NEUROIMMUNE CONSEQUENCES OF CHRONIC ALCOHOL EXPOSURE: RELATIONSHIP TO STRESS

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ABSTRACT

BUDDY A. WHITMAN: Neuroimmune consequences of chronic alcohol exposure: relationship to stress.
(Under the Direction of Dr. George R. Breese)

Alcoholism is characterized by tolerance to alcohol, withdrawal signs or symptoms, and continued use despite detrimental physical or psychological consequences. Studies have illustrated the deleterious effects of prolonged and excessive alcohol use extend to many organs including the liver, lungs, stomach, and brain. While the brain represents the most important organ in the development and maintenance of alcoholism, the neurobiological mechanisms underlying its role are not sufficiently understood to permit effective and timely intervention. Recent work has demonstrated that alcohol-associated changes in neuroimmune function may contribute to the progression of alcoholism. To better understand the role of alcohol in neuroimmune system dysregulation, changes in cytokine expression were determined after alcohol exposure in rats. While acute alcohol administration did not increase proinflammatory cytokines measured in the cerebral cortex, chronic alcohol administration produced a robust effect. Additionally, HMGB1, an endogenous activator of the neuroimmune system, was elevated following chronic alcohol administration. Stress, a major contributor to relapse in abstinent alcoholics, activates the neuroimmune system and, like alcohol, has been shown to increase cytokines in brain. The effects of stress and chronic alcohol on brain cytokines were examined in combination. A stress challenge following chronic alcohol exposure caused a more pronounced increase in

brain cytokines. Because HMBG1 was elevated following chronic alcohol exposure, the possibility that stress was contributing to the release of HMGB1 was examined. Blockade of HMBG1 with two different antagonists reduced stress-induced increases in cytokines following chronic alcohol exposure. This work illustrates that 1) chronic alcohol exposure increases neuroimmune activity and 2) stress following chronic alcohol exposure causes a greater activation of the neuroimmune system. Blockade of HMGB1 blocked the stress-induced production of cytokines following chronic ethanol exposure. Therefore, this work highlights a critical pathway of activation associated with chronic alcohol use and provides a valuable target system for future therapies.

To three special people who helped me along this journey: the late Dr. Alfred M. Dufty Jr., and Drs. Ann Stuart and John Moore. I could not have accomplished this work without your support.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
BAL	blood alcohol levels
CCL2/MCP1	chemokine (C-C motif) ligand 2, or monocyte chemotactic protein-1
CD	control diet
COX2	cyclooxygenase 2
CRF	corticotropin releasing factor
CRID	cytokine release inhibitory drug
DAMP	damage-associated molecular patterns
DNA	deoxyribonucleic acid
ED	ethanol diet
ERK	extracellular signal-related kinases
fMRI	functional magnetic resonance imaging
HMGB1	high-mobility group protein B1
hr	hour or hours
i.g.	intragastric
i.p.	intraperitoneal
IL1-β	interleukin-1 beta
IL-6	interleukin-6
IRF	interferon regulatory factor
LPS	lipopolysaccharide
min	minute or minutes
mRNA	messenger ribonucleic acid

MyD88	myeloid differentiation factor-88
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PAMP	pathogen-associated molecular pattern
PRR	pattern-recognizing receptor
qPCR	quantitative real-time polymerase chain reaction
RAGE	receptor for advanced glycation end product
SEM	standard error of the mean
TLR	toll-like receptor
TLR4	toll-like receptor 4
TNFα	tumor necrosis factor-alpha
TRIF	TIR-domain-containing adaptor-inducing interferon-β

CHAPTER I:

GENERAL INTRODUCTION

Current diagnostic criteria define alcohol dependence by the presence of at least three of the following criteria for more than a year: tolerance, alcohol withdrawal signs or symptoms, drinking more than intended, unsuccessful attempts to reduce drinking, excessive time spent related to alcohol, impaired social or work activities due to alcohol and continued use despite physical or psychological consequences (DSM IV, 1994). Alcohol abuse is defined by the presence of a minimum of one of the following criteria for more than a year: role impairment, hazardous use, legal problems, or social or interpersonal problems due to alcohol (DSM IV, 1994). The lifetime prevalence of alcohol dependence is 12.5%; that of alcohol abuse is 17.8% (Hasin et al., 2007). According to the latest report from the World Health Organization, alcohol is responsible for 4.5% of the total disease burden worldwide (WHO, 2009). These statistics underscore the importance of research into the mechanisms underlying alcoholism.

Although alcohol abuse affects many organs throughout the body, its effects on the brain may represent the most significant component in maintaining this abuse. A number of factors have been implicated in initiating and sustaining alcohol abuse and alcoholism. In addition to neuronal factors, dysregulation of the neuroimmune system has recently come to light as playing a significant role in an adaptive process induced by chronic ethanol exposure (Crews et al., submitted; He and Crews, 2008; Pascual et al., 2011; Qin et al., 2008). Further, chronic ethanol exposure has been found to enhance the effects of stress (Breese et al., 2004;

reviewed in Breese et al., 2011), which may also involve the neuroimmune system (Breese et al., 2008; Knapp et al., 2011).

The Neuroimmune System

The innate immune response in brain consists of a rapid response to stimuli from microglia and infiltrating macrophages (Glezer et al., 2007). Microglia respond to various signals via specific pattern-recognizing receptors (PRR) including the pathogen-associated molecular pattern (PAMP) receptors such as the toll-like receptor (TLR). A key part of the innate immune response, TLRs are responsible for recognizing and responding to pathogens. Activation of toll-like receptor 4 (TLR4) induces a complex signaling cascade resulting in the recruitment of one of two major adaptor molecules myeloid differentiation factor-88 (MyD88) or TIR-domain-containing adaptor-inducing interferon-β (TRIF) (reviewed in Vartanian and Stenzel-Poore, 2010). Differential activation of each pathway is condition dependent and results in a wide array of potential responses (Vartanian and Stenzel-Poore, 2010). For example, recruitment of MyD88 can culminate in transcription of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activation of which induces a proinflammatory response. Recruitment of TRIF can culminate in transcription of interferon regulatory factor (IRF), activation of which induces late-phase NF-κB activation and co-stimulatory molecules (Akira and Takeda, 2004).

Besides responding to exogenously introduced pathogens, TLRs recognize endogenous damage-induced alarmins or damage-associated molecular patterns (DAMPS) (Garg et al., 2010). It has recently been suggested that one of these alarmins, high-mobility group protein B1 (HMGB1), in conjunction with changes in the endogenous receptors for HMGB1, may contribute to the dysregulation of the neuroimmune system by ethanol (Crews

et al., submitted; Pascual et al., 2011). Such changes are accompanied by the prolonged increase in cytokines observed following chronic ethanol exposure in mice (Qin et al., 2008) and human alcoholics (He and Crews, 2008).

TLR4 and Alcohol

Recent experimental studies demonstrate a link between alcohol and activation of the neuroimmune system via TLR4 signaling (Alfonso-Loeches et al., 2010; Blanco et al., 2005; Fernandez-Lizarbe et al., 2008; 2009; Wu et al., 2012). It has been proposed that ethanol's action at TLR4 receptors is similar to that of lipopolysaccharide (LPS) agonist action on this receptor (Fernandez-Lizarbe et al., 2008). Specifically, Fernendez-Lizarbe et al. (2008) provide evidence that low to moderate concentrations of ethanol facilitate the recruitment of TLR4 receptors into lipid rafts in a manner similar to that of LPS stimulation (Triantafilou et al., 2002). The authors suggest that this mechanism is responsible for the increase in brain cytokines associated with TLR4 signaling.

Results of studies of acute ethanol effects on LPS-induced cytokines have been inconsistent. It has been reported that acute ethanol inhibits LPS-induced, TLR4-mediated proinflammatory responses *in vivo* and *in vitro* (Crews et al., 2006; Dai et al., 2005; Pruett and Fan, 2009). For example, Pruett and Fan (2009) found acute ethanol inhibition of LPS-induced cytokines in serum and peritoneal fluid 24 hr following a 6 g/kg (i.g.) dose of ethanol as well as after doses as low as 3 g/kg (i.g.) (Pruett and Pruett, 2006). On the other hand, Qin et al. (2008) found that, 24 hr after a single intragastric (i.g.) dose of ethanol (5 g/kg), the LPS-induced chemokine (C-C motif) ligand 2 (CCL2) and tumor necrosis factor-alpha (TNFα) were not inhibited by acute ethanol in brain, serum, or liver and that the

interleukin-1 beta (IL1-β) response was potentiated. Considered in aggregate, these observations suggest multiple modes of neuroimmune action for acute ethanol.

In contrast to acute ethanol, chronic ethanol exposure is characterized by elevated levels of proinflammatory cytokines in the liver (Qin et al., 2008; Valles et al., 2003), brain (Qin et al., 2008), and serum (McClain and Cohen, 1989; McClain et al., 1999; Qin et al., 2008). Additionally, prior chronic ethanol exposure potentiates the effects of LPS challenge on cytokines (Crews et al., 2006; Qin et al., 2008).

Knockout mouse models of TLR4 exhibit protection from various consequences of chronic ethanol exposure in accord with a relationship to TLR4 function. For example, Uesugi et al. (2001) found TLR4-deficient mice to be protected against ethanol-induced liver injury. The protective nature of TLR4 knockout extends further to prevention of the inflammatory response induced by ethanol in glia (Alfonso-Loeches et al., 2010; Fernandez-Lizabre et al., 2009) and protection from neurodegeneration induced by chronic ethanol intake (Alfonso-Loeches et al., 2010). Further, Pascual et al. (2011) found that the persistent memory deficits and anxiety-related behavioral impairment seen in wild-type mice following chronic ethanol exposure were not observed in TLR4 knockout mice. Wu et al. (2012) found that inhibition of the TLR signaling pathway either by knockout or pharmacological means reduced the sedation and motor impairment associated with ethanol. Taken together, these studies show a critical role of TLR4 signaling in the pathology associated with chronic ethanol exposure.

HMGB1

Originally identified as a nonhistone deoxyribonucleic acid (DNA) binding protein responsible for nucleosome stabilization and facilitation of transcription (Ellwood et al.,

2000; Goodwin et al., 1973), high-mobility group protein B1 (HMGB1) has a second, extracellular role in the mediation of inflammation (Kim et al., 2006; Park et al., 2004; Yang et al., 2005). Extracellular HMGB1 has been implicated in the pathology of many diseases including liver and lung inflammation (Abraham et al., 2000), sepsis (Wang et al., 1999), arthritis (Lotze and Tracey 2005), cancer (Lotze and Tracey 2005), epilepsy (Maroso, et al., 2010), and multiple sclerosis (Andersson et al., 2008). HMGB1, a nuclear associated protein, is released from cells either passively (e.g., necrotic cells; Scaffidi et al., 2002) or actively [e.g., activated macrophages and monocytes (Wang et al., 1999) and neurons and astrocytes (Faraco et al., 2007)]. Active secretion of HMGB1 involves several steps: (1) HMGB1 exclusion from the nucleus due to hyper-acetylation (Palumbo et al., 2004); (2) sequestration into cytoplasmic secretary lysosomes; and (3) exocytosis (Gardella et al., 2002; Yang et al., 2011). Once released, extracellular HMGB1 can bind directly to TLR2/4 and to the receptor for advanced glycation end product (RAGE) to instigate inflammatory responses (Green et al., 2009; Park et al., 2006; Rovere-Querini et al., 2004; Yu et al., 2006). HMGB1 is a key regulator of the innate immune response and works in a feed-forward mechanism to activate RAGE, TLR2, and TLR4 (Yang et al., 2010). Activation of RAGE by HMGB1 leads to activation of ERK and p38 pathways to activate transcription factor NF-κB (Rauvala and Rouhiainen, 2007). Activation of TLR2 or TLR4 by HMGB1 also leads to the activation of NF-κB through the MyD88 pathway (Park et al, 2004). Evidence also suggests that HMGB1 functions as a universal sentinel for nucleic acid-mediated innate immune responses (e.g., nucleic-acid-sensing TLRs 3,7,9) (Yanai et al., 2009) and may also enhance proinflammatory responses by binding to cytokines and potentiating their actions (Sha et al., 2008).

Stress and the Neuroimmune System

Stress differs from traditional pathogenic or traumatic mediators of neuroimmune activation in that it is a "sterile" stimulus. Little is known about this recently recognized role of stress as a "sterile" activator of the neuroimmune system (Andersson and Tracey, 2011). However, several studies have demonstrated that various acute stress protocols increase brain cytokines (Blandino et al., 2009; Deak et al., 2005). Knapp et al. (2011) documented that the protein levels of TNFα were elevated in whole brain 4.5 hr following a 1-hr restraint stress in adult rats. Similar results were obtained in adolescent rats. TNFα protein levels were elevated 5 hr- after 45-min or 90-min restraint stress in adolescent rats (Knapp, unpublished data). Not all stressors activate a central proinflammatory response (Deak et al., 2003; Plata-Salaman et al., 2000), however. The effects of stress on neuroimmune function might differ as a function of factors such as duration, intensity, and type of stressor (Hueston et al., 2011). Sugama et al. (2009) showed that acute stress was associated with concomitant activation of microglia and neurons. This association of microglial activation with neuronal activation may explain the rapid activation of the neuroimmune system following stress.

Neuroimmune Sensitization

Results of several studies suggest that alterations in basal levels of regulatory elements of the neuroimmune system may underlie the increased sensitivity to subsequent neuroimmune system challenge. Barnum et al. (2008) were the first to report that individual differences in basal levels of cytokine expression in the brain predicted neuroimmune consequences to an acute stress challenge. Girotti et al. (2011) demonstrated that neuroimmune reactivity can be sensitized by a chronic stress paradigm leading to increased reactivity to a subsequent stress challenge. Ling et al. (2006) reported priming of microglia by immunological stimulation during critical periods. Godbout et al. (2005) reported priming

of microglia resulting in exaggerated inflammatory responses to additional stimuli in aging. While neuroimmune sensitization has thus been consistently observed, the mechanisms of neuroimmune sensitization have not been elucidated to date.

Research Objective

Several lines of evidence suggest that neuroimmune activation and, ultimately, sensitization to such activation could be persistent consequences of continuous ethanol exposure. Ethanol has been shown to increase cytokines *in vivo* and *in vitro* in animal models. Zou and Crews (2010) showed induction of proinflammatory cytokines TNFα, MCP-1, and IL-1β in brain slice cultures exposed to ethanol. Ten days of ethanol exposure (5g/kg/day) significantly increased both messenger ribonucleic acid (mRNA) expression and protein levels of TNFα and interleukin-6 (IL-6) in mice (Crews et al., submitted). Human alcoholics have abnormalities in basal cytokine tone (He and Crews, 2008) and other neuroimmune regulatory elements (Liu et al., 2006; Okvist et al., 2007). Furthermore, when presented with aversive imagery, abstinent alcoholics show enhanced neural activation compared to nondrinkers measured by functional magnetic resonance imaging (fMRI) (Gilman and Hommer, 2008), a finding consistent with the presence of central sensitization to neural activation. Breese et al., (2004) demonstrated a reemergence of anxiety-like behavior induced by a single acute restraint stress in rats 3 days following cessation of chronic ethanol treatment.

Data presented by Crews et al. (submitted) suggest that HMGB1, in concert with TLRs, underlies ethanol-associated neuroimmune activation and the sensitization of the neuroimmune response following chronic ethanol exposure. However, the potential roles HMGB1 and cytokine tone play in stress activation of the neuroimmune system following

chronic ethanol exposure is not currently understood. The purpose of this research was to explore the means by which ethanol and stress alone and in combination influence neuroimmune mechanisms in the brain.

CHAPTER II:

GENERAL METHODS

Animals

Adult male Sprague Dawley rats (Charles-River, Raleigh, NC) were obtained at 180-200g. Upon arrival, animals were group housed and fed RMH3000 rat chow (TestDiets, Richmond, IN) for 2-3 days prior to the start of the study to acclimate to the new environment (temperatures 70-72˚ F; humidity 40-60% ; and light/dark cycle 12hr:12hr, lights on 7:00-19:00hrs). All animals were singly housed for the duration of the experiments herein. All study methods were approved by the University of North Carolina Chapel Hill Institutional Animal Care and Use Committee.

Liquid Diet

A nutritionally complete and calorically balanced liquid diet was made from a lactalbumin-dextrose-based mixture with added vitamins and minerals (Dyets, Bethlehem, PA) and used for the control diet (CD) or ethanol diet (ED) protocols as previously described and used routinely in the lab (e.g., Frye et al., 1983; Knapp et al., 1998; 2007a; 2011; Overstreet et al., 2002; Wills et al., 2008). CD was calorically balanced to the ED with adjustments of the amount of dextrose in each diet. Rats were fed either CD or 7% ethanol (wt/vol) in a modified pair-feeding system as noted under protocols for experiments in this paper (Fig. 2.1 and 2.2). Volumes consumed each day were measured following the end of the dark cycle (09:00 hr). To minimize weight differences throughout the experiments, the volume of CD the control rats received each day was adjusted to the amount of ED the ethanol exposure group drank on the previous day. Additionally, weights were monitored weekly throughout the experiments. Unless otherwise noted, all animals were 24 hr into

withdrawal when sacrificed, well beyond when blood alcohol levels (BALs) would have returned to zero (Overstreet et al., 2002).

Acute Ethanol Protocol

A single dose of ethanol was administered via oral gavage. The 2.75 g/kg oral dose was chosen (Fig. 2.1A) to approximate peak levels (≈185-200 mg% BAL) previously found with chronic ethanol diet protocols (Overstreet et al., 2002; Wills et al., 2008). Four hr following ethanol or saline administration, the rats were sacrificed and brains collected as described below.

Acute LPS Protocol

To determine the effects of a single exposure to LPS (Calbiochem, La Jolla, CA), rats were injected intraperitoneally (i.p.) (250μg/kg at 125 μg LPS/ml) and sacrificed 4 hr later (n=16, Fig. 2.1B). Brains were collected and tissue processed as described below.

Continuous Ethanol Protocol

To determine the effects of continuous ethanol exposure, rats were provided with liquid diet (7% wt/vol) for 15 continuous days (Frye et al., 1983) and sacrificed 24 hr into withdrawal (n=32, Fig. 2.1C). Brains were collected and processed for biochemistry as described below.

Chronic LPS Protocol

The effects of chronic LPS administration on brain cytokines were determined after 10 consecutive daily i.p. injections of LPS (250 μg/kg) (n=32, Fig. 2.1D). Animals were sacrificed and brains harvested 24 hr following final injection.

Time Course Following Continuous Ethanol Protocol

To determine the time of peak brain cytokine production and the duration of changes, animals were sacrificed 24, 48, and 72 hr and 7 days following withdrawal from the continuous ethanol protocol (n=40, Fig. 2.1E). Brains were collected and processed as described below.

