THE ROLE OF NEURONS AND GLIA IN ETHANOL-INDUCED INNATE IMMUNE SIGNALING

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ABSTRACT

Colleen J. Lawrimore: The role of neurons and glia in ethanol-induced innate immune signaling
(Under the direction of Fulton T. Crews)

The innate immune system is an unexpected and unique addition to signaling pathways in the brain. The brain functions as a largely sterile environment, void of infiltrating infectious agents due to the blood brain barrier. However, in recent years it has been discovered that multiple components of the innate immune system, including Toll-like receptors (TLRs), pro-inflammatory cytokines, and transcription factors such as NFκB that regulate innate immune genes, are upregulated in post-mortem human alcoholic brain. This has further been replicated both in rodent models of alcohol consumption as well as in brain slice cultures. However, it is still unknown how different cell types in brain contribute to this response and how innate immune signaling molecules function as communication mediators between cells.

In this dissertation, we determine the role of neurons and glia (microglia and astrocytes) in ethanol-induced innate immune system by utilizing specific cell lines: SH-SY5Y neurons, BV2 microglia, and U373 astrocytes. In Chapter 2, we treat SH-SY5Y neurons and BV2 microglia with either ethanol, the TLR3 agonist Poly(I:C), or the TLR4-agonist LPS, and discover that ethanol induces a broad and highly sensitive response to ethanol in SH-SY5Y neurons. In Chapter 3, we use a co-culture model of BV2 microglia and SH-SY5Y to determine how co-culture impacts ethanol-induced innate immune signaling between these two cell types. We discover that co-culture modifies multiple innate immune genes in both cell types, as well as

increasing ethanol-induced IL-4/IL-13 signaling, suggesting a novel microglial-neuronal signaling pathway. In Chapter 4, we discover that ethanol induces interferons in SH-SY5Y neurons and U373 astrocytes, but not BV2 microglia, indicating interferons as a neuronal and astrocytic-specific response to ethanol. We further determined using conditioned media experiments that astrocyte-induced TRAIL, an interferon response gene, induces interferons in SH-SY5Y neurons. This suggests novel TRAIL-IFN signaling pathways between astrocytes and neurons.

Overall, these results suggest that neurons have a unique involvement in ethanol-induced innate immune signaling, and that innate immune signaling molecules function as cell-to-cell signaling mediators in brain. In addition, these results indicate that future therapeutic strategies may be utilized to target both specific cell type and cell-to-cell signaling responses.

To my son.

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LIST OF ABBREVIATIONS

ALD – Alcoholic liver disease

AP-1 – Activator protein-1

AUD – Alcohol use disorder

CCR2 – C-C chemokine receptor type 2

Cd11b – Cluster of differentiation molecule 11b

CD200 – Cluster of differentiation 200

CX3CL1 – C-X3-C Motif Chemokine Ligand 1

CX3CR1 – CX3C chemokine receptor 1

DAMP – Damage associated molecular pattern

DR4 – Death receptor 4

DR5 – Death receptor 5

EC – Entorhinal cortex

EtOH – Ethanol

FAS – Fetal alcohol syndrome

HEC – Hippocampal-entorhinal cortex

HMGB1 – High mobility group protein B1

Iba1 – ionized calcium-binding adapter molecule 1

IFNAR1/2 – Interferon α/β receptor 1/2

IFNβ – Interferon β

IFNγ – Interferon γ

IFNγR1/2 – Interferon γ receptor 1/2

IKK –IκB kinase

IL-10 – Interleuken-10

IL-13 – Interleuken-13

IL-1β – Interleuken-1β

IL-4 – Interleuken-4

IL-6 – Interleuken-6

IRF – Interferon regulatory factor

ISG – Interferon stimulated gene

ISM – immune signaling molecule

JAK – Janus kinase

LPS – Lipopolysaccharide

LTP – Long-term potentiation

MAPK – Mitogen activated protein kinase

MCP-1 – Monocyte chemoattractant protein-1

Myd88 – Myeloid differentiation primary response 88

NFκB – Nuclear factor κ-light-chain-enhancer of activated B cells

OFC – Orbitofrontal cortex

PAMP – Pattern associated molecular pattern

Poly(I:C) – Polyinosinic:polycytidylic acid

PRR – Pattern recognition receptor

RAGE – Receptor for advanced glycation end products

STAT – Signal transducer and activator of transcription

TGFβ – Transforming growth factor β

TIR – Toll/IL-1 receptor domain

TLR – Toll-like receptor

TNFα – Tumor necrosis factor α

TRAF6 – TNF receptor associated factor 6

TRAIL – TNF related apoptosis inducing ligand

TRIF – TIR-domain-containing adaptor protein inducing IFNβ

CHAPTER 1: INTRODUCTION

1.1 Alcohol: health and disease

1.1.1 Introduction to alcohol use

Alcohol is a widely used substance, especially in the United States where according to a 2015 survey, 86.4% of adults aged 18 and older consumed alcohol at some point in their lifetime ((SAMHSA), 2015a). Despite the prevalence of alcohol consumption, alcohol is a leading cause of preventable death as well as alcohol use disorder (AUD), fetal alcohol syndrome (FAS), alcoholic liver disease (ALD), leaky gut, various cancers, heart problems, and others. According to NIAAA, AUDs are characterized by compulsive drinking, loss of control over drinking, and negative emotions when not drinking. Interestingly, this definition specifically does not mention exact amounts, implying that even so-called moderate levels of drinking may be problematic. Nonetheless, many of the problems instigated by alcohol are instigated by binge-drinking levels of consumption. According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA), heavy drinking is defined as >= 8 drinks per week for women, or >= 15 drinks per week for men. Binge drinking is defined as having a blood alcohol concentration of >= 80 mg/dL, which is commonly achieved by >= 4 drinks under 2 hours in women, or >= 5 drinks under 2 hours in men. This level of heavy consumption is surprisingly common; a 2015 study found that 26.9% of individuals binge drank within the past month ((SAMHSA), 2015b). Therefore, further research is needed that specifically focuseson binge-level drinking.

Many of the diseases and negative health outcomes caused by heavy alcohol consumption are also linked to dysregulation of the innate immune system (Molina, Happel, Zhang, Kolls, & Nelson, 2010). For example, recent studies have found a strong correlation between maternal proinflammatory cytokines, a mediator of inflammation in the innate immune system, and FAS outcomes in infants with prenatal alcohol exposure (Sowell et al., 2018). Alcoholic liver disease is also associated with increasing levels of inflammation as the disease progresses in severity into cirrhosis (Seitz et al., 2018), which is also associated with alcohol increasing gut permeability, thereby releasing endotoxin into the bloodstream (Keshavarzian et al., 1999). In addition, there is a vast amount of literature suggesting that the addictive pathology that characterizes AUDs is linked to the neuroimmune system (Coleman & Crews, 2018; Crews, Lawrimore, Walter, & Coleman, 2017; Crews & Vetreno, 2011, 2016; Jacobsen, Hutchinson, & Mustafa, 2016; Mayfield, Ferguson, & Harris, 2013; Montesinos, Alfonso-Loeches, & Guerri, 2016). Neuroimmune effects are therefore highly implicated in multiple negative outcomes of alcohol consumption, and will be the focus of the studies in this dissertation.

1.1.2 Alcohol and the brain

The effects that alcohol has on brain function are multiple and obvious even soon after consumption. Intoxication is marked by diminished motor function, slurred speech, lack of impulsivity control, and impaired memory. However, the long-term effects of alcohol consumption are often more sinister and less apparent. In particular, alcohol causes neurodegeneration and an increase of negative affect, which may set off a cascade leading to addiction.

It has been well-documented that heavy alcohol consumption causes neurodegeneration in multiple brain regions (Crews et al., 2004). In rodent models, binge-level drinking causes

neurodegeneration in corticolimbic regions (Collins, Zou, & Neafsey, 1998; Obernier, Bouldin, & Crews, 2002) that is associated with impairments in learning and memory tasks (Obernier, White, Swartzwelder, & Crews, 2002). These changes are also long-lasting, with impairments seen in learning tasks in adulthood following adolescent ethanol exposure (Coleman, He, Lee, Styner, & Crews, 2011; Vetreno & Crews, 2012). Interestingly, there is also a link between neurodegeneration and innate immune signaling in brain (Heneka, Kummer, & Latz, 2014), in particular that of alcohol (Crews & Vetreno, 2014).

Addictive disorders, such as AUD, are thought to be progressed by the "dark side" of addiction, a theory popularized by George Koob (Koob & Le Moal, 2005; Schulteis & Koob, 1994). Addiction is thought to be comprised of three main stages: binge/intoxication, withdrawal/negative affect, and preoccupation/craving (Koob & Volkow, 2010; Volkow, Koob, & McLellan, 2016). Interestingly, there is a strong link between the neuroimmune system and these various stages of addiction (Coleman & Crews, 2018; Crews et al., 2017; Crews & Vetreno, 2011, 2016; Jacobsen et al., 2016; Mayfield et al., 2013; Montesinos et al., 2016), a system which will be described fully in the following section.

Treatment for AUD consist of both behavioral therapies, such as cognitive behavioral therapies and group therapy, and/or medications. There are currently four medications that have been approved to treat alcohol use disorders: disulfiram, acamprosate, and naltrexone. Disulfiram works by increasing aversive withdrawal symptoms of alcohol, whereas acamprosate relieves alcohol cravings (Kranzler & Soyka, 2018). Naltrexone, as an opioid receptor antagonist, works by reducing the rewarding properties of alcohol (Kranzler & Soyka, 2018). Interestingly, naltrexone also functions as an antagonist at innate immune receptor Toll-like

receptor 4 (TLR4) (Hutchinson et al., 2008), receptors which will be further discussed in the next section.

In summary, while it is clear that alcohol has neurodegenerative effects in the brain which correspond with progression of the addiction cycle, treatments remain sparse and neuroimmune signals remain poorly understood. As mentioned previously, there is increasing evidence for involvement in the neuroimmune system as a mediator of alcohol's effects in brain. This system will be described in detail in the following section.

1.2 The innate immune system in brain: effect of alcohol

1.2.1 Overview of the innate immune system

Much of the dogma of the immune system comes from the peripheral immune system, which is comprised of two main systems: the adaptive immune system, which comprises antigen-specific responses, and the innate immune system. Of these, the innate immune system is the older, nonspecific defense against infections (Turvey & Broide, 2010). Activation by pathogen associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) instigates a cascade of cell-mediated responses. PAMPs/DAMPs bind to pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs). Activation of TLRs leads to activation of transcription factors such as NFκB and AP-1, which causes further transcription of cytokines, propagating inflammation through their respective receptors (see Figure 1.1).

The innate immune system has traditionally been studied in the periphery, and mediated by peripheral immune cells such as macrophages in which acute phase activation is followed by the adaptive immune system, e.g., T-cells, which mediate antibody-related responses. The brain, however, is known as an "immune-privileged" organ due to the protection of the blood-brain-barrier (William A. Banks & Erickson, 2010) and lack of these antibody-mediated responses. In

addition, many of the signaling pathways and roles of specific cytokines have not been specifically identified in brain. However, in recent years there has been an increasing focus on understanding the innate immune system in brain (Ransohoff & Brown, 2012). Importantly, the brain features unique innate immune responses. Several cytokines, such as TNFα, IL-6, and IL-1β are capable of crossing the blood-brain barrier (W. A. Banks, Kastin, & Broadwell, 1995; W. A. Banks, Kastin, & Gutierrez, 1994). In addition to responding to peripheral cytokines, the brain is also capable of producing innate immune signals itself. Alcohol in particular has been shown to alter innate immune signaling molecules in brain, with multiple publications from our lab documenting upregulation of innate immune signaling molecules in postmortem human alcoholic brain (see Table 1.1). However, it is unknown which receptors and/or transcription factors alcohol activates to mediate these effects. Also, it is unknown which cell types in brain are responsible for innate immune signaling. Resident innate immune cells are commonly considered to be both microglia and astrocytes, as well as an emerging role for neurons that will be discussed in the following section.

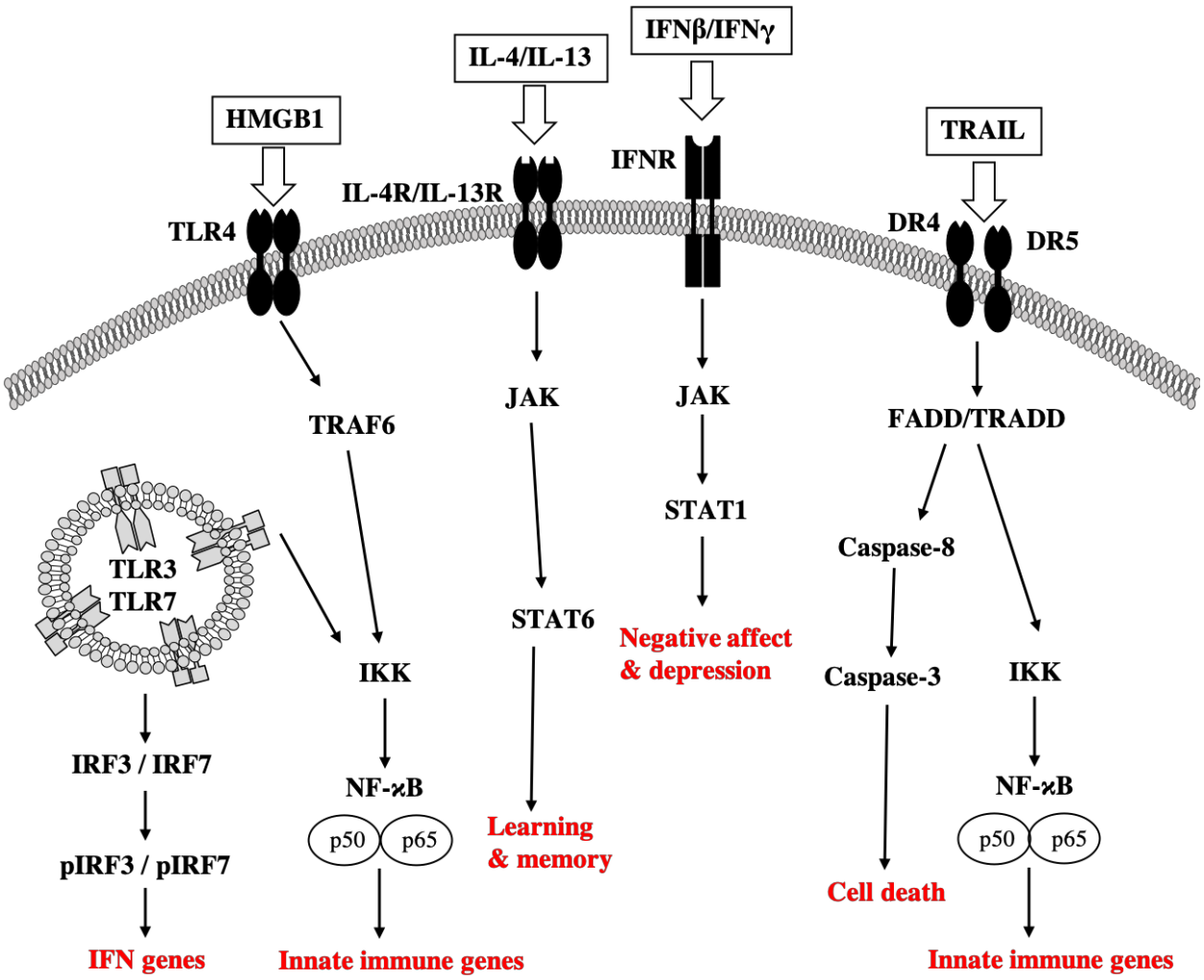

Figure 1.1 Simplified schematic of select innate immune signaling pathways (adapted from Coleman & Crews, 2018). This schematic describes in brief several innate immune signaling pathways that are examined in this dissertation. HMGB1 is a nuclear protein that when released from activated or stressed cells functions as an endogenous TLR agonist. TLR3, 4, and 7 activate transcription factor NFκB to promote innate immune gene induction. TLR3 and TLR7 additionally signal through IRF transcription factors to promote IFN gene transcription. IL-4 and IL-13 function as anti-inflammatory cytokines in the periphery, and signal through their heterodimeric receptor IL-4R/IL-13R. Activation of this pathway in brain has further been linked to enhancement of learning and memory. IFNβ and IFNγ signal through their respective receptors (IFNAR1/2 or IFNγR1/2) to induce IFN stimulated genes (ISGs) via JAK/STAT signaling, promoting negative affect and depression. TRAIL, one such ISG, signals through death receptor (DR) 4 or DR5, leading to either cell death or innate immune gene activation, including interferons. However, it is important to note that while the innate immune signaling pathways diagramed here have been described in peripheral macrophages, it remains unknown how these pathways may vary among different cell types (e.g., microglia, astrocytes, and neurons) in brain.

1.2.2 Unique cell-type responses in innate immune signaling

Microglia and astrocytes are both known to feature immunological responses, although neurons are recently emerging as a novel immune mediator as well. Microglia are the canonical macrophage in brain, which unlike other myeloid cells are derived from yolk sac progenitor cells (Ginhoux, Lim, Hoeffel, Low, & Huber, 2013) and are maintained throughout adulthood via local populations (Ajami, Bennett, Krieger, McNagny, & Rossi, 2011; Ginhoux et al., 2010). However, similar to other macrophages, microglia express multiple markers such as Cd11b, CX3CR1, Iba1, and F4/80. Microglia are commonly understood have two main stages of activation: M1, an inflammatory phase characterized by release of pro-inflammatory cytokines (e.g., TNFα, IL-1β), and M2, an anti-inflammatory phase characterized by anti-inflammatory cytokines (e.g., IL-4, IL-10). However, it has recently been suggested that these descriptions may be an oversimplification which undermines the complex activity of microglia (Ransohoff, 2016). Microglia have also been shown to play a role in ethanol-induced innate immune responses, with ethanol inducing various NFκB-dependent genes in cultured microglia (Fernandez-Lizarbe, Montesinos, & Guerri, 2013; Fernandez-Lizarbe, Pascual, & Guerri, 2009; Lawrimore & Crews, 2017) as well as inducing microglial activation markers *in vivo* (Qin & Crews, 2012a, 2012b) and in post-mortem human alcoholics (He & Crews, 2008).

Astrocytes are also known to play a role in the neuroimmune system (Colombo & Farina, 2016; Farina, Aloisi, & Meinl, 2007). Similar to neurons, astrocytes are derived from the neuroectoderm during embryonic development (Guerout, Li, & Barnabe-Heider, 2014). Astrocytes that are activated by certain pathophysiological events, called reactive gliosis, have varying effects ranging from neuroprotective to degenerative (Pekny & Pekna, 2014). Importantly, astrocytes have been demonstrated to express various cytokines and innate immune

receptors (Jensen, Massie, & De Keyser, 2013). Interestingly, it has also been suggested that astrocytes have pro-inflammatory (A1) and anti-inflammatory (A2) states, similar to microglia (Liddelow et al., 2017). Cultured astrocytes have also been found to have TLR4-dependent innate immune signaling in response to ethanol (Alfonso-Loeches, Pascual-Lucas, Blanco, Sanchez-Vera, & Guerri, 2010; Blanco, Valles, Pascual, & Guerri, 2005), further implicating their role in ethanol-induced innate immune signaling.

Neurons are a controversial newcomer to innate immune signaling. While multiple labs have found that neurons express innate immune signals, such as TLR3, 4, and 7 both *in vitro* and *in vivo* (June et al., 2015; Lawrimore & Crews, 2017; Lehmann et al., 2012; Lok, Basta, Manzanero, & Arumugam, 2015; Tang et al., 2007; Vetreno & Crews, 2012), the role that neurons may play in innate immune signaling is still poorly understood and at times controversial. While TLR4 appears to play a role in neuronal firing, with HMGB1-TLR4 signaling precipitating seizures in rodent models (Maroso et al., 2010) and TLR4 agonist LPS reducing firing of dopaminergic neurons (Blednov et al., 2011), it is less clear whether neurons participate in innate immune signaling. Despite expressing TLR4, some studies find that neurons lack TLR4 agonist LPS-induced innate immune signals (Klegeris & McGeer, 2001; Lawrimore & Crews, 2017; Prehaud, Megret, Lafage, & Lafon, 2005), possibly due to a lack of downstream signaling molecules such as MD2 (Lawrimore & Crews, 2017). In addition, studies by the June and Aurelian labs found that TLR4 and chemokine MCP-1 are localized on dopaminergic neurons in the central nucleus of the amygdala (CeA) and the ventral tegmental area (VTA) of alcohol-preferring P-rats (Balan et al., 2018; June et al., 2015) and that downregulation of TLR4 or MCP-1 using injections of siRNA in the CeA and VTA decreases alcohol consumption in P-rats (Balan et al., 2018; June et al., 2015; Liu et al., 2011).

