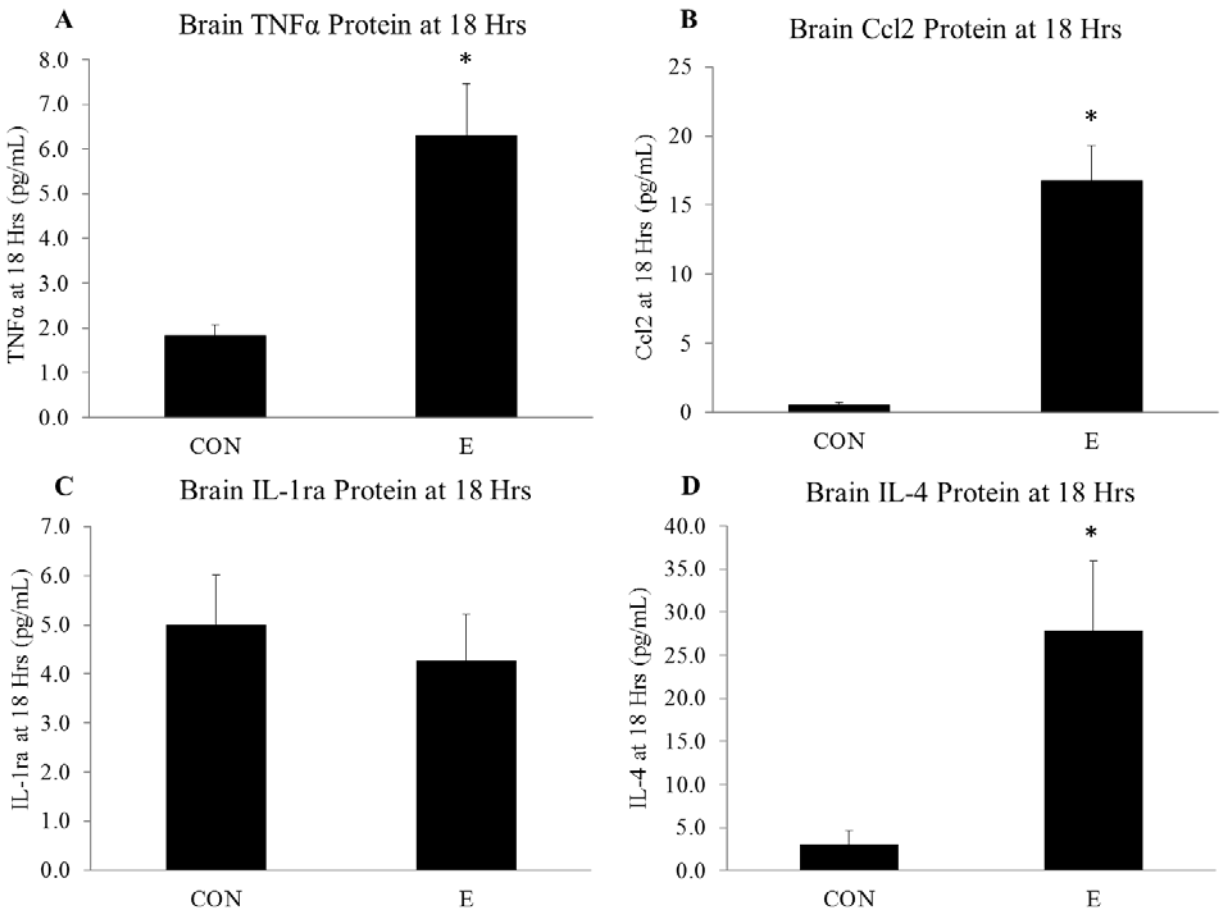


Fig S2.4. Acute binge ethanol increases brain TNFα, Ccl2 and IL-4 protein during withdrawal. Mice were treated with ethanol (6 g/kg, 25% v/v) or and sacrificed 18 hours post-treatment. Brain protein was collected and **A)** TNFα**, B)** Ccl2, **C)** IL-1ra and **D)** IL-4 protein levels were determined via ELISA. Data are represented as mean ± s.e.m. * = $p<0.05$, student's t-test, n=6/group.

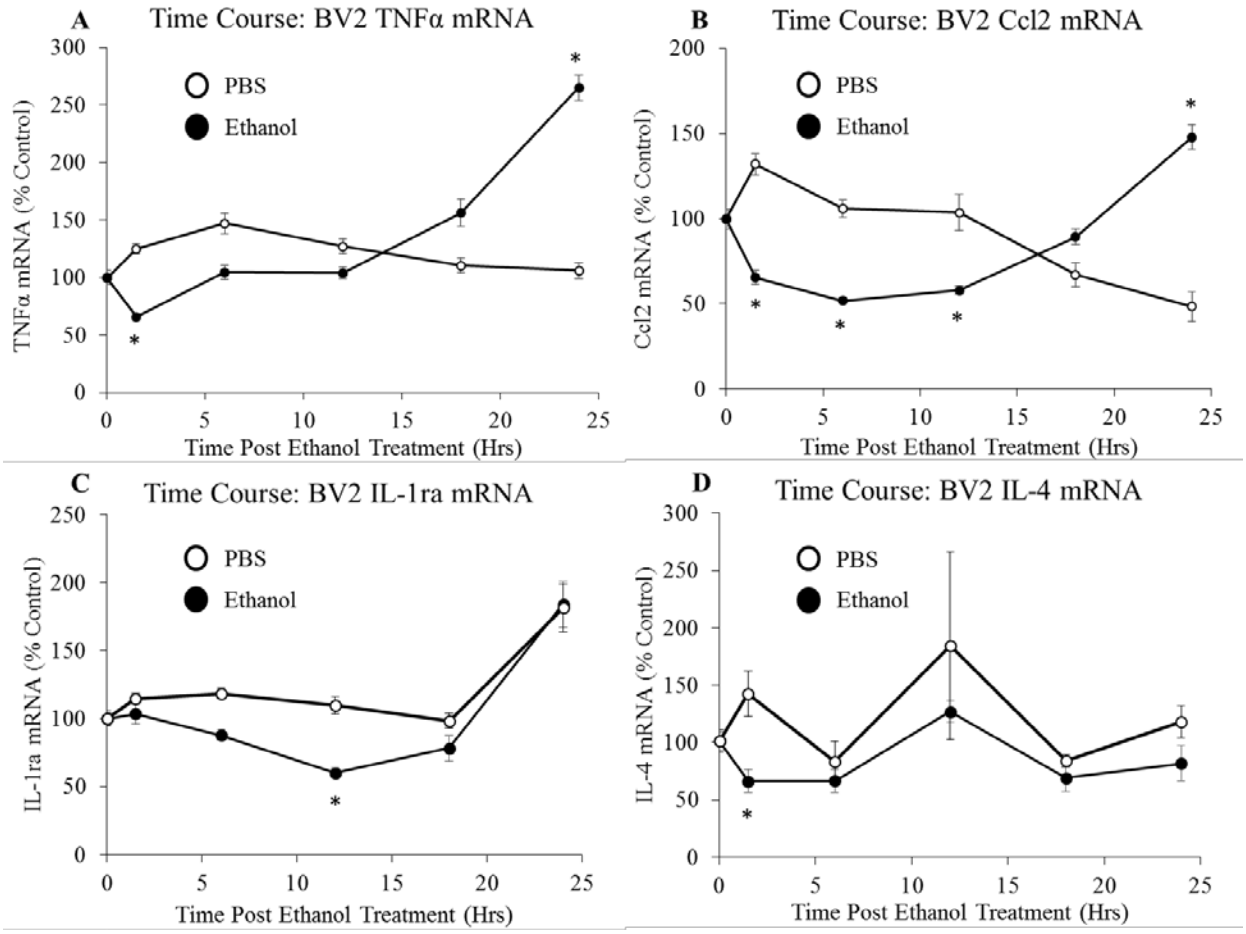


Fig S2.5. Acute ethanol treatment and evaporation changes BV2 TNFα, Ccl2, IL-1ra and IL-4 mRNA compared to controls. Time course of cytokine expression in ethanol-treated and PBS-treated control BV2 cells: Microglia-like BV2 cells were treated with either ethanol (85 mM) or PBS and the ethanol was allowed to evaporate away over time. Transcript levels of **A)** TNFα **B)** Ccl2 **C)** IL-1ra and **D)** IL-4 were assessed in ethanol-treated cells (black dots) and PBS-treated control cells (white dots). Data are represented as mean ± s.e.m. * = $p<0.05$, student's t-test, n=4-6/group.

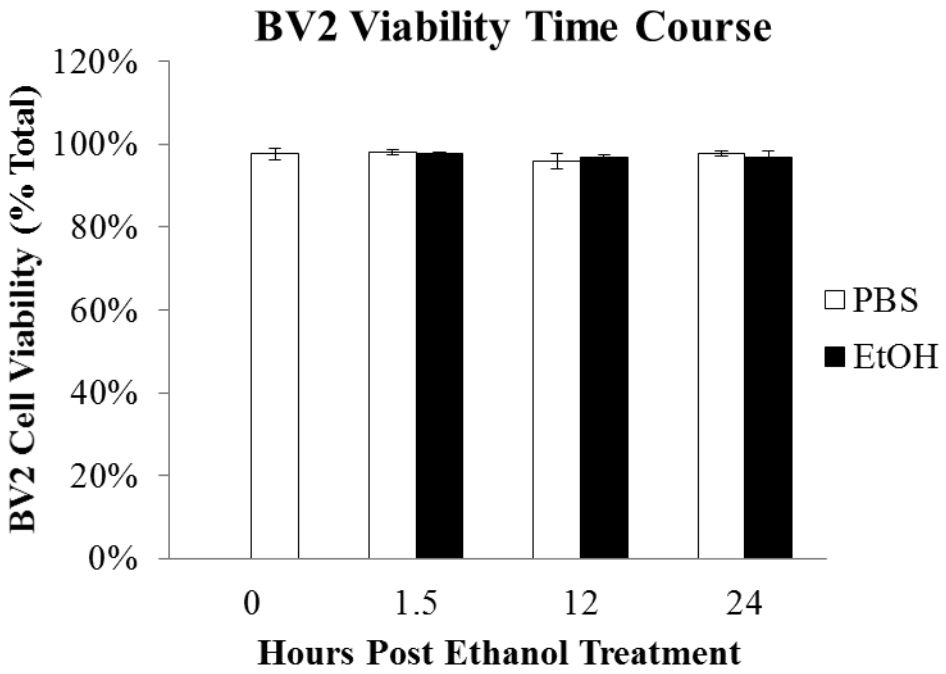


Fig S2.6. BV2 viability following ethanol treatment. Microglia-like BV2 cells were treated with PBS or ethanol (85 mM) and the ethanol was allowed to evaporate away over time. At 0, 1.5, 12 and 24 hours, cell viability was determined with the vital stain, Trypan blue. The number of live and dead cells were counted, and the number of live cells was divided by the number of total cells to calculate percent viability. Note that ethanol treatment did not affect cell viability at any time point. Data are represented as mean ± s.e.m. n=3/group.

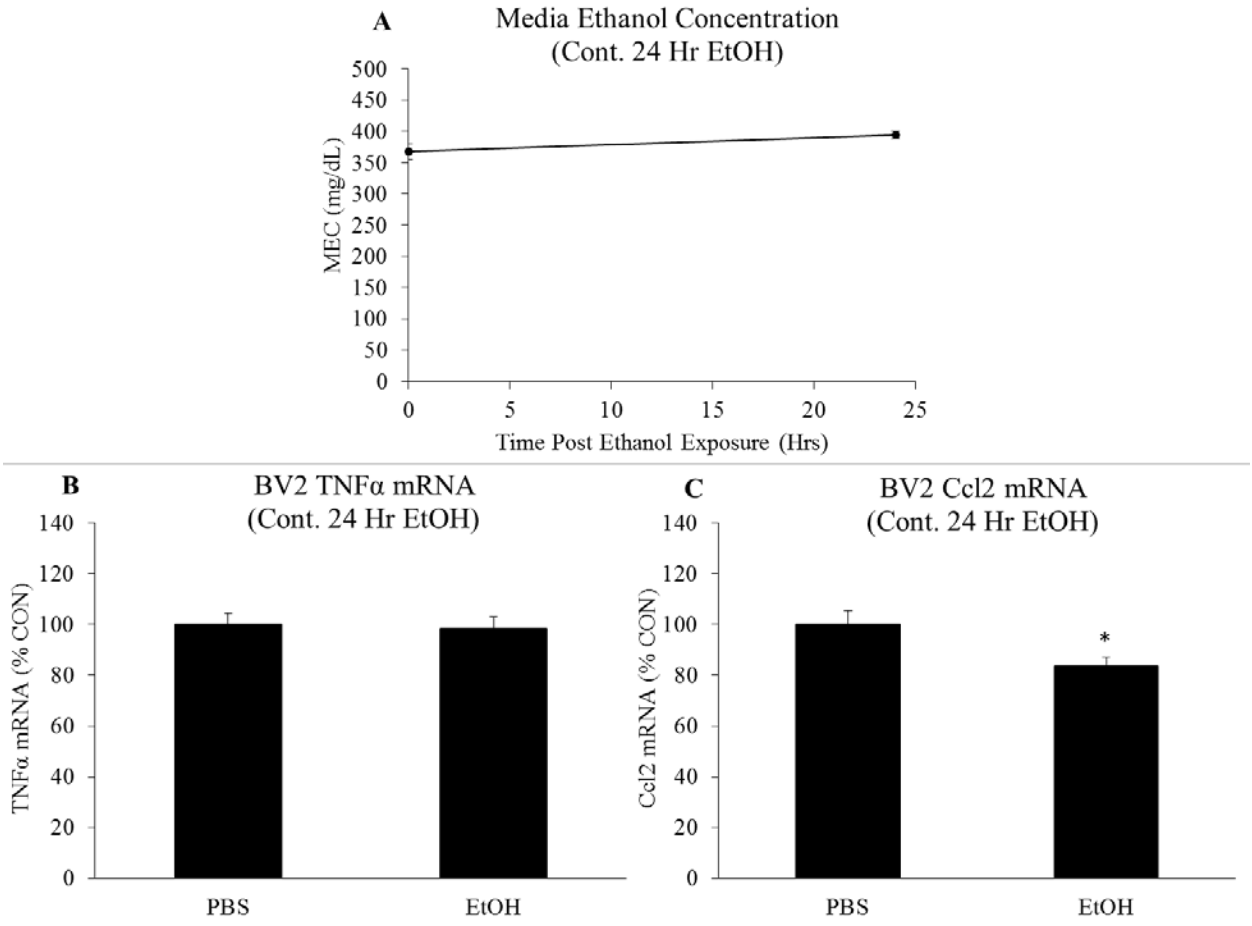


Fig S2.7. Continuous ethanol treatment does not increase BV2 TNFα or Ccl2 expression. Microglia-like BV2 cells were treated with either ethanol (85 mM) or PBS. **A)** For ethanol-treated cells, ethanol was vaporized into the incubator to keep media ethanol concentrations constant. After 24 hours of continuous ethanol exposure, BV2 mRNA was isolated and **B)** TNFα and **C)** Ccl2 gene expression was measured. Note that continuous ethanol exposure does not increase BV2 pro-inflammatory cytokine expression at 24 hours. Data are represented as mean ± s.e.m. * = $p<0.05$, student's t-test, n=6/group.

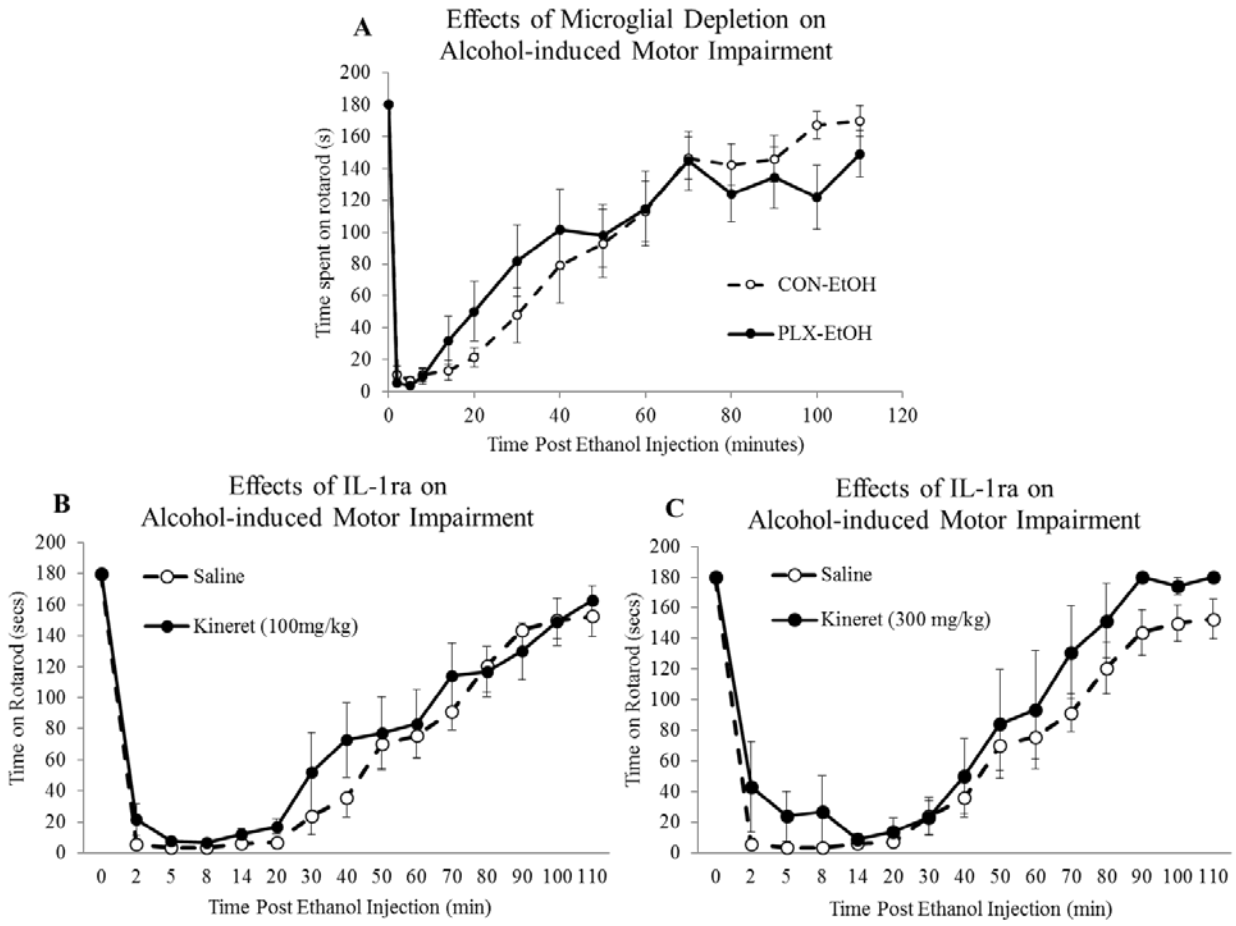


Fig S2.8. The role of microglia in ethanol-induced motor impairment. Mice were treated with compounds that impact microglia – either the CSF1R inhibitor PLX5622 or recombinant IL-1ra (Kineret). **A)** Mice were fed PLX5622 chow for 1 week to deplete microglia and injected i.p. with ethanol (2.0 g/kg, 20% v/v). Mice were then tested on the rotarod at 2, 5, 8, 14, 20 min and every subsequent 10 minutes post injection until 110 minutes had passed. The time the mice remained on the rotarod was recorded. Note that microglial depletion with one week on PLX5622 chow did not alter the ethanol-induced motor impairments. **B,C)** Mice received an intraperitoneal injection of **B)** 100 or **C)** 300 mg/kg IL-1ra 30 minutes prior to an i.p. injection of ethanol (2.0 g/kg, 20% v/v). Mice were then tested on the rotarod at 2, 5, 8, 14, 20 min and every subsequent 10 minutes after ethanol injection until 110 minutes had passed. The time the mice remained on the rotarod was recorded. Note that IL-1ra did not alter the ethanol-induced motor impairments. n=8/group.

CHAPTER 3: ALCOHOL AND STRESS ACTIVATION OF MICROGLIA AND NEURONS: BRAIN REGIONAL EFFECTS

3.1 Introduction

Alcohol use disorders (AUDs) are widespread mental health conditions that contribute to significant morbidity and mortality. Many studies have identified a relationship between AUDs and stress. Indeed, stress is associated with increased alcohol use [29, 30] and excessive alcohol increases the activity of brain stress systems to promote stressful mental states [174]. However, much remains unknown regarding how alcohol and stress interact to contribute to disease. Recent studies suggest the neuroimmune system plays an important role in mediating the relationship between alcohol and stress. Indeed, acute stress increases brain expression of cytokines such as TNFα, IL-1β and Ccl2 [94] and the danger signaling molecule, high mobility group box-1 protein (HMGB1) [116, 175]. Cytokines such as IL-10 and IL-1ra alter ethanol consumption [84, 176], and the cytokine receptors IL1R1 and TNFR1 contribute to stress-induced ethanol consumption [80]. Finally, cytokines such as TNFα enhance stressful emotional states following alcohol withdrawal [177]. Overall, these studies suggest the neuroimmune system plays an important role in the relationship between alcohol and stress; however, the mechanisms mediating this relationship remain poorly understood.

The activity of the neuroimmune system is influenced by signaling from the periphery [178]. Both alcohol and stress increase peripheral levels of immunomodulatory compounds such as the glucocorticoid stress hormone, corticosterone [179], and gut-derived bacterial endotoxins [180, 181]. Glucocorticoids are traditionally considered anti-inflammatory, although they can exacerbate

inflammation under certain circumstances [107], and endotoxins increase inflammation. Glucocorticoids and endotoxins have both been shown to impact the brain neuroimmune system [112, 182]. Therefore, alcohol and stress may impact the neuroimmune system by increasing peripheral levels of glucocorticoids or endotoxins. Among the cell types of the brain, microglia – the resident macrophage-like cells – are the most prominent neuroimmune signaling cells [59]. Microglial function is impacted by both peripheral glucocorticoids [182] and endotoxins [133]. Furthermore, microglia are activated by both alcohol [153] and stress [183], suggesting alcohol and stress may impact microglia through peripheral glucocorticoid or endotoxins. Importantly, microglia can also modulate neuronal function. Studies find that microglia release cytokines such as TNFα [64], neurotrophins such as BDNF [63], and eliminate synapses [151] to impact neuronal activity. These data suggest alcohol and stress may impact neurons through microglia. However, whether alcohol and stress interact to increase peripheral glucocorticoids and endotoxins, impact microglia and subsequently affect neurons is unknown. In this study, we therefore investigated the effects of ethanol and stress on glucocorticoids and endotoxin, as well as microglial and neuronal activation. We performed two experiments: the first studying acute ethanol and acute stress, the second studying chronic ethanol and acute stress. We examined plasma levels of corticosterone and endotoxin, and assessed microglial and neuronal activation by performing immunohistochemical stains for CD11b and c-Fos, respectively. We hypothesized that acute ethanol and acute stress would interact to enhance plasma corticosterone and endotoxin levels, as well as microglial and neuronal activation. We further hypothesized that chronic ethanol would persistently enhance the response of corticosterone, endotoxin, microglia, and neurons to acute stress.