Multiple Withdrawal Protocol

To compare the effects of continuous ethanol exposure with those of cycled ethanol exposure, rats were exposed to three 5-day bouts of ED separated by 2 days of CD (Overstreet et al., 2002) (n=28, Fig. 2.1F). The rats were sacrificed 24 hr into the third withdrawal period and the brains collected for cytokine measurement.

Continuous Ethanol with Stress Challenge Protocol

To determine the effects of stress following continuous ethanol exposure, rats were provided with ED (7% wt/vol) for 15 continuous days (Frye et al., 1983) and withdrawn for 24 hr prior to a 1-hr restraint stress. Four hr following the restraint stress, the animals were sacrificed and brains were immediately collected and frozen for later processing for biochemistry as described below (n=64, Fig. 2.2A).

Acute Stress Time Course Protocol

To determine the time course of changes in cytokines following acute stress application, rats were subjected to 1 hr of restraint stress and brains were collected 2, 4, 8, 24, and 48 hr later (n=40, Fig. 2.2B).

Continuous Ethanol with HMGB1 Antagonism Prior to Stress Challenge Protocol

To determine if the effects of stress following continuous ethanol exposure could be prevented by blockade of HMBG1 signaling, rats were provided with liquid diet for 15 continuous days and withdrawn for 24 hr. Fifteen minutes prior to a 1-hr restraint stress, rats

were treated with either glycyrrhizin or ethyl pyruvate. Four hr following the restraint stress, the animals were sacrificed and brains were collected and processed for biochemistry as described below (n=40, Fig. 2.2C).

Drug Preparations

LPS was prepared in a solution of sterile saline at 125 μg/ml and administered at 2 ml/kg to equal a dose of 250 μg/kg i.p. Glycyrrhizin was prepared in a solution of sterile saline at 25 mg/ml and administered at 2 ml/kg to equal a dose of 50 mg/kg i.p. (Ohnishi et al., 2011). Ethyl pyruvate was prepared in a solution of sterile saline at 37.5 mg/ml and administered at 2 ml/kg to equal a dose of 75 mg/kg i.p. (Su et al., 2011). All control subjects received i.p. injections of sterile saline at 2 ml/kg.

Brain Tissue Collection

All brains were collected following rapid decapitation. Whole brains were extracted and immediately frozen in isopentane at -25˚ C prior to storage at -80˚ C for later microdissection of the cortex. Cortical tissue was divided into two halves to provide tissue for quantitative real-time polymerase chain reaction (qPCR) analysis.

Real-Time RT-PCR Analysis

Total RNA was extracted from homogenized microdissected cortex regions using Trizol (Invitrogen, Carlsbad, CA) and used for reverse transcription PCR analysis as described elsewhere (Qin et al., 2008). The following primer sequences were used: CCL2, 5'-TCACGCTTCTGGGCCTGTTG-3' (forward) and 5'- CAGCCGACTCATTGGGATCATC-3' (reverse); IL1-β, 5'- GAAACAGCAATGGTCGGGAC-3' (forward) and 5'-AAGACACGGGTTCCATGGTG-3' (reverse); TNFα, 5'- ATGTGGAACTGGCAGAGGAG -3' (forward) and 5'- ACGAGCAGGAATGAGAAGAGG-3' (reverse); TLR4, 5'-

GCCGGAAAGTTATTGTGGTGGT-3' (forward) and 5'-ATGGGTTTTAGGCGCAGAGTTT-3' (reverse); NF-κB, 5'- GGCAGCACTCCTTATCAA -3' (forward) and 5'-GGTGTCGTCCCATCGTAG -3' (reverse); MyD88, 5'- GGCAGGCTGCTAGAGTTGCT -3' (forward) and 5'- TGTGGGACACTGCTCTCCAC-3' (reverse); HMGB1, 5'-GAGATCCTAAGAAGCCGAGA-3' (forward) and 5'- CTTCCTCATCCTCTTCATCC-3' (reverse); β-actin, 5'- ATGGTGGGTATGGGTCAGAAGG -3' (forward) and 5'-GCTCATTGTAGAAAGTGTGGTGCC-3' (reverse). SYBR green PCR master mix (Applied Biosystems, Foster City, CA) was used for qPCR analysis. The cycle time (CT) values were normalized with β-actin to assess the relative differences in expression between groups. Calculated values were expressed as relative change compared to controls set as 100%.

Figures

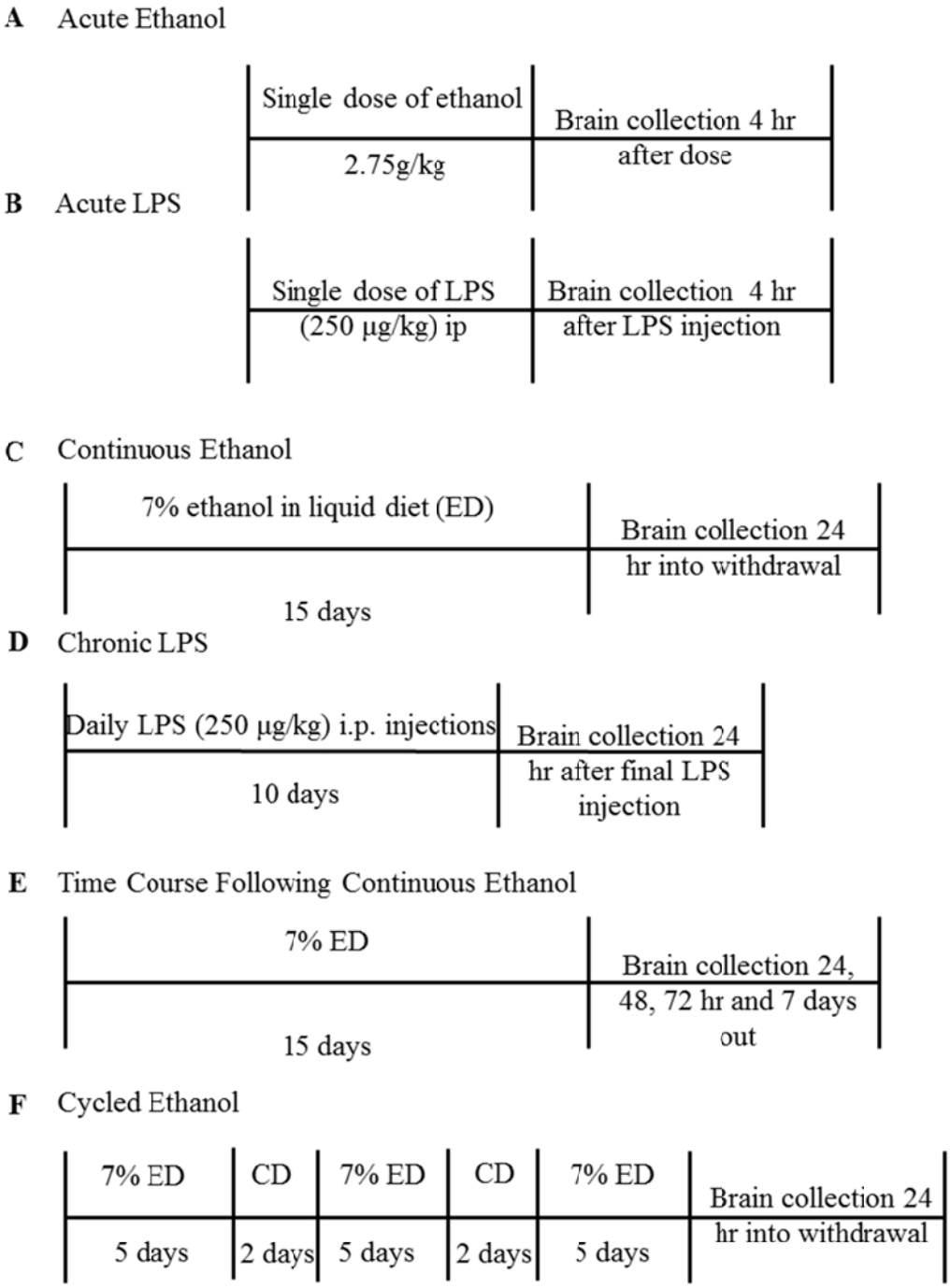

Figure 2.1: Schematics depicting the diet protocols used in Chapter III.

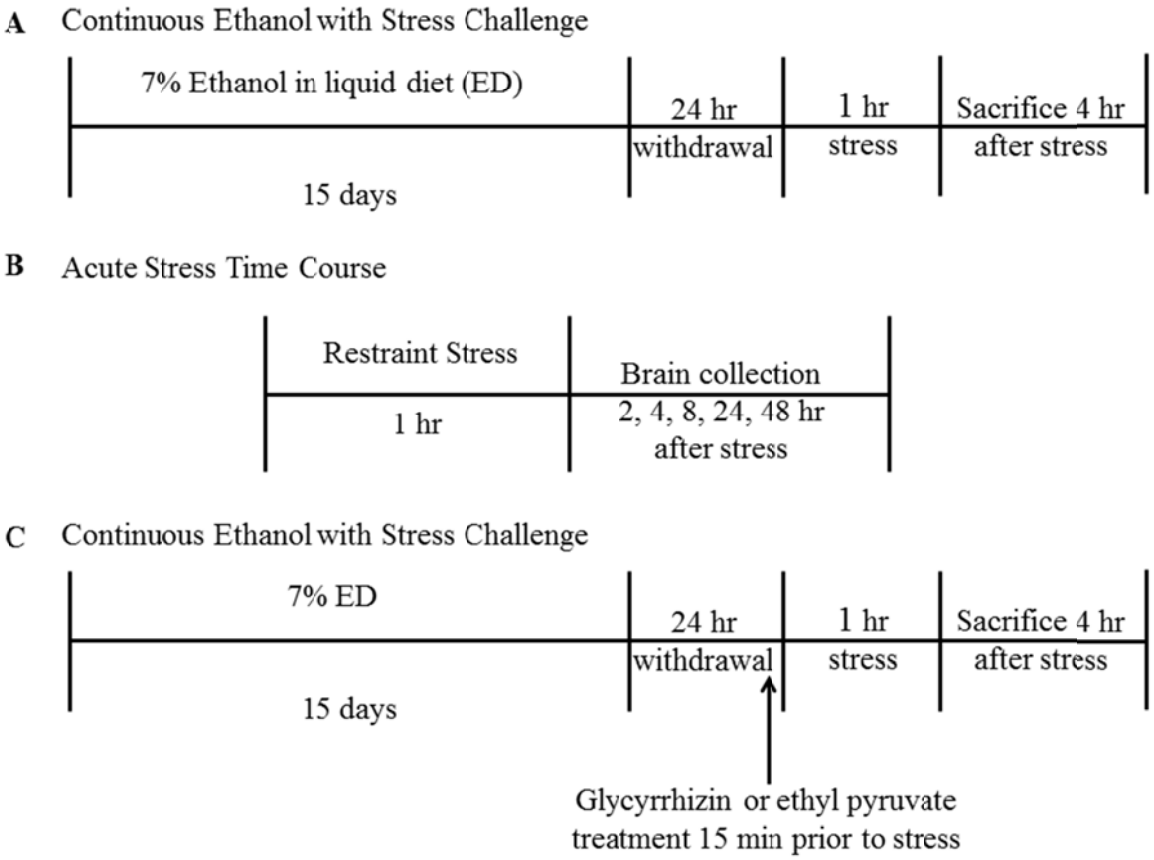

Figure 2.2: Schematics depicting the diet protocols used in Chapter IV.

CHAPTER III:

INCREASED CYTOKINE PRODUCTION IN BRAIN FOLLOWING CONTINUOUS ETHANOL EXPOSURE IS ASSOCIATED WITH ELEVATION OF HMGB1

Introduction

Although alcohol abuse affects many organs throughout the body, its effects on brain contribute most significantly to the pathology of abuse. Among several factors implicated in initiating and sustaining alcohol abuse and alcoholism, dysregulation of the neuroimmune system has recently come to light as playing a significant role in adaptive processes induced by continuous ethanol (Alfonso-Loeches et al., 2010; Breese et al., 2008; Crews et al., submitted; He and Crews 2008; Knapp et al., 2011; Pascual et al., 2011; Qin et al., 2008). Recent experimental studies have demonstrated a link between ethanol and the activation the neuroimmune system via TLR4 signaling (Alfonso-Loeches et al., 2010; Blanco et al., 2005; Fernandez-Lizarbe et al., 2008; Fernandez-Lizarbe et al., 2009; Wu et al., 2012). However, *in vivo* evidence of TLR4 signaling effects of acute and chronic ethanol compared with those of LPS, the prototypical TLR4 ligand, is conflicting (Okun et al., 2009).

It has been proposed that ethanol's action at TLR4 receptors is similar to that of (LPS) agonist action on this receptor (Fernandez-Lizarbe et al., 2008). Specifically, Fernendez-Lizarbe et al. (2008) provide evidence that low to moderate concentrations

of ethanol facilitate the recruitment of TLR4 receptors into lipid rafts in a manner similar to that of LPS stimulation (Triantafilou et al., 2002). Crews et al. (submitted) found increased expression of TLR4 in post-mortem human alcoholic frontal cortex as well as in mice following extended continuous ethanol administration. Further, knockout mouse models of TLR4 have shown protection from various consequences of continuous ethanol exposure in accord with an involvement of TLR4 in ethanol action. For example, Uesugi et al. (2001) found that TLR4- deficient mice were protected from ethanol-induced liver injury. This protective effect of knocking out TLR4 extends to prevention of the inflammatory response induced by ethanol in glia (Alfonso-Loeches et al., 2010; Fernandez-Lizabre et al., 2009) and the neurodegeneration induced by chronic ethanol intake (Alfonso-Loeches et al., 2010). Pascual et al. (2011) found that the persistent memory deficits and anxiety-related behavioral impairment seen in wild-type mice following chronic ethanol exposure were not observed in TLR4 knockout mice. Taken together, these studies implicate TLR4 signaling in the pathology associated with chronic ethanol exposure. Further, Wu et al. (2012) found that inhibition of the TLR signaling pathway either by knockout or pharmacological means reduced the sedation and motor impairment associated with acute ethanol administration. Collectively, these studies provide convincing evidence that TLR4 receptors contribute to ethanol action on brain.

Ethanol is unlikely to be a direct agonist for TLR4 signaling. To shed light on the mechanism of ethanol-initiated, TLR4-mediated changes in cytokines, the present investigation compared the effect of LPS with that of acute and chronic ethanol on brain mRNAs for cytokines and measures of TLR4 signaling.

Materials and Methods

Animals

For standard animal housing and diet procedures, refer to General Methods (Chapter II).

Experimental Protocols

For detailed descriptions of experimental protocols, refer to General Methods (Chapter II). Briefly, acute ethanol exposure consisted of a dose of 2.75 g/kg administered via oral gavage (i.g.) of a 20% wt/vol ethanol and distilled water solution. Control animals for the acute ethanol study received an i.g. volume of water equivalent to the average volume of the ethanol-treated animals. Both control animals and ethanol-treated animals were sacrificed 4 hr following ethanol or water administration and brains collected immediately. For the acute LPS protocol, 250 μg/kg was administered via i.p. injection of 125 μg LPS/ml in sterile water. Control animals for the acute LPS protocol received an i.p. injection of sterile saline equivalent to the average volume of the LPS administered to the LPS treated animals in the study. Both control animals and LPS-treated animals were sacrificed 4 hr following LPS or water administration and brains collected immediately.

The continuous ethanol protocol consisted of ED or CD for 15 consecutive days. ED was provided *ad libitum* to half the animals in the study. CD was adjusted daily to balance calorically the amount the ED animals consumed the previous day. This modified pair-feeding design balances weight gain between control and experimental animals. All animals in this experiment were sacrificed 24 hr into withdrawal for brain collection. Animals in the chronic LPS experiment received i.p. injections of 250 μg/kg for 10 consecutive days. Control animals received i.p. injections of sterile water equivalent to the average volume

given to the LPS-treated animals. All animals in this experiment were sacrificed 24 hr following final LPS injection for brain collection. The protocol for the time course following continuous ethanol exposure is as described above with additional animals sacrificed at 24, 48, and 72 hr and 7 days following removal of ethanol after day 15. All animals were maintained on rat chow following the initial 24-hr withdrawal. Control animals were sacrificed at each time point to account for any differences in age of animals. Animals in the multiple withdrawal experiment received ED for 3 cycles of 5 consecutive days with 2 days of CD between each cycle of ED and were sacrificed 24 hr after withdrawal from the third cycle of ED.

Statistical Analysis

All data were evaluated for statistical significance with student's t-test or analysis of variance (ANOVA) as appropriate using Graph Pad Prism 4.0 (Graph Pad Software, Inc., San Diego, CA) and expressed as mean ± standard error of the mean (SEM). P-values < 0.05 were considered statistically significant.

Results

Comparison of Acute Ethanol to Acute LPS

To evaluate the effects of acute ethanol (Fig. 2.1A) versus acute LPS (Fig. 2.1B) on brain cytokine mRNAs, rats were exposed to an acute dose of either ethanol or LPS. Four hr after administration of the single dose of ethanol (2.75 g/kg), no significant increases in CCL2, IL1-β, or TNFα mRNA ($p>0.05$; Fig. 3.1A) were observed. When an additional group of rats (n=12) was assessed 24 hr following this acute ethanol exposure (2.75 g/kg), no significant increases in CCL2, IL1-β, or TNFα mRNA were observed at this extended period (all $p>0.05$, Fig.3.1, legend). Unlike acute ethanol, which did not affect cytokine mRNA

levels, an acute dose of LPS (250 μg/kg) caused a several-fold increase in CCL2 (432%), IL1-β (337%), and TNFα (920%) mRNAs (all $p<0.0001$ Fig. 3.2B). Neither an acute dose of ethanol nor an acute dose of LPS increased TLR4 mRNA at 4 hr (all $p>0.05$; Fig. 3.1C & 3.1D). Thus, while acute ethanol exposure did not affect cytokine mRNAs at 4 or 24 hr after the exposure, acute LPS caused a several-fold increase in mRNAs for CCL2, IL1-β, and TNFα at 4 hr. Neither exposure affected TLR4 mRNA. The effects of acute ethanol on cytokine mRNAs, thus, clearly differ from those of acute LPS.

Comparison of Continuous Ethanol to Chronic LPS

To assess potential differences in the effect of continuous ethanol and chronic LPS exposure on cytokines in brain, mRNAs for CCL2, IL1-β, and TNFα were measured 24 hr following 15 consecutive days of ethanol exposure (7% ethanol wt/vol in liquid diet) or following 10 daily doses (250 μg/kg) of LPS. The mRNAs for all cytokines showed significant increases over controls (CCL2, 144.9%; IL1-β 97.3%; TNFα 125.0%; all $p<0.05$, Fig. 3.2A) 24 hr following the continuous ethanol protocol. In contrast, 24 hr after the chronic LPS protocol, no significant increases were observed for any of these cytokine mRNAs (all $p>0.05$; Fig. 3.2B). A significant increase in TLR4 mRNA was found 24 hr after both the continuous ethanol protocol (63.3% increase) and the chronic LPS (79.2% increase) protocol (all $p<0.05$, Fig. 3C & 3D, respectively). The effects of continuous ethanol on cytokine mRNAs, thus, clearly differ from those of chronic LPS.