Interestingly, they further found using the N2a neuronal cell line that TLRs that may signal independently of LPS, in particular via protein-protein interactions between TLR4 and the α2 subunit of the $GABA_A$ receptor, in which α2 may mediate NFκB-independent pathways (Balan et al., 2018). In addition, involvement of other molecules, such as corticotrophin releasing factor (CRF), may influence TLR activity, as shown by CRF antagonists decreasing TLR4 in the CeA of P-rats (June et al., 2015).

Other TLRs, in particular TLR3, have also been suggested to have a neuronal role. In particular, there is evidence to suggest that neurons express innate immune signaling molecules in response to TLR3 stimulation (Lawrimore & Crews, 2017; Nessa et al., 2006; Prehaud et al., 2005). Interestingly, endogenous TLR agonist HMGB1 is also highly expressed in neurons (Crews, Qin, Sheedy, Vetreno, & Zou, 2013) and is stimulated following treatment with ethanol (Crews et al., 2013; Lawrimore & Crews, 2017; Wang et al., 2015; Zou & Crews, 2014). However, the role that neurons play in innate immune signaling is still poorly understood, and will be further explored throughout the following chapters of this manuscript.

1.2.3 Neuroimmune signaling across cell-types: paracrine signaling in brain

Signaling between two different cells, or paracrine signaling, is a prominent mechanism of communication in brain between different cell types that is oftentimes overlooked. However, multiple systems of communication between neurons and glia exist. Traditional "immune signals" in particular appear to play an important role in paracrine communication, oftentimes outside of their traditional inflammatory roles. For example, the complement system, which in the immune system enhances elimination of microbes and damaged cells, is important for synaptic pruning during development (Stephan, Barres, & Stevens, 2012). Fractalkine (CX3CL1), which is specific to neurons, binds to its microglial-specific receptor (CX3CR1), also

plays a role in synaptic pruning via microglial recruitment, as well as preventing excessive M1 activation of microglia (Sheridan & Murphy, 2013). However, alternative roles of cytokines and other signals playing a role in neuronal-glial communication remain poorly understood. It is also not well understood how ethanol impacts these signaling pathways. How ethanol-induced effects on innate immune signals can change across cell types needs to be determined, and this will be further explored in the following chapters of this manuscript.

1.2.4 Toll-like receptors and cytokines

Toll-like receptors (TLRs) are a family of pattern recognition receptors (PRRs) that respond to a variety of viral or bacterial components, such as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) that are released from activated or damaged cells. There are currently10 TLRs identified in human (TLR1-10), and 3 additional TLRs in mouse (TLR11-13). TLR1,2,4,5,6,10,11, and 12 are expressed on the cell surface, whereas TLR3,7,8, and 9 are largely localized on intracellular endosomes. TLRs contain 3 main domains: an extracellular N-terminal domain that recognizes PAMPs, a transmembrane domain, and an intracellular C-terminal domain called the Toll/IL-1 receptor (TIR) domain (Beutler, 2009). The TIR domain recruits varying adaptor proteins (dependent on the TLR in question) to further the signaling cascade. TLR4 utilizes one of two pathways: (i) a MyD88-dependent pathway, in which TIR recruits MyD88 adaptor-like (MAL/TIRAP) which recruits MyD88, activating IL-1 receptor-associated kinases (IRAKs) and TNF receptor-associated factor 6 (TRAF6), which then actives IKK and mitogen-activated protein kinase (MAPK) activation, leading to activation of kinases like NFκB and AP-1 and (ii) a Myd88-independent pathway, in which TIR-domain-containing adaptor protein inducing IFNβ (TRIF) associates with TRIF-related adaptor molecule (TRAM), leading to activation of kinases such as

IRF3 (Narayanan & Park, 2015). TLR3, unlike the other TLRs, primarily utilizes a MyD88-independent pathway, recruiting TRIF to its TIR domain (Oshiumi, Matsumoto, Funami, Akazawa, & Seya, 2003) which recruits TRAF3, promoting IRF3 signaling, or recruiting TRAF6 which leads to NFκB/AP-1 activation. However, as canonical TLR3 signaling has been established primarily in peripheral macrophages, signaling in brain may utilize alternative pathways. In addition, since the brain is largely a sterile environment that lacks traditional PAMPs and infectious TLR agonists, endogenous agonists of TLRs, such as HMGB1, are of great interest in the CNS.

Both TLR3 and TLR4 have been demonstrated to play a role in ethanol-induced innate immune responses. Binge-ethanol treatment in mice causes an increase in TLR3 expression that potentiates TLR3-mediated responses (Qin & Crews, 2012a), mediates drinking behavior in rodents (Jang, Lee, Park, Han, & Kim, 2016) and is also upregulated in post-mortem human alcoholic brain (Crews et al., 2013). There is a vast amount of evidence suggesting TLR4 plays a role in ethanol-induced NFκB activation and innate immune signal induction (Alfonso-Loeches et al., 2010; Blanco et al., 2005; Fernandez-Lizarbe et al., 2009). TLR4 deficient mice are also protected from neurodegeneration (Alfonso-Loeches et al., 2010). In addition, TLR4 siRNA injected into the ventral tegmental area (VTA) blunted binge drinking in alcohol preferring P-rats (June et al., 2015). Nonetheless, research from multiple laboratories suggested that TLR4 does not modulate drinking behavior in mice (Harris et al., 2017), suggesting that other factors, including other TLRs, also play an important role in alcohol pathology. Furthermore, this induction of TLRs by ethanol, itself a unique form of plasticity in brain, further implicates multiple endogenous agonists in brain, such as HMGB1, which will be further discussed below.

Both TLR3 and TLR4 activation causes NFκB-dependent transcription of immune signaling molecules such as cytokines (TNFα, IL-1β, IL-6) and chemokines (MCP1). While in the periphery these function as proinflammatory mediators, in brain it has been suggested that they play unique roles. In particular, there have been multiple reports of cytokines impacting long-term potentiation (LTP), a mechanism that underlies learning and memory (Prieto & Cotman, 2017). TNFα is necessary for LTP in rodent visual cortex (Sugimura, Yoshimura, & Komatsu, 2015), but at higher concentrations disrupts LTP (Tancredi et al., 1992), causing increased glutamatergic AMPA receptor expression on the cell surface while internalizing $GABA_A$ receptors, shifting the balance to excitatory signaling (Stellwagen, Beattie, Seo, & Malenka, 2005). Similar to TNFα, IL-1β also enhances LTP at lower concentrations and disrupts it at higher concentrations (Bellinger, Madamba, & Siggins, 1993; Goshen et al., 2007; H. Schneider et al., 1998). Furthermore, TNFα inhibits glial glutamate transporters, increasing glutamate mediated excitotoxicity (Zou & Crews, 2005), suggesting a direct role for cytokines in excitotoxicity-mediated neurodegeneration. Cytokines also appear to play a role in alcohol pathology, with knockout mice for multiple cytokines and receptors (IL-6, CCR2, MCP-1) demonstrate reduced ethanol intake (Blednov et al., 2005; Blednov et al., 2012). In human alcoholics, several cytokines, including TNFα, IL-1β, and IL-6 are elevated in plasma and correlate with alcohol craving (Heberlein et al., 2014; Leclercq, De Saeger, Delzenne, de Timary, & Starkel, 2014). Several different TLRs and other innate immune signaling molecules are also upregulated in post-mortem human alcoholic brain (see Table 1). In addition, increased levels of maternal cytokines are a risk factor for FAS (Sowell et al., 2018). Thus, cytokines are likely linked to both neuroplasticity and neurodegeneration, which can be altered by alcohol exposure.

One cytokine of particular interest in alcohol pathology is High mobility group box protein 1 (HMGB1), a nuclear protein that when released functions as a DAMP, activating multiple TLRs such as TLR2, TLR3, TLR4, TLR7, and TLR9 (Park et al., 2004; Yanai et al., 2009; Yang, Antoine, Andersson, & Tracey, 2013) as well as receptor for advanced glycation end products (RAGE) (Kokkola et al., 2005). HMGB1 is present in all cells, and may be released from cells during or following necrotic cell death, or may be actively secreted from cells. Immune stimulation (e.g., via TLR4 agonist LPS) induces acetylation of HMGB1, causing it to move from the nucleus to cytoplasm, where it then is packed into microvesicles for secretion (Magna & Pisetsky, 2014). HMGB1 has been linked to the pathogenesis of multiple diseases, including lupus (Pisetsky, 2014), rheumatoid arthritis (Pisetsky, Erlandsson-Harris, & Andersson, 2008), and others (Kang et al., 2014). In particular, HMGB1 is released by ethanol in rat hippocampal entorhinal slice culture (Crews et al., 2013; Zou & Crews, 2014), is upregulated in brain of binge ethanol-treated mice, and is increased in postmortem human alcoholic brain (Crews et al., 2013). Therefore, HMGB1, appears to play an important role in ethanol-induced innate immune signaling. However, cell-type specific HMGB1 release still remains poorly understood in brain.

Marker	Brain Region	Effect	Method	Citation
RAGE	Orbitofrontal cortex	↑	IHC, RT-PCR, Western blot	Vetreno et al., 2013
TLR2	Orbitofrontal cortex	↑	IHC, Western blot	Crews et al., 2013
TLR3	Orbitofrontal cortex	↑	IHC, Western blot	Crews et al., 2013
TLR4	Orbitofrontal cortex	↑	IHC, Western blot	Crews et al., 2013
TLR7	Hippocampus	↑	RT-PCR, Western blot	Coleman et al. 2017
HMGB1	Orbitofrontal cortex	↑	IHC, Western blot	Crews et al., 2013
	Hippocampus	↑	ELISA	Coleman et al., 2017; Coleman et al., 2018
IL-1β	Hippocampus	↑	IHC	Zou & Crews, 2012
	Hippocampus	↑	ELISA	Coleman et al., 2018
IFNγ	Prefrontal cortex	↑	Western blot	Johnson et al., 2015
NALP1	Hippocampus	↑	IHC	Zou & Crews, 2012
gp91^{phox} (NOX2)	Orbitofrontal cortex	↑	IHC	Qin & Crews, 2012
MCP-1	Ventral Tegmental Area	↑	ELISA	He & Crews, 2008
	Substantia Nigra	↑	ELISA	
	Hippocampus	↑	ELISA	
	Amygdala	↑	ELISA	
p-NFκB-p65	Hippocampus	↑	IHC	Vetreno et al., 2018
Cd11b	Hippocampus	↑	Western blot	Coleman et al., 2017

Table 1.1 Upregulation of innate immune signaling molecules in post-mortem human alcoholic brain.

1.2.5 Interferons, TRAIL, and depression

Interferons (IFNs) are a class of cytokines consisting of several types: Type I, consisting of IFNα, IFNβ, IFNε, IFNκ, and IFNω; type II, which includes IFNγ; and the more recently described type III, including IFNλ. Type I interferons bind to interferon α/β receptor type 1 or 2 (IFNAR1/IFNAR2), whereas IFNγ binds to the IFNγ receptor 1 or 2 (IFNGR1 or IFNGR2) (Pestka, 2007). Activation of interferon receptors leads to activation of transcription factors such as JAK/STAT, which induce interferon stimulated genes (ISGs).

IFNs have a strong association with the development of depression in humans and in mouse models (Borsini et al., 2018; Callaghan et al., 2018; Fritz, Klawonn, Jaarola, & Engblom, 2018; Mina et al., 2015; Pinto & Andrade, 2016). In particular, IFNs have long been used as a treatment for cancer, although depression is a serious side effect, occurring in 30-70% of patients who undergo interferon therapy (Pinto & Andrade, 2016). Other neuropsychiatric symptoms, such as fatigue, sleep disturbances, irritability, anxiety, and cognitive disturbances are also common (Schaefer et al., 2012). The exact mechanism of both the therapeutic benefits of interferons, as well as the neuropsychiatric adverse effects, remains largely unknown. In addition, IFNγ has been suggested to modulate social behavior in mice (Filiano et al., 2016). IFNγ is also upregulated in postmortem human alcoholic cortex (Johnson et al., 2015) and chronic ethanol treatment *in vivo* rodent models also induces IFNγ (Duncan et al., 2016; Pascual, Balino, Aragon, & Guerri, 2015), suggesting a role for interferons in alcohol pathology as well.

Interferons induce multiple ISGs, largely via STAT transcription factors (Levy & Darnell, 2002). In the periphery, ISGs take on anti-viral activity, as well as modulate IFN signaling both positively and negatively (W. M. Schneider, Chevillotte, & Rice, 2014; Schoggins & Rice, 2011). One of the most prominently studied ISGs is TNF-related apoptosis-inducing

ligand (TRAIL/TNFSF10/APO2L/CD253). TRAIL is a type II transmembrane protein, whose release appears to be mediated by cysteine proteases (Mariani & Krammer, 1998), but may also be secreted in vesicles from its transmembrane form (LeBlanc & Ashkenazi, 2003; Monleon et al., 2001). TRAIL has two known receptors, death receptor 4 and death receptor 5, in addition to several "decoy" receptors that lack a functional intracellular death domain, thereby binding TRAIL without instigating downstream signaling.

TRAIL commonly instigates cell death in cell-death sensitive cells (Wilson, Dixit, & Ashkenazi, 2009), but may also induce interferons and other cytokines in other cell types (Corazza et al., 2004; Croft & Siegel, 2017; Henry & Martin, 2017; Kumar-Sinha, Varambally, Sreekumar, & Chinnaiyan, 2002). In cells in which TRAIL induces apoptosis, the death inducing signaling complex (DISC) is formed in conjunction with TRAILR1/R2, leading to downstream caspase activation and cell death. However, in some cells, TRAIL induces a secondary signaling complex, leading to NFκB activation and promotion of inflammation (Cheng, Zhao, Wang, & Jiang, 2014). In particular, B cells are resistant to TRAIL-mediated cell death (Ursini-Siegel et al., 2002), and TRAIL induces interferons in breast cell carcinomas (Kumar-Sinha et al., 2002) and T-cells (Chou et al., 2001). Neurons also appear to feature a TRAIL-cell-death resistant phenotype that is sensitized by NGF (Ruggeri, Cappabianca, Farina, Gneo, & Mackay, 2016). While it has been suggested that TRAIL may somehow itself function as a receptor to promote interferon and other cytokine signaling (Corazza et al., 2004), the mechanism for this activity of TRAIL remains unclear. It is also unknown the extent of which TRAIL affects neurons, as well as how ethanol affects this pathway.

1.2.6 IL-4/IL-13 signaling in brain

Interleukin-4 (IL-4) and interleukin-13 (IL-13) are two cytokines that in the periphery are known for their ability to promote T-helper type 2 (Th2) cells, commonly mediating allergic inflammation (Haas et al., 1999). IL-4 and IL-13 receptors form a heterodimer, consisting of IL-4Rα1 (IL-4R) and IL-13Rα1 (IL-13R). IL-4 and IL-13 signaling is therefore thought of as heavily interconnected (McCormick & Heller, 2015). Once activated, the receptor activates Janus kinase (JAK), which phosphorylates members of the signal transducer and activator of transcription family (STAT), largely STAT6.

While they have roles as anti-inflammatory cytokines in the periphery, in brain IL-4 and IL-13 also take on unique functions. Mice lacking IL-4 have deficits in learning and memory (Derecki et al., 2010), and IL-4 is decreased in aged rats that have impairment in LTP (Maher, Nolan, & Lynch, 2005), suggesting that IL-4 plays a prominent role in these functions (Gadani, Cronk, Norris, & Kipnis, 2012). Although the mechanism for this role of IL-4 has yet to be fully elucidated, since high levels of TNFα and IL-1β are associated with disrupted LTP (Bellinger et al., 1993; Tancredi et al., 1992), and these cytokines are increased in aged hippocampus, correlating with worsening performance on memory tasks (Doerks, Copley, Schultz, Ponting, & Bork, 2002), the ability of IL-4 to reduce expression of these cytokines (Hart et al., 1989; Wong, Costa, Lotze, & Wahl, 1993) may underlie its ability to enhance learning and memory. Recent data has also suggested a similar role for IL-13, with IL-13 deficient mice demonstrating impaired performance in learning and memory tasks (Brombacher et al., 2017). There has also been a suggested role for the shared receptor subunit IL-13Rα1 in Parkinson's disease, in which IL-13Rα1 knockout mice feature reduced restraint-stress-induced loss of dopaminergic cells in

the substantia nigra, a region in which loss of dopaminergic cells is associated with onset of Parkinson's disease (Mori et al., 2017).

In addition to its direct effects, IL-4 and IL-13 signaling can further induce cytokines such as IL-10 and TGFβ, which are also themselves referred to as Th2 promoting, anti-inflammatory cytokines in the periphery, but are also expressed in brain (Lobo-Silva, Carriche, Castro, Roque, & Saraiva, 2016; Vivien & Ali, 2006). IL-10 has neuroprotective effects as well as roles in learning and memory (Donzis & Tronson, 2014; Lobo-Silva et al., 2016). In particular, IL-10 injection increases neuronal survival after spinal cord injury (Zhou, Peng, Insolera, Fink, & Mata, 2009a, 2009b), mice deficient in IL-10 demonstrate impaired motor learning (Krzyszton et al., 2008), and increased expression of IL-10 via AAV delivery increases spatial learning in a mouse model of Alzheimer's disease (Kiyota et al., 2012). Similarly, TGFβ has also been associated with both neuroprotection (Vivien & Ali, 2006) and learning and memory, with TGFβ enhancing hippocampal-mediated LTP, and blockage of TGFβ is associated with impairment on memory tasks in mouse (Caraci et al., 2015). In terms of their relationship to alcohol pathology, recent studies have shown that injection of IL-10 into the amygdala reduces binge drinking in mice (Marshall, McKnight, Blose, Lysle, & Thiele, 2017). However, it is still poorly understood how ethanol regulates IL-10 and TGFβ in brain.

1.3 Summary and aims

While it is clear that ethanol alters innate immune signaling in brain, the specific roles of neurons and glia is still vastly unknown. In this thesis, we seek to further determine the contribution of neurons, microglia, astrocytes, as well as neuronal-glial signaling in terms of ethanol induction of innate immune signaling molecules.

In Chapter 2, we examine the effects of ethanol relative to both TLR3 and TLR4 stimulation in microglia vs neurons using two cell lines; BV2 microglia and SH-SY5Y neurons. We determine that ethanol induces both TLRs as well as HMGB1 in neurons, a novel finding that brings to the forefront a role for neurons in ethanol-induced innate immune signaling. We also find that ethanol also has a broader range of effect on innate immune gene expression versus either TLR3 or TLR4 stimulation alone, suggesting that ethanol acts through multiple modalities to influence gene expression.

In Chapter 3, we next examine how microglial-neuronal co-culture alters ethanol-induced innate immune signaling. We co-cultured BV2 microglia and SH-SY5Y neurons using Transwell inserts, allowing cells to communicate with one another via secreted signals. Here, we determine that co-culture blunts ethanol-induced HMGB1 induction while increasing IL-4/IL-13 signaling, demonstrating that paracrine signaling between neurons and microglia impact ethanol-induced innate immune signaling.

In Chapter 4, we characterize ethanol-induced innate immune signaling in astrocytes, as well as astrocyte-to-neuron ethanol-induced signaling. Using U373 astrocytes, BV2 microglia, and SH-SY5Y neurons, we discover that ethanol induces interferons in astrocytes and neurons but not microglia, and that IFN-induced gene TRAIL from ethanol-treated astrocytes further induces interferons in neurons. In addition, using an *in vivo* mouse model, we determined that TRAIL is also increased following both ethanol and TLR3 agonist treatment in orbitofrontal and entorhinal cortex.

Overall these data implicate a novel role for neurons in innate immune signaling, as well as for neuronal-glial signaling that is impacted by ethanol.

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CHAPTER 2: ETHANOL, TLR3, AND TLR4 AGONISTS HAVE UNIQUE INNATE IMMUNE RESPONSES IN SH-SY5Y NEURONS AND BV2 MICROGLIA[1]

2.1 Summary

Background: Ethanol (EtOH) consumption leads to an increase of proinflammatory signaling via activation of toll-like receptors (TLRs) such as TLR3 and TLR4 that leads to kinase activation (ERK1/2, p38, TBK1), transcription factor activation (NFκB, IRF3) and increased transcription of pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6. This immune signaling cascade is thought to play a role in neurodegeneration and alcohol use disorders. While microglia are considered to be the primary macrophage in brain, it is unclear what if any role neurons play in ethanol-induced proinflammatory signaling.