3.2 Results

3.2.1 Effects of acute ethanol and acute stress on plasma CORT and endotoxin levels

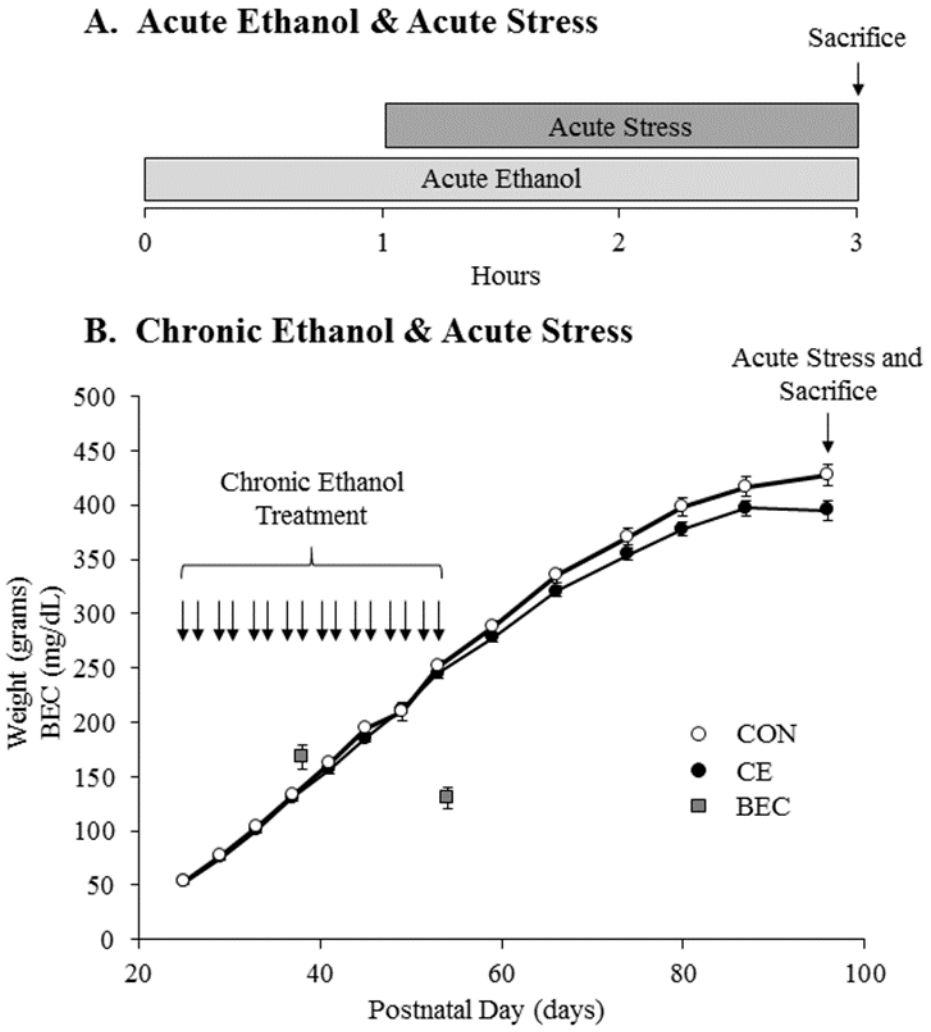


Figure 3-1 Graphical representation of the experimental protocols. A. Rats were treated with acute ethanol (5 g/kg, 25% v/v, i.g.) or a comparable volume of water. One hour later, rats were acutely stressed for two hours using a restraint-water immersion procedure or were left in their home-cage. The rats were sacrificed immediately following the conclusion of the stressor. **B.** Body weights of control- (CON – white dots) and chronic ethanol- (CE – black dots) treated rats are shown over time. Rats were treated from P25 to P54 with either ethanol (5 g/kg, 20-30% v/v, i.g.) or water on a 2-day on/2-day off cycle. Arrows designate days the rats were treated. Blood ethanol concentrations (BECs) were measured one hour after ethanol treatment on P38 and P54 and are represented by gray squares. Rats were acutely stressed on P96 or P97 with a two-hour restraint-water immersion procedure or left in their home-cage. The rats were sacrificed two hours following the conclusion of the stressor. Data are presented as mean ± S.E.M.

Previous studies find that both acute ethanol and acute stress increase plasma corticosterone (CORT) and plasma endotoxin [179-181]. However, whether ethanol and stress interact to impact plasma CORT or plasma endotoxin is unknown. To investigate this, rats were treated with acute ethanol (5 g/kg, 25% v/v) and one hour later treated with a two-hour restraint/water immersion stressor (Fig3.1), as described previously [183]. This stress protocol has been widely used to study

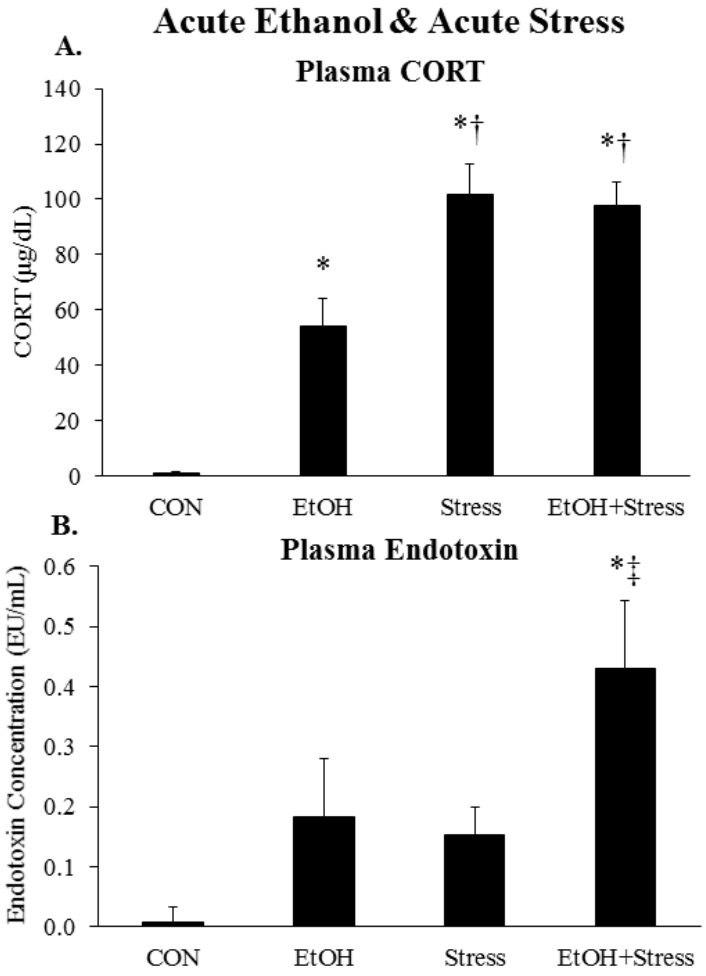


Figure 3-2 Effects of acute ethanol and acute stress on plasma corticosterone and endotoxin. Rats were treated with ethanol (5.0 g/kg, 25% v/v, i.g.) and/or a two-hour restraint-water immersion stressor, and sacrificed immediately following the conclusion of the stressor. **A.** Plasma corticosterone levels were measured. Data are presented as mean ± S.E.M. *p<0.05 compared to CON, †p<0.05 compared to EtOH, ‡p<0.05 compared to Stress (Tukey's *post-hoc* test). **B.** Plasma endotoxin levels were measured. Data are presented as mean ± S.E.M. *p<0.05 compared to CON, †p<0.05 compared to EtOH, ‡p<0.05 compared to Stress (LSD *post-hoc* test) n=4-10/group.

effects of stress, including ulcers [184] and memory impairment [185]. The rats were sacrificed

immediately following the conclusion of the stressor and trunk blood was collected. Analysis of blood ethanol concentrations (BECs) found that rats in the EtOH and EtOH+Stress groups had comparable BECs (191±33 mg/dL in the EtOH group vs. 221±21 mg/dL in the EtOH+Stress group). Acute ethanol increased plasma CORT 41-fold ($p<0.05$), while two hours of acute restraint/water immersion stress increased plasma CORT a marked 78-fold ($p<0.05$) (Fig2A). However, the combination of acute ethanol and acute stress did not further increase plasma CORT beyond the levels induced by acute stress alone (Fig2A). There was a trend for acute ethanol and acute stress to increase plasma endotoxin individually (Fig2B). Interestingly, the combination of acute ethanol and acute stress caused a 50-fold increase in plasma endotoxin that was significantly higher ($p<0.05$) than endotoxin levels in the Stress group (Fig2B). Overall, these results show plasma CORT is markedly increased by acute ethanol and acute stress, while plasma endotoxin shows a modest increase with ethanol or stress alone, and a marked increase when ethanol and stress are combined.

3.2.2 Effects of acute ethanol and acute stress on microglia and neurons in the prefrontal cortex.

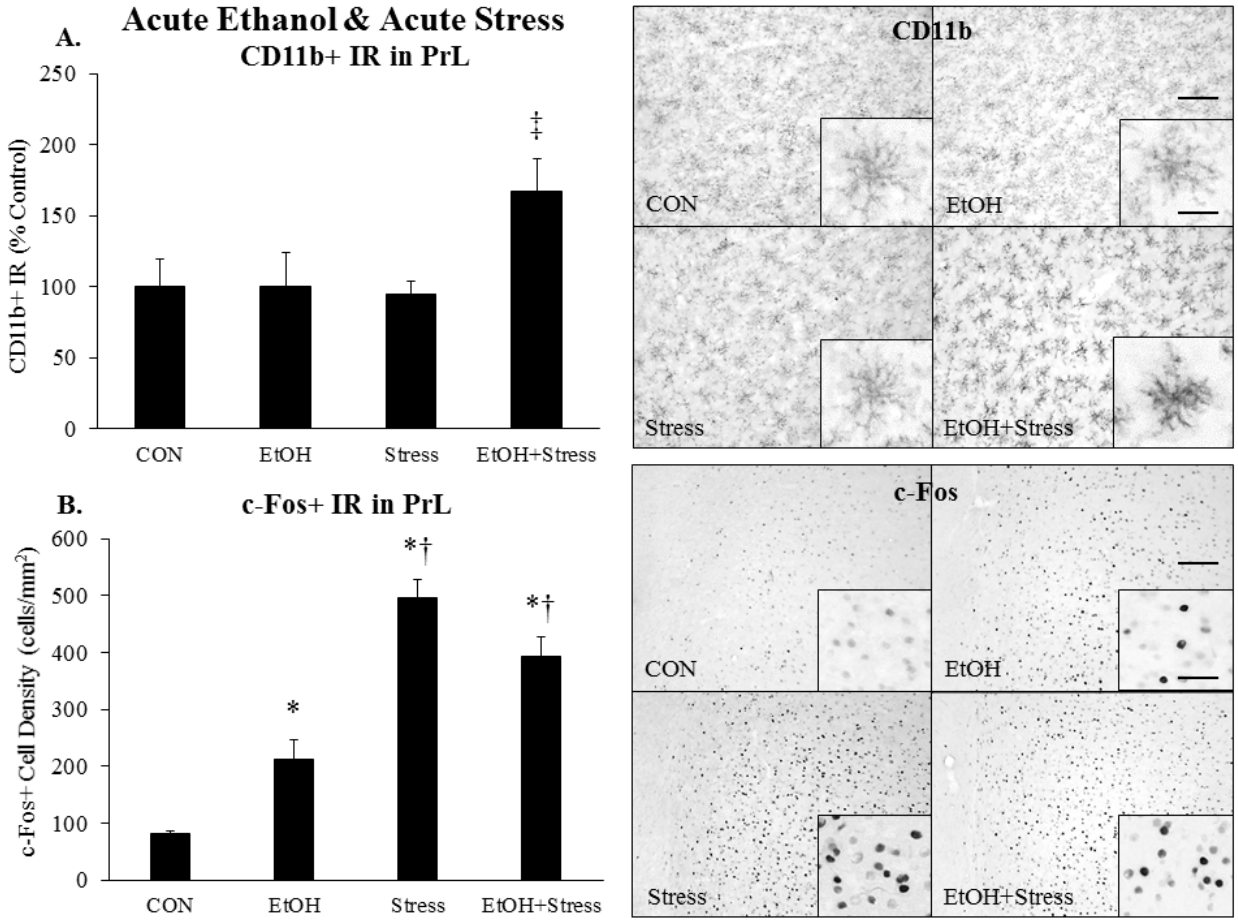


Figure 3-3 Effects of acute ethanol and acute stress on CD11b+ IR and c-Fos+ IR in the prefrontal cortex. Rats were treated with ethanol (5.0 g/kg, 25% v/v, i.g.) and/or a two-hour restraint-water immersion stressor and sacrificed immediately following the conclusion of the stressor. **A.** CD11b+ pixel density in the PrL was assessed in each group. Data are presented as mean ± S.E.M. *p<0.05 compared to CON, †p<0.05 compared to EtOH, ‡p<0.05 compared to Stress (Tukey's *post-hoc* test). n=4-10/group. A representative image of CD11b staining from each group is shown. A higher magnification image is displayed in the inset. The scale bar in the low magnification image measures 100 microns, and the scale bar in the high magnification inset measures 20 microns. **B.** c-Fos+ cell density in the PrL was assessed in each group. Data are presented as mean ± S.E.M. *p<0.05 compared to CON, †p<0.05 compared to EtOH, ‡p<0.05 compared to Stress (Tukey's *post-hoc* test). n=4-10/group. A representative image of c-Fos staining from each group is shown. A higher magnification image is displayed in the inset. The scale bar in the low magnification image measures 100 microns, and the scale bar in the high magnification inset measures 20 microns.

Previous studies show that acute ethanol and acute stress individually activate microglia

[183, 186] and neurons [20, 187] across multiple brain regions. However, whether the two interact to affect microglial or neuronal activation is unknown. To investigate this, we treated rats with acute ethanol and acute stress as described above and examined several brain regions using CD11b and c-Fos as markers of microglial and neuronal activation, respectively (Tables 3.1 & 3.2). We first examined the medial prefrontal cortex (mPFC), a brain region affected by both ethanol and stress. In the prelimbic mPFC (PrL), neither acute ethanol, nor acute stress increased CD11b+ immunoreactivity (IR); however, the two interacted to increase CD11b+ IR 67% ($p<0.05$) (Fig3A). A similar result was seen in the infralimbic mPFC (IL) (Table 3.1). In the PrL, acute ethanol increased c-Fos+ cell density 2.6-fold ($p<0.05$), while acute stress increased c-Fos+ cell density 6.1-fold ($p<0.05$) (Fig3B). However, acute ethanol and acute stress did not further interact to enhance c-Fos+ cell density beyond the levels caused by stress alone (Fig3B). Results were similar in the IL (Table 3.2). Interestingly, we found significant positive correlations between plasma CORT and PrL c-Fos across groups ($R= 0.695$, $p=0.0001$), and between plasma endotoxin and PrL CD11b+ IR across groups ($R= 0.495$, $p=0.019$). However, plasma CORT and PrL CD11b did not correlate, and plasma endotoxin and PrL c-Fos did not correlate. This is consistent with the hypothesis that PrL neuronal activation is related to stress-induced CORT, whereas PrL microglial activation is related to endotoxin-induced immune activation, rather than CORT-induced immune suppression. Overall, these data suggest acute ethanol and acute stress interact to increase mPFC CD11b+ IR, but not mPFC neuronal c-Fos.

3.2.3 Effects of acute ethanol and acute stress on microglia and neurons in the nucleus accumbens and bed nucleus of the stria terminalis.

We next investigated microglial and neuronal activation in the nucleus accumbens (NAc), a brain region involved in reward, and the bed nucleus of the stria terminalis (BNST), a brain region

involved in stress, fear and anxiety. Neither acute ethanol, nor acute stress increased CD11b+ IR in the NAc, although the combination enhanced CD11b+ IR 95% ($p<0.05$) in the NAc core and 69% ($p<0.05$) in the NAc shell (Fig4A). Staining for c-Fos found that acute ethanol did not enhance c-Fos+ cell density in the NAc, but that acute stress increased c-Fos+ cell density 148% ($p<0.05$) in the NAc core and 100% in the NAc shell (Fig4C). The combination of acute ethanol and acute stress did not impact c-Fos+ cell density in the NAc core, but returned c-Fos to control levels in the NAc

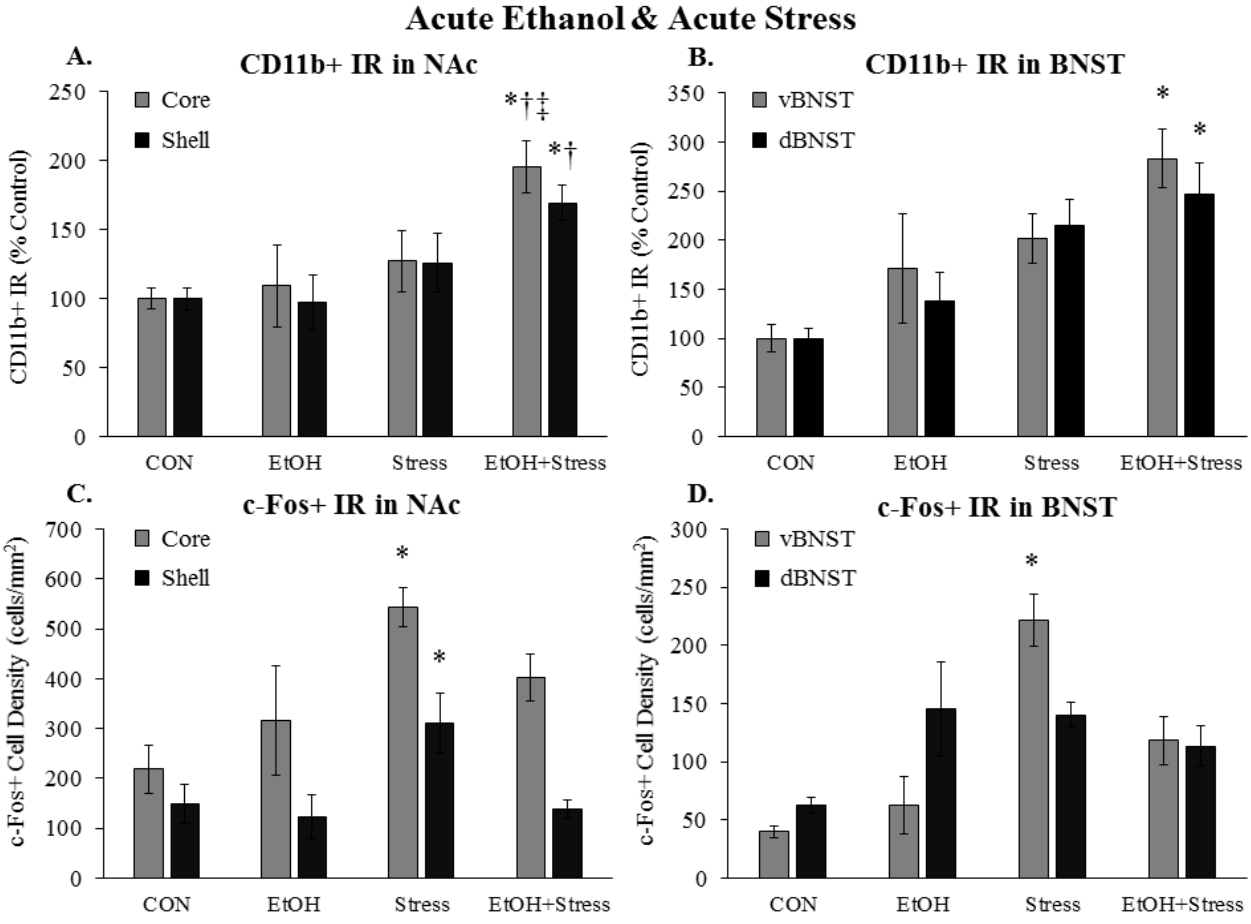


Figure 3-4 Effects of acute ethanol and acute stress on CD11b+ IR and c-Fos+ IR in the nucleus accumbens and bed nucleus of the stria terminalis. Rats were treated with ethanol (5.0 g/kg, 25% v/v, i.g.) and/or a two-hour restraint-water immersion stressor and sacrificed immediately following the conclusion of the stressor. CD11b+ IR was assessed in the **A.** NAc core and shell and **B.** the ventral and dorsal BNST. c-Fos+ cell density in **C.** the NAc core and shell and **D.** the ventral and dorsal BNST were assessed in each group. Data are presented as mean ± S.E.M. *$p<0.05$ compared to CON, †$p<0.05$ compared to EtOH, ‡$p<0.05$ compared to Stress (Tukey's *post-hoc* test). n=4-10/group.

shell (Fig4C). In the BNST, both acute ethanol and acute stress showed a trend to increase CD11b, with the combination further increasing CD11b (Fig4B). Acute stress increased c-Fos+ cell density 5.5-fold ($p<0.05$) in the vBNST, with the combination of acute ethanol and acute stress blunting this response (Fig4D). Neither acute ethanol, acute stress, nor the combination significantly increased c-Fos+ cell density in the dBNST. Overall, these data suggest that acute ethanol and acute stress interact to enhance microglial CD11b in the NAc, but decrease neuronal c-Fos in the NAc shell. Also, acute ethanol and acute stress combined tend to increase microglial CD11b in the BNST, while acute ethanol blunts the effects of acute stress on neuronal c-Fos in the vBNST. Thus, BNST and NAc sub-regions show decreased neuronal c-Fos with combined ethanol and stress compared to stress alone.

3.2.4 Effects of acute ethanol and acute stress on microglia and neurons in the paraventricular nucleus of the hypothalamus and amygdala.