Time Course of Changes in Cytokines Following Continuous Ethanol

In previous studies in mice, increased brain neuroimmune gene expression induced by 5 months of ethanol liquid diet was dependent upon TLR4 receptors (Alfonso-Loaches et al., 2010). In other mouse studies, chronic ethanol exposure increased neuroimmune gene

expression 24 hr after the last exposure (Pascual et al., 2007; Qin, et al., 2008). In rats, 16 hr of abstinence from chronic ethanol exposure was associated with induction of a peak expression of cyclooxygenase 2 (COX2), a proinflammatory oxidase (Knapp and Crews, 1999).

Based upon these previous findings, mRNA expression for CCL2, IL1-β, and TNFα was measured in brain at various times (0 and 24 hr and 3 and 7 days) after 15 days of continuous ED (Fig. 2.1C). No change in cytokines was observed in the rats sacrificed while still exposed to ethanol, (i.e., T=0; no abstinence; Fig. 3.3). However, after 24 hr of abstinence, expression of CCL2 increased by 102%, IL1-β by 93.8%, and TNFα by 107% (all $p<0.05$)—findings in agreement with results in Fig. 3.2. Following the increase after 24 hr of ethanol abstinence, cytokine mRNAs gradually declined toward control levels by 3 days (72 hrs.); CCL2= 67.7%, IL1-β=100.9%, and TNFα=60.7% increase over contols (Fig. 3.3). The mRNA levels for all cytokines had returned to control levels by 7 days (Fig. 3.3). These findings confirm that continuous ethanol exposure followed by abstinence for 24 hr increases mRNA expression of CCL2, IL1-β, and TNFα in brain. Values peak at 24 hr, and changes do not persist beyond 7 days.

Comparison of Continuous and Cycled Ethanol

Cycled ethanol exposure increase withdrawal symptoms such as susceptibility to seizures and anxiety-like behavior more than a comparable continuous alcohol exposure does (McCown and Breese, 1990; Overstreet et al., 2002). This observation prompted studies to determine if differences in proinflammatory gene induction are observed after these different modes of chronic ethanol exposure (Breese et al., 2005, 2011). It was found that CCL2, IL1-β, and TNFα brain mRNA significantly increased with both continuous ethanol exposure

(CCL2, 79.8% increase; IL1-β, 102.3% increase; TNFα, 96.2%) and cycled ethanol exposure (CCL2, 70.5% increase; IL1-β, 82.0% increase; TNFα, 108.4%) 24 hr after ethanol withdrawal (all $p<0.05$ vs. control; Fig. 3.4A). However, no difference between the continuous ethanol group and the cycled ethanol was observed ($p>0.05$; continuous vs. cycled ethanol). Like the cytokine mRNAs, TLR4 mRNA was increased by both the continuous ethanol exposure (122.6%) and the intermittent cycled ethanol exposure (85.2%) ($p<0.05$), but the increases did not differ between groups ($p>0.05$; Fig. 3.4B). Data from this study replicate results from the continuous ethanol investigation (Fig. 3.2), in which CCL2, IL1-β, TNFα, and TLR4 mRNAs were initially found to be increased 24 hr after ethanol withdrawal. Continuous ED and cycled ED increased expression of brain CCL2, IL1-β, TNFα and TLR4 mRNA to similar degrees after 24 hr of abstinence from ethanol.

Effects of Continuous and Cycled Ethanol on HMGB1, My88, and NFκB

In addition to determining mRNAs for cytokines for both continuous and cycled ethanol (Fig. 3.4A), mRNA changes in TLR4 (Fig. 3.4B) and the mRNAs for HMBG1, MyD88 and NFκB (Fig. 3.4C) were determined in these groups. The mRNA for TLR4 was increased by both ethanol protocols (Fig. 3.4B) as was the mRNA for HMGB1 (continuous ethanol, 64.4%; cycled ethanol, 57.3%; $p<0.05$; Fig. 3.4C). However, the difference between the continuous ethanol group and the cycled ethanol group in changes in mRNA for HMGB1 was not statistically significant ($p>0.05$). Further, neither continuous nor cycled ethanol caused a significant change in levels of MyD88 or NF-κB mRNA ($p>0.05$; Fig. 3.4C). Thus, both the continuous and cycled ethanol protocols increased expression of the mRNA for HMGB1, an agonist for TLR4, as well as the mRNA for the TLR4 in brain.

HMGB1 Response After Continuous Ethanol vs. Chronic LPS

Whether the increase in HMGB1 by the continuous ethanol protocol would also be observed after chronic LPS exposure was investigated in a study comparing HMBG1 response after continuous ethanol versus chronic LPS. Consistent with results shown in Fig. 3.4C, the continuous ethanol exposure significantly elevated HMGB1 mRNA by 102.8% (Fig. 3.5A) ($p<0.05$) without inducing a significant change in either MyD88 or NF-κB ($p>0.05$) (Fig. 3.5A). Unlike continuous ethanol, chronic LPS did not increase HMGB1, MyD88, or NF-κB (all $p>0.05$) (Fig. 3.5B).

The time course of the HMGB1 change was determined from the tissue utilized in the experiment described in Fig. 3.4. At the time prior to ethanol removal (T=0), the level of HMGB1 was elevated by 65% (Fig. 3.6). By 24 hr, the HMGB1 was significantly elevated to 119% of control ($p<0.05$) in agreement with the increase noted in Fig. 3.5B. Subsequently, HMGB1 mRNA levels decreased to control levels by 72 hr and were 88% below control levels by 7 days after ethanol removal ($p<0.05$; Fig. 3.6). While TLR4 expression was not elevated at T=0 in the rats that received continuous ethanol exposure (Fig.3.6), the TLR4 level was elevated by 68.3% 24 hr after withdrawal from ethanol ($p<0.05$, Fig.3.6) in corroboration of data in Fig. 3.2C. TLR4 mRNA returned to control levels by 72 hr and was 81% below control levels by 7 days ($p<0.05$; Fig. 3.6).

Discussion

LPS increases cytokines by acting as an agonist at TLR4s (Erridge et al., 2002; Okun et al., 2009). Several studies support a role of TLR4 signaling in ethanol-induced increases in cytokine production. For example, in TLR4 knockout mice, ethanol activation of effects mediated by TLR4 signaling are absent in astrocytes (Blanco et al., 2005; Valles et al., 2004), cultured microglia (Fernandez-Lizarbe et al., 2008; Fernandez-Lizarbe et al., 2009), and

brain (Alfonso-Loeches et al., 2010). TLR4 knockout mice also show altered acute ethanol responses (Wu et al., 2012). Fernendez-Lizarbe et al. (2008) suggested that ethanol induces cytokines in brain by causing accumulation of TLR4s and related signaling molecules into lipid rafts to induce signaling through TLR4s. This hypothesis is compelling but incomplete. The mechanism by which continuous ethanol involves TLR4 function to increase cytokines in brain remains unclear, particularly because ethanol cannot act as an agonist on TLR4s. The present studies were undertaken to explore possible mechanisms by which chronic alcohol might influence TLR4 signaling to increase cytokines in brain. Effects on proinflammatory gene expression and signaling proteins of LPS, the prototype TLR4 direct agonist, were compared with those of acute and continuous ethanol.

The results show that an intoxicating acute dose of ethanol did not increase mRNA for CCL2, TNFα, IL1-β, or TLR4 in cortex of Sprague-Dawley rats 4 or 24 hr after administration. Consistent with these findings, Buck et al. (2011) found no increase in mRNAs for IL-6 or IL1-β in Sprague-Dawley rats 12 hr after 4 g/kg of ethanol. However, Qin et al. (2008) found increased mRNAs for TNFα and CCL2, but not IL1β, 24 hr after a single 5 g/kg oral dose of ethanol. The discrepant results of Qin et al. (2008) might be explained by the use of mice rather than rats and/or by use of a higher dose of ethanol in the mouse studies than in the rat studies.

Unlike acute ethanol, which did not cause acute cytokine changes, acute administration of LPS (250 μg/kg) caused a several-fold increase in mRNAs for CCL2, TNFα, and IL1-β by 4 hr. LPS did not increase mRNA for TLR4. The increase in brain cytokines by acute LPS is consistent with published reports (Qin et al., 2007; 2008; Chen et al., 2005). Qin et al. (2007) suggested that the LPS induction is based upon entrance of

blood-borne TNFα into the brain, where it contributes to the cytokine increase by LPS. Whether this mechanism of TNFα entrance from the periphery to brain is critically responsible for the LPS-induced changes observed in the present study could be determined by administering LPS into brain. Regardless, the present findings provide clear evidence for a fundamental difference between the effects of acute LPS on brain cytokine changes and those of acute ethanol at the doses studied.

Unlike acute ethanol, which did not cause acute cytokine changes, continuous ethanol exposure (continuous 7% alcohol diet for 15 days) approximately doubled CCL2, IL1-β, and TNFα in the cortex of rats 24 hr after ethanol removal (Fig. 3). TLR4 levels were also increased after this continuous ethanol protocol. Previous studies in C57Bl/6 mice found that chronic administration of ethanol (5 g/kg, i.g.) for 10 days followed by 24 hr of ethanol abstinence increased brain CCL2 and TNFα but not IL1-β or other cytokine mRNAs (Qin et al., 2008).

Unlike continuous ethanol, chronic LPS (250 μg/kg daily for 10 days) produced no change in CCL2, IL1β, and TNFα mRNA in the cortex of the rats 24 hr after the final LPS dose. Like continuous ethanol, however, chronic LPS induced a significant increase in TLR4. Thus, continuous ethanol differed from chronic LPS in effects on mRNA changes in cytokines although both increased TLR4 mRNA in brain.

A complete time course for cytokine mRNAs induced by continuous ethanol revealed that mRNAs for TLR4 and cytokines were not different from control when the ED had not been removed (i.e., at T=0). In agreement with initial findings, mRNAs for cytokines were significantly increased by 24 hr after the ethanol removal. However, the mRNA levels for these cytokines gradually declined over 3 days (i.e., by 72 hr) and returned to control levels

by 7 days after cessation of continuous ethanol exposure. These findings indicate that chronic 7% ED increases CCL2, IL1β, TNFα, and TLR4 mRNA in brain with peak effects approximately 24 hr after abstinence from ethanol.

Previous studies in this lab established that multiple intermittent cycles of ethanol exposure are more effective than continuous ethanol exposure at engendering symptoms of ethanol withdrawal (Overstreet et al., 2002; Breese et al., 2011) or supporting an increased sensitivity to kindling of seizures (McCown and Breese, 1990). Based on the possibility that multiple withdrawals from alcohol might have a greater effect on the induction of brain cytokines than continuous alcohol exposure, these two modes of chronic alcohol exposure were compared for their ability to induce cytokine mRNAs 24 hr following withdrawal from ethanol. As found earlier, continuous liquid diet exposure increased cortical CCL2, IL1β, and TNFα mRNAs. The chronic intermittent exposure to ethanol (cycled ethanol) also elevated CCL2, IL1β, and TNFα in cortex—changes almost identical to those observed with the continuous alcohol exposure. Further, both continuous and intermittent ethanol treatments increased the mRNA for the TLR4. Thus, effects of chronic intermittent ethanol exposure were comparable to those of continuous ethanol exposure—a finding inconsistent with the hypothesized "kindling process continuum" responsible for facilitation of withdrawal anxiety or facilitation of kindling of seizures (Breese et al., 2005a; 2011). Possibly, kindling-like effects on mRNAs were not observed because the cortex, the brain site examined in the current investigation, does not mediate the kindling-like effects observed after chronic ethanol exposure in previous studies (i.e., increased susceptibility to seizures [McCown and Breese, 1990], facilitation of anxiety [Breese et al., 2011]). This possibility warrants exploration in future investigations.

In the current investigation, mRNA for the endogenous agonist for TLR4s, HMGB1 (Andersson and Tracey, 2011; Lin et al., 2011), was elevated by both continuous and cycled ethanol protocols without significant changes in mRNAs for MyD88 (Janssens and Beyaert, 2002) or NF-κB (Baeuerle and Henkel, 1994; Fitzgerald et al., 2003)—signaling proteins linked to TLR4 function (Fig.3.5C). Based upon the finding that HMGB1 mRNA was nearly doubled 24 hr after the two modes of chronic alcohol exposure, an investigation was initiated to assess HMGB1 mRNA changes over time during and after the continuous chronic alcohol protocol. HMGB1 mRNA was moderately elevated prior to cessation of the chronic alcohol diet (T=0)—a time when TLR4 and cytokine mRNAs were not different from control (Fig.3.6). By 24 hr after cessation of chronic alcohol, the mRNA for HMGB1 was significantly elevated (Fig.3.6) as were the mRNAs for cytokines (Fig. 3.3). HMGB1 and TLR4 mRNAs gradually fell to control levels by 3 days and were below control levels by 7 days after ethanol removal (Fig. 3.6). The time course, prior to and after the continuous ethanol exposure, of the change in HMGB1 mRNA was similar to the time courses of the changes in the cytokine mRNAs. These changes occurred in the absence of effects on MyD88 or NF-κB 24 hr after cessation of continuous ethanol exposure (Fig. 3.5C). This continuous-ethanol-induced increase in HMGB1 and TLR4s possibly explain the ethanol induction of brain TNF, CCL2, and IL1-β.

Although both continuous ethanol and chronic LPS increased TLR4 mRNA in brain, chronic LPS, unlike continuous ethanol, did not significantly change CCL2, IL1β, or TNFα mRNAs in the cortex of the rats 24 hr after the final dose. Considered in aggregate, the results suggest the possibility that HMGB1 may be the endogenous ligand that activates the TLR4 to increase brain cytokines after abstinence from the continuous ethanol protocol. The

intriguing possibility that HMGB1 released onto TLR4s during withdrawal from chronic alcohol may be responsible for the increase in brain cytokines would help to explain earlier findings relevant to TLR4s and ethanol action (Alfonso-Loeches et al., 2010; Blanco et al., 2005; Fernandez-Lizarbe et al., 2008; 2009; Valles et al., 2004).

The capacity for HMGB1 to induce cytokines is documented in the literature (Faraco et al., 2007; Lin et al., 2012; Andersson and Tracy, 2011; Möller et al., 2001; Yang et al., 2005). In a recent study, Crews et al. (submitted) observed that HMGB1 neutralizing antibodies added to brain slice cultures blunted the ability of ethanol to induce IL1-β, a finding consistent with the possibility that ethanol activation of IL1-β occurs through HMGB1/TLR signaling. Similarly, the *in vivo* increase in brain TNF, CCL2, and IL1-β after withdrawal from the continuous ethanol protocol in the current investigation could be linked to ethanol release of HMGB1 onto TLR4s. The present studies provide a foundation for future work to further elucidate the mechanisms of ethanol-associated increases in brain cytokines during excessive exposure to ethanol, ethanol withdrawal or neural injury.

Figures

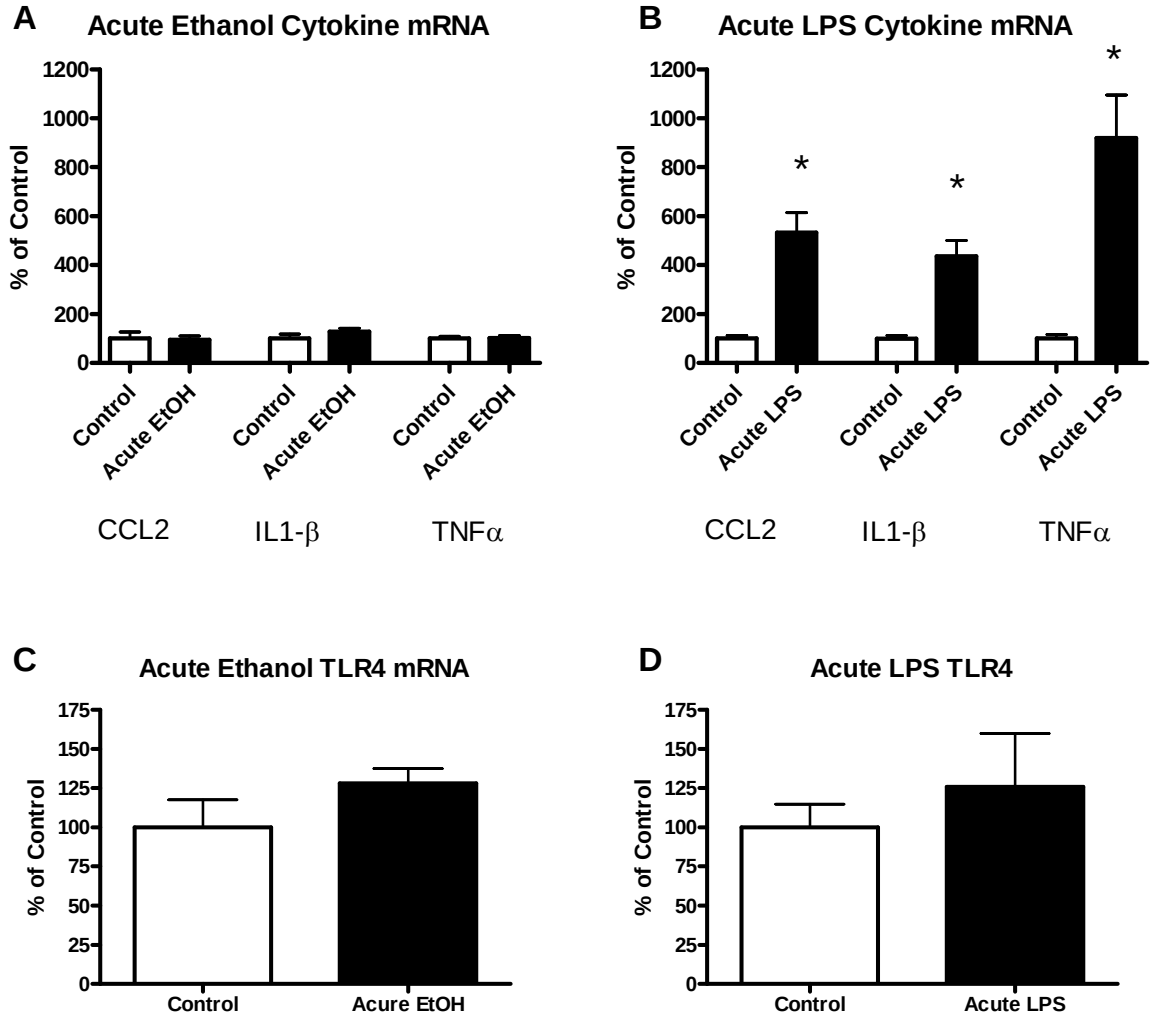

Figure 3.1: Effects of acute ethanol versus acute LPS administration on mRNA for cytokines CCL2, IL1-β, TNFα and for TLR4s in cortex. Cytokine induction was observed following acute LPS (250 mg/kg) but not acute ethanol (2.75 g/kg). A) An acute single dose of ethanol resulted in no significant increase in mRNA of cytokines CCL2, IL1-β, or TNFα (all $p>0.05$) at 4 hr post administration. B) An acute single dose of LPS at 250 mg/kg increased mRNA of cytokines CCL2, IL1-β, and TNFα (CCL2 $t(17)=6.07$, $p<0.0001$; IL1-β $t(16)=6.31$, $p<0.0001$; TNFα $t(13)=4.97$, $p=0.0003$) 4 hr following injection. C) Acute ethanol did not alter TLR4 mRNA expression ($p>0.05$). D) Acute LPS did not alter TLR4 expression ($p>0.05$).