Methods: Microglia-like BV2 and retinoic acid differentiated neuron-like SH-SY5Y were treated with TLR3 agonist Poly(I:C), TLR4 agonist LPS, or EtOH for 10 or 30 minutes to examine proinflammatory immune signaling kinase and transcription factor activation using western blot, and for 24 hours to examine induction of proinflammatory gene mRNA using RT-PCR.

Results: In BV2, both LPS and Poly(I:C) increased p-ERK1/2, p-p38, and p-NFκB by 30 minutes, whereas EtOH decreased p-ERK1/2 and increased p-IRF3. LPS, Poly(I:C), and EtOH

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all increased TNFα and IL-1β mRNA, and EtOH further increased TLR2,7, 8, and MD-2 mRNA in BV2. In SH-SY5Y, LPS had no effect on kinase or proinflammatory gene expression. However, Poly(I:C) increased p-p38 and p-IRF3, and increased expression of TNFα, IL-1β, and IL-6, while EtOH increased p-p38, p-IRF3, p-TBK1 and p-NFκB while decreasing p-ERK1/2 and increasing expression of TLR3,7,8, and RAGE mRNA. HMGB1, a TLR agonist, was induced by LPS in BV2 and by EtOH in both cell types. EtOH was more potent at inducing proinflammatory gene mRNA in SH-SY5Y compared to BV2.

Conclusions: These results support a novel and unique mechanism of ethanol, TLR3, and TLR4 signaling in neuron-like SH-SY5Y and microglia-like BV2 that likely contributes to the complexity of brain neuroimmune signaling.

2.2 Introduction

Ethanol (EtOH) consumption causes increased pro-inflammatory signaling in brain that is linked with neurodegeneration (Collins, Corso, & Neafsey, 1996; F. Crews et al., 2006; Fulton T. Crews et al., 2004; Qin et al., 2008; Reynolds, Berry, Sharrett-Field, & Prendergast, 2015), alcoholism (He & Crews, 2008; Vetreno, Qin, & Crews, 2013), fetal alcohol syndrome disorder (Drew & Kane, 2014), and drinking behavior (Agrawal, Hewetson, George, Syapin, & Bergeson, 2011; Blednov et al., 2005; Blednov et al., 2012). EtOH treatment in mice increases transcription of proinflammatory cytokines such as TNFα, IL-1β, and IL-6 in brain (Alfonso-Loeches, Pascual-Lucas, Blanco, Sanchez-Vera, & Guerri, 2010; Qin & Crews, 2012a; Qin et al., 2008). EtOH is thought to promote this proinflammation in brain through activation of toll-like receptors (TLRs), a family of 11 receptors (13 in mice) that react to viral and bacterial components (pathogen associated molecular patterns, or PAMPs). In particular, TLR4 and TLR3 have been shown to be involved in proinflammatory innate immune signaling by ethanol. EtOH

treatment in mice increases both mRNA expression and number of immunoreactive cells for TLR3 and TLR4 in brain, as well as increased TLR3 and TLR4 immunoreactive cells in postmortem human alcoholic brain (F. T. Crews, Qin, Sheedy, Vetreno, & Zou, 2013). Knockout of TLR4 in mice prevents EtOH-induced increases in pro-inflammatory cytokines TNFα, IL-1β, and IL-6 (Alfonso-Loeches et al., 2010), and knockout of TLR3 in mice decreases EtOH consumption (Jang, Lee, Park, Han, & Kim, 2016). We have previously found that ethanol exposure potentiates both TLR4 agonist (Qin et al., 2008) and TLR3 agonist (Qin and Crews, 2012a) induced expression of proinflammatory innate immune signaling molecules (e.g., TNFα, IL-1β), implying that ethanol activates both TLR3 and TLR4 pathways. However, it is not known to what extent ethanol mimics activation of either TLR3 or TLR4.

Both TLR3 and TLR4 respond to different agonists, yet ultimately both promote proinflammatory signaling through kinase activation. TLR3 is traditionally activated by double-stranded RNA, with polyinosinic:polycytidylic acid [Poly(I:C)] being the most commonly used mimetic, and initiates signaling through a Myd88-independent pathway (Narayanan & Park, 2015). The canonical agonist for TLR4 is lipopolysaccharide (LPS), and unlike TLR3, TLR4 uses co-adaptor proteins (CD14 and MD-2) as well as signaling through both Myd88-dependent and independent pathways (Narayanan & Park, 2015). However, activation of both these receptors leads to activation of downstream signaling kinases (e.g., MAPKs like ERK1/2 and p38; TBK1), leading to activation of kinases such as NFκB and IRF3 which result in increased transcription of various pro-inflammatory cytokines, such as IL-1β, IL-6 and TNFα, as well as additional TLR expression (Narayanan & Park, 2015). These cytokines then signal through their respective receptors to promote a pro-inflammatory state (Newton & Dixit, 2012). EtOH has been shown

to affect various components of this immune signaling pathway in brain, with EtOH increasing expression of Myd88 and CD14 (Alfonso-Loeches et al., 2010) in whole mouse brain. Further, EtOH increases activation of p38, ERK1/2, NFκB and IRF3 in cultured primary microglia (Fernandez-Lizarbe, Pascual, & Guerri, 2009).

Since the brain is a sterile environment, lacking viral and bacterial products (PAMPS) that are present in the periphery, discovery of innate immune signaling in brain has led to increased interest in endogenous TLR agonists. Damage associated molecular patterns, or DAMPs, have been shown to be released from cells following stress, inflammation, or cell death, and lead to activation of TLRs (Chen & Nunez, 2010). One such DAMP, HMGB1, functions as an agonist at TLR2, TLR4, and TLR9 (Park et al., 2004; Yang, Antoine, Andersson, & Tracey, 2013). HMGB1 also binds to receptor for advanced glycation end products (RAGE) (Kokkola et al., 2005), a receptor which leads to increased activation of NFκB (Han, Kim, & Mook-Jung, 2011). Ethanol upregulates both HMGB1 (Crews et al., 2013, Lippai et al., 2013) and RAGE (Vetreno et al., 2013) in brain, suggesting a role for these molecules in EtOH-induced proinflammatory signaling. While DAMPs are commonly thought to be passively released with cell death, HMGB1 is also released actively by immune-competant cells (Andersson & Tracey, 2011). Interestingly, ethanol-induced release of HMGB1 in hippocampal brain slices occurs without any evidence of cell death, and may occur via a histone deacetylase-dependent manner (Zou & Crews, 2014).

The aforementioned TLR signaling pathway has been established in macrophages, such as the canonical macrophage in brain, microglia (Rivest, 2009). Microglia indeed play an integral role in ethanol-induced innate immune signaling (Fernandez-Lizarbe, Montesinos, &

Guerri, 2013; Fernandez-Lizarbe et al., 2009) and neuronal toxicity (Boyadjieva & Sarkar, 2010). While another cell-type, astrocytes, have been implicated alcohol use disorders and display proinflammatory innate immune signaling following EtOH treatment (Adermark & Bowers, 2016; Alfonso-Loeches et al., 2010), neurons have largely been overlooked in having a possible role in the innate immune system. However, TLRs have recently been reported to be on neurons as well. In particular, primary mouse cortical neurons were found to express TLR3 and TLR4 using both single cell PCR and immunocytochemistry (Tang et al. 2007), and expression TLR4 on primary mouse cortical neurons using western blot was also reported (Lok et al. 2015). Previously, we have also shown co-localization of TLR3 and TLR4 on rat neurons using immunohistochemistry (Vetreno & Crews, 2012). Furthermore, there has been evidence that TLR3 agonist Poly(I:C) increases p38 kinase activation and TLR3 expression in neuronal cell line SH-SY5Y (Nessa et al., 2006). In addition, Poly(I:C) increases mRNA expression of TNFα and IL-6 in neuronal cell line NT2-N (Prehaud, Megret, Lafage, & Lafon, 2005). However, despite evidence for neuronal TLR4, previous reports suggest that TLR4 agonist LPS does not affect TNFα, IL-6, or IL-1β release in SH-SY5Y (Klegeris & McGeer, 2001) nor does it increase cytokine release in neuronal cell line NT2-N (Prehaud et al., 2005). In addition, *in vivo* studies using flow cytometry suggest that TLR4 is predominantly located on microglia (Schwarz, Smith, & Bilbo, 2013), suggesting that neurons lack significant TLR signaling. Despite some conflicting evidence, these data suggest that some level of proinflammatory innate immune activity may exist in neurons, but it is unclear if neurons demonstrate a macrophage-like signaling pathway activation (e.g., activation of kinases such as MAPKs that lead to NFκB activation). It is also unknown if ethanol affects innate immune signaling in neurons.

In order to gain insight into the respective involvement of microglia and neurons in ethanol-induced proinflammatory innate immune signaling, we utilized a mouse microglial cell line (BV2) and a retinoic acid-differentiated human neuroblastoma cell line (SH-SY5Y). The BV2 cell line is well-established as a model for microglia, with studies finding that the LPS response in primary glia is highly similar in BV2 (Henn et al., 2009). Neuron-like SH-SY5Y express synaptic proteins and mature-neuronal markers after differentiation with retinoic acid (Cheung et al., 2009), and are commonly used to model dopamine neurons in Parkinson's disease, a condition known to involve neuroinflammation (Korecka et al., 2013). While it is unknown exactly how EtOH affects proinflammatory gene expression in brain (e.g., increases in TNFα, IL-1β, IL-6), it is possible that it has direct effect on transcription through factors such as NFκB, or through release of TLR agonist HMGB1. Therefore, we examined both kinases (ERK1/2, p38, TBK1) and transcription factors (IRF3, NFκB) as well as HMGB1 release in conjunction with mRNA expression of various innate immune signaling molecules, TLRs, and TLR associated proteins, and compared the EtOH findings to both TLR3 and TLR4 stimulation in both microglia-like BV2 and neuron-like SH-SY5Y.

Given the aforementioned data, we hypothesized that (1) neuron-like SH-SY5Y express various components of the innate immune signaling pathway, including TLRs, TLR associated proteins, and cytokines; (2) neuron-like SH-SY5Y display activation of kinases and induction of proinflammatory gene expression in response to TLR3 stimulation, but not TLR4; (3) EtOH causes activation of kinases and upregulation of proinflammatory gene expression in neuron-like SH-SY5Y in a manner unique to that to microglia-like BV2; and (4) EtOH activates kinases and induces of proinflammatory genes unique to either TLR3 or TLR4 stimulation. Our results

indicate that EtOH has a unique and broader pattern of proinflammatory gene induction compared to either TLR3/TLR4 stimulation alone in both cell types, and that EtOH and Poly(I:C) both activate unique proinflammatory innate immune signaling kinase and gene induction in neuron-like SH-SY5Y.

2.3 Materials and Methods

2.3.1 Cell culture and treatment

BV2 were acquired from ICLC (Genoa, Italy, #ATL03001). BV2 were cultured using Dubecco's modified Eagle serum (DMEM, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 1X GlutaMAX (Life Technologies), and 1X antibiotic-antimycotic (Life Technologies). 16hr prior to treatments, media was changed to 2% FBS.

SH-SY5Y were acquired from ATCC (Manassas, Virginia, #CRL-2266). SH-SY5Y were cultured using DMEM/F-12 + GlutaMAX (Life Technologies), 10% FBS, and 1X antibiotic-antimycotic. Prior to treatments, SH-SY5Y were differentiated using 10 uM retinoic acid (RA, Sigma-Aldrich, St. Louis, MO, #R2625) for 4 days in Neurobasal media (Life Technologies) containing 2% B27 supplement (Life Technologies), 0.5 mM GlutaMAX and 1X antibiotic-antimycotic. Media was refreshed 16hr prior to treatments.

Both cell types were maintained in a humidified 5% CO_2 incubator at 37°C. Passage number at the time of treatment did not exceed 10. Cells were treated at ~90% confluency with either 50 ug/mL Poly(I:C) (Amersham/GE Healthcare, Pittsburgh, PA, #27-4732-01), 100 ng/mL LPS (Sigma-Aldrich, #L2630), or varying concentrations of ethanol (EtOH). Concentrations were chosen due to prior literature on Poly(I:C) (Nessa et al., 2006) and LPS

(Shin, Lee, Lee, Jin, & Lee, 2014) treatment in these cells. Cells treated with EtOH were placed in a separate EtOH-saturated chamber to prevent evaporation of EtOH from the media. Cell death following treatments was measured using trypan-blue exclusion assay.

2.3.2 Western blot

Cells were washed with ice-cold PBS and lysed on ice for 15min using cold lysis buffer (20 mM Tris, 0.25 M sucrose, 2 mM EDTA, 10 mM EGTA, 1% Triton X-100) and one tablet of Complete Ultra protease inhibitor cocktail tablets (Roche, Mannheim, Germany) and PhosSTOP phosphatase inhibitor cocktail tablet (Roche)/10 ml. Samples were centrifuged at 16,000xg for 15min and protein-containing supernatant was transferred to a separate tube. Total protein concentration was assessed using a BCA protein assay kit (Pierce, Rockford, IL). 10 ug of protein was mixed with 1X loading buffer (Pierce), boiled for 5 min, and ran on Mini-PROTEAN TGX Stain-Free gels (Bio-Rad, Hercules, CA). Following transfer to a Trans-Blot Turbo nitrocellulose membrane (Bio-Rad), the blot was blocked with Odyssey Blocking Buffer (Li-Cor, Lincoln, NE), and incubated with primary antibodies neuron specific enolase (NSE, Abcam, Cambridge, United Kingdom, #ab53025, 1:500), β-tubulin-III (Tubb3, Abcam, #ab18207, 1:1000), Cd11b (Novus Biologicals, Littleton, CO, #NB110-89474, 1:500), HMGB1 (Abcam, #ab18256, 1:400), TLR4 (Santa Cruz, Dallas, TX, #sc-293072, 1:500), RAGE (Abcam, #ab3611, 1:500), phospho(p)-ERK1/2 (Cell Signaling, #4370, 1:500), p-p38 MAPK (Cell Signaling, #4511, 1:500), p-TBK1 (Cell Signaling, #5483, 1:500), pIRF3 (Cell Signaling, #4947, 1:500), p-NFκB-p65 (Cell Signaling, #3033, 1:500), and β-actin (Santa Cruz, #sc-47778, 1:500) overnight at 4C, followed by IRDye 700DX anti-rabbit (Rockland, Limerick, PA, #611-730-127) or IRDye 800DX anti-mouse (Rockland, #610-731-124) secondary antibodies for 2hr at room temperature. Protein bands were visualized

using an Odyssey fluorescent scanner, with a protein ladder (Odyssey, Lincoln, NE) used as a size reference.

2.3.3 Flow cytometry

Cells were detached and stained with Violet-LD (Invitrogen, #L34955) as a live-dead discrimination marker. Cells were then stained with Cd11b-PE/Cy7 (1:500; eBioscience, #25-0112-82), washed with PBS, then permeabilized using Fix/Perm buffer (BD Biosciences) and stained with primary antibody Tubb3 (1:1000; Abcam, #ab18207) followed by secondary antibody D649 (1:800; Biolegend, #406406). Isotype controls (rabbit IgG, Abcam #171870; rat IgG, eBioscience #25-4031-82) were used to account for background staining. Cells were fixed using 4% paraformaldehyde and analyzed using a CyAN cytometer. FlowJo (Treestar, OR) was used to analyze flow cytometry data.

2.3.4 Real-time (RT)-PCR

Total RNA was extracted from cell lysates using TRIzol (Invitrogen, Carlsbad, CA). RNA concentration was determined using a Nanodrop (Thermo Scientific, Waltham, MA) and was reverse-transcribed to cDNA. The SYBR green PCR master mix (Life Technologies) was used for real-time PCR analysis. The relative differences in expression between groups were expressed using cycle time (Ct) values normalized with β-actin, and relative differences between control and treatment groups were calculated and expressed as relative increases setting control as 100%. For genes that were not detectable within 40Ct in control samples, controls were arbitrarily set at 35Ct to calculate a fold change compared to treated samples, as utilized in previous studies (Tuomela et al., 2013). This method allows an estimated fold change while reducing possible bias (McCall, McMurray, Land, & Almudevar, 2014). Primers used are listed in

Table 2.1.

Gene	Species	Forward (5'-3')	Reverse (5'-3')
β-actin	Human	GAT GCA GAA GGA GAT CAC TGC	ATA CTC CTG CTT GCT GAT CCA
TLR2	Human	GGCTTCTCTGTCTTGTGACC	GGGCTTGAACCAGGAAGACG
TLR3	Human	TTG CCT TGT ATC TAC TTT TGG GG	TCA ACA CTG TTA TGT TTG TGG GT
TLR4	Human	CTC TGG GGA GGC ACA TCT TC	CCC AGG TGA GCT GTA GCA TT
TLR7	Human	GATAACAATGTCACAGCCGTCC	GTTCCTGGAGTTTGTTGATGTTC
TLR8	Human	ATG TTC CTT CAG TCG TCA ATG C	TTG CTG CAC TCT GCA ATA ACT
CD14	Human	AGA GGC AGC CGA AGA GTT CAC	GCG CTC CAT GGT CGA TAA GT
MD-2	Human	GAA GCT CAG AAG CAG TAT TGG GTC	GGT TGG TGT AGG ATG ACA AAC TCC
Myd88	Human	CCGCGCTGGCGGAGGAGATGGAC	GCAGATGAAGGCATCGAAACGCTC
RAGE	Human	CTA CCG AGT CCG TGT CTA CCA	CAT CCA AGT GCC AGC TAA GAG
HMGB1	Human	GGA GAT CCT AAG AAG CCG AGA	CAT GGT CTT CCA CCT CTC TGA
TNFα	Human	CCC AGG CAG TCA GAT CAT CTT CT	ATG AGG TAC AGG CCC TCT GAT
IL-1β	Human	ATG ATG GCT TAT TAC AGT GGC AA	GTCGGAGATTCGTAGCTGGA
IL-6	Human	ACT CAC CTC TTC AGA ACG AAT TG	CCA TCT TTG GAA GGT TCA GGT TG
β-actin	Mouse	GTA TGA CTC CAC TCA CGG CAA A	GGT CTC GCT CCT GGA AGA TG
TLR2	Mouse	GCA AAC GCT GTT CTG CTC AG	AGG CGT CTC CCT CTA TTG TAT T
TLR3	Mouse	GTG AGA TAC AAC GTA GCT GAC TG	TCC TGC ATC CAA GAT AGC AAG T
TLR4	Mouse	ATG GCA TGG CTT ACA CCA CC	GAG GCC AAT TTT GTC TCC ACA
TLR7	Mouse	ATG TGG ACA CGG AAG AGA CAA	GGT AAG GGT AAG ATT GGT GGT G
TLR8	Mouse	GAA AAC ATG CCC CCT CAG TCA	CGTCACAAGGATAGCTTCTGGAA
CD14	Mouse	GCC AAA TTG GTC GAA CAA GC	CCA TGG TCG GTA GAT TCT GAA AGT
MD-2	Mouse	CGC TGC TTT CTC CCA TAT TGA	CCT CAG TCT TAT GCA GGG TTC A
Myd88	Mouse	TCA TGT TCT CCA TAC CCT TGG T	AAA CTG CGA GTG GGG TCA G
RAGE	Mouse	GAA GGC TCT GTG GGT GAG TC	CCG CTT CCT CTG ACT GAT TC
HMGB1	Mouse	CGC GGA GGA AAA TCA ACT AA	TCA TAA CGA GCC TTG TCA GC
TNFα	Mouse	GAC CCT CAC ACT CAG ATC ATC TTC T	CCT CCA CTT GGT GGT TTG CT
IL-1β	Mouse	CTG GTG TGT GAC GTT CCC ATT A	CCG ACA GCA CGA GGC TTT
IL-6	Mouse	ACA AGT CGG AGG CTT AAT TAC ACA T	TTG CCA TTG CAC AAC TCT TTT C

Table 2.1 Primers used for RT-PCR analysis.

2.3.5 Enzyme-linked immunosorbent assays (ELISAs)

Cells were lysed using ice cold lysis buffer (20 mM Tris, 0.25 M sucrose, 2 mM EDTA, 10 mM EGTA, 1% Triton ×-100), with 1 tablet of Complete Ultra protease inhibitor cocktail (Roche) and 1 tablet of PhosSTOP phosphatase inhibitor (Roche)/10 ml. Lysate was spun at 21,000xg for 15min and protein-containing supernatant was collected. Media was collected and spun down at 500xg to eliminate cell debris. Protein concentration was determined using a BCA kit (Thermo Scientific). Cell lysates and/or media were analyzed using HMGB1 (IBL, Hamburg, Germany) and IFNβ (R&D, Minneapolis, MN) ELISAs per manufacturer instructions.