We also examined microglial and neuronal activation in the paraventricular nucleus of the hypothalamus (PVN) and amygdala, key stress-response brain regions that are also activated by ethanol. Acute ethanol did not increase CD11b+ IR in the PVN, whereas acute stress caused a marked 5.5-fold ($p<0.05$) increase in CD11b+ IR that was not altered by the combination of ethanol

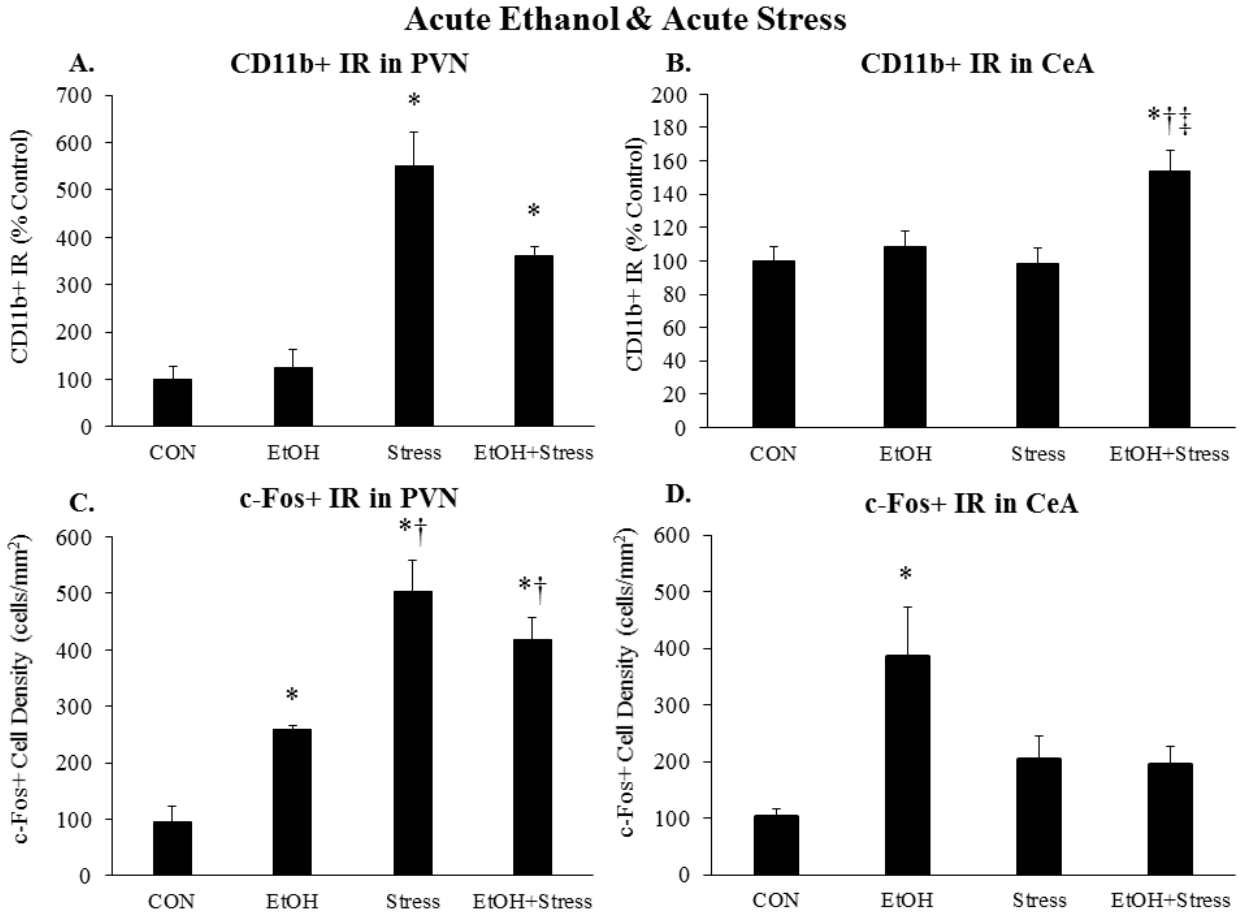


Figure 3-5 Effects of acute ethanol and acute stress on CD11b+ IR and c-Fos+ IR in the paraventricular nucleus of the hypothalamus and the central nucleus of the amygdala. Rats were treated with ethanol (5.0 g/kg, 25% v/v, i.g.) and/or a two-hour restraint-water immersion stressor and sacrificed immediately following the conclusion of the stressor. CD11b+ IR was assessed in **A.** the PVN and **B.** the CeA. c-Fos+ cell density in **C.** the PVN and **D.** the CeA were assessed in each group. Data are presented as mean ± S.E.M. *$p<0.05$ compared to CON, †$p<0.05$ compared to EtOH, ‡$p<0.05$ compared to Stress (Tukey's *post-hoc* test). n=4-10/group.

and stress (Fig5A). Acute ethanol increased PVN c-Fos+ cell density 160% ($p<0.05$), while acute

stress increased c-Fos+ cell density 5-fold ($p<0.05$) in the PVN (Fig5C). There was no interaction of acute ethanol and acute stress on c-Fos in the PVN. In the amygdala, neither acute ethanol nor acute stress significantly increased CD11b+ IR; however, the combination increased CD11b+ IR 107% ($p<0.05$) in the BLA and a 53% ($p<0.05$) in the CeA (Table 3.1, Fig5B). While acute ethanol did not increase c-Fos+ cell density in the BLA, acute stress caused a marked 2.7-fold ($p<0.05$) increase in BLA c-Fos+ cell density (Table 3.2). Interestingly, acute ethanol blunted the stress-induced increase in BLA c-Fos (Table 3.2). While acute ethanol caused a robust 3.8-fold ($p<0.05$) increase in c-Fos+ cell density in the CeA, acute stress had no significant effect (Fig5D). Interestingly, the combination of acute ethanol and acute stress blunted the ethanol-induced increase in CeA c-Fos. Overall, these data suggest that acute ethanol and acute stress cause marked increases in PVN c-Fos, while only acute stress increased PVN CD11b. Furthermore, acute ethanol and acute stress interact to enhance microglial CD11b in the amygdala, whereas the BLA neurons respond to acute stress and CeA neurons respond to acute ethanol.

3.2.5 Persistent effects of chronic intermittent ethanol on the plasma CORT and plasma endotoxin to acute stress.

A large body of research suggests that chronic ethanol alters brain stress systems [188]. However, it is unknown if chronic ethanol leads to long-lasting changes in the acute stress response. To investigate this, we administered chronic ethanol (CE) to male Wistar rats in an intermittent two day-on/two day-off pattern for one month (Fig 1B). Tail blood was drawn to determine BECs. Mean BECs in CE-treated animals were 167 ± 11 mg/dL after 8 doses and 130 ± 10 mg/dL after the final dose (Fig 1B). Following a 40-day period of abstinence, animals were acutely stressed with a two-hour restraint-water immersion stressor or remained in their home cage. The rats were sacrificed two hours following the conclusion of the stressor and plasma CORT and endotoxin were assessed.

Acute stress caused a significant increase in plasma CORT levels ($p<0.05$) (Fig6A); however, this was not altered by chronic ethanol. Chronic ethanol did not persistently change plasma endotoxin levels, while acute stress showed a trend to increase plasma endotoxin (Fig6B). Interestingly, chronic ethanol enhanced acute stress-induced plasma endotoxin 2.7-fold ($p<0.05$) (Fig6B). These results suggest that chronic ethanol sensitizes the gut, resulting in enhanced stress-induced endotoxin release, but does not impact the plasma CORT response.

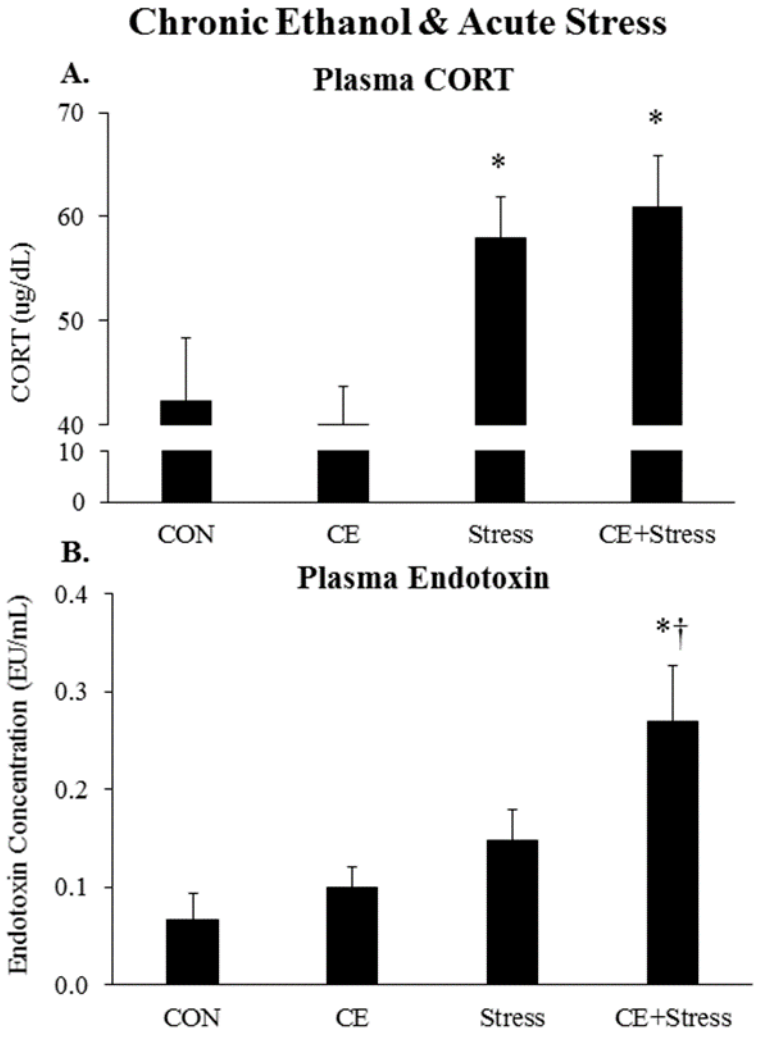


Figure 3-6 Effects of chronic ethanol and acute stress on plasma corticosterone and endotoxin. Rats were treated with chronic ethanol (5.0 g/kg, 20-30% v/v, 2 days-on, 2 days-off from P25-P54) and/or acutely stressed with a two-hour restraint-water immersion stressor following prolonged abstinence on P96/7. The rats were sacrificed two hours following the conclusion of the stressor. **A.** Levels of plasma corticosterone were measured. Data are presented as mean ± S.E.M. *$p<0.05$ compared to CON, †$p<0.05$ compared to CE, (Tukey's *post-hoc* test). n=8-10/group. **B.** Levels of plasma endotoxin were measured. Data are presented as mean ± S.E.M. *$p<0.05$ compared to CON, †$p<0.05$ compared to CE, (Tukey's *post-hoc* test). n=8-10/group.

3.2.6 Persistent effects of chronic intermittent ethanol on the microglial and neuronal response in the prefrontal cortex.

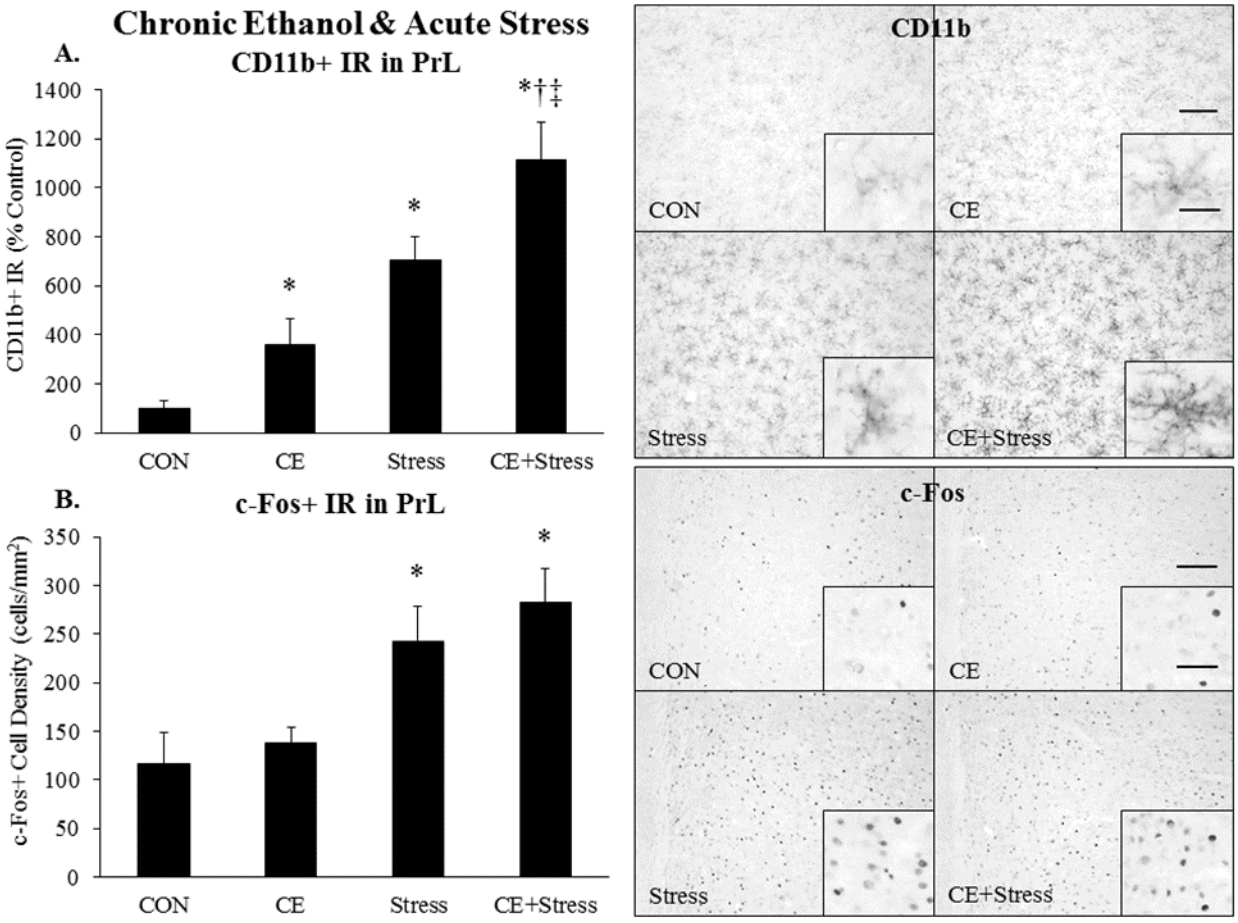


Figure 3-7 Effects of chronic ethanol and acute stress on CD11b+ IR and c-Fos+ IR in the prelimbic cortex. Rats were treated with chronic ethanol (5.0 g/kg, 20-30% v/v, 2 days-on, 2 days-off from P25-P54) and/or acutely stressed with a two-hour restraint-water immersion stressor following prolonged abstinence on P96/7. The rats were sacrificed two hours following the conclusion of the stressor. **A.** CD11b+ pixel density in the PrL was assessed in each group. Data are presented as mean ± S.E.M. *p<0.05 compared to CON, †p<0.05 compared to EtOH, ‡p<0.05 compared to Stress (Tukey's *post-hoc* test). n=8-10/group. A representative image of CD11b staining from each group is shown. A higher magnification image is displayed in the inset. The scale bar in the low magnification image measures 100 microns, and the scale bar in the high magnification inset measures 20 microns. **B.** c-Fos+ cell density in the PrL was assessed in each group. Data are presented as mean ± S.E.M. *p<0.05 compared to CON, †p<0.05 compared to CE, ‡p<0.05 compared to Stress (Tukey's *post-hoc* test). n=8-10/group. A representative image of c-Fos staining from each group is shown. A higher magnification image is displayed in the inset. The scale bar in the low magnification image measures 100 microns, and the scale bar in the high magnification inset measures 20 microns.

To determine whether chronic ethanol exposure persistently altered the microglial or

neuronal response to acute stress, we treated rats with chronic ethanol as described above and stained for CD11b and c-Fos (Tables 3.3 & 3.4). In the PrL, chronic ethanol caused a persistent 3.6-fold increase in CD11b+ IR, while acute stress increased PrL CD11b+ IR 7-fold ($p<0.05$) (Fig7A), with the combination of the two further increasing CD11b+ IR by 11-fold ($p<0.05$) (Fig7A). A similar pattern was observed in the IL (Table 3.3). Similar changes were also observed for the microglial marker Iba1 in the mPFC (FigS3.1). Furthermore, neither chronic ethanol nor acute stress changed microglial cell density in the mPFC (FigS3.2). Finally, staining for markers of robust microglial activation, such as CD68, MHCII and iNOS were all negative (FigS3.3). Staining for c-Fos showed that chronic ethanol had no lasting effect on c-Fos+ cell density in the PrL, while acute stress increased c-Fos+ cell density about two-fold ($p<0.05$) in both control and CE groups (Fig7B). A similar pattern was observed in the IL (Table 3.4). Interestingly, plasma endotoxin and PrL CD11b were correlated (R: 0.508, p:0.005) across groups, consistent with endotoxin-induced microglial activation. Plasma CORT also correlated with PrL CD11b (R: 0.425, p: 0.012) across groups, and plasma endotoxin correlated with PrL c-Fos (R: 0.422, p:0.015) across groups. However, plasma CORT and PrL c-Fos did not correlate. Overall, these data suggest that chronic ethanol persistently sensitizes the microglial CD11b response to acute stress, but not the neuronal c-Fos response to acute stress in the PFC.

3.2.7 Persistent effects of chronic intermittent ethanol on the microglial and neuronal response in the nucleus accumbens and bed nucleus of the stria terminalis.

We also examined whether chronic ethanol alters the microglial or neuronal response to acute stress in the NAc or BNST. In the NAc, there was a trend for chronic ethanol to increase CD11b+ IR (Fig8A). Acute stress increased CD11b+ staining 16-fold ($p<0.05$) in the NAc core and 8-fold in the NAc shell (Fig8A). CD11b+ IR was further increased by the combination of chronic ethanol and

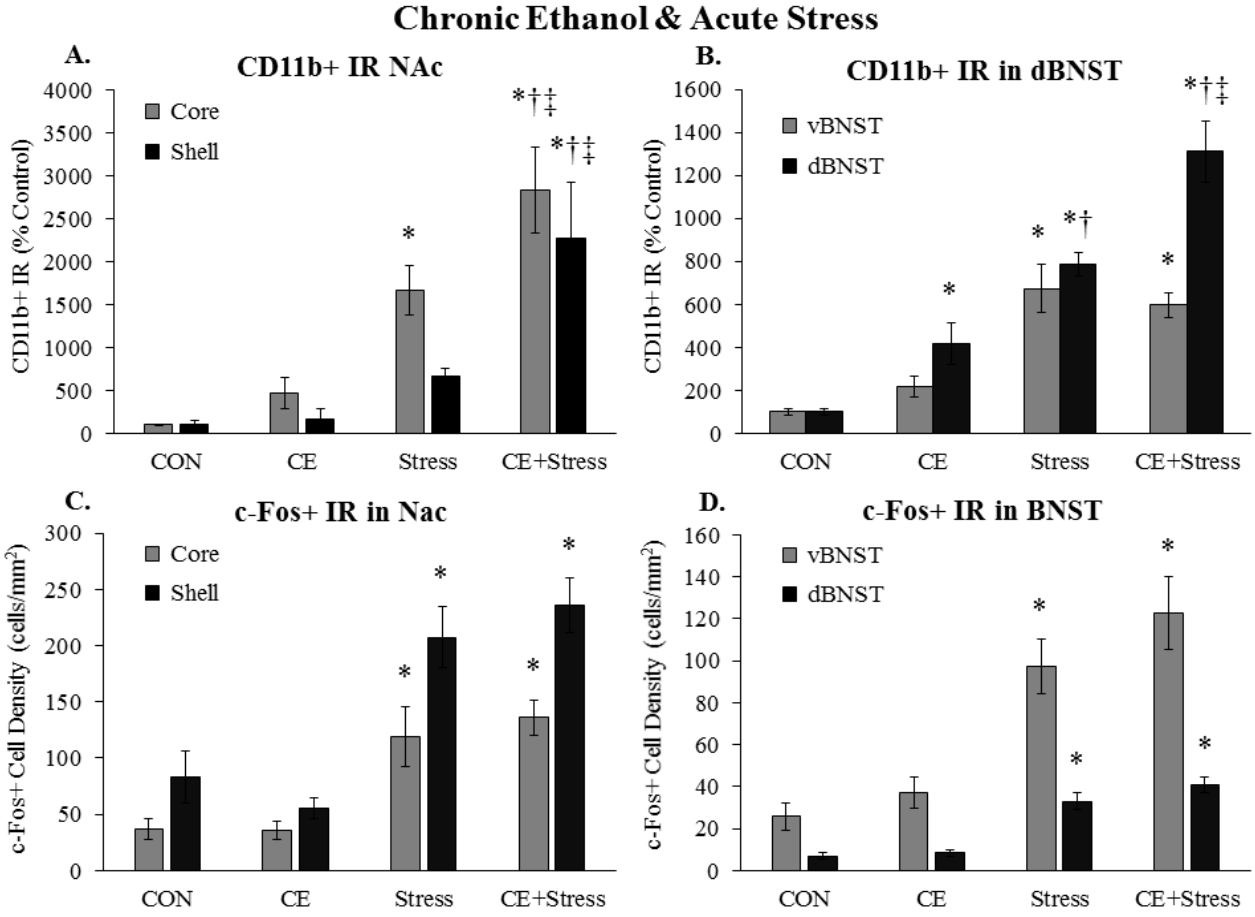


Figure 3-8 Effects of chronic ethanol and acute stress on CD11b+ IR and c-Fos+ IR in the nucleus accumbens and bed nucleus of the stria terminalis. Rats were treated with chronic ethanol (5.0 g/kg, 20-30% v/v, 2 days-on, 2 days-off from P25-P54) and/or acutely stressed with a two-hour restraint-water immersion stressor following prolonged abstinence on P96/7. The rats were sacrificed two hours following the conclusion of the stressor. CD11b+ IR was assessed in the **A.** NAc core and shell and **B.** the ventral and dorsal BNST. c-Fos+ cell density in **C.** the NAc core and shell and **D.** the ventral and dorsal BNST were assessed in each group. Data are presented as mean ± S.E.M. *$p<0.05$ compared to CON, †$p<0.05$ compared to CE, ‡$p<0.05$ compared to Stress (Tukey's *post-hoc* test).n=8-10/group.

acute stress in both the core ($p<0.05$) and shell ($p<0.05$) (Fig8A). Chronic ethanol did not persistently alter c-Fos in the NAc core or shell (Fig8C), while acute stress increased c-Fos+ cell density 3.2-fold ($p<0.05$) in the NAc core and 2.5-fold ($p<0.05$) in the NAc shell (Fig8C). Chronic ethanol and acute stress did not interact to increase c-Fos in the NAc (Fig8C). In the dBNST, chronic ethanol persistently increased CD11b+ IR 315% ($p<0.05$) (Fig8B). Acute stress increased CD11b+ IR 7.8-fold ($p<0.05$) in the dBNST and 6.7-fold ($p<0.05$) in the vBNST (Fig8B). Interestingly, chronic ethanol and acute stress enhanced CD11b staining 13-fold in the dBNST ($p<0.05$) (Fig8B). Chronic ethanol had no persistent effect on c-Fos in the BNST, while acute stress increased c-Fos+ cell density in the dBNST and vBNST (Fig8D). Chronic ethanol and acute stress did not interact to change c-Fos in the BNST. These data suggest that chronic ethanol does not alter the NAc c-Fos response to acute stress, but enhances the NAc CD11b response to acute stress. Also, chronic ethanol persistently increases CD11b in the BNST, and enhances the BNST CD11b response to acute stress, but not the BNST c-Fos response.

3.2.8 Persistent effects of chronic intermittent ethanol on the microglial and neuronal response in the paraventricular nucleus of the hypothalamus and amygdala.