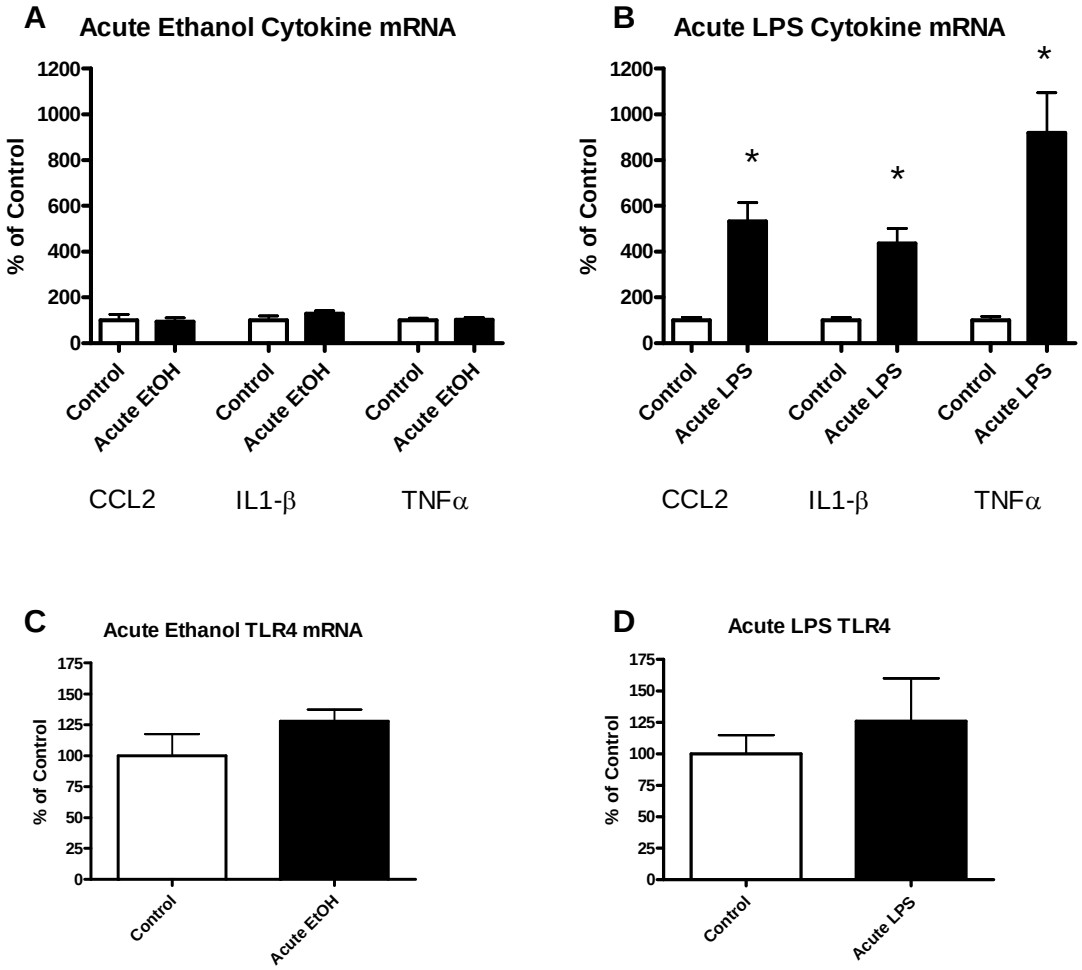

Figure 3.2: Effects of continuous ethanol versus chronic LPS administration on mRNA for cytokines CCL2, IL1-β, TNFα and for TLR4s in cortex. Cytokine induction differed between animals treated with a continuous ethanol protocol and those treated with a chronic LPS protocol. A) Cytokines CCL2, IL1-β, and TNFα were increased 24 hr into withdrawal following continuous ethanol exposure compared to non-treated controls ($t(15)=4.53$, $p=0.0004$; $t(20)=3.42$, $p=0.0027$; $t(21)=5.443$, $p<0.0001$, respectively). B) No significant increase in cytokine mRNA was observed 24 hr after chronic LPS exposure (all $p<0.05$). C) Continuous ethanol exposure significantly increased TLR4 mRNA expression ($t(23)=2.669$, $p=0.0137$). D) Chronic exposure to LPS significantly increased TLR4 mRNA expression ($t(18)=2.83$, $p=0.0112$).

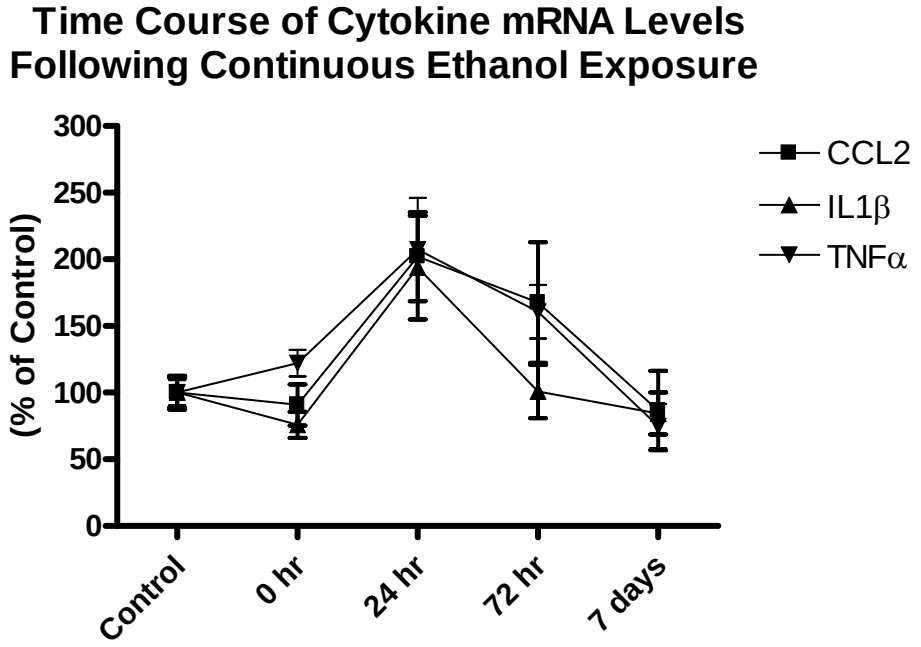

Figure 3.3: Time course of cytokine mRNA following continuous ethanol administration. Peak expression of CCL2, IL1-β, and TNFα was observed 24 hr after withdrawal. A) CCL2 peaked at 24 hr ($F(4,25)= 3.06$, $p=0.035$, Control 100 ± 31.1 vs. 24 hrs. 202.0 ± 33.3, $p=0.025$). IL1-β peaked at 24 hr ($F(4,27)= 5.05$, $p=0.036$, Control 100 ± 11.0 vs. 24 hrs. 193.8 ± 38.9, $p=0.0039$). TNFα also peaked at 24 hr ($F(4,27)=7.30$, $p=0.0004$, Control 100 ± 9.3 vs. 24 hrs. 207.3 ± 38.7, $p=0.0006$).

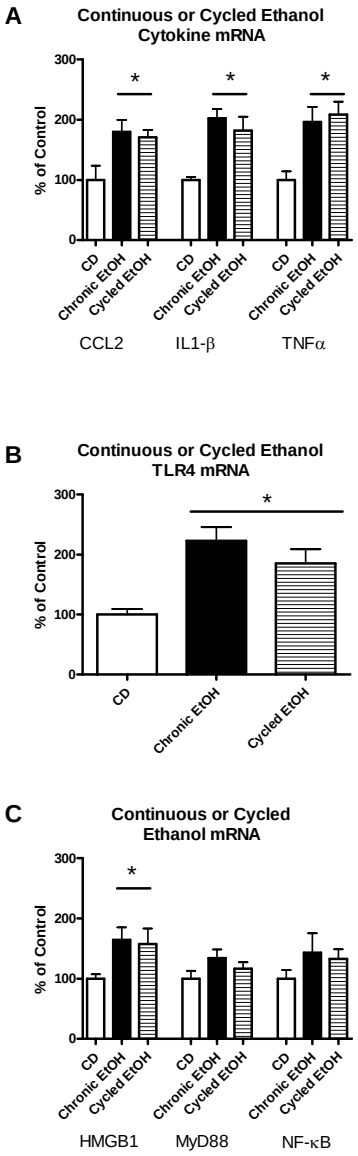

Figure 3.4: Effects of continuous and cycled ethanol exposure on mRNA for cytokines, TLR4 and the signaling molecules HMGB1, MyD88 and NF-κB in cortex. A) No difference in cytokine mRNA was found between continuous ethanol and cycled ethanol exposure. CCL2, IL1-β, and TNFα from both the continuous and cycled ethanol treated groups showed significant increases ($F(2,18)= 5.211$, $p=0.018$; $F(2,24)=9.718$, $p=0.0009$; $F(2,25)=6.475$, $p=0.006$, respectively). No significant differences were observed between the continuous and cycled ethanol treated groups ($p>0.05$). B) TLR4 mRNA was elevated in both the continuous and cycled ethanol groups ($F(2,23)=8.744$, $p=0.0017$), but no differences were observed between the continuous and cycled ethanol exposure groups ($p>0.05$). C) Both continuous and cycled ethanol exposure caused an elevation in HMBG1 mRNA ($F(2,24)=3.787$, $p=0.0466$); no differences were observed between continuous and cycled ethanol groups ($p>0.05$). Neither continuous nor cycled ethanol affected levels of MyD88 or NF-κB ($p>0.05$).

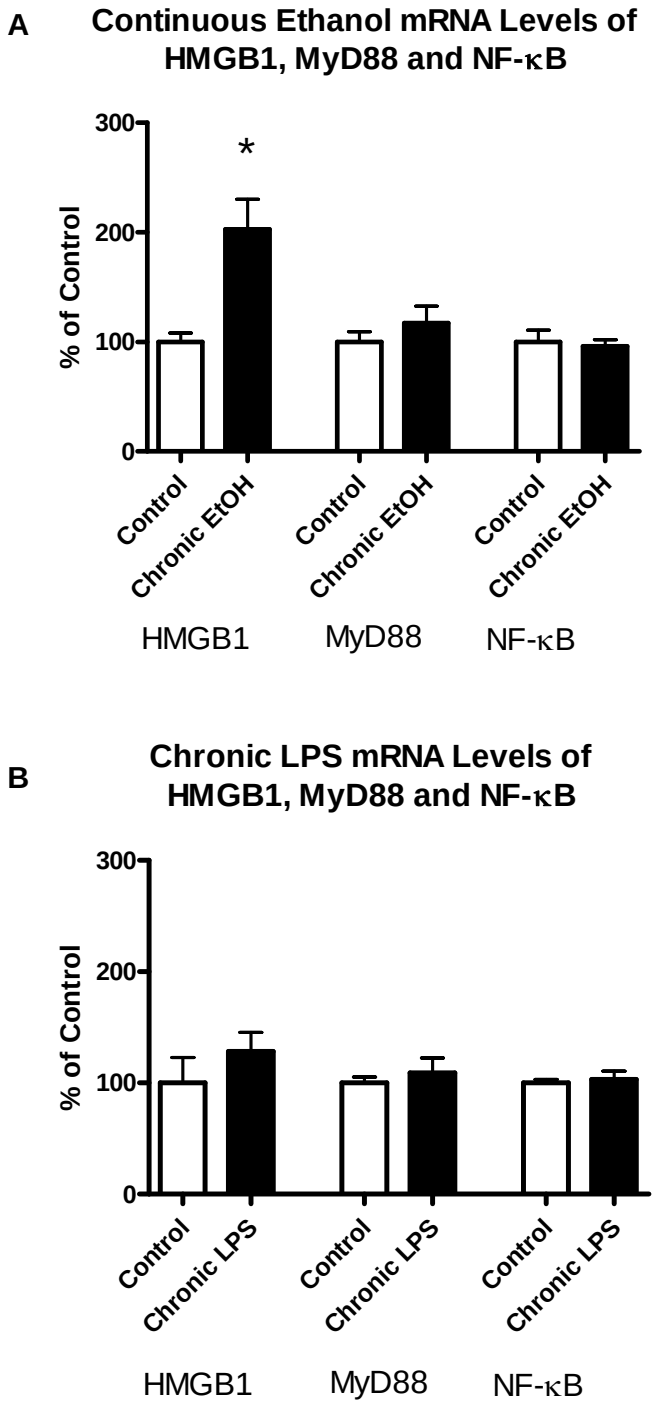

Figure 3.5: Effects of continuous ethanol versus chronic LPS administration on mRNA for HMGB1, MyD88 and NF-κB. A) HMGB1, but not MyD88 or NF-κB, mRNA was elevated following continuous ethanol exposure over nontreated controls (HMGB1; $t(16)=3.618$, $p=0.0023$; MyD88, *ns*; NF-κB, *ns*). B) Chronic LPS did not cause an increase in HMGB, MyD88 or NF-κB mRNA levels (all $p<0.05$).

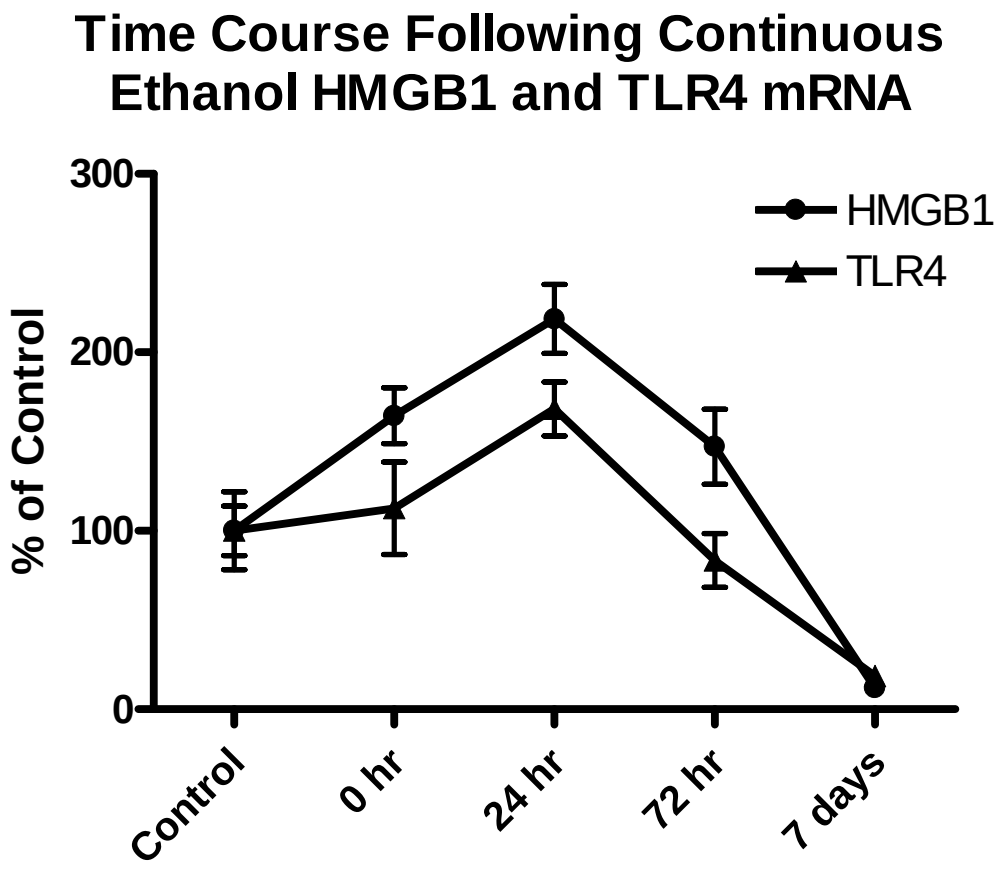

Figure 3.6: Time course of changes to mRNA for HMGB1 and TLR4 following continuous ethanol. TLR4 was also significantly increased following continuous ethanol exposure and peaked at 24 hr ($F(4,27)=8.64$, $p=0.0001$, Control 100 ± 13.9 vs. 24 hr 168.3 ± 15.1, $p=0.012$). HMGB1 was also significantly elevated ($F(2,26)=3.51$, $p=0.02$). HMGB1was significantly elevated at T=0 (Control 100 ± 21.9, vs. 0 hr 164.5 ± 15.6) and peaked at 24 hr (Control 100 ± 21.9, vs. 218.7 ± 19.4).

CHAPTER IV:

ACUTE STRESS FACILITATION OF BRAIN CYTOKINES AFTER CONTINOUS ETHANOL EXPOSURE IS PREVENTED BY HMGB1 ANTAGONISTS

Introduction

Stress has been shown to play a role in craving and risk of relapse (Breese et al., 2005b; 2010; Sinha et al., 2000; Miller et al., 2009; Sinha et al., 2011). In abstinent alcoholics, stress increases activation in brain as measured by fMRI—a change not seen in social drinkers (Gilman and Homer, 2008, reviewed in Breese et al., 2011). In accord with fMRI studies, stress was a more salient initiator of craving than a drug cue (Breese et al., 2011). In rats, Breese et al. (2005c) found that the anxiety-like response to restraint stress was elevated after chronic ethanol exposure. Additionally, Breese et al. (2005a) found that restraint stress could substitute for the first two withdrawal episodes in a chronic intermittent ethanol protocol to facilitate withdrawal-induced anxiety-like behavior (Breese et al., 2005a). Later studies in the lab showed that LPS or specific cytokines could substitute for stress or the first two withdrawal episodes in a chronic intermittent ethanol protocol (Breese et al., 2008).