2.3.6 Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). T-tests were used to compare basal expression of mRNA and protein between BV2 and SH-SY5Y (Figure 2.2). In order to examine the treatment effect in each respective cell-type, one-way ANOVAs were utilized for comparisons between LPS, EtOH, and Poly(I:C) followed by Dunnett's test to account for multiple comparison (Figures 2.3-2.8). Excluding Figure 2.2, which directly compares basal levels of immune signaling molecules, direct statistical comparisons are not made between cell-types. A p value <0.05 was considered statistically significant. Data was analyzed and figures designed using Prism software (GraphPad, La Jolla, CA).

2.4 Results

2.4.1 BV2 microglia and SH-SY5Y neurons display typical microglial and neuronal markers, respectively

To clearly define the cell-type of BV2 and SH-SY5Y, expression of microglial and neuronal protein markers were assessed using Western blot and flow cytometry. RA-differentiated SH-SY5Y expressed neuronal markers neuron-specific enolase (NSE) and β-tubulin III (Tubb3), but

not microglial marker Cd11b (Figure 2.1A). Accordingly, BV2 expressed microglial marker Cd11b but not neuronal markers NSE or Tubb3, whereas SH-SY5Y expressed NSE and Tubb3 but not Cd11b (Figure 2.1B). Flow cytometry analysis confirmed that essentially all BV2 (>99%) express Cd11b, whereas RA-differentiated SH-SY5Y predominantly express neuronal marker Tubb3 (Figure 2.1B). These data confirm that the culture preparations are representative of microglial and neuronal cells.

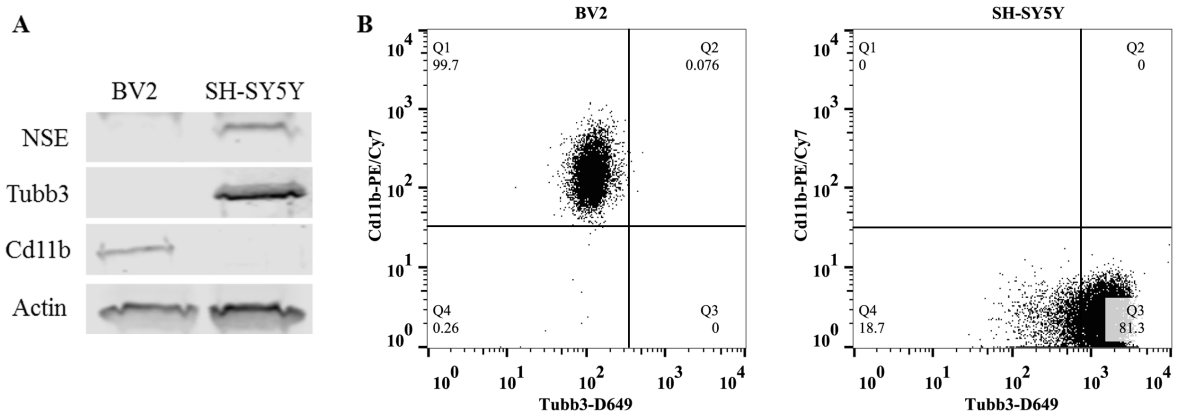

Figure 2.1 BV2 express microglial markers and SH-SY5Y express neuronal markers, indicating they are microglial-like and neuronal-like cells, respectively. (**A**) Western blot analysis of basal expression of microglial and neuronal cell-type markers in cell lysates of BV2 and SH-SY5Y. SH-SY5Y were differentiated into a neuron-like phenotype using retinoic acid (RA) for 4 days. Microglia-like BV2 express microglial marker Cd11b. Neuron-like SH-SY5Y express neuronal markers neuron specific enolase (NSE) and β-III tubulin (Tubb3). β-actin was used as a loading control. (**B**) Flow cytometry analysis of basal expression of microglial and neuronal markers in BV2 and SH-SY5Y. The majority of BV2 cells express microglial marker Cd11b, whereas the majority of SH- SY5Y express neuronal marker Tubb3. n=3 per group. Gates were established using unstained and isotype controls for each antibody.

2.4.2 Microglia-like BV2 and neuron-like SH-SY5Y have unique basal expression of innate immune signaling molecules

The expression of proinflammatory signaling molecules in BV2 and SH-SY5Y was examined using both RT-PCR and Western blot. As expected, microglia-like BV2 were found to express a wide variety of proinflammatory genes (Table 2.2). Neuron-like SH-SY5Y also express most proinflammatory genes examined, although in lower relative amounts compared to BV2 for most genes assessed. Both BV2 and SH-SY5Y were found to express protein and mRNA for TLR4, RAGE, and HMGB1 (Figure 2.2). Consistent with their expected cellular phenotypes, BV2 express more TLR4 protein and mRNA as well as many fold greater levels of mRNA for multiple TLRs, cytokines and other proinflammatory genes. Neuron-like SH-SY5Y had less than 1% of the BV2 level of TLR4 expression (Figure 2.2A). Western blot analysis also found a detectable but relatively lower expression of TLR4 in SH-SY5Y compared to BV2 (Figure 2.2B). SH-SY5Y had greater relative expression of TLR3 than the other TLRs, however this was only 20% of the level in BV2s (Table 2.2). Further, SH-SY5Y had no detectable levels of TLR4 adaptor protein MD-2, or proinflammatory cytokines IL-1β and IL-6 (Table 2.2, using a 40 cycle cut-off). In contrast, SH-SY5Y express more RAGE and HMGB1 compared to BV2. Interestingly, Myd88, a critical adapter signaling transducer for TLRs and cytokine receptor signaling has similar levels in SH-SY5Y and BV2. These data indicate that SH-SY5Y and BV2 both express TLR and other proinflammatory genes, although at different basal levels.

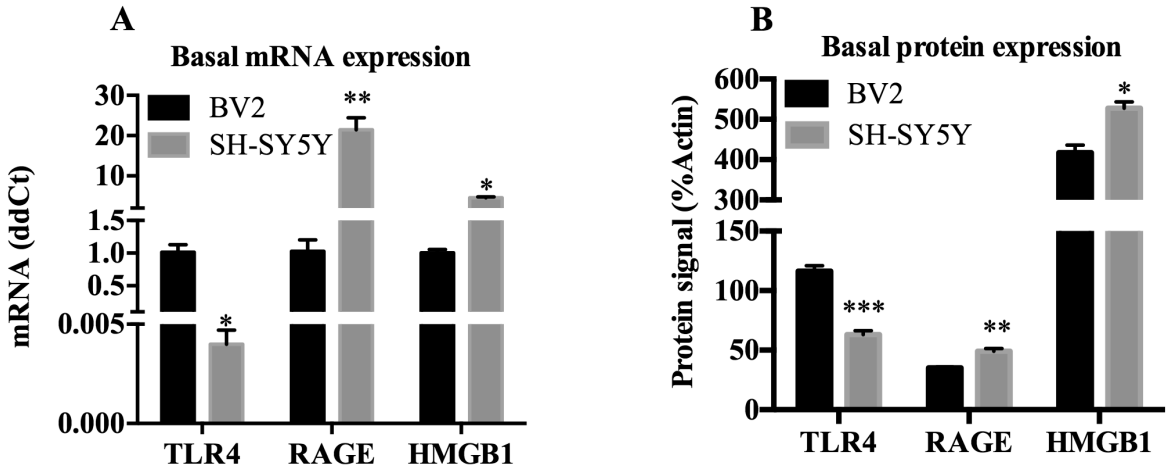

Figure 2.2 Microglia-like BV2 and neuron-like SH-SY5Y express mRNA and protein of various proinflammatory molecules. (**A**) Comparison of basal mRNA expression of TLR4, RAGE, and HMGB1 in BV2 and SH-SY5Y. Data is expressed in ddCt normalized to BV2 expression level. SH-SY5Y express less TLR4 but greater RAGE and HMGB1 mRNA (**B**) Basal protein levels of TLR4, RAGE, and HMGB1 were examined using Western blot in BV2 and SH-SY5Y. Data is expressed in %β-actin units. SH-SY5Y express less TLR4 but greater RAGE and HMGB1 protein. n=3 per group; *p<0.05, **p<0.01, ***p<0.001 vs. BV2.

	ddCt	
	BV2	**SH-SY5Y**
RAGE	1.03	21.4600
HMGB1	1.00	4.6000
Myd88	1.00	0.9500
TLR3	1.03	0.1800
TLR8	1.01	0.1600
CD14	1.01	0.0360
TLR2	1.01	0.0290
TLR7	1.01	0.0035
TLR4	1.01	0.0031
TNFα	1.00	0.0017
MD-2	1.01	n.d.
IL-1β	1.06	n.d.
IL-6	1.02	n.d.

Table 2.2 Summary basal mRNA expression of proinflammatory genes in microglia-like BV2 and neuron-like SH-SY5Y. Average basal mRNA levels of various immune signaling molecules in BV2 and SH-SY5Y (n=3 per group) were analyzed using RT-PCR and expressed as ddCt, setting BV2 as 1.

2.4.3 LPS, Poly(I:C), and EtOH differentially activate kinases/transcription factors that modulate TLR pathways in microglia-like BV2

Activation of TLR3 and TLR4 involves key kinases the lead to activation of NFκB and other transcription factors that mediate induction of proinflammatory genes. To better understand the role of ethanol in relation to TLR4 and TLR3 activation, we treated microglia-like BV2 TLR4 agonist LPS, TLR3 agonist Poly(I:C), or EtOH for 10 or 30min. Activated kinases/transcription factors were measured by examining phosphorylated forms of these proteins in cell lysates using Western blot. As expected, both LPS and Poly(I:C) activated kinases related to TLR signaling. Following 10 minutes of LPS treatment, phospho(p)-ERK1/2 was increased by 29% (±5.9%, $p<0.01$), p-p38 by 98% (±17%, $p<0.001$). p-NFκB, a transcription factor downstream of TLR activation that is involved in transcription of a variety of proinflammatory molecules, was increased by 42% (±20, $p<0.05$) (Figure 2.3A). After 30 minutes, LPS caused an even more robust effect, by increasing p-ERK1/2 by 68% (±5.6%, $p<0.0001$), p-p38 by 568% (±61%, $p<0.0001$), and p-NFκB by 80% (±18%, $p<0.001$) relative to controls (Figure 2.3A). Thus, LPS stimulation displayed a canonical activation of kinases in BV2.

Treatment of BV2 with Poly(I:C) had a slightly less robust effect compared to LPS, with only p-ERK1/2 increased by 21% (±7.8%, $p<0.05$) by 10 minutes of treatment. However, by 30 minutes Poly(I:C) increased p-ERK1/2 by 21% (±9.4, $p<0.05$), p-p38 by 41% (±13, $p<0.05$) and p-NFκB by 40% (±13%, $p<0.05$) (Figure 2.3B), indicating activation of kinases by Poly(I:C) signaling in BV2.

Unlike either Poly(I:C) or LPS, EtOH treatment at 10 and 30 minutes significantly decreased p-ERK1/2 by 38% (±1.6%, $p<0.01$) and 40% (±2.5%, $p<0.001$), respectively, whereas p-IRF3 was increased by 50% (±8.7%, $p<0.001$) after 30 minutes (Figure 2.3C). Thus, LPS,

Poly(I:C), and EtOH activate proinflammatory signaling kinases/transcription factors in BV2. However, EtOH displays a unique pattern of activation compared to either TLR3 or TLR4 signaling pathways.

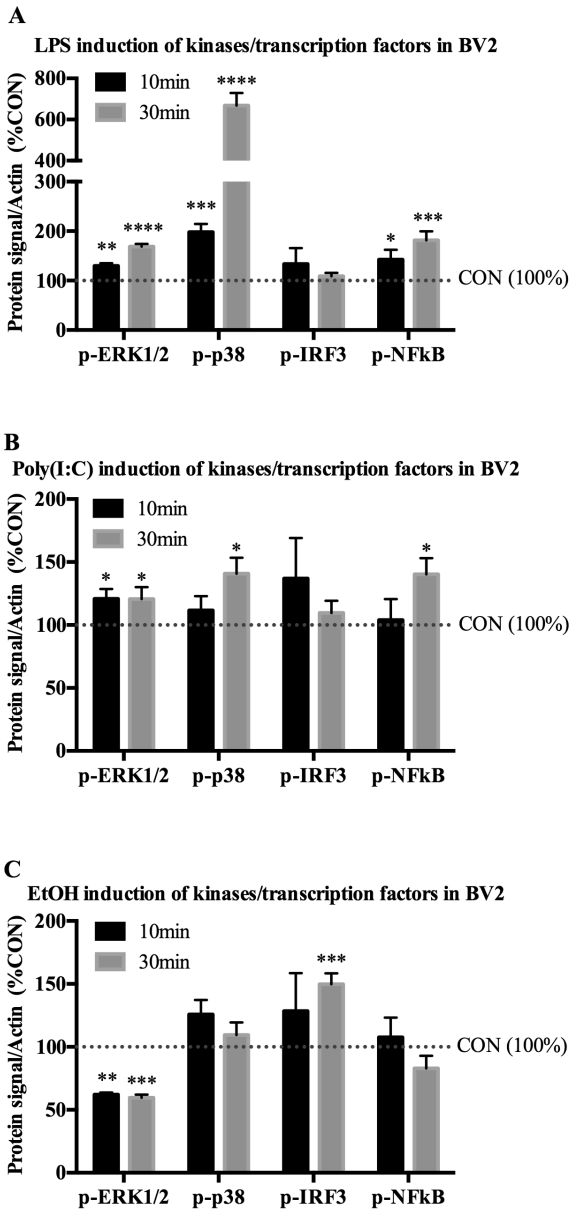

Figure 2.3 LPS, Poly(I:C), and EtOH activate proinflammatory-related kinases/transcription factors in microglia-like BV2. Microglia-like BV2 were treated with indicated drug for 10 or 30min. Cell lysates were collected for Western blot analysis. (**A**) 10 minutes of LPS (100 ng/mL) increased p-ERK1/2 by 29%, p-p38 by 98%, and p-NFκB-p65 by 42%. 30 minutes of LPS increased p-ERK1/2 by 68%, p-p38 by 568%, and p-NFκB by 80%. (**B**) 10 minutes of Poly(I:C) (50 ug/mL) increased p-ERK1/2 by 21%. 30 minutes of Poly(I:C) increased p-ERK1/2 by 21%, p-p38 by 41%, and p-NFκB by 40%. (**C**) 10 minutes of EtOH (150 mM) decreased p-ERK1/2 by 38%. 30 minutes of EtOH decreased p-ERK1/2 by 40% and increased p-IRF3 by 50%. Data is represented as %control (%CON) following β-actin normalization. n=3 per group; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. CON.

2.4.4 LPS, Poly(I:C) and EtOH increase proinflammatory gene expression in microglia-like BV2

Studies of canonical macrophage TLR signaling find that activation of transcription factors such as NFκB and IRF3 leads to further proinflammatory signaling (Narayanan & Park, 2015). Thus, we next assessed subsequent induction of proinflammatory genes following 24hr treatment of TLR3 agonist Poly(I:C), TLR4 agonist LPS, or EtOH in BV2. Cell death was assayed using trypan-blue exclusion and no significant effects were found in any treatment (data not shown). LPS induced a typical microglia-like response in BV2, with significant increases of mRNA expression of TNFα (814 ± 28%, $p<0.001$) IL-1β (2775 ± 370%, $p<0.0001$), IL-6 (209 ± 24%, $p<0.05$) (Figure 2.4A), and TLR4 co-receptor CD14 (242 ± 21%, $p<0.01$) (Table 2.3). LPS treatment also increased HMGB1 in media of BV2 by 37% (±8.5%, $p<0.01$) relative to controls (Figure 2.4B). TLR3 agonist Poly(I:C) treatment increased mRNA expression of TNFα (217 ± 29%, $p<0.05$) and IL-1β (366 ± 33%, $p<0.01$) (Figure 2.4C), whereas Poly(I:C) had no significant effect on HMGB1 release in media (Figure 2.4D). Additionally, TLR8 was downregulated by both Poly(I:C) (49 ± 15%, $p<0.05$), and LPS (21 ± 2.7%, $p<0.01$), whereas LPS upregulated TLR2 (588 ± 69%, $p<0.01$) and CD14 (242 ± 21%, $p<0.01$) as depicted in Table 2.3. Raw Ct values are depicted in Table 2.4. These data are consistent with BV2 displaying a characteristic macrophage-like proinflammatory signaling induction following TLR4 and TLR3 agonist treatment.

BV2 were next treated with EtOH for 24hr to determine whether EtOH causes induction of proinflammatory genes in microglia-like cells. EtOH increased transcription of both cytokines and TLRs. Significant increases were found for TNFα (204 ± 15%, $p<0.01$), IL-1β (304 ± 29%, $p<0.01$), TLR2 (193 ± 28%, $p<0.01$), TLR7 (158 ±8.4%, $p<0.01$), TLR8 (194 ± 9.6%, $p<0.01$), and the TLR4 accessory protein MD-2 (155 ± 4.4%, $p<0.01$) mRNA (Figure 2.5A-B), as well as HMGB1

release in media (Figure 2.5C). A concentration curve indicated that EtOH induces transcription of proinflammatory genes at a concentration of over 50 mM (Figure 2.5D). These data indicate that EtOH treatment of microglia-like BV2 activates proinflammatory gene induction.

BV2

	CON	Poly(I:C)	LPS	EtOH
TLR2	100 ± 10	323 ± 70	**588 ± 69****	**170 ± 15***
TLR3	100 ± 16	158 ± 13	133 ± 9.3	120 ± 9.1
TLR4	100 ± 9.3	100 ± 19	58 ± 2.2	90 ± 8.6
TLR7	100 ± 6.0	121 ± 24	75 ± 3.5	**158 ± 8.4****
TLR8	100 ± 11	**49 ± 15***	**21 ± 2.7****	**194 ± 9.5****
CD14	100 ± 7.5	154 ± 13	**242 ± 21****	104 ± 3.7
MD-2	100 ± 11	118 ± 31	98 ± 13	**156 ± 4.3****
Myd88	100 ± 1.7	113 ± 6.2	96 ± 8.5	89 ± 7.2
RAGE	100 ± 16	134 ± 7.7	98 ± 4.8	104 ± 4.4
HMGB1	100 ± 4.9	104 ± 9.2	90 ± 4.3	67 ± 9.7
TNFα	100 ± 3.6	**217 ± 29***	**440 ± 27*****	**204 ± 15****
IL-1β	100 ± 9.3	**366 ± 33****	**1163 ± 45******	**304 ± 29****
IL-6	100 ± 14	130 ± 39	**210 ± 24***	**10 ± 2.4****

SH-SY5Y

	CON	Poly(I:C)	LPS	EtOH
TLR2	100 ± 19	**230 ± 31***	121 ± 30	177 ± 23
TLR3	100 ± 46	**920 ± 260***	98 ± 35	**398 ± 80***
TLR4	100 ± 23	158 ± 12	67 ± 18	147 ± 14
TLR7	100 ± 46	234 ± 59	153 ± 79	**575 ± 100***
TLR8	100 ± 33	103 ± 22	121 ± 60	**320 ± 17****
CD14	100 ± 18	146 ± 17	61 ± 21	127 ± 11
MD-2	n.d.	n.d.	n.d.	n.d.
Myd88	100 ± 13	**530 ± 30*****	79 ± 4.7	128 ± 5.9
RAGE	100 ± 6.6	117 ± 8.9	61 ± 20	**170 ± 15***
HMGB1	100 ± 4.2	**125 ± 5.2***	84 ± 11	105 ± 2.4
TNFα	100 ± 9.5	**10426 ± 2130****	65 ± 17	67 ± 13
IL-1β	n.d.	**2860 ± 135**	n.d.	n.d.
IL-6	n.d.	**591 ± 100**	n.d.	n.d.

Table 2.3 Summary of Poly(I:C), LPS, and EtOH induction of proinflammatory gene mRNA in BV2 and SH-SY5Y. Microglia-like BV2 and neuron-like SH-SY5Y were treated with TLR3 agonist Poly(I:C) (50 ug/mL), TLR4 agonist LPS (100 ng/mL), or EtOH (150 mM) for 24hr. Cell lysates were collected and analyzed for mRNA expression using RT-PCR. Data for mRNA is expressed as %control (%CON), with CON set at 100%. Data for HMGB1, TNFα, IL-1β, and IL-6 for LPS and Poly(I:C) treatment in BV2 is also represented in Figure 4A and C. Data for HMGB1, TNFα, IL-1β, IL-6, TLR2,3,4,7,8, Myd88, Cd14, MD-2, and RAGE for EtOH treatment in BV2 is also represented in Figure 5A and B. Data for HMGB1, TNFα, IL-1β, and IL-6 for LPS and Poly(I:C) treatment in SH-SY5Y is also represented in Figure 7A and C. Data for HMGB1, TNFα, IL-1β, IL-6, TLR2,3,4,7,8, Myd88, Cd14, MD-2, and RAGE for EtOH treatment in SH-SY5Y is also represented in Figure 8A and B. n.d. = not detectable; n=3 per group; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. CON.