We also determined the effects of chronic ethanol and acute stress on microglia and neurons in the PVN and amygdala. Chronic ethanol had no persistent effect on c-Fos or CD11b in the PVN. However, acute stress increased CD11b+ IR 17-fold ($p<0.05$), with no further increase by chronic ethanol (Fig9A). Acute stress also increased c-Fos+ cell density by 286% ($p<0.05$) in the PVN, with no further increase by chronic ethanol (Fig9C). In the amygdala, there was a trend for chronic ethanol to increase CD11b+ IR. Acute stress increased CD11b+ IR 13.8-fold ($p<0.05$) in the BLA and 4.9-fold ($p<0.05$) in the CeA (Table 3.3, Fig9B). Chronic ethanol plus acute stress increased CD11b+

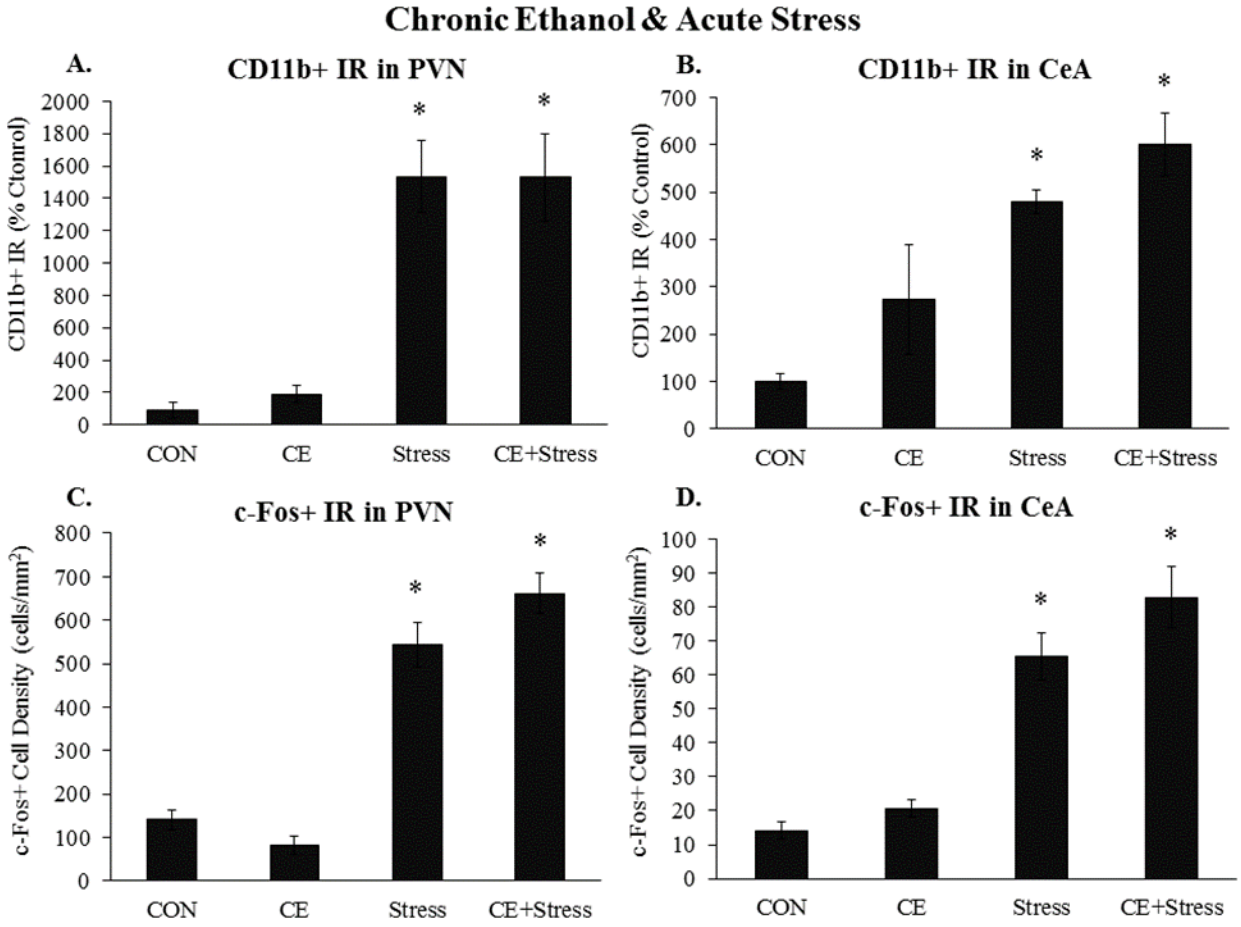


Figure 3-9 Effects of chronic ethanol and acute stress on CD11b+ IR and c-Fos+ IR in the paraventricular nucleus of the hypothalamus and central nucleus of the amygdala. Rats were treated with chronic ethanol (5.0 g/kg, 20-30% v/v, 2 days-on, 2 days-off from P25-P54) and/or acutely stressed with a two-hour restraint-water immersion stressor following prolonged abstinence on P96/7. The rats were sacrificed two hours following the conclusion of the stressor. CD11b+ IR was assessed in **A.** the PVN and **B.** the CeA. c-Fos+ cell density in **C.** the PVN and **D.** the CeA were assessed in each group. Data are presented as mean ± S.E.M. *$p<0.05$ compared to CON (Tukey's *post-hoc* test). n=8-10/group.

IR 25-fold ($p<0.05$) in the BLA and 6-fold in the CeA. Chronic ethanol had no persistent effects on c-Fos in the CeA or BLA. However, acute stress increased c-Fos+ cell density 2-fold ($p<0.05$) in the BLA and 4.7-fold ($p<0.05$) in the CeA (Fig9D), with no further effect of chronic ethanol (Fig9D). Overall, these data suggest chronic ethanol does not persistently alter the c-Fos or CD11b response to acute stress in the PVN. Also, chronic ethanol does not alter the c-Fos response to acute stress, but tends to increase the CD11b response to acute stress in the amygdala.

3.3 Discussion

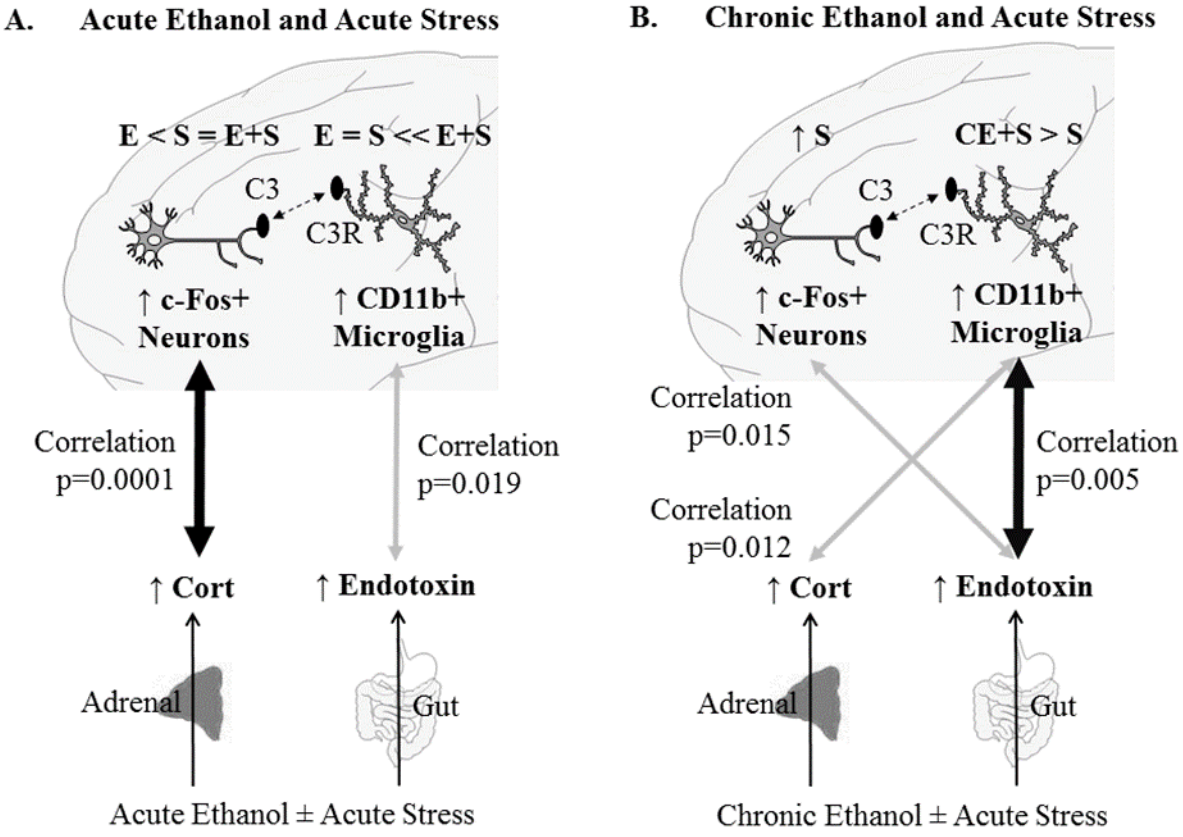


Figure 3-10 Effects of acute and chronic ethanol on the systemic and central nervous system response to acute stress. A. Both acute ethanol and acute stress increase plasma CORT and neuronal c-Fos in the PFC. The magnitude of these effects is represented by E < S = E+S. Furthermore, CORT and PFC c-Fos levels correlate, suggesting these two responses are related. This positive correlation is represented by the thick arrow, and the p-value for the Pearson correlation is displayed. Acute ethanol and acute stress alone have little effect on plasma endotoxin or microglial CD11b in the mPFC. However, acute ethanol and acute stress together interact to enhance plasma endotoxin and microglial CD11b in the PFC. This is represented by E = S << E+S. Plasma endotoxin and PFC CD11b also correlate. This positive correlation is represented by the thin arrow and the p-value for the Pearson correlation is displayed. Finally, a possible interaction between microglial CR3 and neuronal C3 is displayed. Increased CD11b, a component of CR3, may contribute to change synaptic remodeling. **B.** Chronic ethanol exposure does not change the plasma CORT or PFC c-Fos response to acute stress, as indicated by ↑S. However, chronic ethanol enhances the plasma endotoxin and PFC CD11b response to acute stress. This is represented by CE + S > S. Plasma endotoxin and microglial CD11b also correlate, consistent with endotoxin-induced microglial activation. This correlation is represented by the thick gray arrow, and the p-value of the Pearson correlation is shown. Interestingly, the plasma endotoxin and PFC c-Fos response also correlate. This is represented by the thin arrow, and the p-value for the Pearons correlation is shown. Furthermore, the plasma CORT response correlates with the PrL CD11b response. This is represented by the thin arrow, and the p-value of the Pearson correlation is shown. Finally, a possible interaction between microglial CR3 and neuronal C3 is displayed. Increased CD11b, a component of CR3, may contribute to change synaptic remodeling.

In this present study, we examined the effects of acute ethanol and acute stress individually and combined, and the effects of chronic ethanol and acute stress individually and combined on plasma CORT, plasma endotoxin, and brain regional microglial CD11b and neuronal c-Fos. We used models of heavy binge drinking and intense stress to assure measurable responses. We report that acute binge ethanol increased plasma CORT and c-Fos+ IR in multiple brain regions, specifically, the mPFC, PVN and CeA. Acute stress increased plasma CORT about twice as much as acute ethanol, and increased c-Fos+ IR in the mPFC, NAc, BNST, PVN and BLA. These results are consistent with previous studies [20, 189]. The combination of acute ethanol and acute stress did not change plasma CORT, but enhanced plasma endotoxin (Fig3.10A). Acute ethanol and acute stress combined also blunted ethanol-induced c-Fos in the CeA, and blunted stress-induced c-Fos in the NAc shell, vBNST and BLA. The combination of acute ethanol and stress also enhanced microglial CD11b in the mPFC, NAc and amygdala (Fig3.10A). We also report that chronic intermittent ethanol had no persistent effects on plasma CORT, plasma endotoxin or c-Fos, but increased CD11b in the PrL, dBNST and lPAG. Chronic ethanol also persistently enhanced the plasma endotoxin response to acute stress, as well as the microglial CD11b response in multiple brain regions, including the PFC, NAc, dBNST and BLA (Fig3.10B). These studies suggest distinctly different interactions of ethanol and stress on plasma CORT, plasma endotoxin and brain regional microglial and neuronal activation.

CORT release is a key component of the stress response and typically thought to be anti-inflammatory. Also, acute ethanol is typically thought to reduce stress, although chronic stress and ethanol contribute to AUDs [27] and other mental diseases [190]. We report that acute ethanol and acute stress increased plasma CORT individually, consistent with previous studies [179], but the combination was similar to the response induced by stress alone, perhaps due to the stressor causing

a maximal CORT response at the time point studied. A previous study found that ethanol did not enhance plasma CORT after 15 minutes of restraint stress, but did enhance plasma CORT after 60 minutes of restraint stress [191]. Since we only tested one heavy binge ethanol dose, one intense stressor and one time point, we cannot rule out the possibility that interactions occur at other doses, intensities or times. Our results also found that chronic ethanol does not persistently increase plasma CORT, or impact the plasma CORT response to acute stress. We also report that acute ethanol and acute stress increased c-Fos+ IR in several brain regions, consistent with previous studies [20, 189]. Interestingly, acute ethanol blunted stressed-induced c-Fos in the NAc shell, vBNST and BLA. This is consistent with ethanol reducing neuronal excitatory stress responses, perhaps due to ethanol-potentiated GABA inhibition. We also report that chronic ethanol does not affect the c-Fos response to acute stress. However, we cannot exclude the possibility that chronic ethanol and acute stress interact to impact neuronal activation under different circumstances, such as a lower dose of ethanol, more modest stressor or different time point. Overall, these data suggest neither acute ethanol nor chronic ethanol alter the plasma CORT response to acute stress; however, acute ethanol alters the neuronal c-Fos response to acute stress in some brain regions.

The plasma endotoxin response to acute stress is increased by both acute and chronic ethanol. Our results find acute ethanol and acute stress interact to enhance plasma endotoxin. This is consistent with previous studies finding acute ethanol [180] and acute stress [181] increase plasma endotoxin individually. Elevated plasma endotoxin occurs through enhanced gut leakiness leading to bacterial translocation into the peripheral circulation [192]. This causes peripheral inflammation that is thought to impact neuroimmune signaling in the brain [9]. These data suggest exposure to ethanol and stress simultaneously may enhance peripheral inflammation, leading to increased neuroimmune signaling in the brain and potentially exacerbating disease. These studies also find

chronic ethanol enhances stress-induced plasma endotoxin. Notably, this effect persists for several weeks after the last ethanol exposure. This suggests chronic ethanol primes gut leakiness for long periods. Like acute ethanol, chronic ethanol may also increase stress-induced peripheral inflammation and subsequently enhance neuroimmune signaling. This raises the interesting possibility that chronic ethanol makes stressors more "inflammatory," thereby exacerbating the effect of stress on disease processes. Overall, these results suggest that acute and chronic ethanol enhance the effects of acute stress on plasma endotoxin.

This study also finds that acute ethanol and acute stress interact to increase CD11b in multiple brain regions, including the mPFC, the NAc core and the amygdala. CD11b, also known as integrin alpha M (ITGAM), is constitutively expressed in resting microglia and up-regulated when microglia are activated [59]. It complexes with integrin beta 2 to form the heterodimeric integrin $\alpha_M\beta_2$, also known as complement receptor 3 (CR3), and is important for microglial adhesion, migration, and complement-mediated pruning of synapses [151, 193]. Increased CD11b with acute ethanol and acute stress suggests enhanced microglial activation. This is observed in some, but not all examined brain regions, indicating heterogeneity of the microglial responses across brain regions. It is possible the observed increases in plasma endotoxin contribute to the increases in microglial activation. Indeed, previous studies find the peripheral immune response to be critical for activation of microglia [112]. These results suggest the intriguing possibility that alcohol and stress interact to affect the brain through gut-derived endotoxin. This framework could provide a novel way of understanding how alcohol and stress contribute to disease. Overall, these results suggest acute ethanol and acute stress interact to increase microglial CD11b in multiple brain regions, possibly due to enhanced endotoxin release.

This study also found that chronic ethanol persistently increased CD11b in many examined brain regions. This is consistent with chronic ethanol causing a long-lasting change in microglia. These results are consistent with previous studies showing an intense, four-day binge ethanol treatment in rats increased CD11b+IR in the hippocampus immediately following the treatment. This CD11b+IR increase declined over time, but remained persistently increased for at least 28 days [135, 139]. Our studies add to this work by showing less-intense, chronic intermittent ethanol persistently increases CD11b in many brain regions. Increased CD11b following chronic intermittent ethanol may be related to long-term changes in synaptic remodeling, since CD11b is a component of the complement 3 receptor (CR3) that is involved in pruning synapses [151] (Fig 3.10). Thus, the chronic intermittent ethanol exposure and withdrawal of this model may alter synapses and circuits known to be changed by ethanol by changing CD11b levels. Indeed, previous studies find that chronic ethanol exposure increases expression of multiple neuroimmune signaling molecules that could alter neurocircuitry and behavior [136, 194]. Overall, these results show that chronic ethanol persistently increases microglial CD11b in multiple brain regions.

These data also show chronic ethanol enhanced the CD11b response to acute stress in multiple brain regions, including the PrL, IL, dBNST and BLA. This suggests chronic ethanol persistently sensitizes the microglial response to acute stress. Indeed, microglia are known to undergo priming, a process in which an initial stimulus causes a greater response to a secondary stimulus [74]. It is possible chronic ethanol primes microglia. Interestingly, chronic ethanol and acute stress had similar effects on plasma endotoxin and CD11b. Indeed, plasma endotoxin and PrL CD11b correlated across groups, suggesting these responses may be related. It is possible chronic ethanol enhances stress-induced endotoxin, leading to increased peripheral inflammation and consequently increased microglial activation. Indeed, chronic ethanol may make acute stress more

"inflammatory," and enhance its capacity to impact the brain and contribute to disease. Overall, these results suggest chronic ethanol persistently enhances the microglial CD11b response to acute stress.

We observed that plasma endotoxin correlated with PrL CD11b across groups in the acute ethanol and acute stress experiment (Fig3.10A). This suggests a relationship between the plasma endotoxin and PrL microglial response, consistent with endotoxin-induced microglial activation. PrL CD11b did not correlate with plasma CORT, suggesting the PrL microglia are responding to the inflammatory endotoxin, rather than the anti-inflammatory CORT. Interestingly, plasma CORT correlated with PrL c-Fos across groups (Fig3.10A). This suggests a relationship between the CORT response and the PrL neuronal response. We also noticed plasma endotoxin and PrL CD11b correlated across groups (Fig3.10B) in the chronic ethanol and acute stress experiment, consistent with endotoxin-induced microglial activation. Interestingly, plasma endotoxin also correlated with PrL c-Fos (Fig3.10B), suggesting a relationship between plasma endotoxin and PrL neurons following chronic ethanol and acute stress. Plasma CORT also correlated with the PrL CD11b response (Fig3.10B), implying that chronic ethanol may alter the relationship between CORT and microglia. Overall, these results suggest that PrL microglia respond to systemic endotoxin, and that chronic ethanol causes long-lasting changes in the c-Fos response to acute stress.

It is interesting to compare the results of this study to previous studies using an identical model of ethanol administration [195]. These previous studies found chronic intermittent ethanol changes the c-Fos response to an ethanol challenge. Since ethanol acts as a stressor [179], these two studies highlight the effects of chronic ethanol on the response to two different stressors. While chronic ethanol changed the c-Fos response to an ethanol challenge, it did not alter the c-Fos response to a restraint-water immersion stressor. This is consistent with a specific habituation to the

alcohol stressor [196]. Overall, these results suggest that chronic ethanol causes long-lasting, stressor-specific changes in the neuronal c-Fos response.

In this present study, we examined the effects of acute and chronic ethanol on the plasma glucocorticoid, plasma endotoxin, microglial and neuronal response to acute stress. We report that acute ethanol and acute stress interacted to enhance plasma endotoxin levels, as well as microglial CD11b in multiple brain regions, suggesting increased microglial activation (Fig3.10A). We also report that chronic ethanol enhanced the plasma endotoxin response to acute stress. Chronic ethanol also persistently increased CD11b expression in multiple brain regions, suggesting long-lasting activation of microglia. Finally, chronic ethanol persistently sensitized the microglial CD11b response to acute stress in multiple brain regions (Fig3.10B). These results are consistent with acute and chronic ethanol enhancing the effects of stress on the neuroimmune system, and may provide novel insight into how ethanol and stress interact to contribute to disease.

Table 3-1 Effects of acute ethanol and acute stress on CD11b expression across brain regions (% control)

Brain Region	CON	E	S	E+S
Prefrontal Cortex				
PrL	100 ± 19[a]	100 ± 23[a,b]	94 ± 10[a]	167 ± 23[b]
IL	100 ± 21[a]	91 ± 18[a,b]	74 ± 6[a]	149 ± 23[b]
Nucleus Accumbens				
Core	100 ± 8[a]	109 ± 30[a]	127 ± 22[a]	195 ± 19[b]
Shell	100 ± 8[a]	97 ± 20[a]	126 ± 21[a,b]	169 ± 13[b]
Bed Nucleus of the Stria Terminalis				
dBNST	100 ± 14[a]	171 ± 56[a,b]	202 ± 25[a,b]	283 ± 30[b]
vBNST	100 ± 10[a]	138 ± 29[a,b]	215 ± 27[a,b]	247 ± 32[b]
Hypothalamus				
PVN	100 ± 28[a]	126 ± 36[a]	551 ± 70[b]	361 ± 20[b]
Amygdala				
BLA	100 ± 16[a]	112 ± 15[a]	129 ± 13[a]	207 ± 18[b]
CeA	100 ± 8[a]	108 ± 10[a]	99 ± 9[a]	153 ± 13[b]

Rats were treated simultaneously with acute ethanol and a two-hour restraint/water immersion stressor. The rats were then sacrificed immediately following the conclusion of the stressor. Brain regions were stained for CD11b to assess microglial activation. n=4-10 per group. Data are presented as mean ± S.E.M. Statistically significant differences are indicated by different letters, e.g. – means with superscript “a” and “b” are statistically different (Tukey’s post-hoc test), while means with superscript “a” and “a” are not statistically different. BLA: basolateral amygdala; CeA: central amygdala; dBNST: dorsal bed nucleus of the stria terminalis; IL: infralimbic cortex; lPAG: lateral periaqueductal grey; PVN: paraventricular nucleus of the hypothalamus; PrL: prelimbic cortex; vBNST: ventral bed nucleus of the stria terminalis.

Table 3-2 Effects of acute ethanol and acute stress on c-Fos expression across brain regions (cells/mm^2)

Brain Region	CON	E	S	E+S
Prefrontal Cortex				
PrL	81 ± 5^{a}	213 ± 33^{b}	497 ± 31^{c}	394 ± 33^{c}
IL	48 ± 8^{a}	137 ± 32^{b}	224 ± 33^{c}	219 ± 31^{c}
Nucleus Accumbens				
Core	219 ± 48^{a}	$316 \pm 111^{a,b}$	543 ± 39^{b}	$402 \pm 47^{a,b}$
Shell	$149 \pm 40^{a,b}$	122 ± 45^{a}	312 ± 60^{b}	139 ± 19^{a}
Bed Nucleus of the Stria Terminalis				
dBNST	63 ± 7^{a}	145 ± 40^{a}	140 ± 11^{a}	113 ± 7^{a}
vBNST	40 ± 5^{a}	63 ± 25^{a}	222 ± 23^{b}	118 ± 21^{a}
Hypothalamus				
PVN	95 ± 28^{a}	260 ± 6^{b}	503 ± 54^{c}	418 ± 38^{c}
Amygdala				
BLA	113 ± 22^{a}	100 ± 29^{a}	272 ± 31^{b}	143 ± 29^{a}
CeA	104 ± 12^{a}	385 ± 88^{b}	204 ± 41^{a}	195 ± 32^{a}

Rats were treated simultaneously with acute ethanol and a two-hour restraint/water immersion stressor. The rats were then sacrificed immediately following the conclusion of the stressor. Brain regions were stained for c-Fos to assess neuronal activation. n=4-10 per group. Data are presented as mean ± S.E.M. Statistically significant differences are indicated by different letters, e.g. – means with superscript “a” and “b” are statistically different (Tukey’s post-hoc test), while means with superscript “a” and “a” are not statistically different. BLA: basolateral amygdala; CeA: central amygdala; dBNST: dorsal bed nucleus of the stria terminalis; IL: infralimbic cortex; lPAG: lateral periaqueductal grey; PVN: paraventricular nucleus of the hypothalamus; PrL: prelimbic cortex; vBNST: ventral bed nucleus of the stria terminalis.