Chronic exposure to high doses of ethanol can elevate cytokines (Alfonso-Loeches et al., 2010; Blanco et al., 2005; Crews et al., submitted; Fernandez-Lizarbe et al., 2008). In human post-mortem brain tissue, He and Crews (2008) reported elevated levels of CCL2; and Crews et al. (submitted) showed elevated levels of cytokine mRNA and protein following chronic ethanol exposure in mice. Additionally, results from Chapter III demonstrate

increased mRNAs for cytokines CCL2, IL1-β, and TNFα in brain 24 hr following withdrawal from 15 days of continuous ethanol exposure.

Stress is a powerful activator of the neuroimmune system (Blandino et al., 2009; Deak et al., 2005; Girotti et al., 2011; Hueston et al., 2011; Minami et al., 1991; Nguyen et al., 1998, 2000; Shizuya et al., 1997; Suzuki et al., 1997; Knapp et al., 2011). Several studies have demonstrated that proinflammatory cytokines IL-1β and IL-6 are elevated in brain following various acute stress paradigms (Blandino et al., 2009; Deak et al., 2005; Minami et al., 1991; Nguyen et al., 1998; Shizuya et al., 1997; Suzuki et al., 1997), but see Hueston et al. (2011) for comparison of various acute stress paradigms. Knapp et al. (2011) showed that protein levels of TNFα were significantly elevated in whole brain 4.5 hr following a 60-min restraint stress. TNFα levels were also significantly elevated in whole brains collected from adolescent rats 5 hr following 45 or 90 min of restraint stress (Knapp, unpublished data). Activation of the neuroimmune system activates NF-κB transcription, which increases the expression of genes including proinflammatory cytokines and DAMP receptors such as TLR4 (Garg et al., 2010; Okun et al., 2009). Although the mechanism for activation of the neuroimmune system by acute stress remains unclear, several reports suggest that activation of microglia (Miller et al., 2009; Wang and Dow, 2003; Wang et al., 2002) is responsible for the increase in proinflammatory cytokines. These data suggest that the increase in cytokines observed following acute stress may be an important sterile (non-infection-based) neuroimmune challenge (Andersson and Tracey, 2011).

To test changes in sensitivity of the neuroimmune response to stress following chronic ethanol, the present investigation initially established the duration of changes in brain cytokines following a 1-hr restraint stress. Next, whether stress-induced increases in brain

cytokines would be enhanced following continuous ethanol exposure was determined. Finally, whether the activity of HMGB1 reported in Chapter III following continuous ethanol exposure contributes to stress activation of cytokines was tested pharmacologically by blocking HMBG1 just prior to a stress challenge. One antagonist, glycyrrhizin, is a direct inhibitor of HMGB1 and binds HMGB1 in the extracellular space (Girard, 2007; Mollica et al., 2007). This antagonist has been shown to be effective *in vivo* at blocking HMGB1 function in rats (Ohnishi et al., 2011). The second HMGB1 antagonist, ethyl pyruvate, has been shown to block the release of HMGB1 without any decrease in expression of mRNA or protein (Ulloa et al., 2002). Given the upregulation of HMGB1 following continuous ethanol (Chapter III), it was hypothesized that HMGB1 may play a critical role in increased sensitivity of the neuroimmune system following continuous ethanol exposure.

Materials and Methods

Animals

For standard animal housing and diet procedures, refer to General Methods (Chapter II).

Experimental Protocols

For detailed descriptions of experimental protocols, refer to General Methods (Chapter II). Briefly, to test for increased sensitivity of the neuroimmune system following continuous ethanol exposure a 1-hr restraint stress was applied 24 hr following removal of ethanol. Animals were sacrificed and brains harvested as described in Chapter II (Fig. 2.2). This experiment included (1) a group of rats (Controls) that only received CD for the entire procedure and did not receive a stress; (2) a group of rats that received CD for the duration of the experiment and a stress at the end; (3) a group that only received ED for the entire

duration; and (4) a group that received both ED for the entire duration plus a 1-hr restraint stress 24 hr into withdrawal.

To test for the duration of changes in cytokines, TLR4, and HMGB1 following 1 hr of restraint stress, rats were subjected to a 1-h. restraint stress and sacrificed at 2, 4, 8, 24, and 48 hr, and brains harvested as described in Chapter II. Control rats (i.e., non-stressed) were included at all time points to control for differences in time of day and disturbance in animal care facility.

To assess the role of HMGB1 in the increased sensitivity of the neuroimmune system following continuous ethanol exposure, two known blocking agents of HMGB1 were applied 15 min prior to application of stress. This experiment contained six groups: (1) CD/stress group with saline prior to stress; (2) CD/stress group with ethyl pyruvate prior to stress; (3) CD/stress group with glycyrrhizin prior to stress; (4) ED/stress group with saline prior to stress; (5) ED/stress group with ethyl pyruvate prior to stress; and (6) ED/stress group with glycyrrhizin prior to stress.

Statistical Analysis

All data were evaluated for statistical significance with student's t-test or one- or two-way ANOVAs as appropriate using Graph Pad Prism 4.0 (Graph Pad Software, Inc., San Diego, CA) and expressed as mean ± SEM. P-values < 0.05 were considered statistically significant.

Results

Time Course of Changes in Cytokines, TLR4 and HMGB1 Following Restraint Stress

Previous studies have shown an increase in brain cytokines following stress (Blandino et al., 2009; Deak et al., 2005; Knapp et al., 2011, Suzuki et al., 1997). A 1-hr

restraint stress significantly increased CCL2, IL1-β and TNFα mRNA (Fig. 4.1). CCL2 mRNA expression peaked between 2 and 4 hr (120.7% and 111.2% increase over controls, respectively, all $p<0.05$) and returned to control levels by 8 hr following the stress (Fig. 4.1A). IL1-β mRNA was also elevated by the 1-hr restraint stress (Fig. 4.1B). IL1-β mRNA expression peaked at 2 hr (92.1% over controls, $p<0.01$) after restraint stress. Although not statistically significant, at 4 hr, IL1-β mRNA levels remained high (78.2% of control, $p>0.05$) and had returned to control levels by 8 hr following the stress. A 1-hr restraint stress increased TNFα mRNA expression that peaked at 2 hr (98.5% over controls, $p<0.01$) (Fig. 4.1C). TLR4 mRNA expression was also significantly elevated following a 1-hr restraint stress and peaked at 4 hr (68.2% over controls, $p<0.05$) (Fig. 4.1D). HMGB1 mRNA expression was significantly decreased 24 to 48 hrs. following a 1-hr restraint stress, (40.8% and 44.4% below control levels, respectively, $p<0.05$) (Fig. 4.2).

Effect of Stress Challenge Following Continuous Ethanol Exposure on Cytokines, TLR4 and HMGB1

It was established in Chapter III that continuous ethanol exposure for 15 consecutive days at 7% ethanol in liquid diet elevated mRNA for cytokines CCL2, IL1-β, and TNFα. Previous studies have shown that restraint stress is also capable of elevating cytokines in brain (Blandino et al., 2009; Deak et al., 2005; Knapp et al, 2011). To determine whether continuous ethanol exposure sensitizes the neuroimmune system to future challenges, rats were exposed to continuous ethanol and challenged with a 1-hr restraint stress 24 hr later. A 1-hr restraint stress in CD-treated rats caused a 148% ($p<0.05$) increase in CCL2 mRNA. Continuous ethanol exposure with 24 hr of withdrawal alone caused a 145% ($p<0.05$) increase. Stress challenge following continuous ethanol had an additive effect resulting in a

283% ($p<0.01$) increase in CCL2 mRNA (Fig. 4.3A). Restraint stress in CD-treated rats caused an 88% ($p<0.05$) increase in IL1-β mRNA. Continuous ethanol exposure alone caused a 97% ($p<0.05$) increase in IL1-β mRNA. Stress following continuous ethanol exposure had an additive effect and elevated IL1-β expression 186% ($p<0.01$) (Fig. 4.3B). Stress in CD-treated rats caused a 98% ($p<0.05$) increase in TNFα mRNA expression over controls. Ethanol exposure with 24 hr of withdrawal caused a 125% ($p<0.05$) increase in message. A stress challenge following continuous ethanol exposure caused a 126% increase in TNFα mRNA (Fig. 4.3C). Finally, stress in CD-treated rats caused a 78% ($p<0.05$) increase in mRNA for TLR4s. Ethanol exposure with 24 hr of withdrawal alone caused a 76% ($p<0.05$) increase in message. Stress after ethanol exposure caused an 88% ($p<0.05$) increase in TLR4 mRNA (Fig. 4.3D).

To follow up on the observation of elevated HMGB1 mRNA following continuous ethanol exposure (Chapter III), changes in HMGB1 message following a stress challenge were determined in continuous-ethanol-exposed rats. A stress challenge in CD-treated rats did not increase HMGB1 mRNA ($p>0.05$). However, ethanol alone with 24 hr of withdrawal caused a 108 % ($p<0.01$) increase in HMGB1 message. A stress challenge after continuous ethanol exposure did not have an additive effect and resulted in a 98% ($p<0.05$) increase in HMGB1 mRNA over controls (Fig. 4.4).

Blockade of HMGB1 Action with Ethyl Pyruvate and Glycyrrhizin

HMGB1 has been shown to activate TLR4s to induce proinflammatory cytokines (Park et al., 2004). Additionally, continuous ethanol exposure has been shown to increase HMGB1 mRNA expression (Chapter III; Crews et al., submitted). To further explore the idea that HMGB1 is responsible for the neuroimmune system sensitization following chronic

ethanol exposure, HMBG1 was blocked with ethyl pyruvate or glycyrrhizin i.p. 15 minutes prior to a stress challenge after continuous ethanol exposure. Treatment with ethyl pyruvate or glycyrrhizin prior to stress application did not affect CCL2 levels ($p>0.05$) in CD-treated rats. However, in continuous- ethanol-treated rats, ethyl pyruvate and glycyrrhizin significantly reduced CCL2 mRNA expression when applied before the stress (170.2% and 164.5% reduction in mRNA expression, respectively, $p<0.01$) compared to continuous-ethanol-treated rats given stress and treated with saline (Fig. 4.5). Treatment with ethyl pyruvate or glycyrrhizin prior to stress application had no effect on IL1-β levels ($p>0.05$) in CD-treated rats. Treatment with ethyl pyruvate or glycyrrhizin prior to stress in continuous-ethanol-treated rats reduced IL1-β mRNA expression (164% and 177% reduction in mRNA expression, respectively, $p<0.01$) compared to continuous-ethanol-treated rats given stress and treated with saline (Fig. 4.5B). Treatment with ethyl pyruvate or glycyrrhizin prior to stress had no effect on TNFα levels ($p>0.05$) in CD-treated rats. However, a nonsignificant 36.7% decrease in TNFα expression was observed in the CD rats treated with glycyrrhizin prior to stress. Treatment with ethyl pyruvate or glycyrrhizin prior to stress in continuous-ethanol-treated rats reduced TNFα mRNA (ethyl pyruvate, 49% reduction, $p<0.05$; glycyrrhizin, 82% reduction, $p<0.01$) compared to continuous-ethanol-treated rats given stress and treated with saline (Fig. 4.5C). Treatment with ethyl pyruvate or glycyrrhizin prior to stress in CD-treated rats or continuous-ethanol-treated rats did not significantly alter TLR4 mRNA expression (all $p>0.05$) (Fig. 4.5D). Neither ethyl pyruvate nor glycyrrhizin affected mRNA levels for HMGB1 in CD-treated animals or continuous-ethanol-treated animals (all $p>0.05$) (Fig. 4.6).

Discussion

Clinical investigations have demonstrated that stress in abstinent alcoholics can induce craving—a circumstance that can increase the probability of relapse (see Breese et al., 2011; Sinha, 2001). In accord with this observation, basic research has demonstrated that stress-induced anxiety is facilitated following chronic ethanol exposure. Corticotropin releasing factor (CRF) is widely known to play a critical role in stress, but the greater neurochemical milieu in which CRF acts is poorly understood. Stress has been shown to increase cytokines in brain (Blandino et al., 2009; Deak et al., 2005), and cytokines can substitute for stress to facilitate ethanol withdrawal anxiety (Breese et al., 2008). The present effort tested the hypothesis that the increase in cytokines by stress after continuous ethanol exposure would be facilitated by the release of the endogenous TLR4 agonist HMGB1.

To establish the best time for sacrificing animals after stress, the time course of change in cytokines following a 1-hr restraint stress was defined. The stress caused significant increases in CCl2, IL1β, and TNFα mRNAs that peaked in 2 to 4 hr—a finding in agreement with previous demonstrations of stress-induced increases in cytokines (Blandino et al., 2009; Deak et al., 2005; Minami et al., 1991; Nguyen et al., 1998; Shizuya et al., 1997; Suzuki et al., 1997). Stress represents a unique challenge to the neuroimmune system in that it activates cytokine production in a sterile environment; stress in and of itself is not a ligand for known receptors of the neuroimmune system (Andersson and Tracey, 2011). To further elucidate the mechanism by which stress activates the neuroimmune system, the effects of acute stress on the levels of TLR4 and HMGB1 mRNAs were determined. TLR4 mRNA was increased by 68% over controls 4 hr after application of stress. TLR4 mRNA returned to control levels by 8 hr and remained there until at least 48 hr after stress. HMGB1 mRNA did not show an immediate response to acute stress but dropped to 40% of control by 24 hr and

remained at this level through the last post-stress time point assessed at 48 hr. The level of HMGB1 was reduced below control levels 7 days after removal from chronic ethanol, a pattern of results similar to that observed with stress (Chapter III). This finding suggests that some adaptation in this ligand occurs after a challenge to the immune system. Such a detailed assessment of the effects of acute stress on the time course of changes in TLR4 and HMGB1 mRNAs has not previously been reported. The mechanism of the protracted change in HMGB1 warrants further investigation.

To explore whether stress would enhance the effects of chronic ethanol on cytokine levels (Chapter III), restraint stress was applied for 60 min 24 hr after the continuous ethanol protocol. Two recent reports (Barnum et al., 2008; Girotti et al., 2011) provide evidence that the neuroimmune system can have a differential response depending on prior experience—in this case, chronic ethanol prior to the stress. Additionally, Qin et al. (2007) have shown elevated levels of TNFα protein 10 months after a single systemic administration of LPS. In the current investigation, mRNAs for CCL2 and IL1-β were both elevated by stress above the level observed 24 hr after the continuous ethanol exposure. This finding is consistent with a behavioral impact of stress-induced cytokines after chronic ethanol exposure (Breese et al., 2004). Surprisingly, stress did not increase TNFα mRNA beyond the increase seen with chronic ethanol exposure alone. This difference from the other cytokines suggests that TNFα may be controlled by a different mechanism.

Combining stress with the chronic ethanol did not elevate the mRNA for TLR4 over that observed with stress or chronic ethanol alone. Likewise, the increase in HMGB1 by the continuous ethanol protocol was not further increased by stress. Perhaps the failure to

observe stress-associated additive changes in HMGB1 is related to the lack of a stress-associated increase in HMGB1 in controls.

The mRNA results for HMGB1 do not support the hypothesis that HMGB1 mediates cytokine increases via an interaction of stress and chronic ethanol. Therefore, an alternative explanation, based upon evidence that HMGB1 must be actively released from cells (Kim et al., 2006; Park et al., 2004; Yang et al., 2005), was explored. To evaluate directly whether the addition of stress after chronic ethanol induces release of HMGB1, HMGB1 was pharmacologically blocked prior to the stress challenge with two different agents. The first agent, ethyl pyruvate, is a cytokine release inhibitory drug (CRID) (Girard, 2007). Ethyl pyruvate has been shown to block the release of HMGB1 from cells without altering its expression of mRNA or protein (Ulloa et al., 2002). The second agent, glycyrrhizin, is an effective antagonist of HMGB1 function *in vivo* (Ohnishi et al., 2011). Glycyrrhizin inhibits HMGB1 by binding it in the extracellular space after release (Girard, 2007; Mollica et al., 2007). Both ethyl pyruvate and glycyrrhizin profoundly inhibited the stress-induced increases in CCL2 and IL1-β in the rats treated with the continuous ethanol protocol (Fig. 4.5A and B). While stress did not further elevate the TNFα mRNA after continuous alcohol, HMGB1 antagonists still affected TNFα mRNA level—an effect not observed with the other cytokines. Additionally, neither the TLR4 mRNA level nor the HMGB1 mRNA level was affected by the HMGB1 antagonists with or without continuous ethanol exposure although HMGB1 was elevated by ethanol exposure alone. These outcomes are consistent with the previous report that ethyl pyruvate does not alter expression of HMGB1 but does prevent its release from cells (Ulloa et al., 2002).

Surprisingly, neither glycyrrhizin nor ethyl pyruvate altered the stress-induced increase in cytokines in controls although these HMGB1 antagonists reduced mRNA for CCL2 and IL1-β to the level observed with chronic ethanol alone—a change equivalent to that of stress alone (Fig. 4.5A and B). These results suggest that HMGB1antagonism following continuous ethanol exposure blocks the stress-induced release of cytokines as the levels were reduced to the same levels as with continuous ethanol alone. In the absence of prior continuous ethanol exposure stress does not seem to operate by the same mechanism. Specifically, HMGB1 antagonism prior to a stress challenge in CD treated animals had no effect on cytokine production. Taken together, these results suggest that acute stress may operate under two distinct mechanisms in the two different conditions. Without prior continuous ethanol exposure, acute stress uses one of these mechanisms to evoke cytokines in brain. Given the available explanation in the literature, CRF activation of microglia and release of cytokines (Wang et al., 2003) may explain the observed results with regard to acute stress. However, with prior continuous ethanol exposure, the data suggest that stress contributes to the release of already elevated HMGB1 levels to further enhance cytokine production. It is likely that CRF is still playing a role but only in so far as setting the release of HMGB1 in motion. The observation that the HMGB1 antagonists prevented the action of stress after chronic ethanol exposure is consistent with this view. Furthermore, CRF signaling is recruited following alcohol dependence (Heilig and Koob, 2007), which may help explain the apparent dual role of stress following continuous ethanol exposure in the current study. This potential certainly warrants further investigation as the exact mechanism by which stress activates the neuroimmune system is heretofore unknown.

Taken together, these data provide evidence that HMGB1 contributes to the increased sensitivity of the neuroimmune system to stress following continuous ethanol exposure but not to stress-induced increases in cytokines in controls. Such HMGB1-mediated activation of the neuroimmune system following continuous ethanol exposure is consistent with the proposal that non-infection-based central disorders may be mediated in part by HMGB1 (Anderson and Tracey, 2011). In this regard, HMGB1 has been shown to be elevated in many disorders including sepsis (Wang et al., 1999), arthritis and cancer (Lotze and Tracey 2005), multiple sclerosis (Andersson et al., 2008), epilepsy (Moroso et al., 2010) and alcoholism (Crews et al., submitted). Therapeutic options under development based on these findings (Yang et al., 2002; Mollica et al., 2007) may prove to be effective in preventing the consequences of neuroimmune diseases as well as relapse in alcoholics.