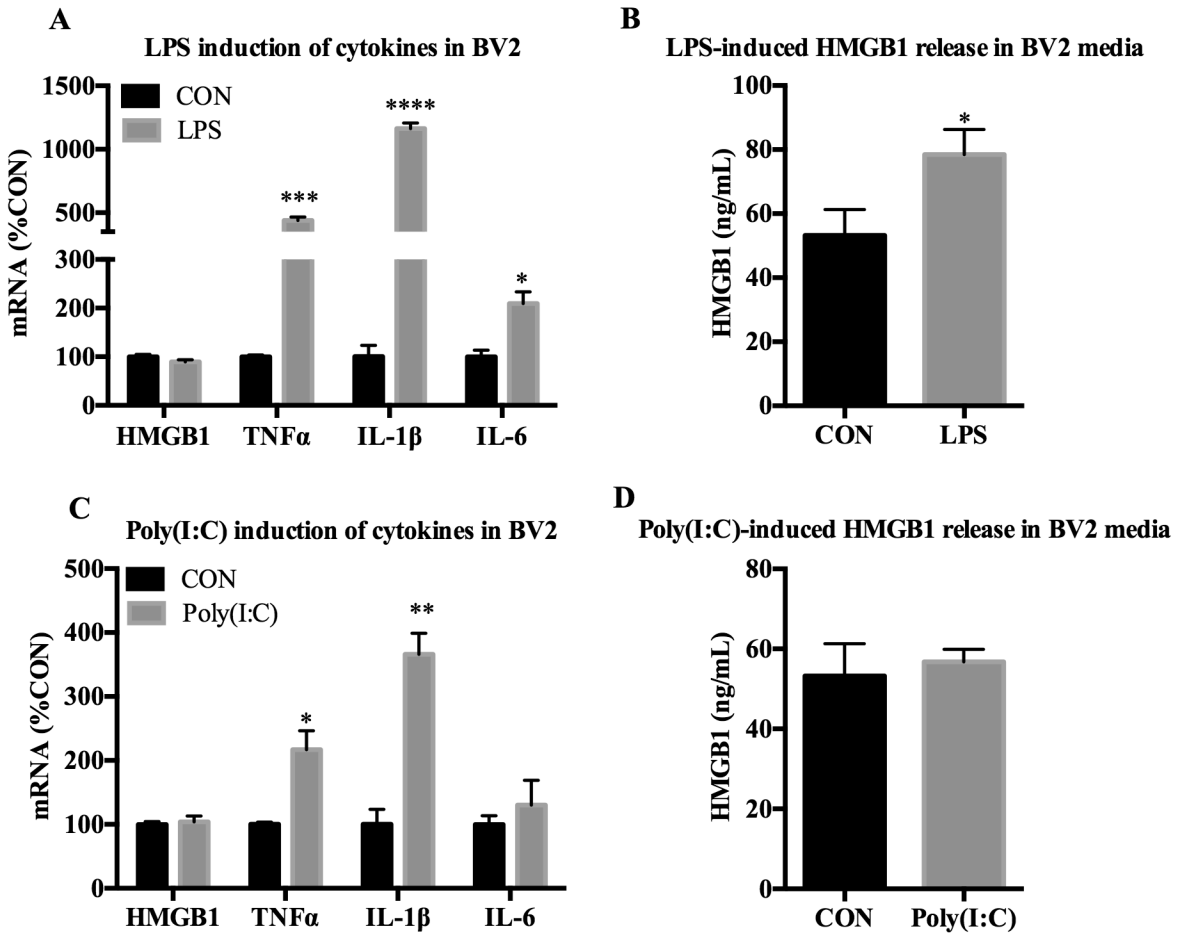

Figure 2.4 LPS and Poly(I:C) upregulates cytokine expression in microglia-like BV2. BV2 were treated with either LPS (100 ng/mL) or Poly(I:C) (50 ug/mL) for 24hr followed by mRNA analysis in cell lysates and analysis of HMGB1 release in media using ELISA. (**A**) LPS increased TNFα (814%), IL-1β (2775%), and IL-6 (209%) mRNA. (**B**) LPS increased HMGB1 release in media by 37%. (**C**) Poly(I:C) increased TNFα (217%) and IL-1β (366%) mRNA. (**D**) Poly(I:C) did not induce HMGB1 release in media. Data for mRNA is expressed as %control (%CON), with CON set at 100%. n=3 per group; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. CON.

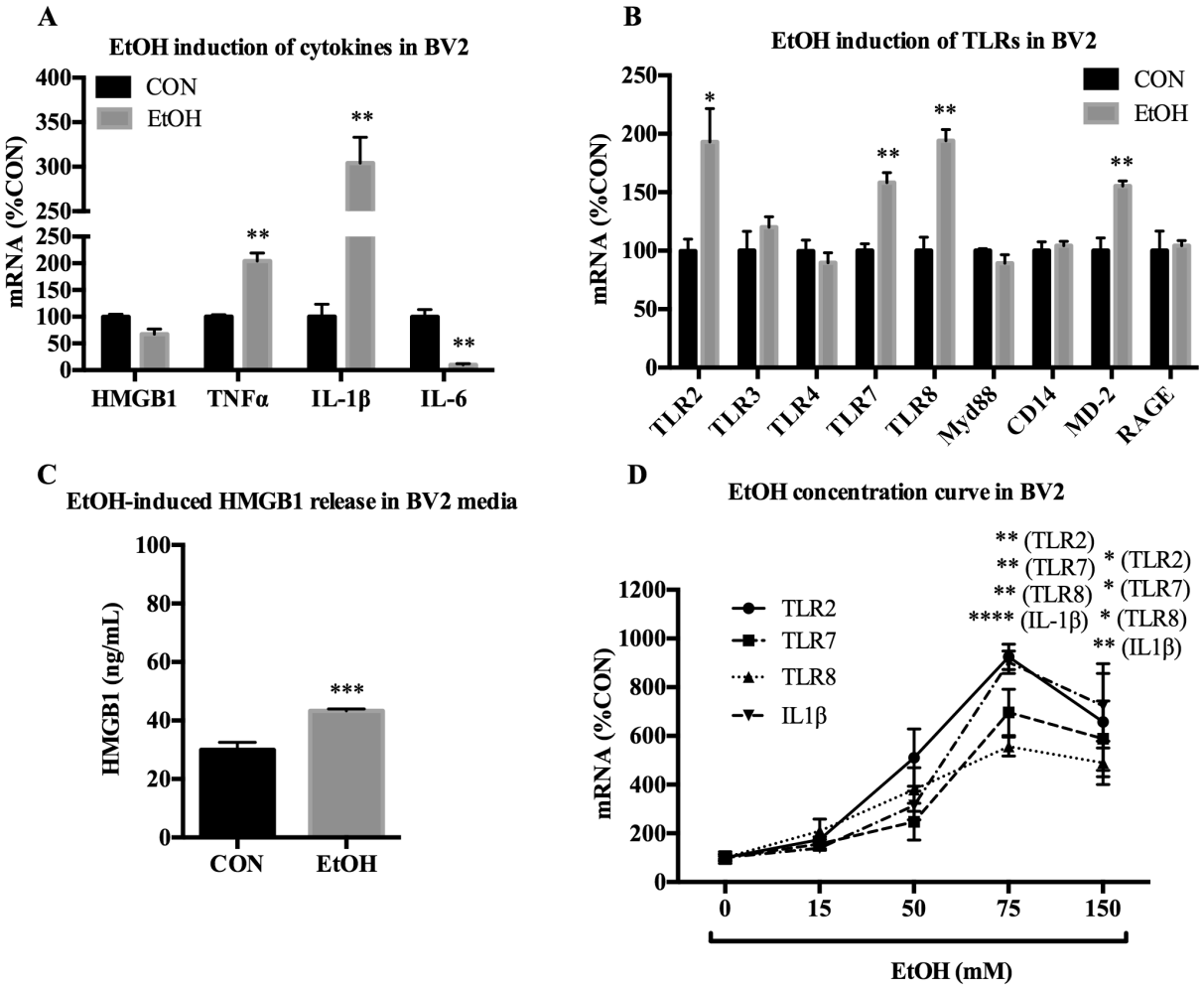

Figure 2.5 EtOH upregulates proinflammatory genes in microglia-like BV2. BV2 were treated with EtOH (150 mM) for 24hr and analyzed for mRNA expression of cytokines, TLRs and TLR-associated signals, and HMGB1 release in media. (**A**) EtOH increased TNFα (204%) and IL-1β (304%), and decreased Il-6 (10%) mRNA. (**B**) EtOH increased TLR2 (193%), TLR7 (158%), TLR8 (194%), and MD-2 (155%) mRNA. (**C**) EtOH increased HMGB1 release in media by 44%. (**D**) Concentration curve of 24hr EtOH treatment on mRNA expression in BV2. EtOH increased TLR2, TLR7, TLR8, and IL-1β at 75 and 150 mM. Data for mRNA is expressed as %control (%CON), with CON set at 100%. n=3 per group; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. CON.

2.4.5 Poly(I:C) and EtOH, but not LPS, activate TLR signaling kinases/transcription factors pathway in neuron-like SH-SY5Y

To determine if TLR3 or TLR4 pathways are activated in neuron-like SH-SY5Y, the cells were treated for 10 or 30min with either TLR4 agonist LPS, TLR3 agonist Poly(I:C), or EtOH followed by Western blot analysis of phosphorylated (activated) kinases. While LPS had no measurable effect on SH-SY5Y at either timepoint (Figure 2.6A), Poly(I:C) increased p-p38 by 88% (±24, $p<0.01$) and p-IRF3 by 38% (±6.6%, $p<0.05$) at 30 minutes (Figure 2.6B). A 10 minute treatment of EtOH decreased p-ERK1/2 by 55% (±4.7%, $p<0.001$), and increased p-p38 by 49% (±17%, $p<0.05$), p-IRF3 by 41% (±19%, $p<0.05$), p-TBK1 by 74% (±17%, $p<0.001$), and p-NFκB by 56% (±17%, $p<0.01$) (Figure 2.6C). By 30 minutes of treatment, EtOH decreased p-ERK1/2 by 49% (±6.7, $p<0.01$), and caused a broad range of effects, increasing p-p38 by 222% (±24, $p<0.0001$), p-IRF3 by 125% (±14%, $p<0.0001$), p-TBK1 by 94% (±11%, $p<0.0001$), and p-NFκB by 72% (±13%, $p<0.001$) (Figure 2.6C). These data indicate that Poly(I:C) but not LPS activates kinases in SH-SY5Y. Importantly, EtOH induces a broad range of activation of proinflammatory signaling kinases/transcription factors in SH-SY5Y, with several effects unique to ethanol.

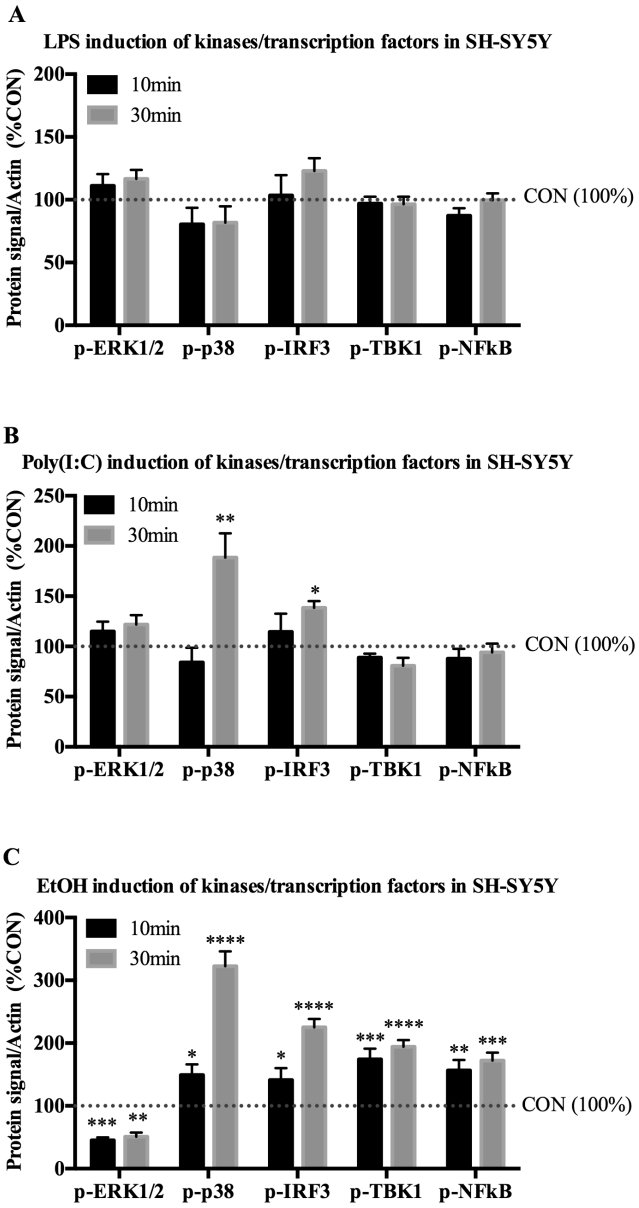

Figure 2.6 Poly(I:C) and EtOH, but not LPS, activate proinflammatory-related kinases/transcription factors in neuron-like SH-SY5Y. SH-SY5Y were treated with indicated drug for 10 or 30min. Cell lysates were collected for Western blot analysis. (**A**) LPS (100 ng/mL) had no detectable effect on any of the kinases/transcription factors examined. (**B**) Poly(I:C) (50 ug/mL) increased p-p38 by 88% and p-IRF3 by 38% by 30 minutes. (**C**) 10 minutes of EtOH (150 mM) decreased p-ERK1/2 by 55%, and increased p-p38 by 49%, p-IRF3 by 41%, p-TBK1 by 74%, and p-NFκB by 56%. 30 minutes of EtOH decreased p-ERK1/2 by 49%, and increased p-p38 by 222%, p-IRF3 by 125%, p-TBK1 by 94%, and p-NFκB by 73%. Data is represented as %CON following β-actin normalization. n=3 per group; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. CON.

2.4.6 Poly(I:C) and EtOH, but not LPS increase proinflammatory gene expression in neuron-like SH-SY5Y

Subsequent proinflammatory gene expression was then examined in neuron-like SH-SY5Y. Cell death was assayed using trypan-blue exclusion and no significant effects were found in any treatment (data not shown). In accordance with the lack of kinase activation, SH-SY5Y did not have any detectable changes in proinflammatory cytokine expression or HMGB1 release in response to LPS (Figure 2.7A-B). However, Poly(I:C) increased mRNA expression of TNFα (10426 ± 2132%, $p<0.01$) and HMGB1 (125 ± 5.2%, $p<0.05$), and further increased levels of IL-1β and IL-6 from non-detectable (>40 Ct) to detectable ranges, with estimated fold changes of 2860 (± 135%) and 591 (± 100%), respectively (Figure 2.7C). Poly(I:C) also increased expression of its own receptor, TLR3 (920 ± 260%, $p<0.05$) as well as TLR2 (230 ± 31%, $p<0.05$) and TLR adaptor protein Myd88 (530 ± 30%, $p<0.001$), as shown in Table 2.3. Poly(I:C) had no effect on HMGB1 release (Figure 2.7D), and no change in IFNβ protein in cell lysates was observed (data not shown). Raw Ct values are depicted in Table 2.4. These data indicate that neuron-like SH-SY5Y appear to lack a detectable LPS response. However, SH-SY5Y are highly responsive to TLR3 stimulation. Poly(I:C) induced strong upregulation of cytokines in SH-SY5Y, as well as TLR receptors and HMGB1.

SH-SY5Y were next treated with EtOH to examine whether EtOH causes induction of proinflammatory genes in neuron-like cells. While no significant changes were seen in mRNA of the cytokines we assessed (Figure 2.8A), EtOH did increase TLR3 (398 ± 80%, $p<0.05$), TLR7 (574 ± 100%, $p<0.05$), TLR8 (319 ± 17%, $p<0.01$), and RAGE (170 ± 15%, $p<0.05$) (Figure 2.8B). Ethanol also caused HMGB1 release in media (Figure 2.8C). Concentrations as low as 15mM EtOH caused increases in TLR3, TLR7, and RAGE mRNA (Figure 2.8D). In summary, EtOH causes

induction of TLR3, TLR7, TLR8, and RAGE in SH-SY5Y even at low concentrations, and in a unique manner, different from either TLR3 or TLR4 stimulation alone.

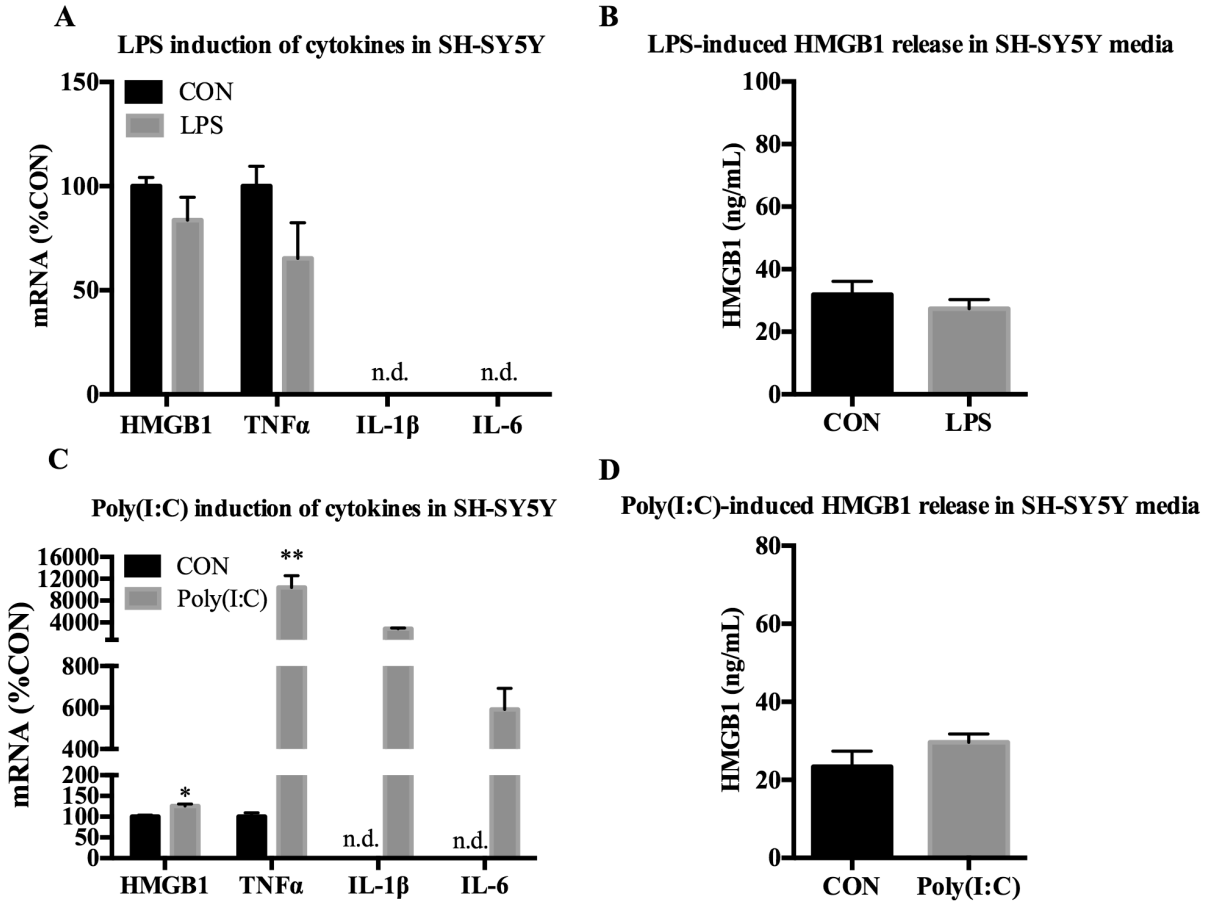

Figure 2.7 Poly(I:C), but not LPS, upregulates cytokine expression in neuron-like SH-SY5Y. SH-SY5Y were treated with either LPS (100 ng/mL) or Poly(I:C) (50 ug/mL) for 24hr followed by mRNA analysis in cell lysates and analysis of HMGB1 release in media using ELISA. (**A**) LPS had no detectable effect on expression of HMGB1, TNFα, IL-1β, or IL-6 mRNA. (**B**) LPS had no effect on HMGB1 release in media. (**C**) Poly(I:C) increased expression of HMGB1 (125%), TNFα (10426%), IL-1β (2860%), and IL-6 (591%) mRNA. (**D**) Poly(I:C) had no effect on HMGB1 release in media. Data for mRNA is expressed as %control (%CON), with CON set at 100%. n=3 per group; n.d. = not detectable; *p<0.05, **p<0.01 vs. CON.