Table 3-3 Effects of chronic ethanol and acute stress on CD11b expression across brain regions (% control)

Brain Region	CON	CE	Stress	CE+Stress
Prefrontal Cortex				
PrL	100 ± 29^{a}	358 ± 105^{a}	702 ± 95^{b}	1115 ± 154^{c}
IL	100 ± 19^{a}	156 ± 35^{a}	236 ± 25^{b}	322 ± 26^{c}
Nucleus Accumbens				
Core	100 ± 11^{a}	464 ± 183^{a}	1664 ± 289^{b}	2835 ± 501^{c}
Shell	100 ± 47^{a}	170 ± 110^{a}	824 ± 184^{a}	2266 ± 664^{b}
Bed Nucleus of the Stria Terminalis				
dBNST	100 ± 12^{a}	415 ± 97^{b}	778 ± 51^{c}	1300 ± 139^{d}
vBNST	100 ± 15^{a}	218 ± 49^{a}	674 ± 113^{b}	597 ± 59^{b}
Hippocampus				
CA1	100 ± 20^{a}	$249 \pm 114^{a,b}$	$331 \pm 37^{a,b}$	429 ± 80^{b}
CA3	100 ± 17^{a}	$164 \pm 50^{a,b}$	211 ± 16^{b}	248 ± 35^{b}
DG	100 ± 17^{a}	$172 \pm 66^{a,b}$	$211 \pm 17^{a,b}$	282 ± 35^{b}
Hypothalamus				
PVN	100 ± 56^{a}	211 ± 55^{a}	1700 ± 244^{b}	1700 ± 300^{b}
Amygdala				
BLA	100 ± 22^{a}	222 ± 22^{a}	1378 ± 144^{b}	2489 ± 433^{c}
CeA	100 ± 16^{a}	277 ± 116^{a}	488 ± 25^{b}	610 ± 67^{b}

Periaqueductal Gray

lPAG	100 ± 15^{a}	225 ± 39^{b}	325 ± 11^{c}	350 ± 41^{c}

Rats were treated with chronic intermittent ethanol and, after a period of prolonged abstinence, were acutely stressed with two hours of restraint/partial water immersion. Rats were sacrificed two hours following the termination of the stressor. Brain regions were stained for CD11b to assess microglial activation. n=6-10 per group. Data are presented as mean ± S.E.M. Statistically significant differences are indicated by different letters, e.g. – means with superscript “a” and “b” are statistically different (Tukey’s post-hoc test), while means with superscript “a” and “a” are not statistically different. BLA: basolateral amygdala; CeA: central amygdala; dBNST: dorsal bed nucleus of the stria terminalis; DG: dentate gyrus; IL: infralimbic cortex; lPAG: lateral periaqueductal grey; PVN: paraventricular nucleus of the hypothalamus; PrL: prelimbic cortex; vBNST: ventral bed nucleus of the stria terminalis.

Table 3-4 Effects of chronic ethanol and acute stress on c-Fos expression across brain regions (cells/mm^2)

Brain Region	CON	CE	Stress	CE+Stress
Prefrontal Cortex				
PrL	142 ± 39^a	144 ± 24^a	302 ± 52^b	297 ± 33^b
IL	116 ± 33^a	138 ± 16^a	242 ± 37^b	282 ± 35^b
Nucleus Accumbens				
Core	37 ± 9^a	35 ± 8^a	119 ± 27^b	136 ± 16^b
Shell	83 ± 23^a	55 ± 9^a	207 ± 27^b	236 ± 24^b
Bed Nucleus of the Stria Terminalis				
dBNST	7 ± 2^a	8 ± 2^a	33 ± 4^b	41 ± 4^b
vBNST	26 ± 7^a	37 ± 8^a	97 ± 13^b	123 ± 17^b
Hippocampus				
CA1	5 ± 1^a	4 ± 1^a	4 ± 1^a	4 ± 1^a
CA3	9 ± 2^a	11 ± 2^a	6 ± 1^a	6 ± 1^a
DG	9 ± 1^a	10 ± 1^a	14 ± 2^a	11 ± 1^a
Hypothalamus				
PVN	141 ± 24^a	83 ± 20^a	544 ± 51^b	661 ± 47^b
Amygdala				
BLA	11 ± 2^a	12 ± 2^a	22 ± 3^b	20 ± 3^b
CeA	14 ± 2^a	21 ± 3^a	66 ± 7^b	83 ± 9^b

Periaqueductal Gray

lPAG	46 ± 11^{a}	74 ± 7^{a}	160 ± 10^{b}	171 ± 12^{b}

Rats were treated with chronic intermittent ethanol and, after a period of prolonged abstinence, were acutely stressed with two hours of restraint/partial water immersion. Rats were sacrificed two hours following the termination of the stressor. Brain regions were stained for c-Fos to assess neuronal activation. n=6-10 per group. Data are presented as mean ± S.E.M. Statistically significant differences are indicated by different letters, e.g. – means with superscript “a” and “b” are statistically different (Tukey’s post-hoc test), while means with superscript “a” and “a” are not statistically different. BLA: basolateral amygdala; CeA: central amygdala; dBNST: dorsal bed nucleus of the stria terminalis; DG: dentate gyrus; IL: infralimbic cortex; lPAG: lateral periaqueductal grey; PVN: paraventricular nucleus of the hypothalamus; PrL: prelimbic cortex; vBNST: ventral bed nucleus of the stria terminalis.

3.4 Materials and Methods

3.4.1 Animals

For the acute ethanol and acute stress studies, male Wistar rats (P75, Harlan Sprague-Dawley, Indianapolis, IN) were acclimated to the animal facility for 3 weeks prior to experimental procedures. Subjects were group-housed (n=2/cage). For the chronic ethanol and acute stress studies, young pregnant female Wistar rats (embryonic day 17; Harlan Sprague-Dawley, Indianapolis, IN) were acclimated to the animal facility prior to birthing. On postnatal day (P)1, 24 hours after birth, litters were culled to 10 pups per dam and housed with their mother until group housing with same-sex littermates at weaning on P21. All animals were housed in a temperature- (20°C) and humidity-controlled vivarium on a 12 hr/12 hr light/dark cycle (light onset at 0700 hr), and provided *ad libitum* access to food and water. Experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill, and conducted in accordance with National Institutes of Health regulations for the care and use of animals.

3.4.2 Acute Ethanol & Acute Stress Experiment

Male Wistar rats (P96) were treated with ethanol (5.0 g/kg, 25% v/v, i.g.) or a comparable volume of water. One hour later, the rats were either acutely stressed or left in their home cage, resulting in a total of four treatment groups: water-gavaged, non-stressed (CON), ethanol-gavaged, non-stressed (EtOH), water-gavaged, stressed (Stress) and ethanol-gavaged, stressed (EtOH+Stress). Animals in the Stress groups received an acute stressor consisting of restraint/partial water immersion as previously described [183]. Briefly, the rats were restrained in DecapiCone restrainers (Braintree Scientific, Braintree, MA, Cat.# DCL-200), and immersed up to their zyphoid processes in chilled water (21±2°C) for two hours. The animals were sacrificed immediately following the termination of the stressor (see Fig1A). Non-stressed animals remained in their home-

cages until sacrifice. Trunk blood was collected at sacrifice, and blood ethanol concentration (BECs) were calculated using a GM7 Analyzer (Analox; London, UK).

3.4.3 Chronic Ethanol & Acute Stress Experiment

On P21, male subjects were weaned and randomly assigned to either chronic ethanol (CE) or water control (CON) treatment, as described previously [136]. Beginning on P25, animals in the CE condition received intragastric (i.g.) administration of ethanol (5.0 g/kg, 20-30% v/v) on a two-day on/two-day off schedule until P54 (see Fig 1B). Animals in the control condition received comparable volumes of water on an identical schedule. Tail blood was collected one hour after ethanol administration on P38 and P54 for the assessment of BECs. Following the conclusion of CE treatment, subjects were pair-housed (n = 2/cage), and maintained on *ad libitum* access to food and water for the duration of experiments. One group of animals was sacrificed on P55, 24 hours after the last ethanol exposure. The remaining animals continued to mature until P96 or P97, when animals were randomly assigned to either a stress or control treatment group, for a total of four groups: water-gavaged, non-stressed (CON), ethanol-gavaged, non-stressed (CE), water-gavaged, stressed (Stress) and ethanol-gavaged, stressed (CE+Stress). Stressed animals received a two-hour restraint/water immersion stressor as described above. The rats were sacrificed two hours after the conclusion of the two-hour acute stressor. Non-stressed animals remained in their home cages until sacrifice.

3.4.4 Perfusion and Brain Tissue Preparation

Rats were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4.0% paraformaldehyde in PBS. Brains were extracted, post-fixed in 4.0% paraformaldehyde/PBS solution for 24 hours and then placed in a 30% sucrose solution in PBS. Brain tissue was sectioned coronally at a thickness of 40 μm on a sliding microtome (MICROM HM450; ThermoScientific, Austin, TX). Sections were

sequentially collected into well plates and stored at −20 °C in a cryoprotectant solution consisting of 30% glycol/30% ethylene glycol in PBS for immunohistochemistry.

3.4.5 Corticosterone Measurements

Trunk blood was collected during perfusion. Blood was drawn from the left ventricle into a syringe, placed in BD Microtainer™ Plastic Capillary Blood Collector with lithium heparin (Fisher Scientific, Hampton, NH, Cat.# 02-668-75), and placed on ice. Plasma was collected by spinning down the whole blood twice at 4°C for 15 minutes at 13800 g. Corticosterone measurements were done using an RIA kit (MP Biomedical, Santa Ana, CA, Cat.# 07120102)**.**

3.4.6 Endotoxin Measurements

Trunk blood was collected during perfusion. Blood was drawn from the left ventricle of the heart into a syringe, place in BD Microtainer™ Plastic Capillary Blood Collector with lithium heparin (Fisher Scientific, Hampton, NH, Cat.# 02-668-75), and placed on ice. Plasma was collected by spinning down the whole blood twice at 4°C for 15 minutes at 13800 g. Endotoxin was measured using an Endotoxin Detection kit (Lonza, Basel, Switzerland, Cat.# 50-647U). Briefly, plasma samples were heated for 10 minutes at 70°C. Samples were then processed following the kit instructions, with the exception that color was allowed to develop for longer than recommended by the kit (i.e. – for 30 – 120 minutes).

3.4.7 Immunohistochemistry

Free-floating sections were washed in 0.1 M PBS, incubated in 0.3% H_2O_2 for 30 minutes, washed again in PBS and blocked for one hour at room temperature in 0.25% Triton-X100/5% normal serum (MP Biomedicals, Solon, OH, Cat.# 19135680). Sections were transferred directly from the block to primary antibody diluted in blocking solution and were incubated overnight at 4°C. Sections were then washed in PBS, incubated for one hour in biotinylated secondary antibody

(1:200; Vector Laboratories, Burlingame, CA), washed and incubated for one hour in avidin–biotin complex solution (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, Cat.# PK6100). The chromogen, nickel-enhanced diaminobenzidine (Sigma–Aldrich, St. Louis, MO, Cat.# D5637), was used to visualize immunoreactivity. Tissue was mounted onto slides, dehydrated, and coverslipped.

3.4.8 Microscopic Quantification and Image Analysis

For staining in the various brain regions, tissue was drawn as follows using the atlas of Paxinos and Watson [197]: for PrL and IL, a 1:6 series from approximately Bregma +3.70 to +2.20 mm (~4 slices per animal); for NAc Core and NAc Shell, a 1:6 series from approximately Bregma +1.70 to +0.70 mm (~4 slices per animal); for dBNST and vBNST, a 1:4 series from approximately Bregma -0.26 to -0.40 mm (~3 slices per animal); for PVN, a 1:3 series from approximately Bregma -1.40 to -2.12 mm (~3 slices per animal); for CA1, CA3, and DG, a 1:6 series from approximately Bregma -2.56 to -3.60 mm (~4 slices per animal); for CeA and BLA, a 1:3 series from approximately Bregma -1.88 to -2.80 mm (~3 slices per animal); and for lPAG, a 1:6 series from approximately Bregma -5.80 to -6.72 mm (~3 slices per animal) (see FigM1). The only exception was staining in the PVN, for which 1 slice per animal was used due to limited tissue. A modified stereological profile quantification method was used to quantify immunopositive cell counts and pixel density within the regions of interest. We have previously published that a comparison of this method with unbiased stereological methodology yielded nearly identical values relative to control subjects [198]. Images of the regions of interest were captured using an Olympus BX50 microscope and Sony DXC-390 video camera linked to a computer. BioQuant Nova Advanced Image Analysis (R&M Biometric, Nashville, TN) was used for image analysis. The threshold was rigorously determined by calculating the average of the darkest and lightest values from each region of interest from control subjects. Sections were imaged under identical conditions [199]. Immunopositive cell counts or pixel density

were then determined by the BioQuant program. The area of the outlined regions of interest was determined and immunoreactivity was calculated by dividing either cell counts or pixel density by the overall area (mm^2).

3.4.9 Statistical Analyses

The Statistical Package for the Social Sciences (SPSS; Chicago, IL) was used for all statistical analyses. The data from each experiment was analyzed using a two-by-two ANOVA. Significant main effects and interactions were further investigated using Tukey's *post hoc* test for multiple comparisons, unless otherwise noted. Pearson correlations (r) were used to determine significant correlations. All values are reported as mean ± S.E.M., and significance was defined at a level of $p \leq 0.05$.

3.5 Supplemental Figures

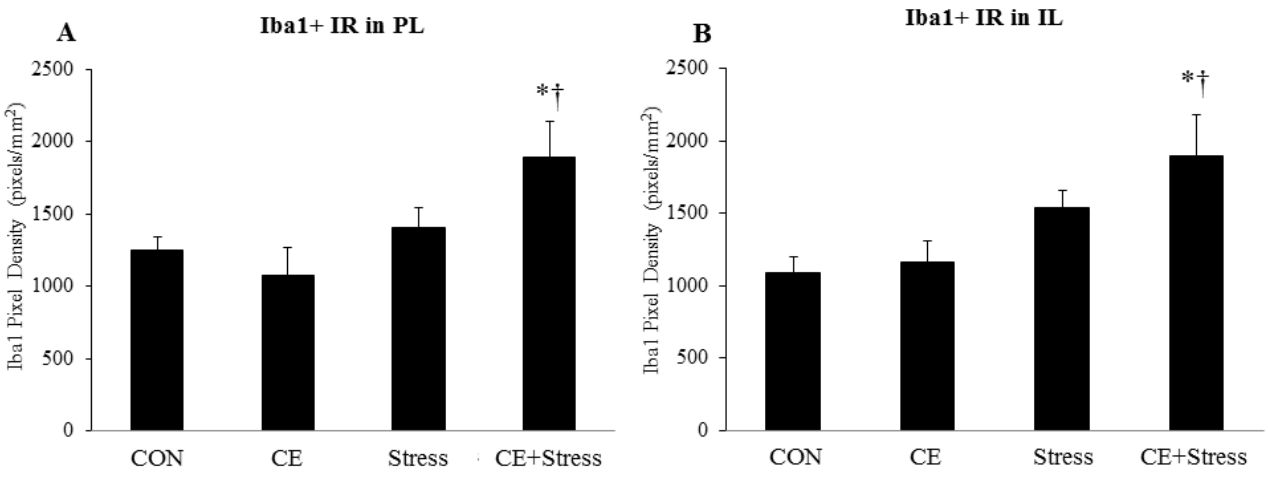


Fig S3.1. Effects of chronic ethanol and acute stress on Iba1+ IR in the prelimbic cortex. Rats were treated with chronic ethanol (5.0 g/kg, 20-30% v/v, 2 days-on, 2 days-off from P25-P54) and/or acutely stressed with a two-hour restraint-water immersion stressor following prolonged abstinence on P96/7. The rats were sacrificed two hours following the conclusion of the stressor. **A.** Iba1+ IR in the PrL was assessed in each group. Data are presented as mean ± S.E.M. *p<0.05 compared to CON, †p<0.05 compared to CE (Tukey's *post-hoc* test). n=8-10/group. **B.** Iba1+ IR in the PrL was assessed in each group. Data are presented as mean ± S.E.M. *p<0.05 compared to CON, †p<0.05 compared to CE (Tukey's *post-hoc* test). n=8-10/group.

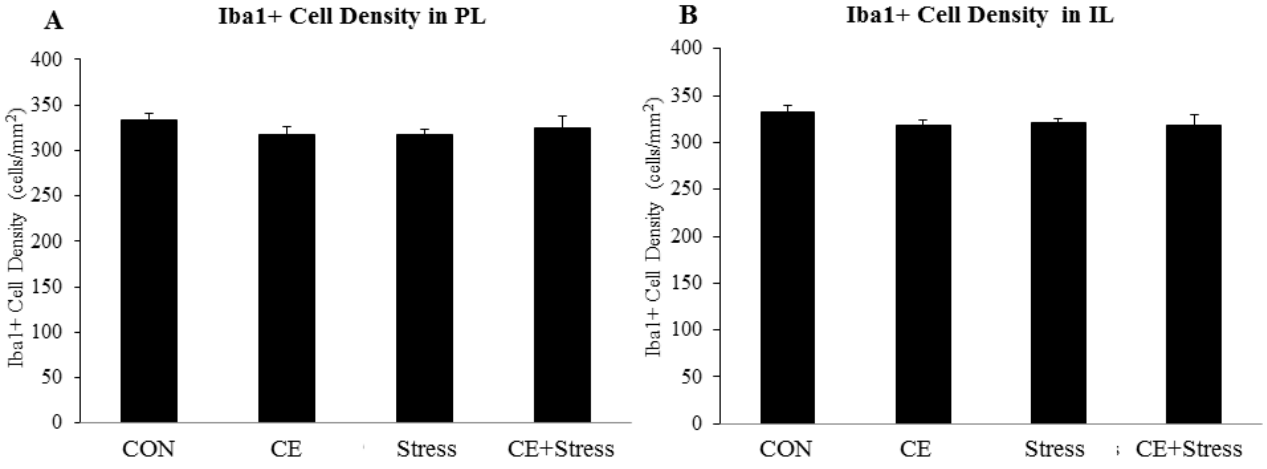


Fig S3.2. Effects of chronic ethanol and acute stress on Iba1+ cell density in the prelimbic cortex. Rats were treated with chronic ethanol (5.0 g/kg, 20-30% v/v, 2 days-on, 2 days-off from P25-P54) and/or acutely stressed with a two-hour restraint-water immersion stressor following prolonged abstinence on P96/7. The rats were sacrificed two hours following the conclusion of the stressor. **A.** Iba1+ cell density in the PrL was assessed in each group. Data are presented as mean ± S.E.M. n=8-10/group. **B.** Iba1+ cell density in the IL was assessed in each group. Data are presented as mean ± S.E.M. n=8-10/group.

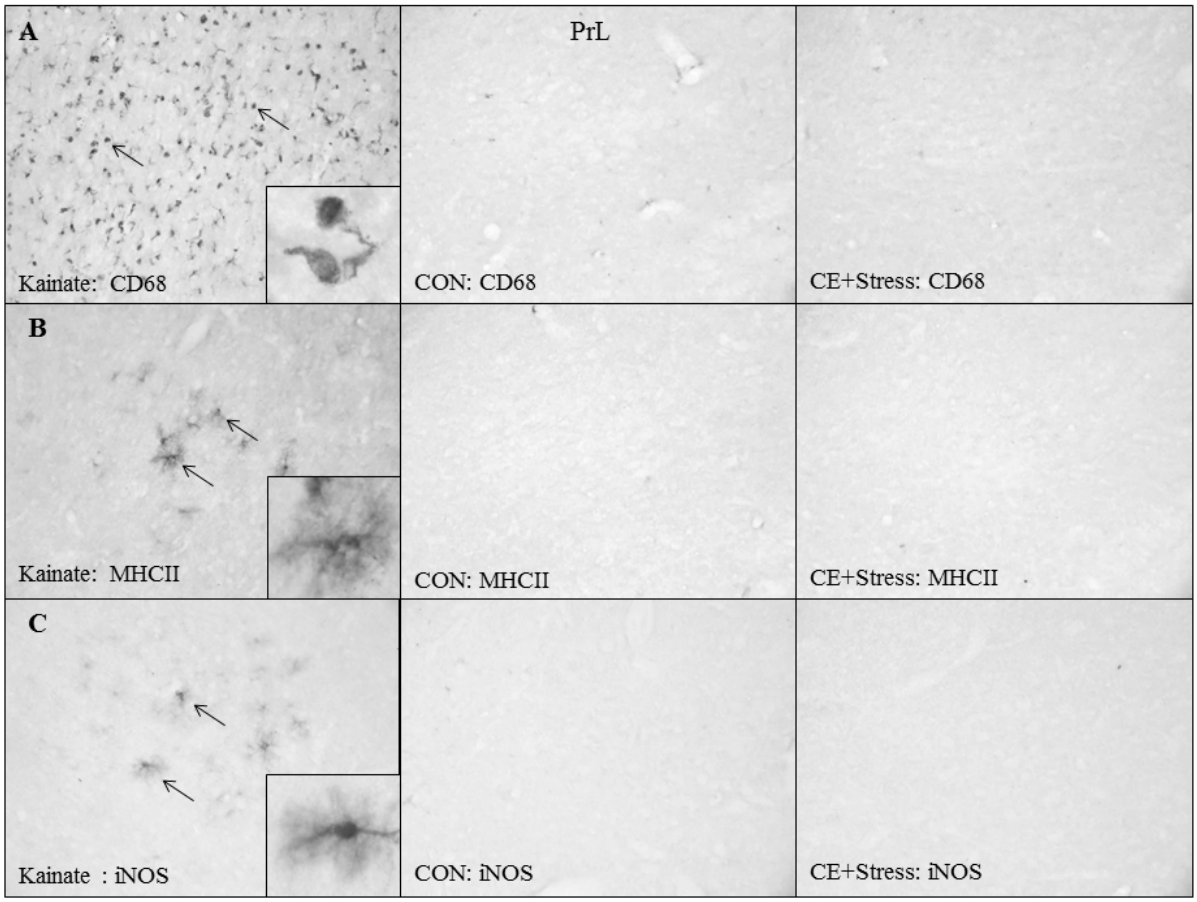


Fig S3.3. Effects of chronic ethanol and acute stress on CD68, MHCII and iNOS in the prelimbic cortex. Rats were treated with chronic ethanol (5.0 g/kg, 20-30% v/v, 2 days-on, 2 days-off from P25-P54) and/or acutely stressed with a two-hour restraint-water immersion stressor following prolonged abstinence on P96/7. The rats were sacrificed two hours following the conclusion of the stressor. **A.** CD68+ IR, **B.** MHCII+ IR, and **C.** iNOS+ IR in the PrL was assessed in each group. Representative sections from the CON and CE+Stress group are shown. Note the absence of CD68, MHCII and iNOS staining in each of the groups.