Figures

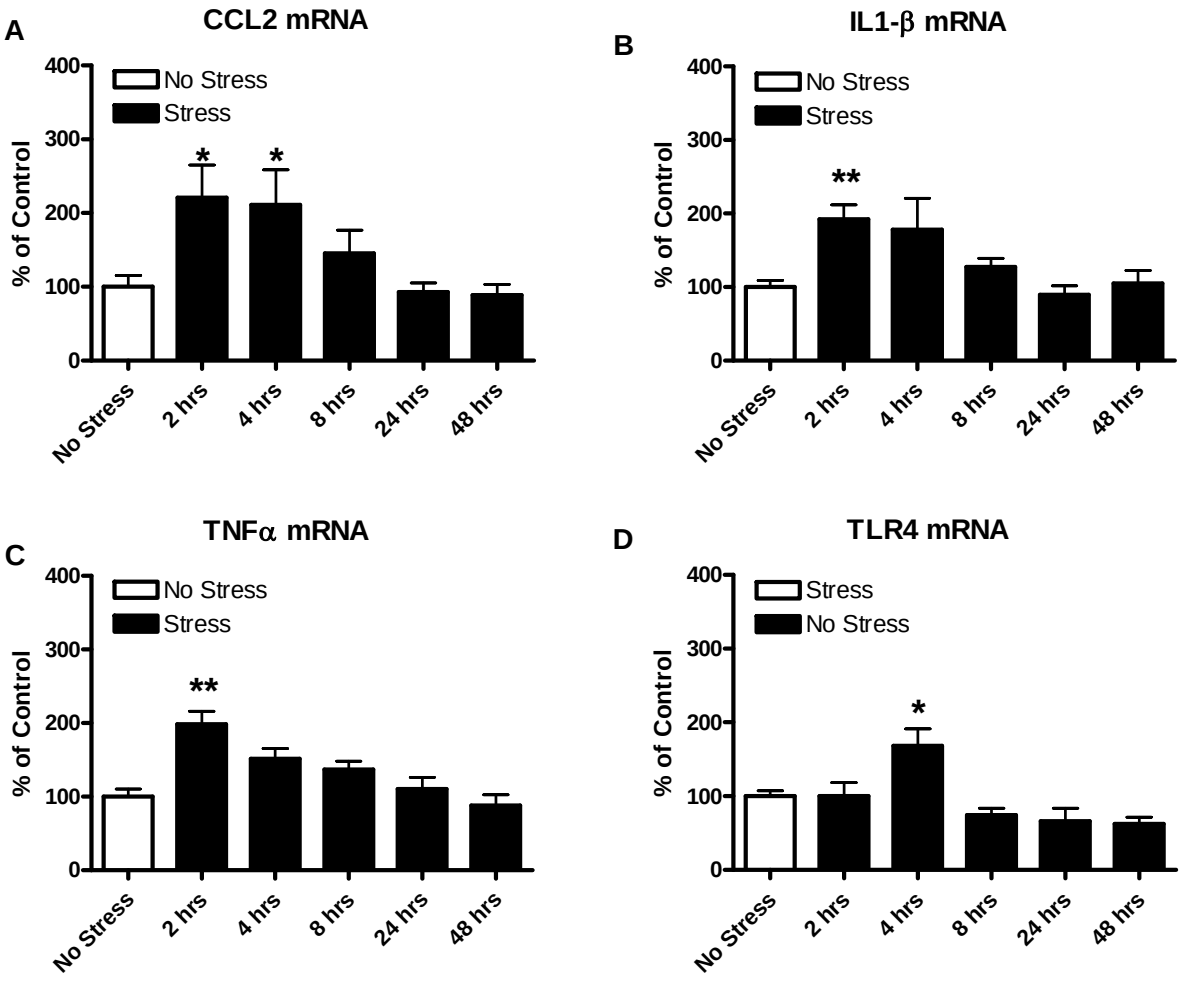

Figure 4.1: Time course of changes in mRNAs for CCL2, IL1-β, and TNFα and for TLR4s following 1-hr restraint stress. Stress increased mRNAs for cytokines CCL2, IL1-β, and TNFα and for TLR4s. A) CCL2 mRNA was elevated by stress ($F(5,32)=3.82$, $p=0.008$) and peaked at 2 to 4 hr after the stress. B) IL1-β mRNA was elevated by stress ($F(5,33)=5.04$, $p=0.015$) and peaked 2 hr after the stress. C) TNFα mRNA was elevated by stress ($F(5,38)=8.5$, $p<0.0001$) and peaked 2 hr after the stress. D) TLR4 mRNA was elevated by stress ($F(5,39)=6.26$, $p=0.0002$) and peaked at 4 hr after the stress. *$p<0.05$ compared to controls, **$p<0.01$ compared to controls.

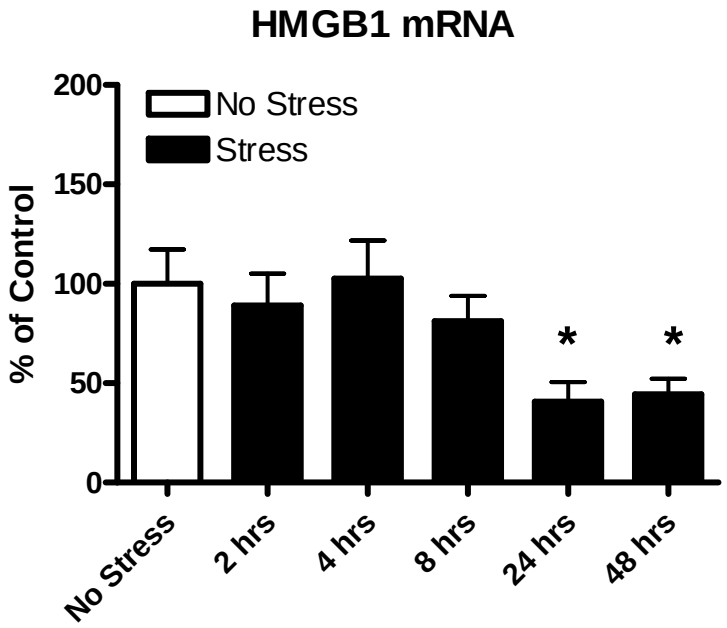

Figure 4.2: Time course of changes in mRNA for HMGB1 following 1-hr restraint stress. Stress causes a decrease in HMGB1 by 24 hr, an effect that persisted through at least 48 hr ($F(5,36)=5.35$, $p=0.0061$). *$p<0.05$ compared to controls

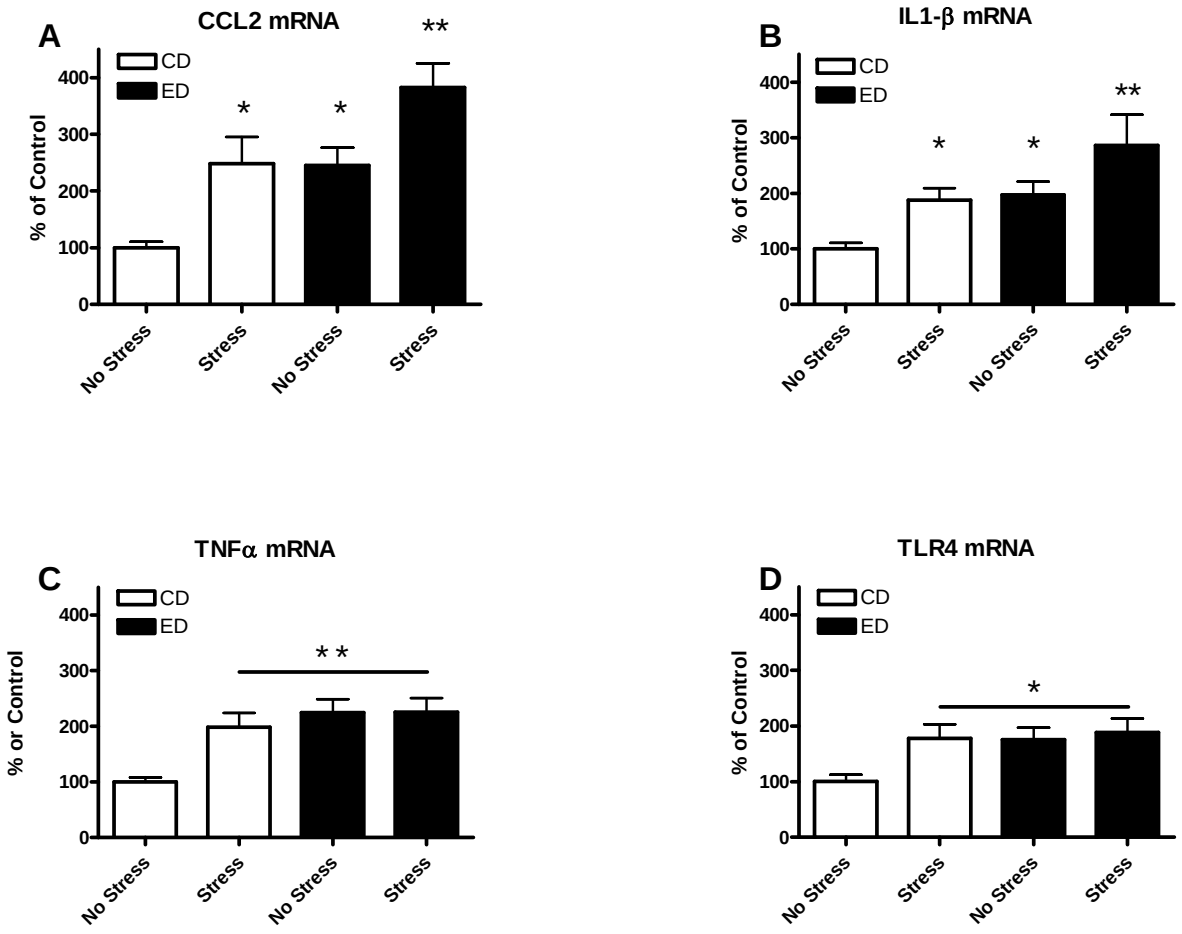

Figure 4.3: Effects of continuous ethanol and stress on mRNA for cytokines CCL2, IL1-β, TNFα and for TLR4s. Both stress and continuous ethanol increased CCL2, IL1-β, TNFα, and for TLR4 mRNA. A) Both stress ($F(1,28)=16.0$, $p=0.0004$) and ethanol ($F(1,28)=15.3$, $p=0.0005$) increased CCL2. No interaction of stress and ethanol was observed ($p>0.05$). B) Both stress ($F(1,31)=10.9$, $p=0.0024$) and ethanol exposure ($F(1,31)=13.4$, $p=0.0009$) increased IL1-β. No interaction of stress and ethanol was observed ($p>0.05$). C) Both stress ($F(1,44)=4.9$, $p=0.031$) and ethanol ($F(1,44)=11.7$, $p=0.031$) increased TNFα. The interaction of stress and ethanol was significant ($F(1,44)=4.8$, $p=0.033$). D) Both stress ($F(1,41)=4.4$, $p=0.04$) and ethanol ($F(1,41)=4.0$, $p=0.05$) increased TLR4. No interaction of stress and ethanol was observed ($p>0.05$). *$p<0.05$ compared to controls, **$p<0.01$ compared to controls.

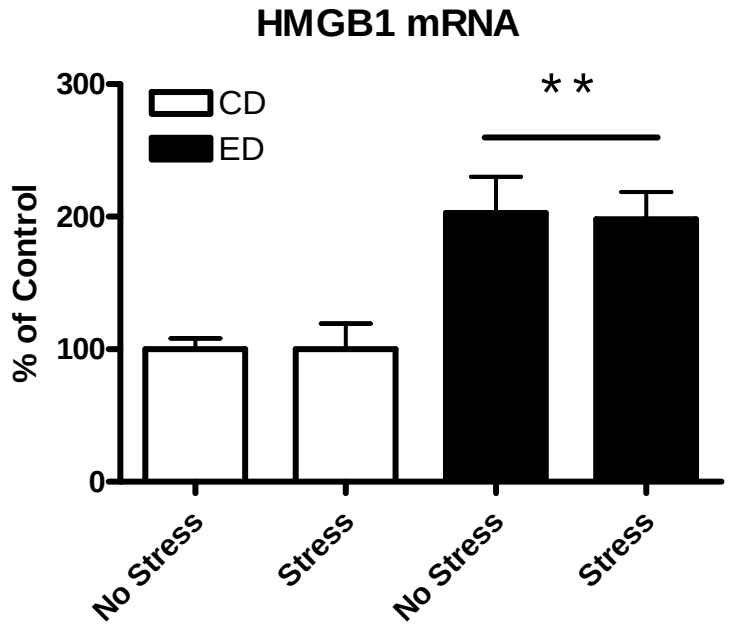

Figure 4.4: Effects of continuous ethanol and stress on mRNA for HMGB1. HMGB1 was elevated by continuous ethanol ($F(1,36)=24.65$, $p<0.0001$) but not by stress ($p>0.05$). No interaction between ethanol exposure and stress was observed ($p>0.05$). **$p<0.01$ compared to controls.

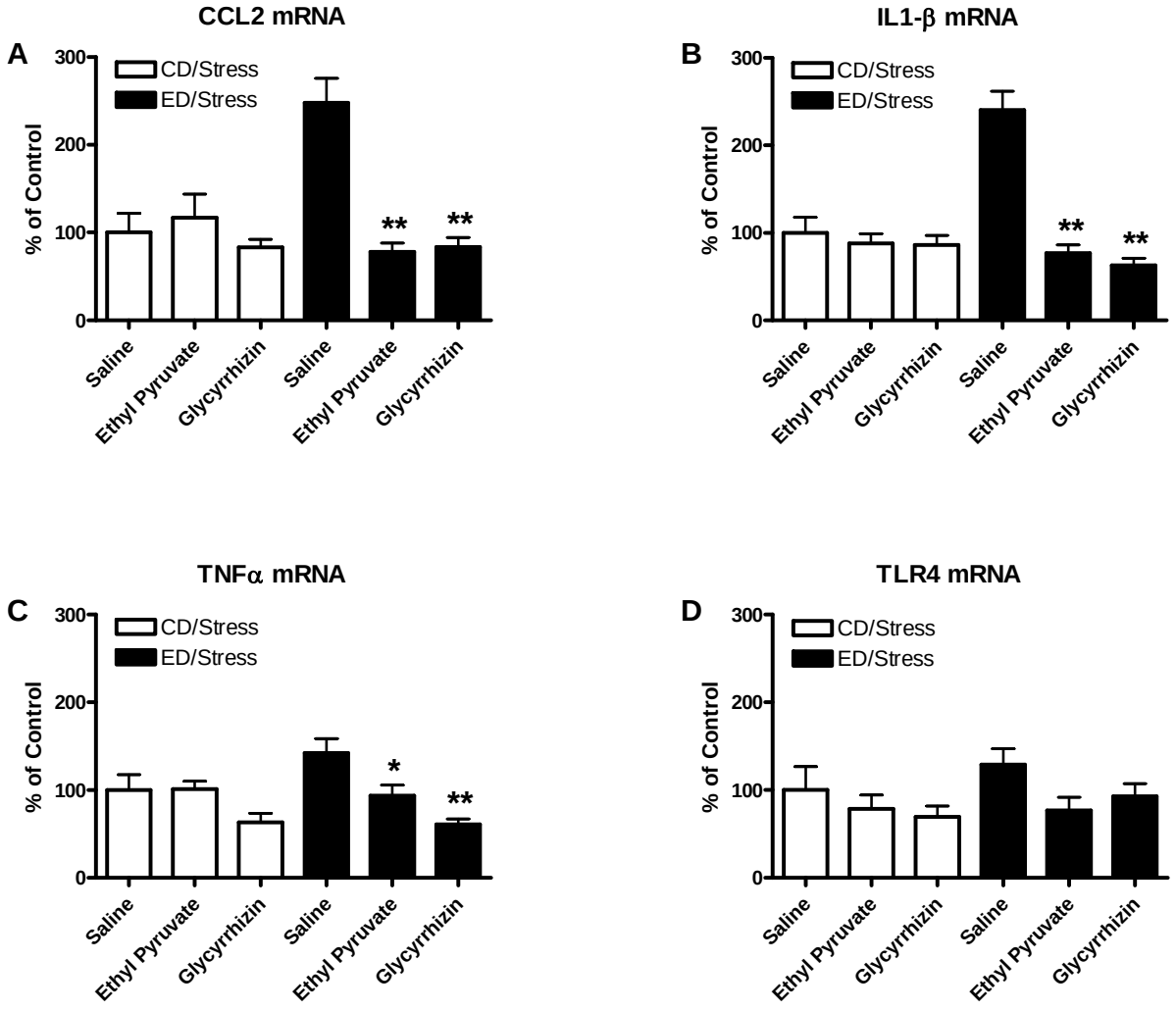

Figure 4.5: Effect of HMGB1 blockade with ethyl pyruvate and glycyrrhizin on mRNAs for CCL2, IL1-β, and TNFα and for TLR4s following a stress challenge after continuous ethanol exposure. A) Neither ethyl pyruvate nor glycyrrhizin blocked the increase of CCL2 mRNA caused by stress in CD-treated rats ($p>0.05$), but both caused a significant decrease in the CCL2 mRNA caused by stress in ED-treated rats ($F(2,24)=8.22$, $p=0.0019$). B) IL1-β mRNA was not reduced by ethyl pyruvate or glycyrrhizin after stress in CD-treated animals, but both caused a decrease in mRNA caused by stress in ED-treated rats ($F(2,20)=44.48$, $p<0.001$). C) TNFα mRNA was not reduced by ethyl pyruvate or glycyrrhizin after stress in CD- treated animals, but both caused a significant decrease in mRNA caused by stress in ED-treated rats ($F(2,24)=8.22$, $p=0.0019$). D) Neither ethyl pyruvate nor glycyrrhizin affected mRNA for TLR4 in either CD- or ED-treated animals. *$p<0.05$ compared to controls, **$p<0.01$ compared to controls.