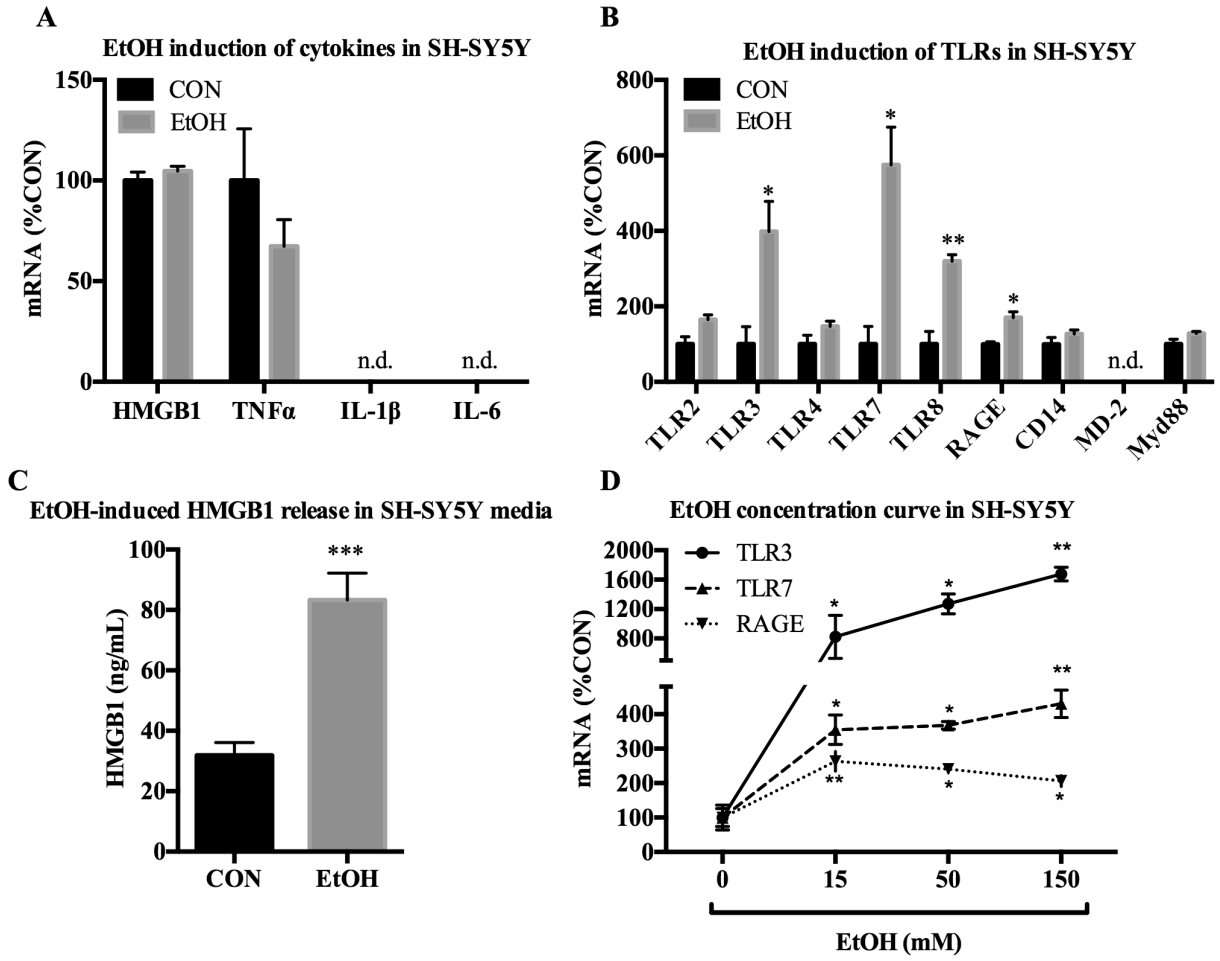

Figure 2.8 EtOH upregulates proinflammatory genes in neuron-like SH-SY5Y. SH-SY5Ywere treated with EtOH (150 mM) for 24hr and analyzed for mRNA expression of cytokines, TLRs and TLR-associated signals, and HMGB1 release in media. (**A**) EtOH had no detectable effect on HMGB1, TNFα, IL-1β, or IL-6 mRNA. (**B**) EtOH increased expression of TLR3 (398%), TLR7 (574%), TLR8 (319%), and RAGE (170%) mRNA. (**C**) EtOH increased release of HMGB1 in the media by 161%. (**D**) Concentration curve of 24hr EtOH treatment on mRNA expression in SH-SY5Y. EtOH increased expression of TLR3, TLR7, and RAGE mRNA by 15 mM. Data for mRNA is expressed as %control (%CON), with CON set at 100%. n=3 per group; n.d. = not detectable; *p<0.05, **p<0.01, ***p<0.001 vs. CON.

2.5 Discussion

This study compares TLR4, TLR3, and ethanol induced kinase activation and proinflammatory gene induction in BV2 microglia-like cells and differentiated SH-SY5Y neuron-like cells (Figure 2.9). As expected, BV2 express greater levels of most proinflammatory signaling molecules compared to SH-SY5Y. BV2 responded to all treatments as indicated by activation of proinflammatory signaling kinases and induction of proinflammatory signaling gene mRNA (Figure 2.9A-C, red labels). Although all 3 treatments induced a BV2 response, TLR3 and TLR4 activation increased canonical kinases, e.g. p-ERK1/2, p-p38, and p-NFκB, whereas ethanol uniquely increased p-IRF3 in BV2, a different canonical proinflammatory signaling pathway not changed by LPS or Poly(I:C) treatment. Further, EtOH decreased p-ERK1/2 in BV2, whereas both LPS and Poly(I:C) increased p-ERK1/2, further distinguishing the signaling of ethanol from TLR3 and TLR4. Proinflammatory cytokines TNFα and IL-1β were increased by all three treatments in BV2, but ethanol further increased TLR2, TLR7, TLR8, and MD-2 (Figure 2.9A-C, red labels). In neuron-like SH-SY5Y, we found TLR4 protein and mRNA, but no detectable LPS responses (Figure 2.9A). However, Poly(I:C) and ethanol both increased p-p38 and p-IRF3, with EtOH also increasing p-NFκB and decreasing p-ERK1/2 in SH-SY5Y (Figure 2.9B-C, blue labels). Interestingly, Poly(I:C) increased proinflammatory cytokine mRNA, e.g. TNFα, IL-1β, and IL-6 in SH-SY5Y, some from non-detectable levels, without increasing p-NFκB, the key proinflammatory transcription factor in monocytes, whereas ethanol increased p-NFκB, but did not induce TNFα, IL-1β, or IL-6 in SH-SY5Y. Both Poly(I:C) and ethanol increased multiple TLR mRNA in SH-SY5Y, but only ethanol increased RAGE expression, which uniquely had greater basal expression in SH-SY5Y than BV2. HMGB1, a TLR and RAGE agonist, was induced and

released by LPS in BV2, but not SH-SY5Y, whereas EtOH released HMGB1 from both cell types (Figure 2.9A,C). EtOH induced TLR mRNA at lower concentrations in SH-SY5Y (15mM, Figure 2.8D) compared to BV2 (75mM, Figure 2.5D). This suggests greater neuronal sensitivity to ethanol induction of TLR mRNA and unique cellular differences in ethanol induction of TLR and other proinflammatory genes that contribute to the complexity in brain.

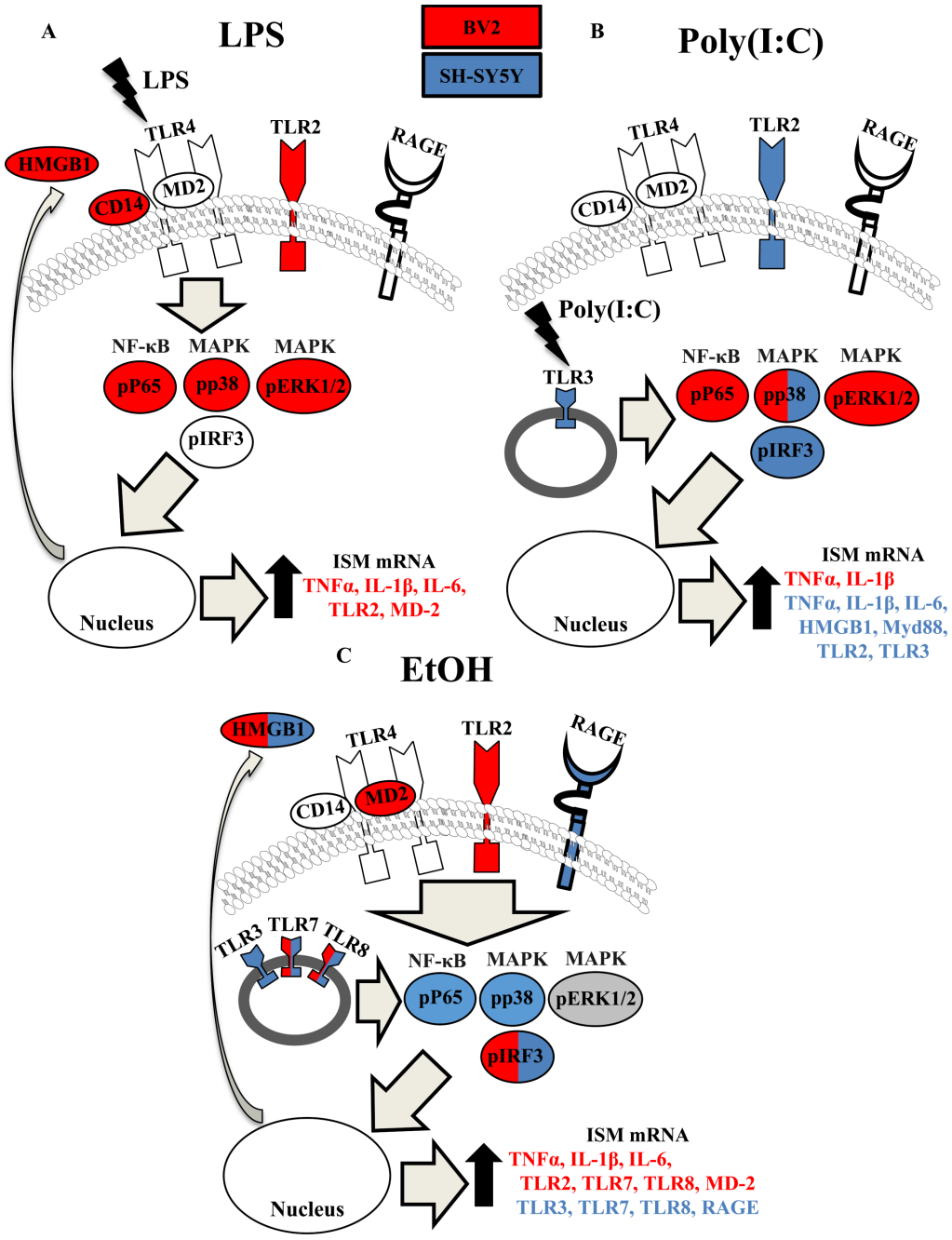

Figure 2.9 EtOH instigates broader neuroimmune changes than TLR4-LPS or TLR3-Poly(I:C) alone in either microglia-like BV2 or neuron-like SH-SY5Y. Red = increased in BV2; blue = increased in SH-SY5Y; gray = decreased in both cell types; white = unchanged in both cell types.

Ethanol is known to modulate innate immune responses in brain (Coller & Hutchinson, 2012; F. T. Crews & Vetreno, 2016; Mayfield, Ferguson, & Harris, 2013; Montesinos, Alfonso-

Loeches, & Guerri, 2016), and drinking behavior (Agrawal et al., 2011; Blednov et al., 2005; Blednov et al., 2012). Microglia and astrocytes have been found to play critical roles in the proinflammatory responses to ethanol, particularly through TLR4 activation (Alfonso-Loeches et al., 2010; Blanco, Valles, Pascual, & Guerri, 2005; Fernandez-Lizarbe et al., 2013; Fernandez-Lizarbe et al., 2009). However, neuronal proinflammatory signaling in response to ethanol has not previously been described. This study utilized microglial and neuronal cell lines to determined commonalities and differences in proinflammatory signaling to ethanol. As expected, microglia-like BV2 have canonical activation to ethanol. EtOH treatment increased activation of IRF3 and increased transcription of NFκB dependent genes (e.g. TNFα, IL-1β, TLR2) consistent with previous reports (Alfonso-Loeches et al., 2010; F. T. Crews et al., 2013; Fernandez-Lizarbe et al., 2013). However, we now report that ethanol causes proinflammatory signaling in neuronal cells that is distinct from microglia. In neuron-like SH-SY5Y, EtOH activated a broader range of transcription factors and proinflammatory modulating kinases (NFκB, p38, TBK1) than in microglia-like BV2. Ethanol also increased transcription of immune receptors RAGE, TLR3, TLR7, and TLR8 in neuron-like SH-SY5Y. Although microglia-like BV2. cells basally express more TLRs and proinflammatory genes than neuron-like SH-SY5Y cells, SH-SY5Y cells had a lower concentration threshold of response to EtOH compared to BV2 cells (15mM vs. 75mM), indicating a higher sensitivity of neurons to EtOH-induced induction of molecules that in the periphery function as proinflammatory. We further found that EtOH has a unique pattern of activation of kinases/proinflammatory genes compared to either TLR3 or TLR4 stimulation alone, again pointing towards a broader effect of EtOH on immune signaling.

Our data support a role for ethanol-induced proinflammatory innate immune signaling in neurons unique from that of microglia as well as TLR3 or TLR4 activation alone.

Previously, we found that EtOH treatment of mice for 10 days (5 g/kg/day) induces brain TLR3 and TLR4 expression, as well as increased induction of proinflammatory cytokines following systemic treatment with LPS (Qin et al., 2008), and Poly(I:C) (Qin & Crews, 2012a), implying that EtOH sensitizes TLR responses through upregulation of these pathways to instigate proinflammatory signaling. Our findings here expand upon these data by suggesting that EtOH has a broad range of proinflammatory activation rather than through TLR3 or TLR4 alone. In BV2, EtOH had a less robust effect on kinases/transcription factors compared to treatment with TLR3 agonist Poly(I:C) or TLR4 agonist LPS. In SH-SY5Y, however EtOH actually had a broader range of effects, with EtOH activating several kinases/transcription factors (TBK1 and NFκB) that were not activated by Poly(I:C). We also found that ERK1/2 was decreased by EtOH in both cell types, whereas p38 was increased in EtOH-treated SH-SY5Y. A similar study showed that EtOH (100 mM, 10 min) inhibited p-ERK1/2 in cultured human cortical neurons (Kalluri & Ticku, 2003), and p-ERK1/2 is decreased in cortex in brains of adolescent intermittent ethanol-treated rats (W. Liu & Crews, 2015). Poly(I:C) increased activation of IRF3, a transcription factor that controls interferon production (Hiscott, 2007), in SH-SY5Y, and EtOH increased IRF3 in both BV2 and SH-SY5Y. Consistent with our studies, EtOH (50 mM, 1hr) increases activated IRF3 in cultured primary mouse microglia (Fernandez-Lizarbe et al. 2009). However, we observed no Poly(I:C) or EtOH-induced changes in IFNβ in SH-SY5Y cell lysates, suggesting that either different IFNs are being targeted or that a non-canonical role for IRF3 exists in neurons. It is important to note that while we observed that 30 minutes of EtOH

increased p-NFκB and p-p38 in SH-SY5Y, no change was seen in BV2 at this timepoint, although other studies in our lab find an increase in p-NFκB at 6 hours in BV2 (data not shown). This may suggest greater importance of other transcription factors (e.g., IRF3) early after EtOH exposure in BV2. Given these results, we surmise that EtOH causes a unique activation of kinases/transcription factors in SH-SY5Y and BV2, with a broader range of activation in neuron-like SH-SY5Y. While it is not clear why these neuron-like and microglia-like cells show different responses to ethanol, it may be related to differential kinase activation in neurons versus microglia. Immune induction by ethanol is complex and includes TLR clustering in lipid rafts, release of endogenous TLR agonists, and oxidative stress activation of NFκB (Fernandez-Lizarbe et al., 2013; Qin & Crews, 2012b; Zou & Crews, 2014). Although complex, our findings suggest ethanol uniquely activates proinflammatory signaling pathways in both microglia-like BV2 and neuron-like SH-SY5Y.

Microglia-like BV2 and neuron-like SH-SY5Y demonstrated unique responses to TLR3 and TLR4 agonists. Microglial responses were canonical with both TLR3 agonist Poly(I:C) and TLR4 agonist LPS increasing activated p-NFκB, p-p38 and p-ERK1/2, consistent with findings in primary microglia cultures (Town, Jeng, Alexopoulou, Tan, & Flavell, 2006). However, neuron-like SH-SY5Y displayed a response only to TLR3 stimulation. TLR3 agonist Poly(I:C) activated p38 and IRF3 in SH-SY5Y, consistent with previous studies in SH-SY5Y (Nessa et al., 2006). We also found that Poly(I:C) greatly upregulated TNFα, IL-1β, and IL-6 mRNA in SH-SY5Y, whereas LPS had no effect, consistent with previous studies (Klegeris & McGeer, 2001; Prehaud et al., 2005). Importantly, Poly(I:C) can bind other receptors (RIG-I, MDA5) in some cells, but studies in human neuronal cells indicate that extracellular Poly(I:C) specifically utilizes TLR3 (Peltier,

Simms, Farmer, & Miller, 2010). Though we detected TLR4 mRNA and protein, we were unable to detect MD-2 expression in SH-SY5Y. MD-2 is a critical accessory protein that is necessary for both LPS and HMGB1 induced TLR4 signaling (Nagai et al., 2002; Yang et al., 2015) in macrophages. This absence of MD-2 might explain the lack of detectable TLR4 response to LPS in SH-SY5Y. However, the α2 subunit of the $GABA_A$ receptor appears to bind to and stimulate neuronal TLR4 activation and TLR4-mediated drinking behavior in alcohol-preferring P-rats (Balan et al., 2018; J. Liu et al., 2011), suggesting alternative roles of TLR4 in neurons. Thus, our findings indicate LPS and Poly(I:C) activate canonical immune signaling kinases and induce immune signaling mRNA in microglia-like BV2, but only Poly(I:C) and not LPS activates TLR-dependent signaling in neuron-like SH-SY5Y.

Ethanol did not increase TLR4 expression in either cell type at the concentration and timepoint (24hr) utilized in this study, although our previous *in vivo* study found TLR4 mRNA upregulated in mouse brain following 10 daily binges of 5 g/kg EtOH (F. T. Crews et al., 2013). Primary rodent microglia, however, have maximally upregulated TLR4 protein at 30min that decreases to non-significantly elevated levels by 24hr (Fernandez-Lizarbe et al., 2013), indicating a timepoint-specificity of EtOH-induced TLR4 expression in cultured microglia. Despite the lack of TLR4 induction, we found that TLR3 mRNA was increased by EtOH and Poly(I:C) in SH-SY5Y, suggesting possible EtOH activation of TLR3 signaling in neurons. Fewer studies have examined possible EtOH proinflammatory signaling through TLR3, although a recent study showed that TLR3 KO mice consume more EtOH (Jang et al., 2016). More research is needed to elucidate the possible neuronal role of TLR3 in EtOH-induced innate immune signaling. Furthermore, the upregulation of both TLR7 and TLR8 by EtOH in both BV2 and SH-

SY5Y indicates a commonalty shared between the two cell-types, indicating more research is needed to understand the complexity of EtOH proinflammatory signaling.

We also found that EtOH increased HMGB1 release in both BV2 and SH-SY5Y, consistent with EtOH-induced HMGB1 in hippocampal brain slices, brains of binge EtOH-treated mice, and post-mortem human alcoholic brain (F. T. Crews et al., 2013). Similar to our data, HMGB1 is also released from EtOH-treated (50 mM, 24hr) non-differentiated neuroblastoma SH-SY5Y (Wang et al., 2015). Since HMGB1 activates multiple TLRs (Park et al., 2004; Yang et al., 2013), RAGE (Kokkola et al., 2005), and can enhance signaling of cytokines such as IL-1β (Sha, Zmijewski, Xu, & Abraham, 2008), the EtOH-induced HMGB1 release we observe may play a role in proinflammatory signaling in both microglia and neurons. We also found that RAGE, a receptor that binds HMGB1 and enhances NFκB signaling (Han et al., 2011), was upregulated in EtOH-treated SH-SY5Y but not in BV2, consistent with an increase in neuronal RAGE in adolescent-intermittent ethanol treated rats (Vetreno et al., 2013). Overall, we report here that EtOH upregulates proinflammatory genes and releases HMGB1 in both cell types, as well as specific TLR3 and RAGE mRNA upregulation in neuron-like SH-SY5Y but not microglia-like BV2.