CHAPTER 4: MICROGLIAL DEPLETION ALTERS THE NEURONAL RESPONSE TO ACUTE BINGE ETHANOL WITHDRAWAL AND DECREASES VOLUNTARY ETHANOL CONSUMPTION

4.1 Introduction

Alcohol use disorders (AUDs) are common mental health diseases in which individuals drink alcohol despite negative consequences. Excessive alcohol use has several detrimental effects on the brain, contributing to neurodegeneration [146], neuronal dysfunction [147] and dependence. Moreover, studies suggest excessive alcohol changes the brain to promote further alcohol consumption [13]. Chronic alcohol alters stress circuitry to promote aversive withdrawal states, such as irritability, anxiety, depression and dysphoria. These unpleasant withdrawal states motivate further alcohol consumption to provide relief from these states. While research has uncovered many of the molecular events associated with these processes, much remains unknown. Interestingly, recent studies find the neuroimmune system contributes to alcohol drinking behavior. Injection of TLR4 siRNA into the central amygdala reduces alcohol self-administration [82], and intracerebroventricular infusion of the pro-inflammatory cytokine Ccl2 increases alcohol self-administration. Furthermore, injections of IL-1ra, the IL-1 receptor antagonist, into the basolateral amygdala (BLA) reduced alcohol self-administration in mice [84]. Another study found that injection of the anti-inflammatory cytokine IL-10 into the BLA also reduces alcohol consumption. These studies demonstrate a role for the neuroimmune system in alcohol consumption.

Additional studies suggest that microglia, the primary cells of the neuroimmune system, may

play an important role in alcohol use disorders. Indeed, alcohol activates microglia in animal models [137, 200], and activated microglia are observed in the brains of post-mortem human alcoholics [10, 144]. Studies also find that microglia impact neuronal activity and subsequently behavior. Microglia strip synapses from neurons, thereby altering neuronal activity [72]. Microglia also release cytokines such as TNFα to alter synaptic strength [64] and neurotrophins such as BDNF to impact spine formation and neuronal excitation [63]. Other studies find that microglial processes contact highly active neurons and reduce their activity [201]. Furthermore, these studies also find microglia can influence behavior. Cocaine increases microglial TNFα in the nucleus accumbens, thereby altering NAc neuronal activity and the behavioral sensitization to cocaine. Deletion of BDNF specifically from microglia also reduces learning. Other studies find that deletion of microglial CX_3CR1 caused decreased social interaction and increased repetitive behavior. However, whether microglia mediate the effects of alcohol on neurons or alcohol drinking behavior is unknown. To investigate this, we sought to deplete microglia from the brains of mice using the colony stimulating factor 1 receptor (CSF1R) inhibitor PLX5622. In our first experiment, microglial-depleted mice were gavaged with an acute binge dose of ethanol and neuronal activity was examined using a marker of neuronal activation, c-Fos. We hypothesized that microglial depletion would block ethanol-induced c-Fos. In our second experiment, microglial-depleted mice were allowed intermittent access to ethanol and voluntary ethanol consumption was recorded. We hypothesized that microglial depletion would decrease ethanol consumption.

4.2 Results

4.2.1 Effects of PLX5622 chow on microglial cell density in various brain regions

Previous studies found that microglia impact the brain response to acute binge ethanol withdrawal [200]. However, whether microglia specifically impact the neuronal response to acute binge ethanol withdrawal is unknown. To investigate this, we used the CSF1R inhibitor PLX5622 to deplete microglia from the brains of mice. Mice were treated with PLX5622 for 3 weeks, a time previously shown to result in maximal microglial depletion [152]. The mice were then gavaged with an acute binge dose of ethanol (6 g/kg, 25% v/v) or water control. This treatment protocol has previously been shown to cause microglial and neuroimmune activation, but no morbidity or mortality [200]. Tail blood was collected 1 hour post-gavage to verify PLX5622 had no effect on

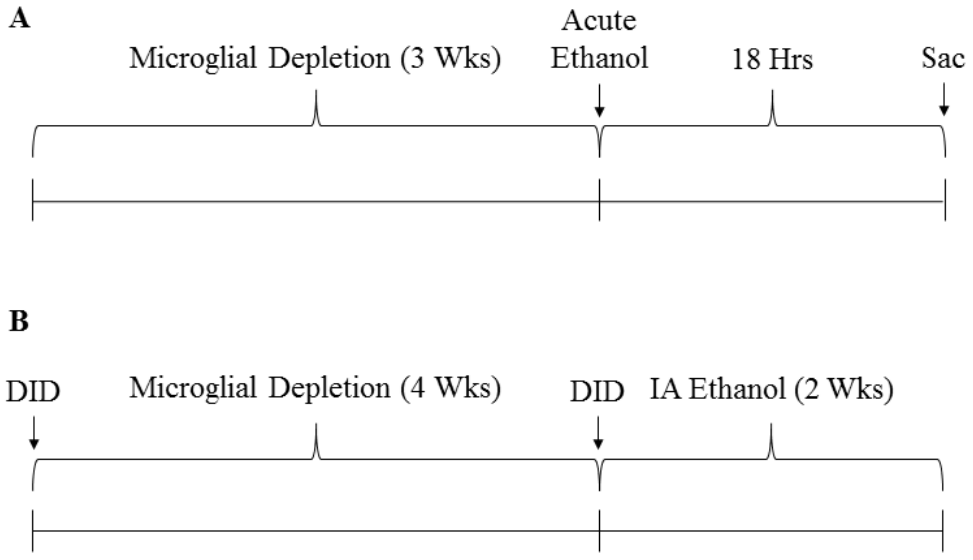


Figure 4-1 Outline of experimental designs. A. Mice were fed either control or PLX5622 chow for three weeks to deplete microglia. Mice were then treated with acute binge ethanol (6 g/kg, 25% v/v, i.g.) and sacrificed 18 hours later during withdrawal. **B.** Another cohort of mice underwent the "Drinking-in-the-Dark" procedure to assess baseline drinking. Mice were then randomly assigned to either control or PLX5622 chow for four weeks to deplete microglia. The mice then received intermittent access to free choice ethanol or water for two weeks.

peak blood ethanol concentrations (426±28 mg/dL in the ethanol-exposed group vs. 419±21 mg/dL in the microglial-depleted and ethanol-exposed group; p=0.82). Animals were then sacrificed 18 hours post-gavage during withdrawal (Fig 4.1). This time point was chosen because previous studies showed maximal brain inflammatory cytokine expression at this time [200].

To verify microglial depletion across the brain, we performed immunohistochemical stains for Iba1, a microglial marker, in several regions, including the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), bed nucleus of the stria terminalis (BNST), paraventricular nucleus of the hypothalamus (PVN), the CA3 region of the hippocampus and the central amygdala (CeA) (Fig 4.2). Microglial cell density in each region was reduced by 95% or more (Table 4.1). Furthermore, acute ethanol withdrawal did not significantly change microglial cell density in any examined region

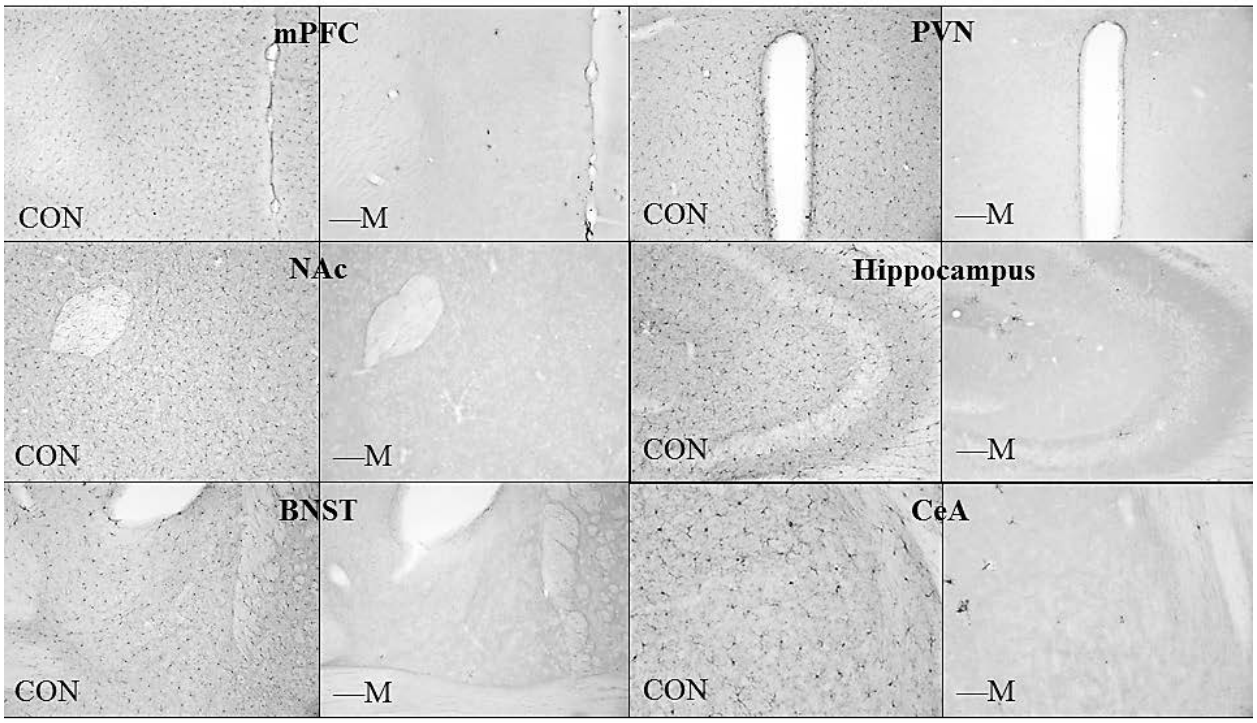


Figure 4-2 Microglia are depleted across several brain regions. Mice were fed either control or PLX5622 chow for three weeks to deplete microglia. The mice were then sacrificed, and their brains were processed for immunohistochemistry. Staining for Iba1, a microglial marker, showed depletion of microglia across several brain regions, including the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), bed nucleus of the stria terminalis (BNST), paraventricular nucleus of the hypothalamus (PVN), hippocampus, and central nucleus of the amygdala (CeA). CON: Control Chow. —M: PLX5622 Chow.

(Table 4.1). The density of Iba1+ cells in the microglial-depleted and ethanol-treated group was also reduced by 95% or more (Table 4.1). Overall, these data show PLX5622 chow substantially depleted microglia in every examined brain region.

4.2.2 Effects of microglial depletion on acute ethanol withdrawal-induced neuronal activation in various brain regions

Previous studies find that microglia can alter neuronal activity [63, 64, 201]. However, it is unknown whether microglia play a role in ethanol withdrawal-induced neuronal activation. To examine this, brains from the same microglial-depleted and ethanol-exposed mice as above were stained for c-Fos, a marker of neuronal activation. We examined c-Fos staining at 18 hours following acute ethanol gavage, a time when brain microglial markers and cytokine expression was maximally increased. This allowed us to examine the effects of microglia on withdrawal-induced neuronal

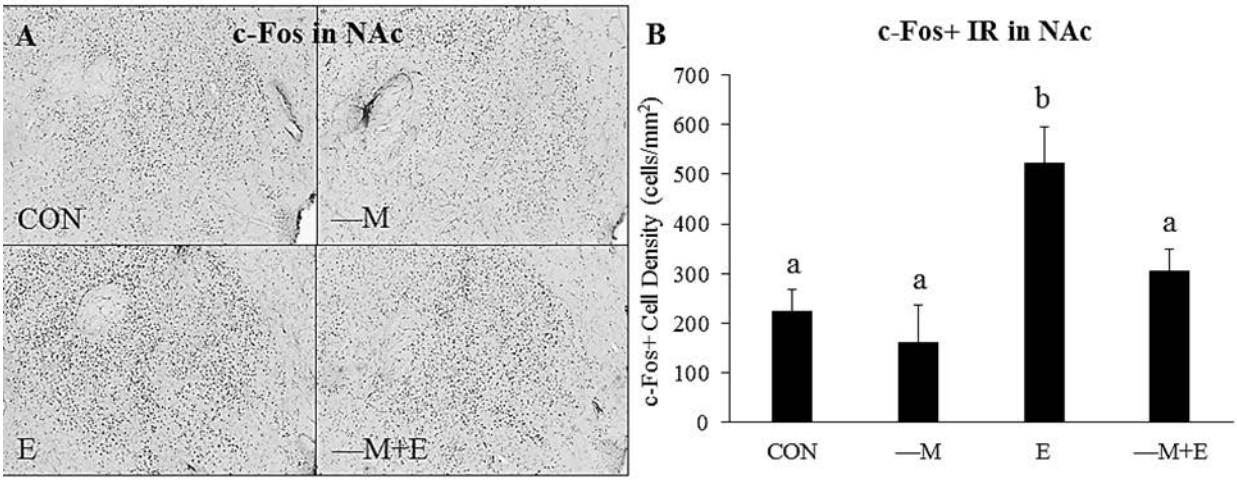


Figure 4-3 The nucleus accumbens neuronal response to acute ethanol withdrawal is blunted by microglial depletion. Microglia were depleted from the brains of mice. Mice were then treated with acute binge ethanol (6 g/kg, 25% v/v, i.g.) and sacrificed 18 hours later during withdrawal. Brains were isolated and processed for immunohistochemistry. Sections of NAc were stained for the marker of neuronal activation, c-Fos. **A.** Representative images of c-Fos staining in the NAc from control mice (CON), microglial-depleted mice (—M), ethanol-treated mice (E), and microglial-depleted and ethanol-treated mice (—M+E) are displayed. **B.** The regional density of c-Fos+ cells was determined. Data are presented as mean ± s.e.m. a,b = Means with different letters are significantly different from each other ($p<0.05$, Tukey's post-hoc). Means with the same letters are not significantly different from each other. n=6-12/group.

activation. We assessed c-Fos+ cell density in several regions implicated in alcohol use disorders (Table 4.2). Microglial depletion had no effect on baseline c-Fos in any of the brain regions examined. However, acute ethanol-withdrawal significantly increased c-Fos+ cell density in several of these regions (Table 4.2), consistent with previous studies [20]. Microglial depletion did not significantly alter ethanol withdrawal-induced c-Fos in several brain regions, including the mPFC, BNST, PVN and CeA. However, we identified two regions in which microglial depletion significantly decreased the c-Fos response to ethanol withdrawal: the hippocampus and the NAc. There was a trend for acute ethanol withdrawal to increase c-Fos+ cell density in the CA3 region of the hippocampus. Interestingly, microglial depletion significantly decreased ethanol withdrawal-induced c-Fos by approximately 50% ($p<0.05$) (Table 4.2). In the NAc, ethanol withdrawal increased c-Fos+ cell density 234% ($p<0.05$). Interestingly, microglial depletion significantly decreased withdrawal-induced c-Fos by 42% ($p<0.05$) in the NAc (Fig 4.3). To verify ethanol-induced changes in c-Fos occurred in neurons in the NAc, we performed co-localization studies in the ethanol-treated and microglial-depleted/ethanol-treated groups. Immunofluorescent staining for c-Fos and NeuN, a neuronal marker, in the NAc showed that >95% of c-Fos+ cells were also NeuN+ in both groups (Fig S4.1). These data suggest that withdrawal-induced c-Fos is localized to neurons in the NAc. To determine whether decreased c-Fos in the microglial-depleted/ethanol-treated group is due neuronal cell death, we also performed immunohistochemical stains for cleaved caspase-3, a marker of cell death. There was no observable staining for cleaved caspase-3 in any of the four treatment groups (Fig S4.2). Furthermore, positive control stains in sections of kainite-treated brains showed robust staining (Fig S4.2), suggesting a true lack of staining in the microglial-depleted and ethanol-treated brains. These results suggest that decreases in withdrawal-induced c-Fos in the microglial-depleted/ethanol-treated group is not due to cell death. Overall, these results suggest that

microglia may modulate the activity of neurons during acute binge ethanol withdrawal.

4.2.3 Effects of microglial depletion on voluntary ethanol consumption

Since microglial depletion altered the neuronal response to ethanol in the NAc – a brain region involved in alcohol consumption – we next investigated whether microglial depletion impacted voluntary ethanol consumption. Mice were first tested in the "Drinking in the dark" (DID) procedure as previously described [202] to assess baseline ethanol consumption. This was done by providing access to 20% ethanol for 2 hours for three days, followed by 20% ethanol for 4 hours for a fourth day. The amount of ethanol consumed on the fourth day was measured, and the mice were then randomly assigned to either control or PLX5622 chow. There was no significant difference in body weight (30.1±0.6 g vs 30.1±0.7 g) or fourth-day DID ethanol consumption (4.8±0.2 g/kg/4 hrs vs. 4.8±0.3 g/kg/4 hrs) between groups prior to microglial depletion. The mice remained on control or PLX5622 chow for 4 weeks. After these 4 weeks, the mice were tested again in the DID procedure as described above to determine whether microglial depletion impacted baseline ethanol consumption. There was no difference in baseline ethanol consumption between groups. To

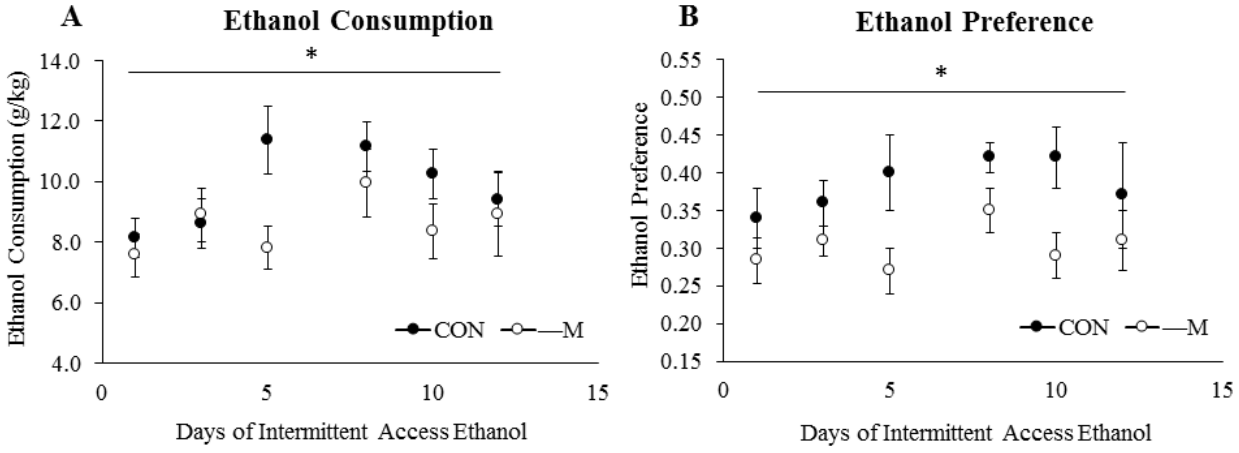


Figure 4-4 Microglial depletion decreased free choice ethanol consumption and ethanol preference. Mice received either control or PLX5622 chow for 4 weeks to deplete microglia. The mice then received intermittent access to free choice ethanol on the first, third and fifth day of each week for two weeks. The **A.** ethanol consumed and **B.** ethanol preference for each mouse over time were calculated. CON: control chow, —M: microglial-depletion. Data are presented as mean ± s.e.m. n=13-14/group. *p<0.05: main effect of microglial depletion.

determine whether microglial depletion impacts ethanol consumption over time, the mice were then randomly assigned to either Water or Intermittent Access Ethanol groups for a total of four groups: CON Chow – Water, PLX Chow – Water, CON Chow – IA Ethanol, and PLX Chow – IA Ethanol. The mice then received either water or intermittent access ethanol as previously described [203] while remaining on the PLX5622 chow to keep microglia depleted. On Monday, Wednesday and Friday, mice were provided one bottle of 20% ethanol and one bottle of water for 24 hours. On Tuesday, Thursday, Saturday and Sunday, mice were provided two water bottles for 24 hours. Body weights, ethanol consumption, water consumption, and total fluid intake were measured over the next two weeks. Microglial depletion did not change body weights or total fluid intake (Fig S4.3). However, microglial depletion caused a significant reduction in ethanol consumption ($p<0.05$) and ethanol preference ($p<0.05$) (Fig 4.4). To verify microglial depletion in these mice, brains were collected after intermittent ethanol exposure and mRNA was collected. RT-PCR was performed for various microglial markers. Results showed substantially decreased microglial markers (Fig S4.4), suggesting microglia remained depleted throughout the duration of the experiment. Overall, these results suggest microglia contribute to voluntary ethanol consumption and preference.

4.3 Discussion

In this study, we examined the role of microglia in the neuronal response to ethanol, and how microglia impact ethanol consumption. We report that the CSF1R inhibitor PLX5622 depleted microglia across several brain regions. Furthermore, acute ethanol withdrawal induces c-Fos across the brain, consistent with widespread neuronal activation. Microglial depletion blocked withdrawal-induced c-Fos in some brain regions, including the nucleus accumbens and hippocampus, but not all brain regions. This is consistent with microglia contributing to withdrawal-induced neuronal

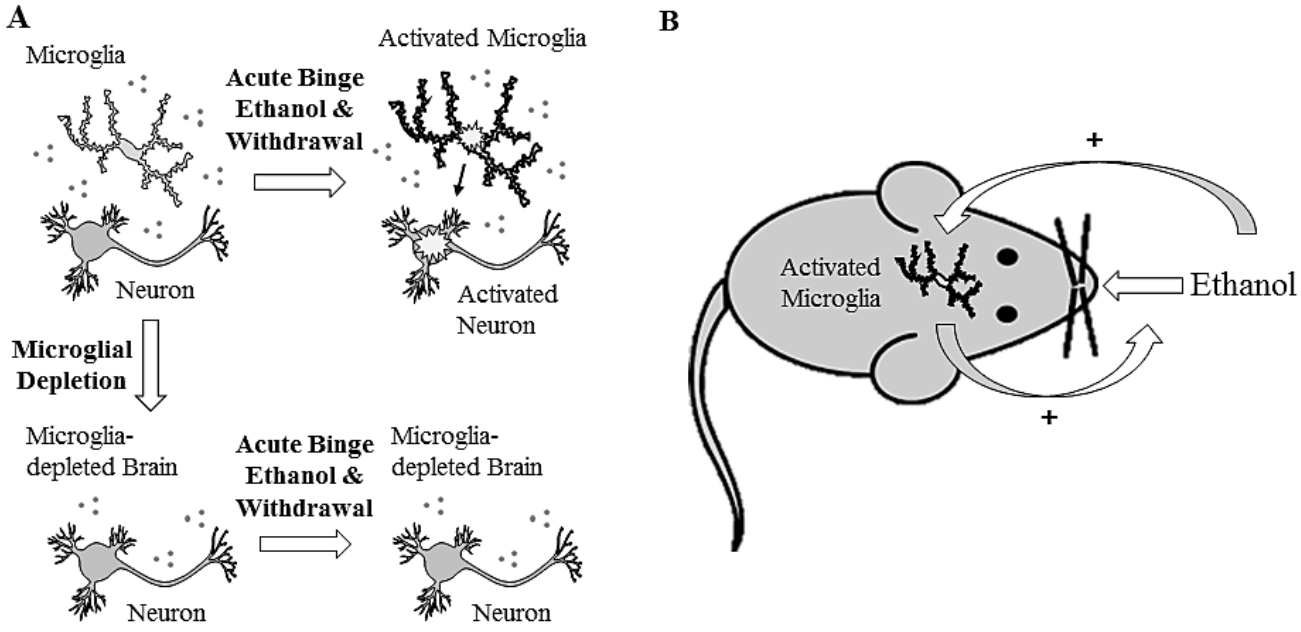


Figure 4-5 Effects of microglial depletion on ethanol-induced neuronal activation and voluntary ethanol consumption. A. Withdrawal from acute binge ethanol activates microglia and neurons across the brain. Interestingly, microglial depletion decreased ethanol-induced neuronal activation in the nucleus accumbens, suggesting microglia contribute to ethanol-induced neuronal activation in this brain region **B.** Ethanol activates microglia. Furthermore, microglia were found to contribute to ethanol consumption. This suggests a possible novel molecular mechanism by which alcohol abuse contributes to additional alcohol abuse, i.e. – alcohol activates microglia which contributes to further alcohol consumption, furthering activating microglia, etc.

activation (Fig 4.5A). We also report that microglial depletion decreased voluntary ethanol consumption and ethanol preference, without changing total fluid intake. This suggests microglia contribute to the reinforcement of alcohol consumption (Fig 4.5B). Overall, these results suggest

that microglia play a role in cellular and behavioral pathogenesis of alcohol use disorders.