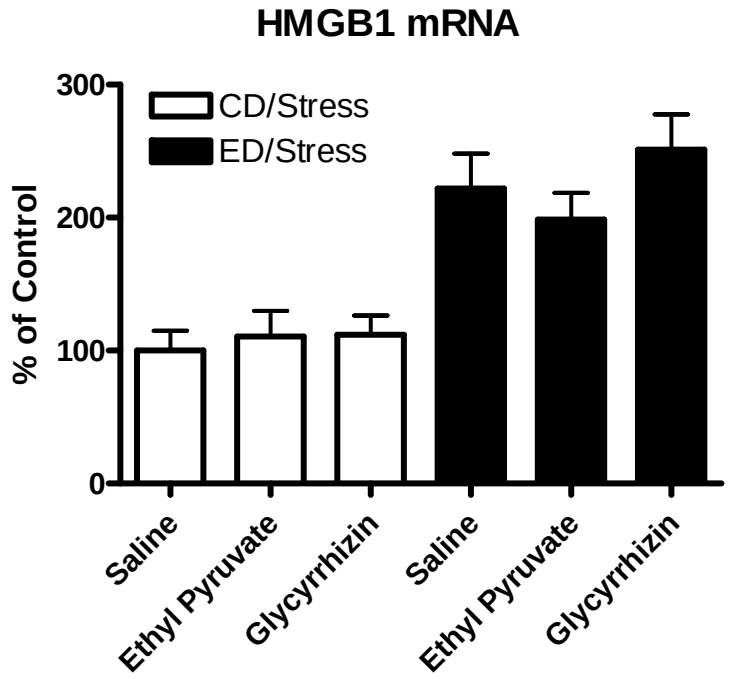

Figure 4.6: Effect of HMGB1 blockade with ethyl pyruvate and glycyrrhizin on mRNA for HMGB1 following a stress challenge 24 hr after withdrawal from continuous ethanol exposure. Treatment with ethyl pyruvate or glycyrrhizin prior to stress in CD-treated rats did not affect HMGB1 mRNA levels ($p>0.05$). Neither ethyl pyruvate nor glycyrrhizin blocked the effects of stress on HMGB1 mRNA levels after ED exposure in rats ($p>0.05$).

CHAPTER V:

GENERAL DISCUSSION

Clinically, stress has been shown to play a role in craving and risk of relapse (Breese et al., 2005b; 2010; Sinha et al., 2000; Miller et al., 2009; Sinha et al., 2011). In abstinent alcoholics, stress increases fMRI signals in brain that are accompanied by craving—changes not apparent in social drinkers (Breese et al., 2011 for review). Consistent with this finding, chronic intermittent alcohol exposure facilitated the anxiety response to restraint stress in rats (Breese et al., 2005b). Stress powerfully activates the neuroimmune system (Blandino et al., 2009; Deak et al., 2005; Girotti et al., 2011; Hueston et al., 2011; Minami et al., 1991; Nguyen et al., 1998, 2000; Shizuya et al., 1997; Suzuki et al., 1997; Knapp et al., 2011). Cytokines, which are increased in brain by various types of stressors (Blandino et al., 2009; Deak et al., 2005; Girotti et al., 2011; Hueston et al., 2011; Minami et al., 1991; Nguyen et al., 1998, 2000; Shizuya et al., 1997; Suzuki et al., 1997; Knapp et al., 2011), are potential mediators of the negative affect associated with the interaction of chronic ethanol and stress.

Like stress, chronic alcohol administration increases cytokines in brain (Alfonso-Loeches et al., 2010; Blanco et al., 2005; Crews et al., submitted; Fernandez-LIzarbe et al., 2008). While infection is known to activate cytokines as part of the immune response, both stress and ethanol increase cytokines in a "sterile" environment in the absence of infection. The present body of work for this dissertation explored the possible basis of how alcohol and stress could increase cytokines in brain.

Previous literature provides clear evidence that ethanol's influence on cytokines involves the TLR4 (Alfonso-Loeches et al., 2010; Blanco et al., 2005; Fernandez-Lizarbe et

al., 2009). The Guerri laboratory proposed that ethanol activates cytokines by influencing a lipid raft to activate TLR4 (Fernandez-Lizarbe et al., 2008). However, while agents such as LPS are direct agonists at the TLR4 (Triantafilou et al., 2002), ethanol has not been shown to date to be a direct agonist of any known receptor. A non-agonist (ethanol) should not be capable of stimulating TLR4s directly; however, several studies using TLR4 knockout mice reveal a lack of sensitivity to ethanol (Alfonso-Loeches et al., 2010; Fernandez-Lizabre et al., 2009; Pascual et al., 2011; Uesugi et al., 2001, Wu et al., 2012). Because ethanol is unlikely to have a direct agonist action on TLR4, the acute and chronic actions of LPS and ethanol were compared to explore other possible modes by which ethanol could activate TLR4 and the neuroimmune system.

A comparison of the effects of LPS and ethanol on brain cytokines provided considerable insight into the *in vivo* action of ethanol. This effort (Chapter III) clearly differentiated the effects of LPS from those of ethanol on brain cytokines**.** A moderate acute dose of ethanol did not affect brain cytokine mRNA whereas an acute dose of LPS did. This finding is important in demonstrating fundamental differences in the mechanism by which LPS, a direct TLR4 agonist, and ethanol activate TLR4s to increase brain cytokines, the work of Fernandez-Lizabre (2008) notwithstanding. The comparison of chronic LPS exposure to continuous ethanol further highlights the difference in the mechanisms by which ethanol and LPS act on TLR4s. Continuous ethanol caused considerable elevation in brain cytokines whereas chronic LPS caused endotoxin tolerance. This marked difference between ethanol and LPS in neuroimmune system effects is consistent with a corresponding difference in potential mechanisms by which each affects the TLR4.

Possible mechanisms of the differential activation of the neuroimmune system by ethanol and LPS were explored. An endogenous TLR4 agonist, HMGB1, was discovered to be significantly elevated following 15 days of continuous ethanol exposure but not following chronic LPS exposure. This finding is consistent with the possibility that ethanol could elevate brain cytokines via HMGB1 when LPS was no longer able to do so. This finding is of considerable importance both to understanding targets for intervening in the progression of alcoholism and to further defining the mechanism by which ethanol is capable of influencing TLR4 signaling. Figure 5.1A depicts a model of the increase in HMGB1 following continuous ethanol exposure. In this model, HMGB1 is elevated by continuous ethanol and further elevated by withdrawal to provide a potent potential for enhanced cytokine expression in the instance of a further challenge.

Building on the findings from Chapter III, Chapter IV focused on whether HMGB1 could be involved in the facilitation of the stress-induced cytokine response after continuous ethanol exposure. Clinically, stress has been shown to play a role in craving and risk of relapse (Breese et al., 2005b; 2010; Sinha et al., 2000; Miller et al., 2009; Sinha et al., 2011). Additionally, stress is known to be a powerful activator of the neuroimmune system (Blandino et al., 2009; Deak et al., 2005; Girotti et al., 2011; Hueston et al., 2011; Minami et al., 1991; Nguyen et al., 1998, 2000; Shizuya et al., 1997; Suzuki et al., 1997; Knapp et al., 2011). As predicted, the addition of a stress challenge following continuous ethanol exposure caused an additive increase in cytokines CCL2 and IL1-β. No further increase in TLR4s or HMGB1 in response to stress following continuous ethanol exposure was observed. Stress in the absence of prior ethanol exposure had no effect on HMGB1 mRNA levels until 24 hr, at which time levels were reduced to less than half those of controls. The

latter finding potentially reflects adaptive regulation of HMGB1. To further define the role of HMGB1 following continuous ethanol exposure, HMGB1 was pharmacologically blocked just prior to the stress challenge. Blocking HMGB1 signaling had a profound effect on the stress-induced increase in CCL2 and IL1-β mRNA and also reduced the level of TNFα, which was not further elevated by stress following continuous ethanol exposure. Based on these results, a model of the influence of stress on the already primed neuroimmune system following continuous ethanol exposure is depicted in Figure 5.1B. Since blockade of HMGB1 did not abrogate the increase in cytokines observed in stress-only controls, it is hypothesized that (1) continuous ethanol elevates HMGB1; and (2) the addition of stress to an already primed system is responsible for HMGB1 release from cells in order to activate the cell surface TLR4s. Thus, stress following continuous ethanol exposure further stimulates the neuroimmune system to cause a more pronounced increase in brain cytokines.

Future Work

The time course of changes in brain cytokines following withdrawal from continuous ethanol exposure in Chapter III indicated that HMGB1 mRNA levels were elevated prior to ethanol removal. However, because HMGB1 must be released from the cell in order to activate cell surface receptors like TLR4 (Kim et al., 2006), it is unclear whether this early rise in HMGB1 contributes to the increase in brain cytokines, which are not elevated until 24 hr following withdrawal from continuous ethanol. Future studies should define the role of HMGB1 in stimulating brain cytokines following continuous ethanol exposure.

Data from Chapter IV indicate that stress in the absence of previous ethanol exposure had no early effects on HMGB1 and served to reduce levels at time points beyond 24 hr. Stress under various conditions has been shown to cause increases in brain cytokines

(Minami et al., 1991; Nguyen et al., 1998). However, neither the literature nor the current data provide a good mechanism for stress activation of the neuroimmune system. Sugama et al. (2009) describe data showing that stress can differentially activate microglia when compared to LPS activation in the same brain area. This research implicates a specialized role for neurons to contribute directly to stress-induced increased in brain cytokines. Additionally, Wang et al. (2002; 2003) demonstrated functional expression of CRF receptors and induction of TNFα release following application of CRF in cultured microglia cells. The exact mechanism by which stress induces brain cytokines warrants further investigation.

Previous studies indicate that ethanol sensitivity might vary by brain area (Huang et al., 2010; Overstreet et al., 2006). The present effort focused on changes in the cortex, an area previously shown to have clinical relevance to human alcoholics (He and Crews, 2008; Crews et al., submitted). It remains to be determined whether areas of the brain associated with withdrawal-induced anxiety (Huang et al., 2010) or stress (Deak et al., 2005; Suguma et al., 2009) are differentially affected by continuous ethanol.

Antagonism of HMGB1 blocked the stress-induced increase in cytokines following continuous ethanol exposure. HMGB1 can signal though several receptors including TLR2 and receptor for advanced glycation end product (RAGE) (Green et al., 2009; Park et al., 2006; Rovere-Querini et al., 2004 Yu et al., 2006) and can act as a universal sentinel for nucleic acid-mediated immune response (e.g., TLRs 3, 7, 9) Yanai et al., 2009). Blocking HMGB1 with ethyl pyruvate or glycyrrhizin prevents its action on all of these receptors. However, blocking HMGB1 directly provides little evidence as to the concomitant contribution of other neuroimmune receptors to cytokine induction after continues ethanol

exposure. Therefore, the relative contribution of TLR4s could be determined by directly antagonizing the TLR4 following continuous ethanol exposure.

Figures

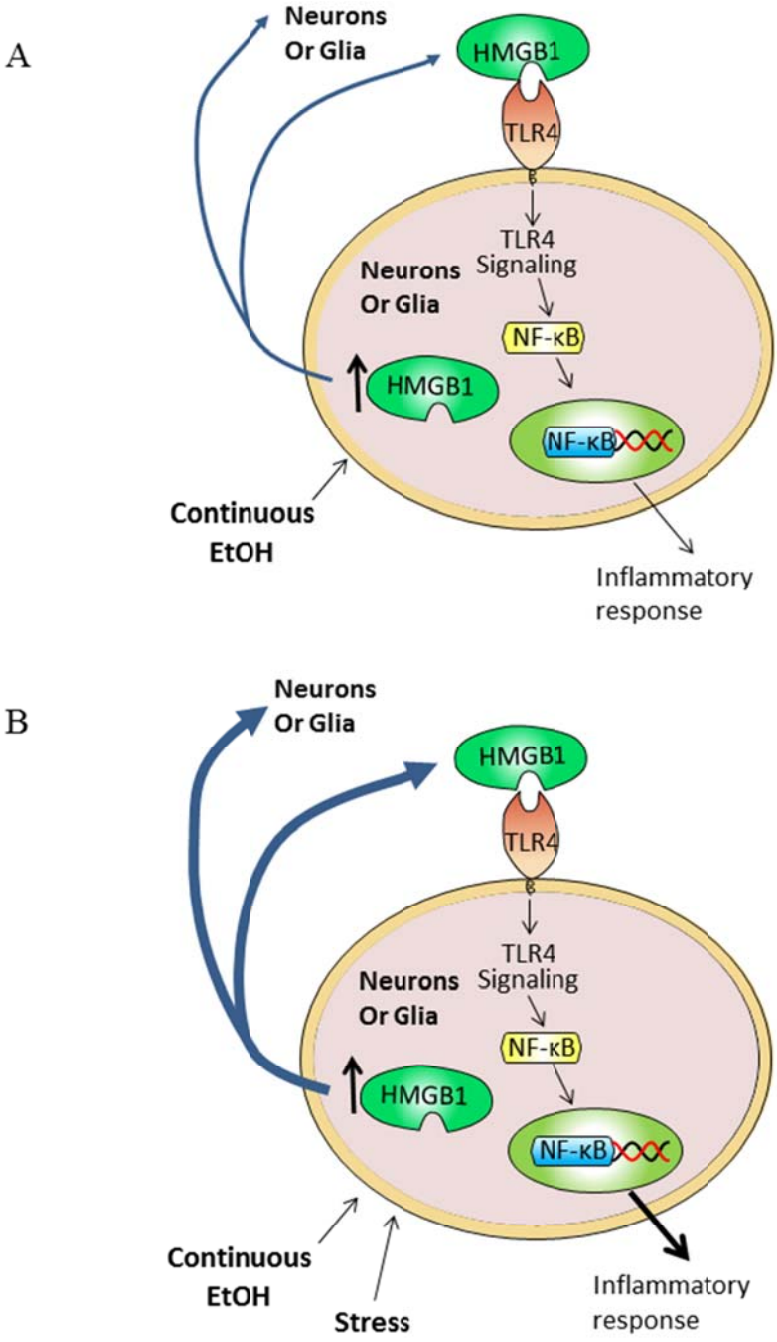

Figure 5.1: Models depicting the influence of HMGB1 on neuroimmune signaling following continuous ethanol exposure. A) HMGB1 is elevated following continuous ethanol exposure and may contribute to the elevated levels of cytokines. B) The elevated levels of HMGB1 can be used to further stimulate the neuroimmune system when a challenge is presented to the system following chronic ethanol exposure.

REFERENCES

Abraham E, Arcaroli J, Carmody A, Wang H, Tracey KJ. (2000) HMGB1 as a mediator of acute lung inflammation. J Immunol. 165(6):2950-4.

Akira S, Takeda K. (2004) Toll-like receptor signaling. Nat Rev Immunol. 4(7):499-511.

Alfonso-Loeches S, Pascual-Lucas M, Blanco AM, Sanchez-Vera I, Guerri C. (2010) Pivotal role of TLR4 receptors in alcohol-induced neuroinflammation and brain damage. J Neurosci. 30(24):8285-95.

Andersson A, Covacu R, Sunnemark D, Danilov AI, Dal Bianco A, Khademi M, Wallström E, Lobell A, Brundin L, Lassmann H, Harris RA. (2008) Pivotal advance: HMGB1 expression in active lesions of human and experimental multiple sclerosis. J Leukoc Biol. 84(5):1248-55.

Andersson U, Tracey KJ. (2011) HMGB1 is a therapeutic target for sterile inflammation and infection. Annu Rev Immunol. 29:139-62.

Baeuerle PA, Henkel T. (1994) Function and activation of NF-kappa B in the immune system. Annu Rev Immunol. 12:141-79.

Barnum CJ, Blandino P Jr, Deak T. (2008) Social status modulates basal IL-1 concentrations in the hypothalamus of pair-housed rats and influences certain features of stress reactivity. Brain Behav Immun. 22(4):517-27.

Blanco AM, Vallés SL, Pascual M, Guerri C. (2005) Involvement of TLR4/type I IL-1 receptor signaling in the induction of inflammatory mediators and cell death induced by ethanol in cultured astrocytes. J Immunol. 175(10):6893-9.

Blandino, P, CJ Barnum, LG Solomon, Y Larish, BS Lankow and T Deak. 2009. Gene expression changes in the hypothalamus provide evidence for regionally-selective changes in IL-1 and microglial markers after acute stress. Brain Behav Immun. 23:958–968.

Breese GR, Chu K, Dayas CV, Funk D, Knapp DJ, Koob GF, Lê DA, O'Dell LE, Overstreet DH, Roberts AJ, Sinha R, Valdez GR, Weiss F. (2005b) Stress enhancement of craving during sobriety: a risk for relapse. Alcohol Clin Exp Res. 29(2):185-95.

Breese GR, Knapp DJ, Overstreet DH, Navarro M, Wills TA, Angel RA. (2008) Repeated lipopolysaccharide (LPS) or cytokine treatments sensitize ethanol withdrawal-induced anxiety-like behavior. Neuropsychopharmacology. 33(4):867-76.

Breese GR, Knapp DJ, Overstreet DH. (2004) Stress-induced anxiety-like behavior during abstinence from previous chronic ethanol exposure. Alcohol Clin Exp Res. 28:191A.

Breese GR, Overstreet DH, Knapp DJ (2005a) Conceptual framework for the etiology of alcoholism: a "kindling"/stress hypothesis. Psychopharmacology (Berl). 178:367-380.

Breese GR, Overstreet DH, Knapp DJ, Navarro M. (2005c) Prior multiple ethanol withdrawals enhance stress-induced anxiety-like behavior: inhibition by CRF1- and benzodiazepine-receptor antagonists and a 5-HT1a-receptor agonist. Neuropsychopharmacology. 30(9):1662-9.

Breese GR, Sinha R, Heilig M (2011) Chronic alcohol neuroadaptation and stress contribute to susceptibility for alcohol craving and relapse. Pharmacology & Therapeutics. 129:149-171.

Buck HM, Hueston CM, Bishop C, Deak T. (2011) Enhancement of the hypothalamic-pituitary-adrenal axis but not cytokine responses to stress challenges imposed during withdrawal from acute alcohol exposure in Sprague-Dawley rats. Psychopharmacology 218(1):203-15.

Carson, E J and SB Pruett. 1996. Development and characterization of a binge drinking model in mice for evaluation of the immunological effects of ethanol. Alcohol Clin Exp Res. 20:1: 132-138.

Chen R, Zhou H, Beltran J, Malellari L, Chang S. (2005) Differential expression of cytokines in the brain and serum during endotoxin tolerance. Journal of Neuroimmunology.163:53–72.

Crews FT, Bechara R, Brown LA, Guidot DM, Mandrekar P, Oak S, Qin L, Szabo G, Wheeler M, Zou J. (2006) Cytokines and alcohol. Alcohol Clin Exp Res. 30(4):720-30.

Crews, F, Qin L, Sheedy D, Vetreno, Zou J. (submitted). Increased DANGER Signaling through HMGB1 and TLR Receptors in Alcohol. Biol Psychiatry.

Dai Q, Zhang J, Pruett SB. (2005) Ethanol alters cellular activation and CD14 partitioning in lipid rafts. Biochem Biophys Res Commun. 332(1):37-42.

Deak T, Bellamy C, D'Agostino LG. (2003) Exposure to forced swim stress does not alter central production of IL-1. Brain Res. 972(1-2):53-63.

Deak T, Bordner KA, McElderry NK, Barnum CJ, Blandino P Jr, Deak MM, Tammariello SP. (2005) Stress-induced increases in hypothalamic IL-1: a systematic analysis of multiple stressor paradigms. Brain Res Bull. 64(6):541-56.