It is important to note that SH-SY5Y is a neuroblastoma cell-line derived from human tissue and may be different than rodent neurons. However, primary rodent cortical neuronal cultures express and release HMGB1 similar to our findings in human SH-SY5Y (Perez-Carrion & Cena, 2013). We also found that TLR2, 3, 4, 7 and 8 mRNA are expressed in neuron-like SH-SY5Y, consistent with previous studies in mouse (Lehmann et al., 2012; Lok, Basta, Manzanero, & Arumugam, 2015; Tang et al., 2007). However, since BV2 and SH-SY5Y are an *in vitro* model consisting of immortalized and cancer-derived cells, respectively, their responses may differ

from a microglial or neuronal phenotype *in vivo*. Nonethless, reports suggest a negligible difference between gene induction by LPS in primary microglia and BV2 (Henn et al., 2009), and SH-SY5Y are a well-accepted model for studying dopamine neurons in Parkinson's disease (Korecka et al., 2013). Furthermore, examination of any single cell-type outside of its intact environment may alter results, making it worthwhile for our future studies to examine both interaction between these cell-types as well using *in vivo* studies.

Although not utilized in this study, astrocytes are also involved in alcohol use disorders and display proinflammatory immune signaling following EtOH treatment (Adermark & Bowers, 2016; Alfonso-Loeches et al., 2010). Astrocytes would then make an interesting future point of comparison of innate immune signaling vs. the canonical macrophage signaling in microglia.

In summary, this study supports that neurons may contribute to proinflammatory signaling, and suggests a novel role of neurons in ethanol-induced innate immune signaling. Microglia-like BV2 displayed activated kinases following LPS, Poly(I:C), and EtOH treatment, as well as induction of cytokines like TNFα and IL-1β. Although neuron-like SH-SY5Y had no detectable response to LPS, Poly(I:C) and EtOH increased activated kinases and proinflammatory genes. EtOH displayed a different pattern of activated kinases/transcription factors compared to Poly(I:C) or LPS treatment in either cell type. EtOH also increased release of HMGB1 in both cell types and increased expression of RAGE in SH-SY5Y, indicating a possible role of HMGB1/RAGE signaling in this model. These findings are consistent with EtOH activating proinflammatory signaling in both neuron-like SH-SY5Y and microglia-like BV2. Further work is needed to examine ethanol-induced immune signaling in both neurons and microglia.

BV2

	CON	Poly(I:C)	LPS	EtOH
TLR2	24.47 ± 0.26	22.84 ± 0.33	21.92 ± 0.18	23.68 ± 0.12
TLR3	26.12 ± 0.36	25.42 ± 0.12	25.67 ± 0.11	25.87 ± 0.10
TLR4	22.23 ± 0.09	22.26 ± 0.26	23.01 ± 0.06	22.34 ± 0.04
TLR7	22.65 ± 0.17	22.43 ± 0.32	23.07 ± 0.07	22.02 ± 0.06
TLR8	24.22 ± 0.15	25.43 ± 0.58	26.48 ± 0.19	23.37 ± 0.07
CD14	24.46 ± 0.21	23.84 ± 0.12	23.19 ± 0.13	24.37 ± 0.06
MD-2	22.83 ± 0.27	22.70 ± 0.45	22.86 ± 0.20	22.21 ± 0.04
Myd88	23.74 ± 0.11	23.58 ± 0.08	23.81 ± 0.12	23.84 ± 0.12
RAGE	34.57 ± 0.32	34.53 ± 0.08	34.61 ± 0.12	34.43 ± 0.07
HMGB1	23.25 ± 0.22	23.20 ± 0.15	23.42 ± 0.07	23.77 ± 0.23
TNFα	24.30 ± 0.20	23.20 ± 0.19	22.16 ± 0.09	23.25 ± 0.11
IL-1β	25.93 ± 0.40	24.50 ± 0.53	22.31 ± 0.06	24.30 ± 0.15
IL-6	23.45 ± 0.20	23.22 ± 0.55	22.37 ± 0.16	26.83 ± 0.37

SH-SY5Y

	CON	Poly(I:C)	LPS	EtOH
TLR2	29.22 ± 0.26	27.67 ± 0.27	28.89 ± 0.17	28.33 ± 0.13
TLR3	29.06 ± 0.67	25.59 ± 0.50	28.83 ± 0.55	26.75 ± 0.27
TLR4	31.37 ± 0.17	30.70 ± 0.11	32.04 ± 0.44	30.81 ± 0.14
TLR7	33.25 ± 0.71	31.74 ± 0.41	31.93 ± 1.36	29.20 ± 0.24
TLR8	28.28 ± 0.58	28.12 ± 0.35	28.89 ± 1.67	26.37 ± 0.08
CD14	29.13 ± 0.24	28.53 ± 0.18	29.99 ± 0.53	28.75 ± 0.13
MD-2	n.d.	n.d.	n.d.	n.d.
Myd88	23.72 ± 0.25	21.36 ± 0.08	24.05 ± 0.08	23.35 ± 0.07
RAGE	27.11 ± 0.28	26.89 ± 0.12	27.98 ± 0.48	26.35 ± 0.13
HMGB1	20.35 ± 0.32	20.00 ± 0.07	20.63 ± 0.19	20.28 ± 0.03
TNFα	33.57 ± 0.39	27.11 ± 0.27	34.03 ± 0.42	32.61 ± 0.12
IL-1β	n.d.	29.85 ± 0.12	n.d.	n.d.
IL-6	n.d.	32.17 ± 0.35	n.d.	n.d.

Table 2.4 Summary of mRNA CT values in BV2 and SH-SY5Y. Microglia-like BV2 and neuron-like SH-SY5Y were treated with TLR3 agonist Poly(I:C), TLR4 agonist LPS (100 ng/mL), or EtOH (150 mM) for 24hr. Cell lysates were collected and analyzed for mRNA expression using RT-PCR. Actin was used as an endogenous control, with no significant differences in actin expression between treatment groups. Data for mRNA is expressed as raw CT value. n.d. = not detectable.

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CHAPTER 3: NEURONAL-MICROGLIAL CO-CULTURE ALTERS ETHANOL-INDUCED INNATE IMMUNE SIGNALING MOLECULES: A ROLE FOR IL-4/IL-13

3.1 Summary

Background: Innate immune signaling molecules have been found to function as a microglial-neuronal signaling mechanism in brain and play unique roles in learning and memory. However, it is unknown how ethanol impacts these cell-to-cell signaling pathways. Various innate immune signaling molecules, such as Toll-like receptors (TLRs), cytokines, and transcription factor NFκB are upregulated in post-mortem human alcoholic brain, suggesting the importance of further studying these pathways.

Methods: BV2 microglia and SH-SY5Y neurons were co-cultured using a Transwell system followed by ethanol treatment. Effects of co-culture and ethanol on immune signaling molecules were examined using RT-PCR.

Results: Co-culture modified a variety of innate immune signaling molecule expression in both BV2 microglia and SH-SY5Y. Furthermore, co-culture prevented ethanol-induction of endogenous TLR agonist HMGB1, corresponding with a lack of induction of several NFκB genes. Cytokines IL-4 and IL-13 were upregulated in co-cultured BV2 microglia, as well as IL-4 and IL-13 receptors in SH-SY5Y neurons, suggesting a novel microglial-neuronal signaling pathway.

Conclusion: Co-culture of microglia and neuronal cell lines impacts expression of various innate immune signaling genes. Furthermore, we discovered that co-culture increased expression of IL-4/IL-13 ligands and receptors in BV2 microglia and SH-SY5Y neurons,

indicated a novel cell-to-cell signaling pathway in brain that may be a target for future therapeutic strategies.

3.2 Introduction

The innate immune system, which consists of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) that facilitate cytokine induction, takes on unique roles in brain. In particular, cytokines often function as signaling molecules between neurons and glia. Several of these systems have been previously described for their roles in development and synaptic pruning, such as neuronal fractalkine signaling through its microglial receptor, or the complement system (Sheridan & Murphy, 2013; Stephan, Barres, & Stevens, 2012). However, the ability of other cytokine systems to function as microglial-neuronal signaling mechanisms is less well described. In particular, IL-4/IL-13 signaling is an emerging glia to neuron signaling pathway. IL-4 and IL-13 are cytokines which in the periphery commonly function as anti-inflammatory mediators that are produced by Th2 cells (Haas et al., 1999). The receptors for these cytokines form a heterodimer, consisting of IL-4Rα1 (IL-4R) and IL-13Rα1 (IL-13R). In brain, microglia appear to express low levels of IL-4 and IL-13 basally, but can induce expression under certain conditions (Shin et al., 2004; Zhou, Spittau, & Krieglstein, 2012). Interestingly, IL-13 receptor is located on dopaminergic neurons, and overactivation of this receptor has been linked to loss of dopaminergic cells (Mori et al., 2017). Furthermore, IL-4 and IL-13 also have roles in learning and memory (Brombacher et al., 2017; Gadani, Cronk, Norris, & Kipnis, 2012). Stimulation of this pathway leads to activation of transcription factor STAT6 which is linked to IL-10 and TGFβ expression, cytokines which themselves have roles in neuroprotection and learning/memory (Caraci et al., 2015; Donzis & Tronson, 2014; Lobo-Silva, Carriche, Castro, Roque, & Saraiva, 2016; Vivien & Ali, 2006).

In brain, dysregulation of the neuroimmune system caused by alcohol has been linked to both neurodegeneration and addiction pathology (Coleman & Crews, 2018; Crews et al., 2004; Crews, Lawrimore, Walter, & Coleman, 2017; Mayfield, Ferguson, & Harris, 2013). Alcohol consumption is correlated with a variety of negative health outcomes, including but not limited to onset of alcohol use disorders (AUD), fetal alcohol syndrome, alcoholic liver disease, and cancers. In particular, TLR signaling has been suggested to be implicated in alcohol pathology. While in the periphery TLRs respond to viral and bacterial components, in brain they respond to endogenous agonists such as high mobility group box protein-1 (HMGB1), which activates multiple TLRs (Park et al., 2004; Yanai et al., 2009; Yang, Antoine, Andersson, & Tracey, 2013). Activation of TLRs leads to stimulation of transcription factors like NFκB, which stimulates transcription of cytokines, propagating inflammation through their respective receptors (Narayanan & Park, 2015). Multiple components of this pathway have been found to be upregulated in post-mortem human alcoholic brain; in particular, there is increased expression of TLR3, TLR4, TLR7 (Coleman, Zou, & Crews, 2017; Crews, Qin, Sheedy, Vetreno, & Zou, 2013), as well as phosphorylated (activated) NFκB (Vetreno, Lawrimore, Rowsey, & Crews, 2018), and cytokines like IL-1β and HMGB1 (Coleman, Zou, Qin, & Crews, 2018; Crews et al., 2013; J. Zou & Crews, 2012). Furthermore, alcohol-potentiated increases in TLR3 and TLR4 increases TLR agonist-induction of cytokines (Qin & Crews, 2012; Qin et al., 2008). These data therefore suggest that both TLR pathways are implicated in alcohol pathology, but exact mechanisms remain to be elucidated, such as the respective involvement of various cell types as well as cell-to-cell interactions that bring about ethanol-induced innate immune signaling.

Microglia, the canonical macrophage in brain, are thought to be the main mediator of innate immune signaling in brain. Indeed, previous studies have shown that ethanol induces

various cytokines in microglial cultures (Fernandez-Lizarbe, Montesinos, & Guerri, 2013; Fernandez-Lizarbe, Pascual, & Guerri, 2009). However, we have previously reported neuronal localization of TLR3, TLR4, and HMGB1 in rat brain (Vetreno & Crews, 2012), as well as neuronal IL-1β in mouse brain (Coleman et al., 2018). In addition, we recently found that ethanol increases both TLR3 and TLR7 in SH-SY5Y neurons, and at lower ethanol concentrations than in BV2 microglia, in addition to HMGB1 release in both cell types (Lawrimore & Crews, 2017). However, as these experiments were conducted in single cell types, whereas in brain there is on-going communications between neurons and microglia. Such signaling mechanisms have been demonstrated in settings such as it remains unknown how microglial-neuronal interactions can affect these ethanol-induced changes in innate immune gene expression. In addition, while IL-13 was found to be increased in serum of cirrhotic alcoholic patients (González-Reimers et al., 2012), little is known about how IL-4/IL-13 signaling is affected by alcohol.

In this study, we examine how neuronal-microglial co-culture influences ethanol-induced innate immune signaling. We expanded upon previous findings by discovering that co-culture modifies cytokine and TLR induction in BV2 microglia and SH-SY5Y neurons. We next found that IL-4 and IL-13 signaling is upregulated by ethanol in co-cultured BV2 microglia and SH-SY5Y neurons, and that IL-4/IL-13 diminishes expression of cytokines like TNFα and IL-1β in hippocampal-entorhinal slice culture, suggesting a role for this pathway in alcohol pathology.

3.3 Materials and Methods

3.3.1 Cell lines and treatment

BV2 were acquired from ICLC (Genoa, Italy, #ATL03001). BV2 were cultured using Dubecco's modified Eagle serum (DMEM, Life Technologies, Carlsbad, CA) supplemented with

10% fetal bovine serum (FBS, Life Technologies), 1X GlutaMAX (Life Technologies), and 1X antibiotic-antimycotic (Life Technologies). In the BV2 alone group, media was changed to 2% FBS approximately 16 hours prior to treatment.

SH-SY5Y was acquired from ATCC (Manassas, Virginia, #CRL-2266). SH-SY5Y were cultured using DMEM/F-12 + GlutaMAX (Life Technologies), 10% FBS, and 1X antibiotic-antimycotic. Prior to treatments, SH-SY5Y were differentiated using 10 uM retinoic acid (RA, Sigma-Aldrich, St. Louis, MO, #R2625) for 4 days in Neurobasal media (Life Technologies) containing 2% B27 supplement (Life Technologies), 0.5 mM GlutaMAX and 1X antibiotic-antimycotic. In the SH-SY5Y alone group, media was refreshed 16 hours prior to treatment.

In all groups treated with EtOH, a concentration of 100 mM for 24 hours was utilized. Cells exposed to ethanol were placed into an ethanol-saturated chamber. Control groups were given an equal volume of vehicle (PBS). Following treatment, cells lysates were harvested for RT-PCR analysis.

3.3.2 Transwell co-culture model

SH-SY5Y were plated on 6-well plates. After the differentiation procedure, media was refreshed to 10% FBS containing DMEM, and Transwell inserts (Corning, Corning, NY) were placed on top of the SH-SY5Y-containing wells. BV2 were plated on top of the Transwell inserts; see Figure 3.1 for a schematic. 16 hours prior to EtOH treatment (30 hours following plating of BV2), media was changed to 2% FBS. See Figure 3.1 for a diagram of the treatment outline.

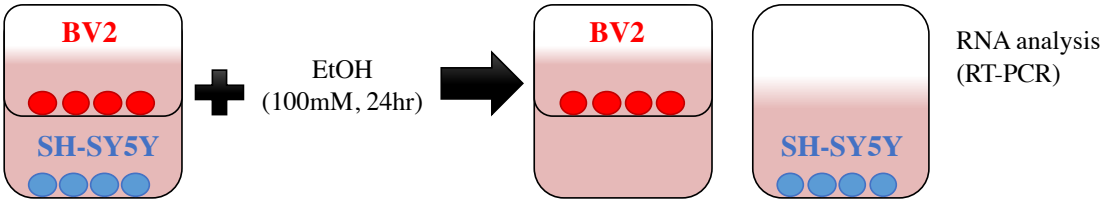

Figure 3.1 Transwell co-culture model for examining ethanol-induced changes in BV2 microglia and SH-SY5Y neurons. SH-SY5Y neurons were plated on the bottom of 6-well culture plates, while Transwell inserts inserted in the wells. BV2 microglia were plated on top of the Transwells. Cells were treated with ethanol (100 mM) for 24 hours, followed by RNA isolation in both BV2 microglia and SH-SY5Y neuron cell lysates.

3.3.3 Hippocampal entorhinal (HEC) slice culture

All protocols followed in this study were approved by the Institutional Animal Care Use Committee at UNC and were in accordance with National Institute of Health regulation for the care and use of animal in research. Organotypic brain slice cultures are prepared as described previously (J. Y. Zou & Crews, 2014). Briefly, the hippocampal entorhinal region is dissected and sliced transversely (375 μm thick) from postnatal day 7 rat pups. HEC slices were placed onto tissue insert membrane (10 slices/insert) and cultured with medium containing 75% MEM with 25 mM HEPES and Hank's salts, 25% horse serum (HS), 5.5 g/L glucose, 2 mM L-glutamine in a humidified 5% CO2 incubator at 36.5 °C for 7 days in vitro (DIV), followed by 4 DIV in medium containing 12.5% HS and then 3 DIV in serum-free medium supplemented with N2. The cultures after 14 DIV were used for experiments and drug treatments with serum-free N2- supplemented medium. For ethanol exposures, slices were exposed to ethanol (100 mM) for 48 hours. Recombinant rat IL-4 protein (500 ng/mL; R&D Systems, #504-RL-005) and recombinant rat IL-13 protein (1 ug/mL; R&D Systems, #1945-RL-025) were also added to the indicated groups for 48 hours.

3.3.4 Real-time (RT)-PCR

For SH-SY5Y and BV2, RNA was extracted from cell lysates using TRIzol (Invitrogen, Carlsbad, CA). RNA was extracted from HEC slices using RNeasy Mini Kit (Qiagen, CA). RNA concentration was determined using a Nanodrop (Thermo Scientific, Waltham, MA) and was reverse-transcribed to cDNA. The SYBR green PCR master mix (Life Technologies) was used for real-time PCR analysis. The relative differences in expression between groups were expressed using cycle time (Ct) values normalized with β-actin, and relative differences between

control and treatment groups were calculated and expressed as relative increases setting control as 100%.

3.3.5 Enzyme-linked immunosorbent assay (ELISA)

Media was collected after ethanol treatment and spun at 500xg for 10 minutes to remove cell debris. HMGB1 protein concentration in media was detected using an HMGB1 ELISA kit (IBL, Hamburg, Germany), per manufacturer's directions.

3.3.6 Statistical analysis

One-way or two-way ANOVAs followed by post-hoc Tukey's test were used as appropriate. A p-value of less than 0.05 was considered significant. All data analysis was conducted using Prism (Graphpad, La Jolla, CA).

3.4 Results

3.4.1 Co-culture modifies BV2 microglia and SH-SY5Y gene expression and ethanol induced cytokines and TLRs

Co-cultures provide a model to understand glial-neuronal signaling (Lin, Uang, Lin, Chen, & Lo, 2007) and how it may modify responses to ethanol. We compared BV2 microglia alone and SH-SY5Y neurons alone to co-cultured BV2/SH-SY5Y (see Figure 3.1 for co-culture schematic). Interestingly, co-culture of BV2 microglia with SH-SY5Y neurons caused significant changes in microglial gene expression (Figure 3.2, Table 3.1). Multiple neuroimmune receptors were increased in BV2 microglia by SH-SY5Y neuronal co-culture including TLR4 (15-fold, $p<0.0001$), TLR7 (6.8-fold, $p<0.001$), IL-13R (10-fold, $p<0.05$) RAGE (3-fold, $p<0.0001$) as well as statistically significant increases in iNOS, IL4R, CXCR1, CD200R, Arg1, TNFα, TGFB and multiple immune receptor intracellular signaling proteins, (MD-2, TBK1, IKKβ). Among the genes measured in BV2, only DAP12 was decreased. In SH-SY5Y

neurons, co-culture robustly increased several neuroimmune genes, such as RAGE (8.1-fold, $p<0.0001$) and TGFβ (8.8-fold, $p<0.001$), as well as neuronal markers such as doublecortin (DCX; 7.3-fold, $p<0.0001$) and tyrosine hydroxylase (TH; 6.7-fold, $p<0.0001$). Multiple other genes, including CXCL10, CD200, MCP-1, IL-13R, CD200, CX3CL1, DAP12, and HDAC2 were increased by co-culture in the SH-SY5Y neurons (Table 3.2). Of particular interest in these findings is the increase of microglial-neuronal signaling pathways, such as fractalkine (CX3CL1) and CD200, by co-culture alone. These findings are consistent with the complex known interactions of microglia and neurons.