These studies find that the CSF1R inhibitor PLX5622 depletes microglia across several brain regions, consistent with previous studies [152, 200]. Indeed, microglial cell density was reduced by 95% or more in every brain region examined, including the mPFC, NAc, BNST, PVN, Hippocampus and CeA. Furthermore, our results also show that PLX5622 persistently depletes microglia for up to 16 weeks. Indeed, the microglial markers Iba1, CD11b and CD68 were substantially reduced in the brains of mice 16 weeks after starting the PLX5622 chow. This is to our knowledge the longest period of microglial depletion reported. Overall, these results find that PLX5622 deplete microglia across the brain for extended periods of time.

These studies also find acute ethanol withdrawal induces c-Fos across the brain, consistent with previous studies [20]. c-Fos is an immediate early gene that is up-regulated following neuronal activation [204]. These results suggest acute ethanol withdrawal activates neurons across the brain. Indeed, ethanol withdrawal is known to cause aversive emotional states that are mediated by neuronal activation in regions such as the BNST and CeA. We observed withdrawal-induced c-Fos in almost every brain region examined, including the mPFC, NAc, BNST, PVN and CeA. Overall, these data show that acute ethanol withdrawal induces c-Fos across the brain, suggesting neuronal activation.

Our results also find that microglial depletion decreased withdrawal-induced c-Fos in the nucleus accumbens and hippocampus. This suggests microglia contribute to changes in neuronal activation following ethanol exposure. Indeed, our results find that c-Fos colocalizes with the neuronal marker NeuN in the nucleus accumbens, suggesting that c-Fos induction occurs in neurons. It is possible that microglial depletion blocks withdrawal-induced c-Fos because of neuronal cell death. However, our studies failed to find increased levels of the cell death marker, cleaved caspase-

3, in the nucleus accumbens. It is possible that neuronal cell death occurs before the examined 18 hour withdrawal time point; however, previous studies find that signs of neurodegeneration continue for at least 20 hours after heavy ethanol exposure [205]. These results suggest c-Fos is not decreased because neurons are dying. It is possible c-Fos is decreased because microglia impact the neuronal response to ethanol. Indeed, this study finds increased c-Fos at a time when microglial markers and cytokines are increased. Previous studies also support the idea that microglia impact neuronal activity. Microglia have been found to sense and regulate the activity of neurons [201]. Furthermore, microglial TNFα alters the plasticity of neurons in the nucleus accumbens and subsequently impacts behavior [64]. Microglial BDNF is also important for spine remodeling, changes in neuronal plasticity and learning behavior [63]. The regions in which microglial depletion altered the c-Fos response, namely, the NAc and the hippocampus, have been implicated in alcohol use disorders. The NAc is critical for reward and reinforcement, and the hippocampus is involved in learning and memory. Dysfunction in these brain regions contributes to substance abuse and dependence [11]. Overall, our results suggest microglia promote ethanol withdrawal-induced neuronal activity in brain regions such as the nucleus accumbens.

We also report that microglial depletion reduces voluntary ethanol consumption over time. Following four weeks of PLX5622 treatment to deplete microglia, mice were given intermittent access to ethanol while remaining on the PLX5622 chow to keep microglia depleted. Over the next two weeks, microglial-depleted mice drank significantly less ethanol and had a significantly lower ethanol preference compared to controls. Importantly, microglial depletion did not significantly change body weights, water intake or total fluid intake. These results suggest microglia contribute to the reinforcing properties of ethanol. Our results are also consistent with previous studies finding effects of the neuroimmune system on ethanol consumption. For example, deletion of *Ccr2*, the gene

encoding the receptor for the pro-inflammatory cytokine Ccl2, reduced voluntary alcohol consumption in mice without reducing overall fluid intake [78]. Deletion of the *IL1rn* and *IL-6* genes also decreased voluntary alcohol consumption without changing total fluid intake [79]. Additional studies show that double knock-outs of the IL-1 receptor and TNF receptor decrease stress-induced ethanol intake [80]. Other studies show that administration of neuroimmune molecules directly into the brain can change alcohol consumption. Indeed, injection of TLR4 siRNA into the central amygdala reduces alcohol self-administration [82]. Intracerebroventricular infusion of the pro-inflammatory cytokine Ccl2 increases self-administration of sweetened alcohol, but does not increase self-administration of sucrose [83]. Furthermore, injections of IL-1ra, the IL-1 receptor antagonist, into the basolateral amygdala (BLA) reduced alcohol self-administration in mice [84]. Another study found that injection of the anti-inflammatory cytokine IL-10 into the BLA also reduced alcohol consumption. These studies demonstrate a role for the neuroimmune system in alcohol consumption behavior. As the primary cells of the neuroimmune system, microglia may play a role in these processes. Overall, our results suggest a role for microglia in voluntary ethanol consumption.

In this study, we examined the role of microglia in the neuronal response to ethanol and ethanol consumption. We report that the CSF1R inhibitor PLX5622 depleted microglia across several brain regions. Furthermore, acute ethanol withdrawal induces c-Fos across the brain, consistent with widespread neuronal activation. Microglial depletion blocked withdrawal-induced c-Fos in the NAc and hippocampus, but not in all brain regions. This is consistent with microglia promoting ethanol-induced neuronal activation in certain brain regions. We also report that microglial depletion decreased voluntary ethanol consumption and ethanol preference, without changing total fluid intake. This suggests microglia contribute to the reinforcement of alcohol

consumption. Overall, these results suggest that microglia play a role in cellular and behavioral pathogenesis of alcohol use disorders.

Table 4-1 Iba1+ Cell Density in Brain Regions of Microglial-Depleted & Ethanol-Treated Mice

	CON	—M	E	—M+E
mPFC	301 ± 27^{A}	14 ± 6^{B}	301 ± 22^{A}	5 ± 2^{B}
NAc	343 ± 32^{A}	6 ± 2^{B}	306 ± 13^{A}	2 ± 1^{B}
BNST	177 ± 25^{A}	2 ± 1^{B}	246 ± 21^{A}	0 ± 0^{B}
PVN	329 ± 14^{A}	0 ± 0^{B}	328 ± 17^{A}	0 ± 0^{B}
Hippocampus	174 ± 7^{A}	3 ± 1^{B}	181 ± 11^{A}	3 ± 1^{B}
CeA	297 ± 15^{A}	20 ± 9^{B}	277 ± 17^{A}	3 ± 1^{B}

Mice were depleted of microglia, gavaged with an acute binge dose of ethanol (6 g/kg, 25% v/v) or water and sacrificed 18 hours later during withdrawal. Brains were prepared for immunohistochemistry and stained for Iba1, a microglial marker. Microglial cell density was assessed in several brain regions. Data are presented as mean ± s.e.m. and were analyzed by two-by-two ANOVA for each region followed by Tukey's *post-hoc* test for significant main effects and/or interactions. n=6-12/group. Means that are significantly different are labeled with different letters (e.g. – "A" and "B"). Means that are not significantly different are labeled with the same letters (e.g. – "A" and "A"). CON: control mice gavaged with water, —M: microglial-depleted mice gavaged with water, E: control mice gavaged with ethanol, —M+E: microglial-depleted mice gavaged with ethanol. mPFC: medial prefrontal cortex. NAc: nucleus accumbens. BNST: bed nucleus of the stria terminalis. PVN: paraventricular nucleus of the hypothalamus. CeA: central nucleus of the amygdala.

Table 4-2 c-Fos+ Cell Density in Brain Regions of Microglial-Depleted & Ethanol-Treated Mice

	CON	—M	E	—M+E
mPFC	111 ± 27^{A}	97 ± 9^{A}	333 ± 51^{B}	278 ± 35^{B}
NAc	224 ± 44^{A}	162 ± 75^{A}	524 ± 73^{B}	304 ± 46^{A}
BNST	56 ± 17^{A}	44 ± 16^{A}	134 ± 11^{B}	191 ± 38^{B}
PVN	395 ± 61^{A}	658 ± 179^{A}	2256 ± 195^{B}	2405 ± 331^{B}
Hippocampus	$623 \pm 55^{A,B}$	$572 \pm 21^{A,B}$	709 ± 134^{A}	319 ± 74^{B}
CeA	97 ± 18^{A}	76 ± 8^{A}	199 ± 25^{B}	249 ± 44^{B}

Mice were depleted of microglia, gavaged with an acute binge dose of ethanol (6 g/kg, 25% v/v) and sacrificed 18 hours later during withdrawal. Brains were prepared for immunohistochemistry and stained for c-Fos, a marker of activated neurons. c-Fos+ cell density was assessed in several brain regions. Data are presented as mean ± s.e.m. and were analyzed by two-by-two ANOVA for each region followed by Tukey's *post-hoc* test for significant main effects and/or interactions. n=6-12/group. Means that are significantly different are labeled with different letters (e.g. – "A" and "B"). Means that are not significantly different are labeled with the same letters (e.g. – "A" and "A"). CON: control mice gavaged with water, —M: microglial-depleted mice gavaged with water, E: control mice gavaged with ethanol, —M+E: microglial-depleted mice gavaged with ethanol. mPFC: medial prefrontal cortex. NAc: nucleus accumbens. BNST: bed nucleus of the stria terminalis. PVN: paraventricular nucleus of the hypothalamus. CeA: central nucleus of the amygdala.

4.4 Methods

4.4.1 Animals

Male C57BL/6J mice were ordered from the Jackson Laboratory and housed in an animal facility at the University of North Carolina at Chapel Hill. All mice were group housed (n=3-4) in a temperature- (20°C) and humidity-controlled vivarium on a 12 hr/12 hr light/dark cycle, and provided *ad libitum* access to food and water. Experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill, and conducted in accordance with National Institutes of Health regulations for the care and use of animals.

4.4.2 Microglial Depletion and Acute Ethanol Experiment

The CSF1R inhibitor PLX5622 was provided by Plexxikon Inc. (Berkeley, CA) formulated at a dose of 1200 mg/kg in AIN-76A chow by Research Diets (New Brunswick, NJ). Control chow was also provided. Twelve week old mice received either control or PLX5622 chow for 3 weeks. Mice were then acutely gavaged with ethanol (6 g/kg, 25% v/v, i.g.) or an equivalent volume of water and sacrificed 18 hours later (at 15 weeks of age) (Fig1A). All mice were sacrificed mid-morning (9-10 AM).

4.4.3 Microglial Depletion and Intermittent Access (IA) Ethanol Experiment

Fifteen week old mice were acclimated to single housing with no enrichment and a reverse light-dark cycle (lights off at 8 AM) for 2 weeks before undergoing the “Drinking-in-the-Dark” (DID) procedure as previously described [202]. Briefly, for three consecutive days, mice were provided a bottle with 20% ethanol 3 hours into the dark cycle (11 AM) for 2 hours. On the fourth day, mice were provided a bottle with 20% ethanol 3 hours into the dark cycle (11 AM) for 4 hours. No water was available during the ethanol exposure. Tail blood was collected at the end of the

ethanol exposure on the fourth day to assess blood ethanol concentrations (BECs). On each day, the bottles containing ethanol were weighed before and after the procedure, and the grams of ethanol consumed per body weight (g/kg) were calculated for each mouse. The mice were then randomly assigned to either control (n=22) or PLX5622 (n=20) chow. There was no significant difference in body weight (30.1±0.6 g vs 30.1±0.7 g) or ethanol consumption (4.8±0.2 g/kg/4 hrs vs. 4.8±0.3 g/kg/4 hrs) between groups prior to microglial depletion. The mice remained on chow for 4 weeks to ensure maximal microglial depletion [152]. While remaining on the PLX5622 chow, mice underwent another round of DID to determine whether the diet formulation of the CON and PLX Chow altered ethanol consumption. Tail blood was collected at the end of the ethanol exposure on the fourth day to assess blood ethanol concentrations (BECs). While remaining on the PLX5622 chow, mice were then randomly assigned to either a Water or Intermittent Access Ethanol group for a total of four groups: CON Chow – Water, PLX Chow – Water, CON Chow – IA Ethanol and PLX Chow – IA Ethanol. There was no significant differences in body weight (32.8±0.7 g, 32.6±1.5 g, 32.2±1.0g and 32.7±1.0 g, respectively) or ethanol consumption (3.7±0.2 g/kg, 3.4±0.4 g/kg, 3.6±0.2 g/kg and 3.3±0.4 g/kg, respectively) between groups prior to starting Intermittent Access Ethanol. The CON Chow – IA Ethanol mice (n=14) and PLX Chow – IA Ethanol mice (n=12) then received intermittent access ethanol as previously described [203]. Briefly, mice were provided 24 hour access to a bottle of 20% ethanol and a bottle of water 3 hours into the dark cycle (11 AM) on Monday, Wednesday, and Friday. The placement of the ethanol bottle was alternated each day to avoid side preferences. Mice were provided 24 hour access to two bottles of water 3 hours into the dark cycle (11 AM) on Tuesday, Thursday and Saturday. The ethanol and water bottles were weighed before being put on the cage and after being taken off. Body weights of each mouse were measured on Monday and Friday. The grams of ethanol and water consumed per body weight (g/kg)

were calculated for each mouse. Ethanol preference was calculated according to the following formula: ethanol preference = (mL/kg of 20% ethanol)/(mL/kg of 20% ethanol + mL/kg of water). Ethanol consumption was measured for two weeks (Fig1B). Brains were assessed using RT-PCR to verify microglial depletion.

4.4.4 Perfusion and Brain Tissue Preparation

Mice were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4). Brains used for RT-PCR or ELISAs were extracted and immediately frozen in liquid nitrogen. Brains used for immunohistochemistry were perfused with 4.0% paraformaldehyde in PBS, extracted, post-fixed in 4.0% paraformaldehyde/PBS solution overnight and then placed in a 30% sucrose solution in PBS for a few days. Brain tissue was sectioned coronally at a thickness of 40 μm on a sliding microtome (MICROM HM450; ThermoScientific, Austin, TX). Sections were sequentially collected into well plates and stored at −20 °C in a cryoprotectant solution consisting of 30% glycol/30% ethylene glycol in PBS for immunohistochemistry.

4.4.5 mRNA Isolation, Reverse Transcription, and RT-PCR

Total mRNA was extracted from frozen half-brains by homogenization in TRI reagent (Sigma-Aldrich, St. Louis, MO, Cat.# T9424) following the single-step method [173]. Total mRNA was reverse transcribed. Primers used for RT-PCR are listed in Table M1. In all experiments, 18S rRNA was used as a reference gene. SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA, Cat.# 4367659) was used for the RT-PCR. The real time RT-PCR was run with an initial activation for 10 min at 95°C, followed by 40 cycles of denaturation (95°C, 15 s), annealing/extension (57-58°C, 1 min) and finally a melt curve. The threshold cycle (C_T) of each target product was determined and the $\Delta\Delta C_T$ method was used to calculate the percent change

compared to the control group.

4.4.6 Immunohistochemistry

Free-floating sections were washed in 0.1 M PBS, incubated in 0.3% H_2O_2 for 30 minutes and washed again in PBS. For c-Fos and cleaved caspase-3 staining, sections were antigen retrieved by incubating in 10X Antigen Retrieval Citra Buffer (Fisher Scientific, Hampton, NH, Cat.# HK086-9K) diluted to 1X in PBS. Sections were then blocked for one hour at room temperature in 0.25% Triton-X100/5% normal serum (MP Biomedicals, Solon, OH, Cat.# 19135680). Sections were transferred directly from the block to primary antibody diluted in blocking solution and were incubated overnight at 4°C. For Iba1 staining, rabbit anti-Iba1 (WAKO, Japan, Cat.# 019-19741) was used at a dilution of 1:1000. For c-Fos staining, mouse anti-c-Fos (Santa Cruz, Dallas, TX, Cat.# sc-166940) was used at a dilution of 1:300. Finally, for cleaved caspase-3 staining, rabbit anti-cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, Cat.# 9661) was used at a dilution of 1:150. Sections were then washed in PBS, incubated for one hour in biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA), washed and incubated for one hour in avidin–biotin complex solution (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, Cat.# PK6100). The chromogen, nickel-enhanced diaminobenzidine (Sigma–Aldrich, St. Louis, MO, Cat.# D5637), was used to visualize immunoreactivity. Tissue was mounted onto slides, dehydrated, and coverslipped.

4.4.7 Immunofluorescence

Free-floating sections were washed in 0.1 M PBS, incubated in 0.3% H_2O_2 for 30 minutes and washed again in PBS. Sections were antigen retrieved by incubating in 10X Antigen Retrieval Citra Buffer (Fisher Scientific, Hampton, NH, Cat.# HK086-9K) diluted to 1X in PBS, and then blocked for one hour at room temperature in 0.25% Triton-X100/5% normal serum (MP Biomedicals, Solon, OH, Cat.# 19135680). Sections were transferred directly from the block to

primary antibodies diluted in blocking solution and were incubated for 48 hours at 4°C. Primary antibodies included mouse anti-c-Fos (Santa Cruz, Dallas, TX, Cat.# sc-166940) used at a dilution of 1:300, and rabbit anti-NeuN (Abcam, Cambridge, UK, Cat.# ab104225) used at a dilution of 1:3000. Sections were then washed in PBS and incubated for two hours in fluorescent secondary antibodies (Alexa Fluor 594 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse, Molecular Probes, Eugene, OR, Cat.#s A11012 and A11001) used at a dilutions of 1:500. Sections were washed in PBS, then mounted onto slides and immediately coverslipped using Vectashield Antifade Mounting Media with DAPI (Vector, Olean, NY, Cat.# H-1200). Sections were protected from light in a slide box and kept at 4°C until analysis.

4.4.8 Microscopic Quantification and Image Analysis

For staining in the various brain regions, tissue was drawn using the atlas of Paxinos and Watson [197]. For each brain region, approximately two sections were analyzed. A modified stereological profile quantification method was used to quantify immunopositive cell counts within the regions of interest. We have previously published that a comparison of this method with unbiased stereological methodology yielded nearly identical values relative to control subjects [198]. For immunohistochemical stains, BioQuant Nova Advanced Image Analysis (R&M Biometric, Nashville, TN) was used for image analysis. Images of the regions of interest were captured using an Olympus BX50 microscope and Sony DXC-390 video camera linked to a computer. The threshold for positive cells was determined based on control subjects. Sections were imaged under identical conditions, and immunopositive cells were counted by the BioQuant program. The area of the outlined regions of interest was determined and immunopositive cell densities were calculated by dividing cell counts by the overall area (mm^2). For immunofluorescent stains, NIS – Elements AR software (Nikon, Tokyo, Japan) and the Eclipse Ni-E Motorized Microscope System (Nikon,

Tokyo, Japan) were used to capture and analyze images. A threshold for Alexa Fluor 488 intensity (i.e. – c-Fos+ cells) and for Alexa Fluor 594 intensity (i.e. – NeuN+ cells) was determined. Sections were imaged and analyzed under identical conditions. The number c-Fos+ cells that were also NeuN+ positive was divided into the total number c-Fos+ cells to calculate the percent co-localization.

4.4.9 Statistical Analyses

The Statistical Package for the Social Sciences (SPSS; Chicago, IL) was used for all statistical analyses. Data from the Microglial Depletion and Acute Ethanol Experiment was analyzed using a two-by-two ANOVA with significant main effects and/or interactions being further investigated using Tukey's *post hoc* test for multiple comparisons. Data from the Microglial Depletion and Intermittent Access Ethanol Experiment was analyzed using a two-by-two ANOVA to determine significant main effects of microglial depletion on body weight, total fluid intake, water consumption, ethanol consumption and ethanol preference. Note that of a total of 162 data points, 8 CON data points and 13 PLX data points were missing for water and ethanol consumption due to leaked bottles. Where two means are compared, a student's t-test was used. All values are reported as mean ± S.E.M., and significance was defined at a level of $p \leq 0.05$.

4.5 Supplemental Figures

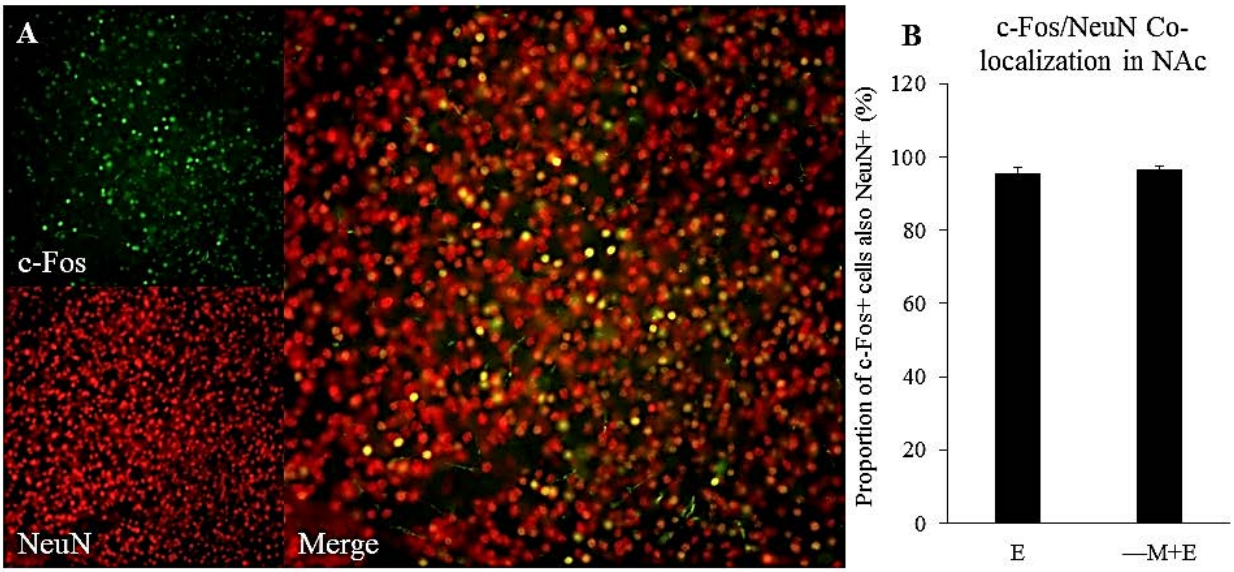


Fig S4.1. c-Fos co-localizes with NeuN in the nucleus accumbens. Microglia were depleted from the brains of mice. Mice were then treated with acute binge ethanol (6 g/kg, 25% v/v, i.g.) and sacrificed 18 hours after treatment during withdrawal. Brains were isolated and processed for immunofluorescence. Sections of NAc were stained for the marker of activated neurons, c-Fos, and the neuronal marker, NeuN. **A.** Representative images of c-Fos and NeuN staining in the NAc are shown separately and merged. c-Fos is displayed in green, while NeuN is displayed in red. Yellow represents co-localization of c-Fos and NeuN in the merged image. **B.** The percentage of c-Fos+ cells that were also NeuN+ were quantified in the ethanol-treated (E) and microglial-depleted and ethanol-treated groups (—M+E). Data are presented as mean ± s.e.m. n=9-12/group.