DSM IV. American Psychiatric Association. (1994). Diagnostic and statistical manual of mental disorders (4th ed.). Washington, DC.

Ellwood KB, Yen YM, Johnson RC, Carey M. (2000) Mechanism for specificity by HMGB1 in enhanceosome assembly. Mol Cell Biol. 20:4359–4370.

Erridge C, Bennett-Guerrero E, Poxton IR. (2002) Structure and function of lipopolysaccharides. Microbes Infect. 4(8):837-51.

Faraco G, Fossati S, Bianchi ME, Patrone M, Pedrazzi M, Sparatore B, Moroni F, Chiarugi A. (2007) High mobility group box 1 protein is released by neural cells upon different stresses and worsens ischemic neurodegeneration in vitro and in vivo. J Neurochem. 103(2):590-603.

Fernandez-Lizarbe S, Pascual M, Gascon MS, Blanco A, Guerri C. (2008) Lipid rafts regulate ethanol-induced activation of TLR4 signaling in murine macrophages. Mol Immunol. 45(7):2007-16.

Fernandez-Lizarbe S, Pascual M, Guerri C. (2009) Critical role of TLR4 response in the activation of microglia induced by ethanol. J Immunol. 183(7):4733-44.

Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, Monks B, Pitha PM, Golenbock DT. (2003) LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J Exp Med. 198(7):1043-55.

Frye GD, McCown TJ, Breese GR. (1983) Characterization of susceptibility to audiogenic seizures in ethanol-dependent rats after microinjection of gamma-aminobutyric acid (GABA) agonists into the inferior colliculus, substantia nigra or medial septum. J Pharmacol Exp Ther. 227(3):663-70.

Gardella S, Andrei C, Ferrera D, Lotti LV, Torrisi MR, Bianchi ME, Rubartelli A. (2002) The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. EMBO Rep. 3(10):995-1001.

Garg AD, Nowis D, Golab J, Vandenabeele P, Krysko DV, Agostinis P. (2010) Immunogenic cell death, DAMPs and anticancer therapeutics: an emerging amalgamation. Biochim Biophys Acta. 1805(1):53-71

Gilman JM, Hommer DW. (2008) Modulation of brain response to emotional images by alcohol cues in alcohol-dependent patients. Addict Biol. (3-4):423-34.

Girard JP. A direct inhibitor of HMGB1 cytokine. (2007) Chem Biol. 14(4):345-7.

Girotti M, Donegan JJ, Morilak DA. (2011) Chronic intermittent cold stress sensitizes neuro-immune reactivity in the rat brain. Psychoneuroendocrinology. 36(8):1164-74

Glezer I, Simard AR, Rivest S. (2007) Neuroprotective role of the innate immune system by microglia. Neuroscience. 147(4):867-83

Godbout JP, Chen J, Abraham J, Richwine AF, Berg BM, Kelley KW, Johnson RW. (2005) Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system. FASEB J. 19(10):1329-31

Goodwin, GH, C Sanders and EW Johns (1973) A new group of chromatin-associated proteins with a high content of acidic and basic amino acids. Eur J Biochem, 38:14–19

Green DR, Ferguson T, Zitvogel L, Kroemer G. (2009) Immunogenic and tolerogenic cell death. Nat Rev Immunol. 9(5):353-63.

Hasin DS, Stinson FS, Ogburn E, Grant BF. (2007) Prevalence, correlates, disability, and comorbidity of DSM-IV alcohol abuse and dependence in the United States: results from the National Epidemiologic Survey on Alcohol and Related Conditions. Arch Gen Psychiatry. 64(7):830-42.

He J, and Crews FT. (2008) Increased MCP-1 and microglia in various regions of the human alcoholic brain. Exp. Neurol. 210(2):349-58.

Heilig M, Koob GF. (2007) A key role for corticotropin-releasing factor in alcohol dependence. Trends Neurosci. 30(8):399-406.

Hueston, CM, C J Barnum, JA Eberle, FJ Ferraioli, HM Buck, T Deak. (2011) Stress-dependent changes in neuroinflammatory markers observed after common laboratory stressors are not seen following acute social defeat of the Sprague Dawley rat. Physiol Behav. 104(2- 3):187-198.

Janssens S, Beyaert R. (2002) A universal role for MyD88 in TLR/IL-1R-mediated signaling. Trends Biochem Sci. 27(9):474-82.

Kim JB, Sig Choi J, Yu YM, Nam K, Piao CS, Kim SW, Lee MH, Han PL, Park JS, Lee JK. (2006) HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. J Neurosci. 26(24):6413-21.

Knapp DJ, Crews FT (1999) Induction of cyclooxygenase-2 in brain during acute and chronic ethanol treatment and ethanol withdrawal. Alcohol Clin Exp Res. 23:633-643.

Knapp DJ, Duncan GE, Crews FT, Breese GR. (1998) Induction of Fos-like proteins and ultrasonic vocalizations during ethanol withdrawal: further evidence for withdrawal-induced anxiety. Alcohol Clin Exp Res. 22(2):481-93.

Knapp DJ, Overstreet DH, Angel RA, Navarro M, Breese GR. (2007) The amygdala regulates the antianxiety sensitization effect of flumazenil during repeated chronic ethanol or repeated stress. Alcohol Clin Exp Res. 31(11):1872-82.

Knapp DJ, Whitman BA, Wills TA, Angel RA, Overstreet DH, Criswell HE, Ming Z, Breese GR. (2011) Cytokine involvement in stress may depend on corticotrophin releasing factor to sensitize ethanol withdrawal anxiety. Brain Behav Immun. 25 Suppl 1:S146-54.

Lin Q, Yang XP, Fang D, Ren X, Zhou H, Fang J, Liu X, Zhou S, Wen F, Yao X, Wang JM, Su SB. (2011) High-mobility group box-1 mediates toll-like receptor 4-dependent angiogenesis. Arterioscler Thromb Vasc Biol. 31(5):1024-32.

Ling Z, Zhu Y, Tong C, Snyder JA, Lipton JW, Carvey PM. (2006) Progressive dopamine neuron loss following supra-nigral lipopolysaccharide (LPS) infusion into rats exposed to LPS prenatally. Exp Neurol. 199(2):499-512.

Liu J, Lewohl JM, Harris RA, Iyer VR, Dodd PR, Randall PK, Mayfield RD. (2006) Patterns of gene expression in the frontal cortex discriminate alcoholic from nonalcoholic individuals. Neuropsychopharmacology. 31(7):1574-82.

Lotze MT, Tracey KJ. (2005) High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol. 5(4):331-42.

Maroso M, Balosso S, Ravizza T, Liu J, Aronica E, Iyer AM, Rossetti C, Molteni M, Casalgrandi M, Manfredi AA, Bianchi ME, Vezzani A. (2010) Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. Nat Med. 16(4):413-9.

McClain CJ, and Cohen DA. (1989) Increased tumor necrosis factor production by monocytes in alcoholic hepatitis. Hepatology. 9(3):349-51.

McClain CJ, Barve S, Deaciuc I, Kugelmas M, Hill D. (1999) Cytokines in alcoholic liver disease. Semin Liver Dis. 19(2):205-19.

McCown TJ, Breese GR. (1990) Multiple withdrawals from chronic ethanol "kindles" inferior collicular seizure activity: evidence for kindling of seizures associated with alcoholism. 34(5):777- Alcohol Clin Exp Res. 14(3):394-9.

Miller AH, Maletic V, Raison CL. (2009) Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. Biol Psychiatry. 65(9):732-41.

Minami M, Kuraishi Y, Yamaguchi T, Nakai S, Hirai Y, Satoh M. (1991) Immobilization stress induces interleukin-1 beta mRNA in the rat hypothalamus. Neurosci Lett. 123(2):254-6.

Mollica L, De Marchis F, Spitaleri A, Dallacosta C, Pennacchini D, Zamai M, Agresti A, Trisciuoglio L, Musco G, Bianchi ME. (2007) Glycyrrhizin binds to high-mobility group box 1 protein and inhibits its cytokine activities. Chem Biol.14(4):431-41.

Müller S, Scaffidi P, Degryse B, Bonaldi T, Ronfani L, Agresti A, Beltrame M, Bianchi ME. (2001) New EMBO members' review: the double life of HMGB1 chromatin protein: architectural factor and extracellular signal. EMBO J. 20(16):4337-40.

Nguyen KT, Deak T, Owens SM, Kohno T, Fleshner M, Watkins LR, Maier SF. (1998) Exposure to acute stress induces brain interleukin-1beta protein in the rat. J Neurosci. 18(6):2239-46.

Nguyen KT, Deak T, Will MJ, Hansen MK, Hunsaker BN, Fleshner M, Watkins LR, Maier SF. (2000) Timecourse and corticosterone sensitivity of the brain, pituitary, and serum interleukin-1beta protein response to acute stress. Brain Res. 859(2):193-201.

Nguyen, MD, JP Julien and S Rivest. (2002) Innate immunity: the missing link in neuroprotection andneurodegeneration? Nature reviews. Neuroscience 3(3): 216-227.

Ohnishi M, Katsuki H, Fukutomi C, Takahashi M, Motomura M, Fukunaga M, Matsuoka Y, Isohama Y, Izumi Y, Kume T, Inoue A, Akaike A. (2011) HMGB1 inhibitor glycyrrhizin attenuates intracerebral hemorrhage-induced injury in rats. Neuropharmacology. 61(5-6):975-80.

Okun E, Griffioen KJ, Lathia JD, Tang SC, Mattson MP, Arumugam TV. (2009) Toll-like receptors in neurodegeneration. Brain Res Rev. 59(2):278-92.

Okvist A, Johansson S, Kuzmin A, Bazov I, Merino-Martinez R, Ponomarev I, Mayfield RD, Harris RA, Sheedy D, Garrick T, Harper C, Hurd YL, Terenius L, Ekstro¨m TJ, Bakalkin G, Yakovleva T (2007) Neuroadaptations in human chronic alcoholics: dysregulation of the NF-kappaB system. PLoS ONE 2:e930.

Overstreet DH, Knapp DJ, Breese GR. (2002) Accentuated decrease in social interaction in rats subjected to repeated ethanol withdrawals. Alcohol Clin Exp Res. 26(8):1259-68.

Overstreet DH, Knapp DJ, Angel RA, Navarro M, Breese GR. (2006) Reduction in repeated ethanol-withdrawal-induced anxiety-like behavior by site-selective injections of 5-HT1A and 5-HT2C ligands. Psychopharmacology (Berl). 187(1):1-12.

Palumbo R, Sampaolesi M, De Marchis F, Tonlorenzi R, Colombetti S, Mondino A, Cossu G, Bianchi ME. (2004) Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation. J Cell Biol. 164(3):441-9.

Park JS, Gamboni-Robertson F, He Q, Svetkauskaite D, Kim JY, Strassheim D, Sohn JW, Yamada S, Maruyama I, Banerjee A, Ishizaka A, Abraham E. (2006) High mobility group box 1 protein interacts with multiple Toll-like receptors. Am J Physiol Cell Physiol. 290(3):C917-24.

Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A, Abraham E. (2004) Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. J Biol Chem. 279:7370–7377.

Pascual M, Baliño P, Alfonso-Loeches S, Aragón CM, Guerri C. (2011) Impact of TLR4 on behavioral and cognitive dysfunctions associated with alcohol-induced neuroinflammatory damage. Brain Behav Immun. 25 Suppl. 1:S80-91.

Pascual M, Blanco AM, Cauli O, Minarro J, Guerri C (2007) Intermittent ethanol exposure induces inflammatory brain damage and causes long-term behavioural alterations in adolescent rats. Eur J Neurosci. 25:541-550.

Plata-Salamán CR, Ilyin SE, Turrin NP, Gayle D, Flynn MC, Bedard T, Merali Z, Anisman H. (2000) Neither acute nor chronic exposure to a naturalistic (predator) stressor influences the interleukin-1beta system, tumor necrosis factor-alpha, transforming growth factor-beta1, and neuropeptide mRNAs in specific brain regions. Brain Res Bull. 51(2):187-93.

Pruett BS, and Pruett SB. (2006) An explanation for the paradoxical induction and suppression of an acute phase response by ethanol. Alcohol. 39(2):105-10.

Pruett SB, and Fan R. (2009) Ethanol inhibits LPS-induced signaling and modulates cytokine production in peritoneal macrophages in vivo in a model for binge drinking. BMC Immunol. 10:49.

Qin L, He J, Hanes RN, Pluzarev O, Hong JS, Crews FT. (2008) Increased systemic and brain cytokine production and neuroinflammation by endotoxin following ethanol treatment. J Neuroinflammation. 18: 5-10.

Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong JS, Knapp DJ, Crews FT. (2007) Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. Glia. 55(5):453-62.

Rauvala H, Rouhiainen A. (2007) RAGE as a receptor of HMGB1 (Amphoterin): roles in health and disease. Curr Mol Med. 7(8):725-34.

Rovere-Querini P, Capobianco A, Scaffidi P, Valentinis B, Catalanotti F, Giazzon M, Dumitriu IE, Müller S, Iannacone M, Traversari C, Bianchi ME, Manfredi AA. (2004) HMGB1 is an endogenous immune adjuvant released by necrotic cells. EMBO Rep. 5(8):825-30

Scaffidi P, Misteli T, Bianchi ME. (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature. 418(6894):191-5.

Sha Y, Zmijewski J, Xu Z, Abraham E. (2008) HMGB1 develops enhanced proinflammatory activity by binding to cytokines. J Immunol. 180(4):2531-7.

Shizuya K, Komori T, Fujiwara R, Miyahara S, Ohmori M, Nomura J. (1997) The influence of restraint stress on the expression of mRNAs for IL-6 and the IL-6 receptor in the hypothalamus and midbrain of the rat. Life Sci. 61(10):PL 135-40.

Sinha R, Fox HC, Hong KI, Hansen J, Tuit K, Kreek MJ. (2011) Effects of adrenal sensitivity, stress- and cue-induced craving, and anxiety on subsequent alcohol relapse and treatment outcomes. Arch Gen Psychiatry. 68(9):942-52.

Su X, Wang H, Zhao J, Pan H, Mao L. (2011) Beneficial effects of ethyl pyruvate through inhibiting high-mobility group box 1 expression and TLR4/NF-κB pathway after traumatic brain injury in the rat. Mediators Inflamm. 2011:807142.

Sugama S, Takenouchi T, Fujita M, Conti B, Hashimoto M. (2009) Differential microglial activation between acute stress and lipopolysaccharide treatment. J Neuroimmunol. 207(1-2):24-31.

Suzuki E, Shintani F, Kanba S, Asai M, Nakaki T. (1997) Immobilization stress increases mRNA levels of interleukin-1 receptor antagonist in various rat brain regions. Cell Mol Neurobiol. 17(5):557-62.

Triantafilou M, Miyake K, Golenbock DT, Triantafilou K. (2002) Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation. J Cell Sci.115(Pt 12):2603-11.

Uesugi T, Froh M, Arteel GE, Bradford BU, Thurman RG. (2001) Toll-like receptor 4 is involved in the mechanism of early alcohol-induced liver injury in mice. Hepatology. 34(1):101-8.

Ulloa L, Ochani M, Yang H, Tanovic M, Halperin D, Yang R, Czura CJ, Fink MP, Tracey KJ. (2002) Ethyl pyruvate prevents lethality in mice with established lethal sepsis and systemic inflammation. Proc Natl Acad Sci U S A. 99(19):12351-6

Valles SL, Blanco AM, Azorin I, Guasch R, Pascual M, Gomez-Lechon MJ, Renau-Piqueras J, Guerri C. (2003) Chronic ethanol consumption enhances interleukin-1-mediated signal transduction in rat liver and in cultured hepatocytes. Alcohol Clin Exp Res. 27(12):1979-86.

Vartanian K, Stenzel-Poore M. (2010) Toll-like receptor tolerance as a mechanism for neuroprotection. Transl Stroke Res. 1(4):252-260.

Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ. (1999) HMG-1 as a late mediator of endotoxin lethality in mice. Science. 285(5425):248-51.

Wang W, Ji P, Dow KE. (2003) CRF induces proliferation and TNFα release in cultured rat microglia via MAP-kinase signaling pathways. J Neurochem. 84:189-195.

Wang W, Ji P, Riopelle RJ, Dow KE. (2002) Functional expression of CRH receptor 1 in

WHO (2009) Alcohol and Drugs. Report on the meeting on indicators for monitoring alcohol, drugs and other psychoactive substance use, substance-attributable harm and societal responses. In: Abuse, D.o.M.H.a.S. (Ed.), WHO Press, Geneva.

Wills TA, Knapp DJ, Overstreet DH, Breese GR. (2008) Differential dietary ethanol intake and blood ethanol levels in adolescent and adult rats: effects on anxiety-like behavior and seizure thresholds. Alcohol Clin Exp Res. 32(8):1350-60.

Wu Y, Lousberg EL, Moldenhauer LM, Hayball JD, Coller JK, Rice KC, Watkins LR, Somogyi AA, Hutchinson MR. (2012) Inhibiting the TLR4-MyD88 signalling cascade by genetic or pharmacological strategies reduces acute alcohol-induced sedation and motor impairment in mice. Br J Pharmacol. 165(5):1319-29.

Yanai H, Ban T, Wang Z, Choi MK, Kawamura T, Negishi H, Nakasato M, Lu Y, Hangai S, Koshiba R, Savitsky D, Ronfani L, Akira S, Bianchi ME, Honda K, Tamura T, Kodama T, Taniguchi T. (2009) HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses. Nature. 462:99-103.

Yang H, Wang H, Czura CJ, Tracey KJ. (2002) HMGB1 as a cytokine and therapeutic target. J Endotoxin Res. 2002;8(6):469-72.

Yang H, Wang H, Czura CJ, Tracey KJ. (2005) The cytokine activity of HMGB1. J Leukoc Biol. 78(1):1-8.

Yang QW, Lu FL, Zhou Y, Wang L, Zhong Q, Lin S, Xiang J, Li JC, Fang CQ, Wang JZ. (2011) HMGB1 mediates ischemia-reperfusion injury by TRIF-adaptor independent Toll-like receptor 4 signaling. J Cereb Blood Flow 31(2):593-605.

Yang QW, Wang JZ, Li JC, Zhou Y, Zhong Q, Lu FL, Xiang J. (2010) High-mobility group protein box-1 and its relevance to cerebral ischemia. J Cereb Blood Flow Metab. 30(2):243-54

Yu M, Wang H, Ding A, Golenbock DT, Latz E, Czura CJ, Fenton MJ, Tracey KJ, Yang H. (2006) HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. Shock. 26(2):174-9.

Zou J, Crews F. (2010) Induction of innate immune gene expression cascades in brain slice cultures by ethanol: key role of NF-κB and proinflammatory cytokines. Alcohol Clin Exp Res.