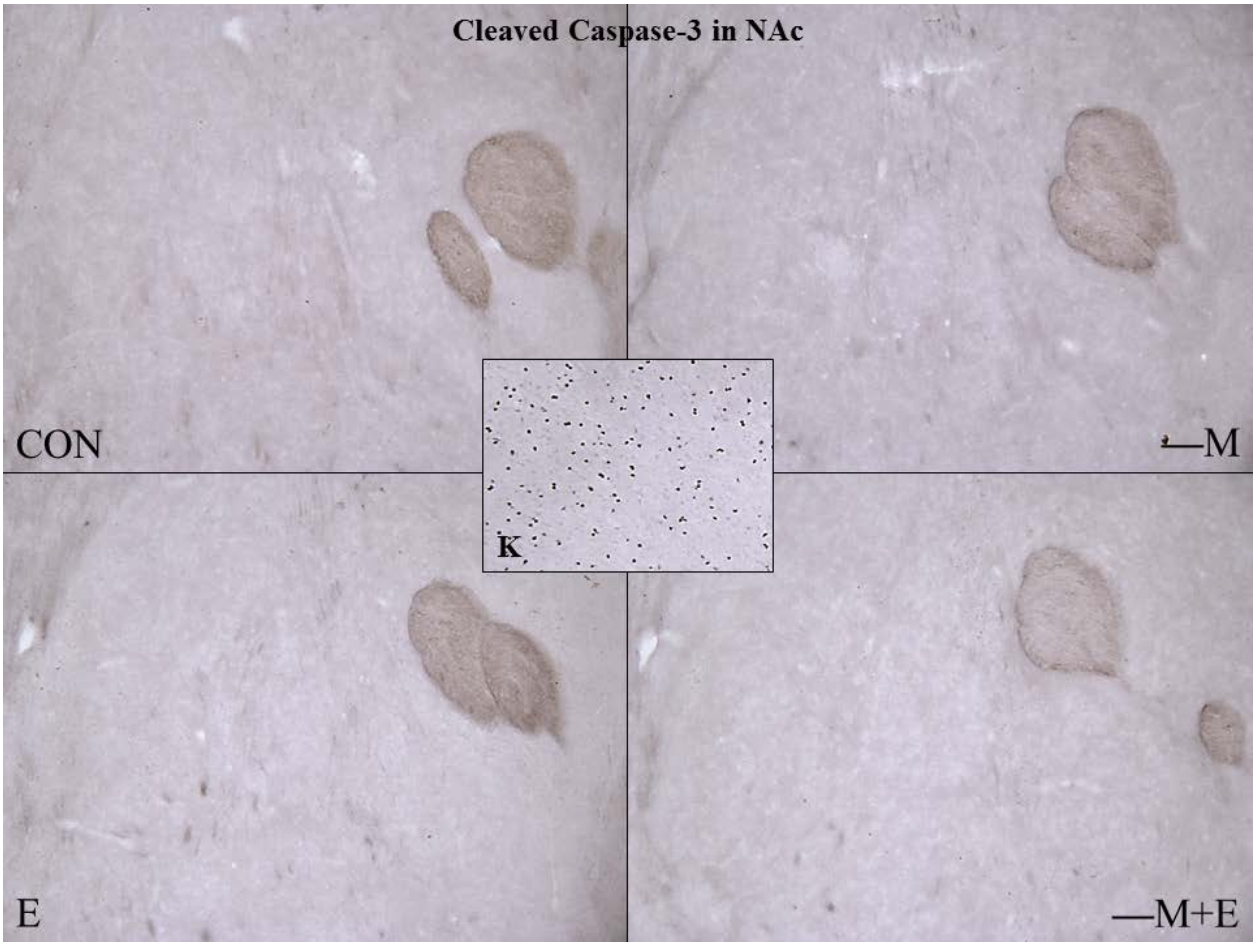


Fig S4.2. Cleaved caspase-3 is not detectable in microglial-depleted and/or ethanol-treated brains. Microglia were depleted from the brains of mice. Mice were then treated with acute binge ethanol (6 g/kg, 25% v/v, i.g.) and sacrificed 18 hours after treatment during withdrawal. Brains were isolated and processed for immunohistochemistry. Sections of NAc were stained for the cell death marker, cleaved caspase-3. Representative images of cleaved caspase-3 staining in the NAc from control mice (CON), microglial-depleted mice (—M), ethanol-treated mice (E), and microglial-depleted and ethanol-treated mice (—M+E) are displayed. Also note the inset in the middle depicting a positive control stain of an animal treated with kainate (15 mg/kg, i.p.).

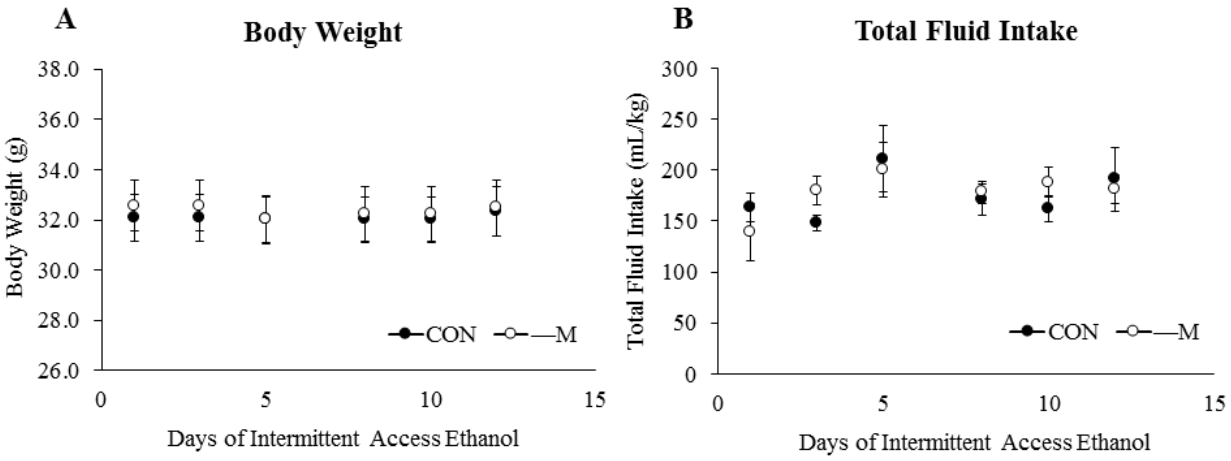


Fig S4.3. Body weight and total fluid intake are unchanged in microglial-depleted mice. Mice received either control or PLX5622 chow for 4 weeks to deplete microglia. The mice then received intermittent access to free choice ethanol on the first, third and fifth day of each week for two weeks. The **A.** body weights and **B.** total fluid intake for each mouse over time were calculated. CON: control chow, —M: microglial-depletion. Data are presented as mean ± s.e.m. n=13-14/group.

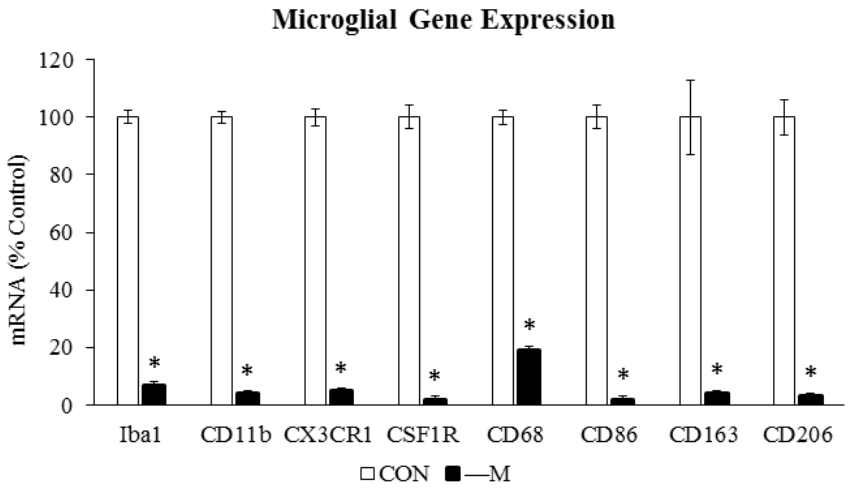


Fig S4.4. Microglial depletion does not alter ethanol-induced anxiety-like behavior or locomotor activity. Mice received either control or PLX5622 chow for 4 weeks to deplete microglia. The mice then received intermittent access to free choice ethanol on the first, third and fifth day of each week for eight weeks. Eight hours after ethanol removal, mice were tested in the open field assay. **A,B.** Center time was recorded for two five-minute intervals as a measure of anxiety-like behavior. Data are presented as mean ± s.e.m. Note that intermittent access ethanol caused an increase in anxiety-like behavior during the first five-minute intervals as evidenced by decreased center time (*$p<0.05$: main effect of ethanol). **C,D.** Locomotor activity was also recorded for two five-minute intervals. Note that neither microglial depletion, nor ethanol impacted locomotor activity.

CHAPTER 5: DISCUSSION

5.1 Summary

In this dissertation, we described work investigating the relationship between microglia and alcohol use disorders. Post-mortem human alcoholic brains show increased microglial markers, implicating microglia in AUDs [10]. Furthermore, a low response to acute alcohol is associated with increased risk for alcoholism [206]. Therefore, in Chapter 2, we examined the effects of acute ethanol exposure and withdrawal on microglia, and how microglial depletion alters the brain neuroimmune response to acute ethanol withdrawal. We found that acute ethanol biphasically changed microglial marker gene expression, with initial decreases during intoxication, followed by later increases during withdrawal. Acute binge ethanol withdrawal dose-dependently increased neuroimmune gene expression, starting at high doses. Cultured microglial-like cells also showed biphasic changes in pro-inflammatory gene expression with ethanol treatment *in vitro*, consistent with direct effects on microglia. Furthermore, microglial depletion changed the expression of some (TNFα, NOX2, etc.), but not all (IL-1β, TLR4, etc.) brain neuroimmune genes. In general, microglial depletion blunted the pro-inflammatory response and enhanced the anti-inflammatory response to acute ethanol withdrawal. Thus, microglial depletion alters the acute response to ethanol.

In Chapter 3, we examined the interactions of acute ethanol and acute stress on microglia and neurons. Cycles of alcohol and stress are thought to jointly drive increased drinking and risk of AUDs; however, much remains unknown. We also examined the long-lasting effects of chronic ethanol on the microglial and neuronal response to acute stress. We found that acute ethanol and acute stress interacted to increase CD11b in multiple brain regions, suggesting enhanced microglial

activation, and decrease neuronal activation in some regions. Acute ethanol and acute stress also increased plasma endotoxin, implying a potential mechanism for the increased microglial activation. We also report that chronic ethanol persistently increased CD11b across the brain and enhanced the microglial CD11b response to acute stress in multiple brain regions without changing the neuronal response. Furthermore, chronic ethanol enhanced the plasma endotoxin response to acute stress. Overall, this work suggests ethanol and stress may interact to enhance gut leakiness, leading to increased plasma endotoxin and enhanced microglial activation. Thus, endotoxin and brain microglia contribute to ethanol and stress-induced responses.

Post-mortem human alcoholic brains show increased microglial markers [10] and immune gene expression [207], and the neuroimmune system has been found to impact alcohol intake [84]. In Chapter 4, we therefore examined the role of microglia in the neuronal response to acute ethanol withdrawal, and how microglia impact ethanol consumption. We found that acute ethanol withdrawal induces c-Fos across the brain, consistent with widespread neuronal activation. Microglial depletion blocked withdrawal-induced c-Fos in some, but not all brain regions, notably the nucleus accumbens and hippocampus. Furthermore, induction of c-Fos occurred in neurons, and the blunting of ethanol-induced c-Fos was not due to cell death. This is consistent with microglia impacting neuronal activity during acute binge ethanol withdrawal. We also report that microglial depletion decreased voluntary ethanol consumption and ethanol preference, without changing body weight or total fluid intake. These findings suggest microglia contribute to the neuronal response to ethanol and can alter ethanol drinking.

5.2 Neuroimmune Gene Expression & Effects of Alcohol on Microglia

These studies find that microglial depletion changes expression of some, but not all neuroimmune genes in the brain. Indeed, genes such as TNFα, NOX2, TLR2 and TLR7 are

decreased with microglial depletion, but genes such as IL-1β, IL-6, TLR3 and TLR4 are not. This suggests that many neuroimmune genes are expressed in other cell types of the brain. This is surprising, as microglia are the primary immune cells of the brain. One might therefore expect microglia to the main cell type expressing these genes. However, our results suggest neuroimmune genes are expressed by other brain cell types. Indeed, astrocytes have been observed to produce IL-1β [39]. Other studies suggest IL-6 is produced by many brain cell types, including neurons [163, 164], astrocytes [40], and endothelial cells [165]. TLR3 and TLR4 have been observed to co-localize with cortical neurons using immunohistochemistry [136, 208]. Furthermore, sequencing studies of the various cell types of the brain find that TLR3 and TLR4 are prominently expressed in cell types other than microglia, including astrocytes and endothelial cells [209]. Overall, these data suggest other cells types such as astrocytes, oligodendrocytes, endothelial cells and even neurons express neuroimmune genes. Future studies could further define the contribution of different brain cell types to neuroimmune gene expression, as clearly neuroimmune signaling in the brain involves multiple cell types.

These studies also sought to define the effects of acute ethanol on microglia. Indeed, understanding the effects of ethanol on microglia *in vivo* is critical, as microglia interact extensively with other cell types of the brain to impact brain function. Microglia alter synaptic plasticity via release of pro-inflammatory cytokines such as TNFα [64] and synaptic formation via release of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) [63]. Microglia can also promote or inhibit neurogenesis [65, 66], and protect against or contribute to neuronal cell death [73, 74] depending on their activation state. In Chapter 2, we find that acute ethanol withdrawal induces pro-inflammatory gene expression (e.g. – TNFα and Ccl2) in microglia *in vitro* and *in vivo*, consistent with an M1 phenotype. Previous studies find varying results regarding the microglial

phenotype induced by ethanol, with some studies suggesting M1 [134] and others suggesting M2 [135]. Microglia are known to exhibit complex, dynamic responses to stimuli [97], and our results suggest this is also true with ethanol. Indeed, acute intoxication decreased microglial markers, while acute withdrawal increased microglial markers. It is possible ethanol induces different microglial activation states depending on the time following ethanol exposure, as well as the dose, duration and pattern of ethanol administration. Consistent with this notion, previous studies find that a single ethanol exposure causes a partial, homeostatic microglial activation, while a second ethanol exposure causes a more robust, pro-inflammatory activation [138]. A complete understanding of the effects of ethanol on microglia will require examining the entire microglial transcriptome. Indeed, recent articles call into question the validity of using one or two markers to assess microglial activation state [69], as emerging evidence suggests microglial activation is far more complex than the M1-M2 theory would suggest. Indeed, microglial activation is probably better understood as a cluster of several different states, rather than a spectrum between two states [70]. Therefore, potential future directions include using RiboTag mice [210] to collect the entire *in vivo* microglial transcriptome following ethanol exposure over time. Data from these experiments would yield novel insights into the dynamic response of the microglial transcriptome to ethanol. Overall, these studies show acute ethanol biphasically modulates microglial markers, inducing a pro-inflammatory state during withdrawal.

Our studies also examine the persistent effects of chronic ethanol on microglia. Chronic ethanol treatment caused long-lasting upregulation of microglial marker, CD11b. CD11b is a component of CR3, which is involved in synaptic pruning. Increased CD11b may therefore contribute to altered synaptic remodeling. This suggests chronic ethanol may cause persistent microglial changes that contribute to altered brain plasticity. Chronic ethanol also sensitized the

microglial CD11b response to acute stress. It is possible enhanced CD11b also leads to enhanced synaptic remodeling. Therefore, chronic ethanol may persistently impact the effect of stress on brain plasticity. Further investigation into potential effects of microglia on synaptic remodeling may provide novel insights into AUDs and treatment strategies. Overall, these data suggest chronic ethanol has persistent effects on microglia that may contribute to changed plasticity.

5.3 Role of Microglia in the Molecular and Cellular Effects of Alcohol

It is also important to understand how alcohol-activated microglia impact the rest of the brain. Indeed, previous studies find that microglia play important roles in the effects of drugs of abuse on the brain [64]. In Chapters 2 and 3, we examined the effects of microglial depletion on the brain response to acute ethanol withdrawal. Our results suggest microglia promote the pro-inflammatory response and suppress the anti-inflammatory response to ethanol withdrawal. This is a significant finding because brain inflammation is associated with decreased neurogenesis [211], increased neuronal cell death [212, 213], and depression-like behavior [98]. The pro-inflammatory effects of ethanol withdrawal may therefore have behaviorally relevant consequences. Various studies find increased inflammatory signaling promotes ethanol consumption [81, 83]. Future directions include sequencing the entire transcriptome of microglial-depleted and ethanol-treated brains. This would enhance our understanding of the role of microglia in the effects of ethanol on the brain. Other directions include microglial depletion and chronic ethanol treatment. It is likely ethanol impacts microglia differently over time, and studying how chronically ethanol-exposed microglia impact the brain could provide valuable information. It would also be interesting to explore whether anti-inflammatory medications block the effects of ethanol withdrawal on the brain. Overall, further investigation into how microglia impact the brain immune response to ethanol may provide novel insights into alcohol use disorders and treatment strategies.

Our results also suggest microglia contribute to withdrawal-induced neuronal activation. Indeed, microglial depletion blunted ethanol-induced c-Fos in the nucleus accumbens and the hippocampus. c-Fos induction occurred in neurons and decreases were not due to cell death. This suggests microglia mediate the effects of ethanol withdrawal on neuronal activity. This finding is significant because it provides a potential link between microglia and alcohol-related behaviors. Indeed, the nucleus accumbens is involved in reward and reinforcement, and the hippocampus is involved in learning and memory, processes that are dysregulated with addiction. Future studies could determine which neuronal populations in these brain regions are being affected and how. The nucleus accumbens contains several different kinds of neurons, including D1-expressing and D2-expressing GABAergic medium spiny neurons, and parvalbumin and cholinergic interneurons [214], while the CA3 region of the hippocampus contains glutamatergic pyramidal neurons. Performing slice electrophysiology in microglial-depleted sections exposed to ethanol would provide fascinating insights into the relationship between ethanol, microglia and neuronal activity. Other future directions include treating mice with anti-inflammatory drugs to see if they reverse the effects of ethanol withdrawal on neuronal activation. Overall, further investigation into how microglia impact the neuronal response to ethanol may provide novel insights into alcohol use disorders and treatment strategies.

5.4 Interactions of Alcohol and Stress on Microglia

Whether stress and alcohol interact to affect microglia could provide novel insights into the development of alcohol use disorders. Indeed, both stress [183] and alcohol [200] activate microglia, and activated microglia impact neuronal function [72]. Therefore, stress and alcohol may interact to enhance disease processes in the brain. Our results find that acute ethanol and acute stress increase microglial CD11b across the brain. Ethanol and stress also enhance plasma endotoxin, suggesting a

mechanism for increased microglial activation. Furthermore, chronic ethanol persistently sensitized stress-induced microglial CD11b in several brain regions. This was associated with increased plasma endotoxin. Overall, these results suggest ethanol and stress interact to enhance gut leakiness, increase plasma endotoxin and induce microglial activation across the brain. Previous research finds that alcohol increases gut permeability, leading to increased leakage of bacterial products into the periphery [215]. This contributes to increased peripheral inflammation, which is thought to alter neuroimmune signaling and contribute to the symptoms of alcohol use disorders [9, 215]. Our results suggest that stress enhances these processes. This raises the intriguing possibility that stress makes alcohol more "inflammatory," thereby exacerbating disease processes. Additional experiments to test this hypothesis include measuring immune molecules in the periphery and central nervous system following acute ethanol and acute stress. Measuring plasma levels of other bacterial products, such as peptidoglycan, would further test the hypothesis of increased gut leakage. One fascinating future experiment would be to sterilize the gut with antibiotics and determine whether microglial activation with alcohol and/or stress still occurs. Other future directions include testing whether anti-inflammatory compounds block the interaction of ethanol and stress. Overall, further investigation into the relationship between alcohol, stress and microglia may provide additional insight into alcohol use disorders and treatment strategies.

5.5 Role of Microglia in Alcohol Drinking Behavior

Whether microglia contribute to alcohol drinking behavior is critical to understanding if they would make tractable targets for the treatment of alcohol use disorders. Indeed, microglia are unique, not only among brain cells, but also among macrophage-like cells [60]. Microglia selectively express a variety of receptors, such as TREM2 and Gpr34 [60], that are involved in microglial activation and the neuroimmune response [216, 217]. Microglia may therefore provide novel and relatively

selective targets for the treatment of alcohol use disorders. Indeed, our results find microglial depletion reduced ethanol consumption and ethanol preference in mice without altering body weights or total fluid intake. The specific decrease in ethanol consumption suggests microglia contribute to the reinforcing effects of ethanol, rather than generally reducing consumptive behavior. Previous studies find that the immune system impacts voluntary drinking behavior [79, 176]. These data are also consistent with microglia impacting the ethanol response of nucleus accumbens neurons. Indeed, microglial depletion may reduce ethanol consumption through effects on NAc neurons. Overall, these findings are significant because they suggest microglia may be therapeutic targets for alcohol use disorders. Future directions include studies investigating the mechanisms by microglia impact drinking behavior. Indeed, previous studies have deleted genes such as TNFα and BDNF specifically from microglia and found effects on behavior [63, 64], including behavioral sensitization to substances of abuse [64]. Examining the drinking behavior of mice carrying deletions of TNFα or BDNF specifically in microglia could provide insight into the mechanism by which microglia reduce drinking. Indeed, while CSF1R inhibitors are in human clinical trials for treatment of various cancers, it is unclear if microglial depletion would be a viable strategy for treating alcohol use disorders in humans. Targeting microglia or the neuroimmune system in other ways may help with treating alcohol use disorders. For example, if microglial inflammatory activity contributes to alcohol consumption, treating with anti-inflammatory compounds may be effective. Overall, further investigation into how microglia reduce ethanol consumption could provide novel insights into treatment strategies.

5.6 Conclusion

In this dissertation, we described work investigating the relationship between microglia and alcohol use disorders. In Chapter 2, we examined the effects of acute binge ethanol

on microglia and how microglia impact the brain immune response to acute ethanol withdrawal. We found that acute ethanol biphasically changed microglial marker and immune gene expression *in vitro* and *in vivo*. Furthermore, microglial depletion blunted the pro-inflammatory response and enhanced the anti-inflammatory response to acute ethanol withdrawal. In Chapter 3, we examined the role of microglia in the neuronal response to ethanol, and how microglia impact ethanol consumption. Microglial depletion blocked ethanol withdrawal-induced neuronal activation in the nucleus accumbens and hippocampus. Microglial depletion also decreased voluntary ethanol consumption and ethanol preference. In Chapter 4, we examined the effects of acute and chronic ethanol on microglia and their response to acute stress. We found that acute and chronic ethanol interact with acute stress to increase plasma endotoxin and microglial activation in multiple brain regions. Overall, this work suggests microglia play an important role in the molecular, cellular and behavioral aspects of alcohol, and may represent novel targets for AUD therapy.

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