Previously, we found that ethanol treatment for 24 hrs. upregulates cytokines IL1β and TNFα, as well as TLR7 in BV2 microglia, whereas in SH-SY5Y neurons, cytokine expression was not affected but both TLR3 and TLR7 expression was increased (Lawrimore & Crews, 2017). Interestingly, while we observed that in BV2 microglia cultures alone ethanol increases IL-1β (1.9-fold, $p<0.01$) and TNFα (1.2-fold, $p<0.0001$) expression, in BV2/SH-SY5Y co-cultures, ethanol did not induce IL-1β and TNFα in BV2 microglia (Figure 3.2A-B). Similarly, pro-inflammatory mediator iNOS was decreased by ethanol in BV2 microglia cultures only in cells co-cultured with SH-SY5Y (Figure 3.2C). Both TLR4 and TLR7, interestingly, were increased by co-culture alone (14-fold, $p<0.0001$ and 6.8-fold, $p<0.001$, respectively) whereas the ethanol-induced increase in TLR7 (3-fold, $p<0.05$) was blocked in the co-culture (Figure 3.2D-F).

In SH-SY5Y neurons, we found that even when co-cultured with BV2 microglia, ethanol had little effect on IL-1β and TNFα expression (Figure 3.3A-B). However, we did observe a small increase in iNOS expression by ethanol in SH-SY5Y neurons alone (1.4-fold, $p<0.05$), which was blocked by co-culture with the BV2 microglia (Figure 3.3C). Interestingly, the

increased expression of TLR3 (2-fold, $p<0.05$) and TLR7 (4.3-fold, $p<0.01$) by ethanol in SH-SY5Y neurons alone was blocked in co-cultured cells, although TLR4 expression was actually increased by ethanol (3.1-fold, $p<0.01$) in co-cultured SH-SY5Y neurons (Figure 3.3D-F). We further examined a multitude of innate immune and other signaling genes in both BV2 microglia (Table 3.1) and SH-SY5Y neurons (Table 3.2). These data indicate that co-culture between neurons and microglia modifies ethanol-induced changes in innate immune gene expression.

HMGB1, an endogenous TLR agonist with cytokine-like activity when released, was previously found to be released by ethanol treatment in both BV2 microglia and SH-SY5Y neurons (Lawrimore & Crews, 2017). In order to investigate how microglial-neuronal signaling alters this ethanol-induced release of HMGB1, we examined HMGB1 levels in the media of ethanol-treated BV2 microglia alone, SH-SY5Y neurons alone, as well as BV2/SH-SY5Y co-cultures. We confirmed our previous findings, with EtOH increasing HMGB1 release in both BV2 microglia and SH-SY5Y neurons, but interestingly, ethanol treatment in the co-cultured cells did not cause a release of HMGB1 (Figure 3.4A). As diagramed in Figure 3.4B-C, our findings indicate that ethanol-induced HMGB1 release is correlated with an increase of multiple NFκB-regulated proinflammatory innate immune genes, with co-culture blocking both HMGB1 and downstream transcription of these genes.

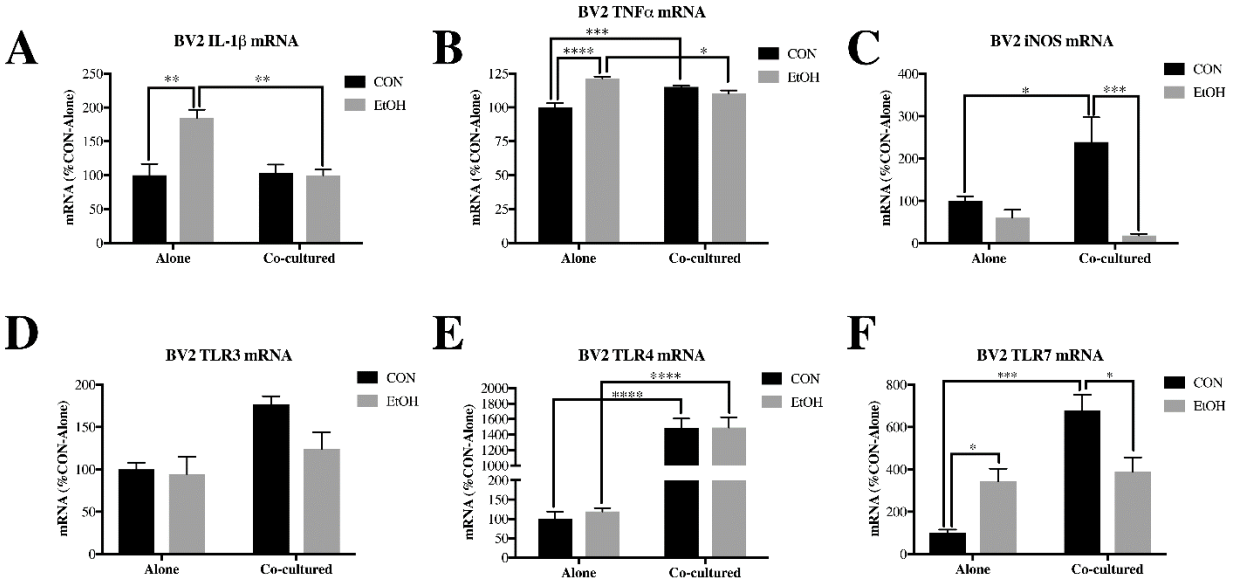

Figure 3.2 Co-culture alters ethanol-induced cytokines and TLRs in BV2 microglia. BV2 microglia were treated with ethanol (EtOH, 100 mM) for 24 hours either alone or while co-cultured with SH-SY5Y neurons. Cell lysates were examined for mRNA expression. (**A**) IL-1β expression was increased by EtOH in BV2 alone (185 ± 12%), but not in co-cultured BV2. Two-way ANOVA indicated significant main effects of EtOH ($p<0.01$), co-culture ($p<0.01$), and the interaction ($p<0.01$). (**B**) TNFα expression was increased by EtOH in BV2 alone (121 ± 1.2%) and by co-culture (115 ± 0.9%), but not by EtOH in co-cultured BV2. Two-way ANOVA indicated significant main effect by EtOH ($p<0.01$) and interaction ($p<0.0001$). (**C**) iNOS was increased by co-culture in BV2 (239 ± 58%), while EtOH significantly reduced iNOS in co-cultured cells (18 ± 4.0%). Two-way ANOVA indicated significant main effect by EtOH ($p<0.01$) and their interaction ($p<0.01$). (**D**) TLR3 expression in BV2; two-way ANOVA indicated significant main-effect of co-culture ($p<0.05$). (**E**) TLR4 expression was increased by co-culture ($p<0.0001$); two-way ANOVA indicated a significant main effect of co-culture ($p<0.0001$). (**F**) TLR7 expression was increased by EtOH in BV2 alone (308 ± 59%), as well as by co-culture (678 ± 74%), but was significantly decreased by EtOH in co-cultured BV2 (389 ± 68%). Two-way ANOVA indicated significant main effect of co-culture ($p<0.001$) and interaction ($p<0.01$). Data is represented as %CON (control)-Alone ± SEM, n=5-6 per group. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ vs. indicated group via Tukey's post-hoc.

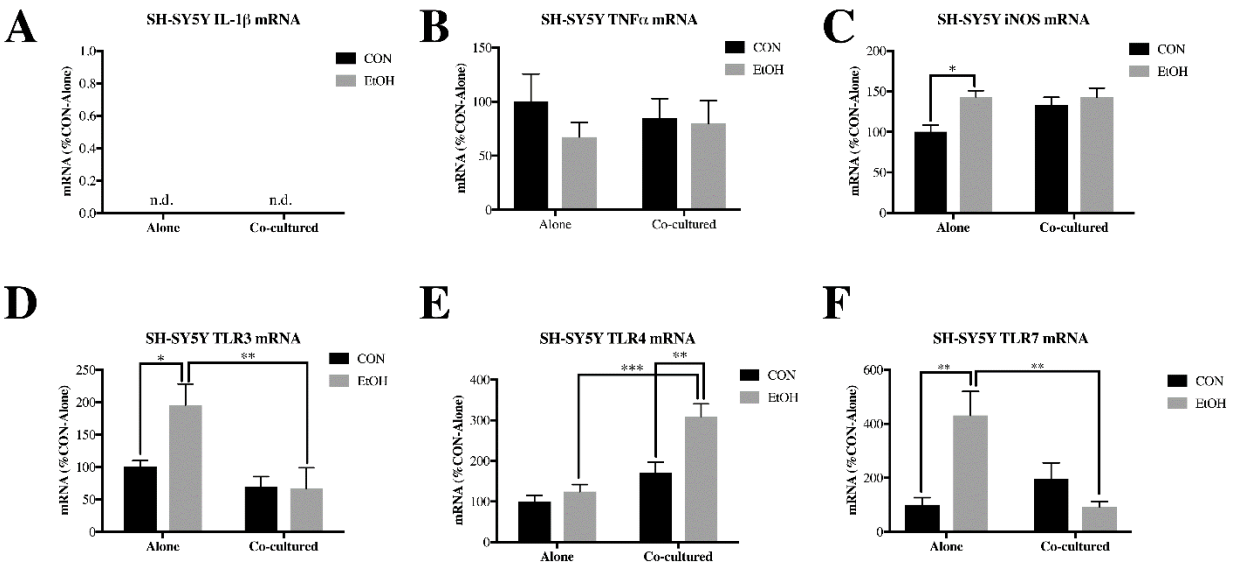

Figure 3.3 Co-culture alters ethanol-induced TLRs in SH-SY5Y neurons. SH-SY5Y neurons were treated with ethanol (EtOH, 100 mM) for 24 hours alone or while co-cultured with BV2 microglia. Cell lysates were examined for mRNA expression. (**A**) IL-1β mRNA was not detected in SH-SY5Y cell lysates. (**B**) TNFα expression was not significantly affected by EtOH or co-culture in SH-SY5Y. (**C**) iNOS expression was significantly increased by EtOH in SH-SY5Y alone (143 ± 8.0%). Two-way ANOVA indicated a significant main effect of EtOH ($p<0.05$). (**D**) TLR3 expression was significantly increased by EtOH in SH-SY5Y alone (196 ± 32%), but not in co-cultured SH-SY5Y. Two-way ANOVA indicated a significant main effect of co-culture ($p<0.01$), EtOH ($p<0.05$), and their interaction ($p<0.05$). (**E**) TLR4 expression was significantly increased by EtOH in co-cultured SH-SY5Y (310 ± 31%). Two-way ANOVA indicated a significant main effect of co-culture ($p<0.0001$), EtOH ($p<0.01$), and interaction ($p<0.05$). (**F**) TLR7 was significantly increased by EtOH in SH-SY5Y alone (431 ± 89%), but not in co-cultured SH-SY5Y. Two-way ANOVA indicated a significant interaction ($p<0.01$). Data is represented as %CON (control)-Alone ± SEM, n=5-6 per group. n.d. = not detected (>40 cycles) *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. indicated group via Tukey's post-hoc.

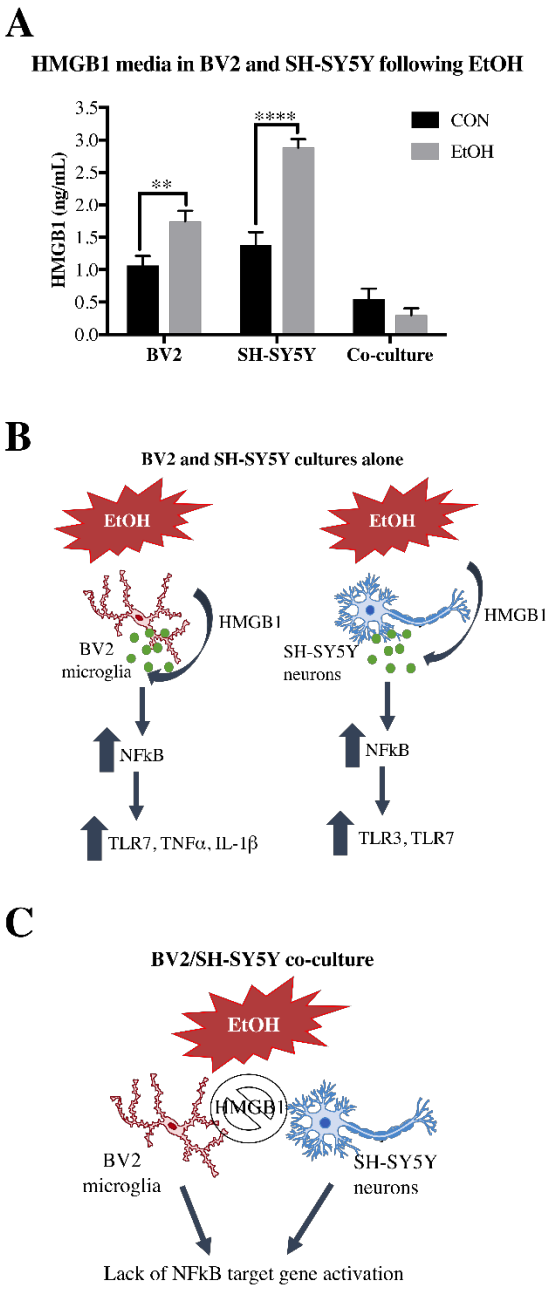

Figure 3.4 Co-culture blocks ethanol-induced HMGB1 release. BV2 microglia alone, SH-SY5Y neurons alone, and co-cultured BV2/SH-SY5Y were treated with ethanol (EtOH, 100 mM) for 24 hours. Media was collected and analyzed for HMGB1 protein using ELISA. (**A**) HMGB1 was increased in the media of EtOH-treated BV2 microglia and SH-SY5Y, but not in co-cultured cells. (**B**) Schematic diagraming EtOH increasing HMGB1 release in BV2 and SH-SY5Y alone, leading to increased expression of NFκB genes. (**C**) Schematic diagraming a lack of HMGB1 release in co-cultured BV2 and SH-SY5Y, preventing transcription of NFκB genes. n=5-6 per group; **p<0.01, ****p<0.0001.

Gene	CON-Alone	EtOH-Alone	CON-CC	EtOH-CC
NFkB signaling genes				
CCR2	100 ± 11	185 ± 29*	177 ± 23	294 ± 16@@,##
iNOS	100 ± 11	60 ± 19	239 ± 58*	18 ± 4.0###
MCP1	100 ± 15	62 ± 21	58 ± 5.6	19 ± 0.7#
MD-2	100 ± 21	147 ± 22	187 ± 5.9*	173 ± 22
RAGE	100 ± 8.5	117 ± 12	342 ± 55***	340 ± 33@@@
TBK1	100 ± 23	106 ± 22	631 ± 61****	666 ± 65@@@@
TGFβ	100 ± 19	94 ± 30	217 ± 20**	319 ± 19@@@@,#
HMGB1	100 ± 12	109 ± 13	107 ± 6.9	67 ± 8.3
IKKβ	100 ± 29	90 ± 21	367 ± 11****	500 ± 45@@@@,#
Interleukin genes				
IL-1β	100 ± 17	185 ± 12**	103 ± 12	100 ± 8.9@@
IL-4	100 ± 19	98 ± 11	94 ± 8.7	207 ± 27@@,##
IL-4R	100 ± 6.1	91 ± 12	198 ± 15*	412 ± 38@@@@,####
IL-10	100 ± 26	93 ± 8.8	165 ± 26	182 ± 17@@@,##
IL-13	n.d.	n.d.	100 ± 12.4	220 ± 15####
IL-13R	100 ± 18	468 ± 135	997 ± 107*	2034 ± 261@@@@,##
Toll-like receptor (TLR) genes				
TLR3	100 ± 8.2	94 ± 21	177 ± 9.3	124 ± 20
TLR4	100 ± 19	120 ± 8.5	1483 ± 124****	1491 ± 132@@@@
TLR7	100 ± 16	308 ± 59*	678 ± 74***	389 ± 68#
Microglial markers				
Arg1	100 ± 21	73 ± 15	160 ± 5.6*	137 ± 5.3@
CD200R	100 ± 27	217 ± 46	320 ± 27*	658 ± 71@@@@,###
CX3CR1	100 ± 23	122 ± 20	203 ± 32*	234 ± 20@
TREM signaling genes				
DAP12	100 ± 6.6	187 ± 8.2****	75 ± 2.3**	124 ± 4.4@@@@,####
TREM1	100 ± 11	114 ± 18	401 ± 34	1144 ± 199@@@,##
TREM2	100 ± 13	138 ± 3	86 ± 8.9	125 ± 7.1#
TNF superfamily genes				
TNFα	100 ± 3.4	121 ± 1.2****	115 ± 0.9***	100 ± 2.4@
DR3	100 ± 12	199 ± 36	341 ± 24	2261 ± 429@@@@,####

Table 3.1 Summary of effects of ethanol and co-culture on gene expression in BV2 microglia. (red) *p<0.05, **p<0.1, ***p<0.001, ****p<0.0001 vs. CON-Alone; (blue) @p<0.05, @@p<0.01, @@@p<0.001, @@@@p<0.0001 vs. EtOH-Alone; (green) #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 vs. CON-CC. CON = control, EtOH = ethanol, CC = co-culture. Data was analyzed via two-way ANOVA followed by post-hoc Tukey's as appropriate.

Gene	CON-Alone	EtOH-Alone	CON-CC	EtOH-CC
NFkB signaling genes				
CCR2	100 ± 24	187 ± 26	98 ± 14	142 ± 45
CXCL10	100 ± 32	84 ± 12	210 ± 32*	85 ± 18#
HMGB1	100 ± 5.9	71 ± 8.9	42 ± 12*	102 ± 22#
HMOX1	100 ± 8.5	580 ± 95****	126 ± 7.5	186 ± 25
IKKβ	100 ± 7.5	258 ± 57*	202 ± 12	490 ± 47@@,###
iNOS	100 ± 8.2	143 ± 8.0*	134 ± 9.2	143 ± 11
MCP1	100 ± 29	42 ± 11	180 ± 19*	77 ± 7.7##
MD-2	n.d	n.d	n.d	n.d
RAGE	100 ± 6.2	233 ± 38*	810 ± 63****	796 ± 114@@@
TBK1	100 ± 33	185 ± 47	243 ± 24	642 ± 107@@@,##
TGFβ	100 ± 16	308 ± 48	880 ± 53***	2571 ± 211@@@@,####
Interleukin genes				
IL-1β	n.d.	n.d.	n.d.	n.d.
IL-4	n.d.	n.d.	n.d.	n.d.
IL-4R	100 ± 8.6	238 ± 18**	164 ± 11	480 ± 37@@@@,####
IL-10	100 ± 24	239 ± 55	194 ± 37	231 ± 31
IL-10R	n.d.	n.d.	n.d.	n.d.
IL-13	n.d.	n.d.	n.d.	n.d.
IL-13R	100 ± 35	136 ± 28	1723 ± 117****	4147 ±119@@@@,####
Toll-like receptor (TLR) genes				
TLR3	100 ± 9.6	196 ± 32*	70 ± 15	67 ± 32@@
TLR4	100 ± 15	124 ± 17	171 ± 25	310 ± 31@@,##
TLR7	100 ± 27	431 ± 89**	197 ± 58	93 ± 20
TNF superfamily and death receptor pathway genes				
Caspase 3	100 ± 19	243 ± 34	525 ± 78**	636 ± 134@@
DR3	100 ± 33	178 ± 33	561 ± 28****	702 ± 70@@@@
FADD	100 ± 7.1	124 ± 11.5	171 ± 18	330 ± 58@@@,#
Fas	100 ± 11.8	861 ± 352	1203 ± 357	2422 ± 238@
TL1A	n.d.	n.d.	100 ± 19	43 ± 7.1#
TNFα	100 ± 26	67 ± 13	85 ± 18	80 ± 21
NGFR	100 ± 12	5127 ± 471****	42 ± 1.7	331 ± 80@@@@
Neuronal-specific genes				
CD200	100 ± 26	183 ± 61	290 ± 14**	604 ± 67@@@@,###
CX3CL1	100 ± 6.3	123 ± 7.0	213 ± 17****	274 ± 12@@@@, ##
ChAT	100 ± 16	144 ± 43	177 ± 29	234 ± 38
DCX	100 ± 26	108 ± 32	729 ± 40****	732 ± 54@@@@
TH	100 ± 15	124 ± 40	668 ± 113****	110 ± 27####
TREM signaling genes				
DAP12	100 ± 20	70 ± 5.2	13701 ± 3140***	11800 ± 1552@@@
TREM1	100 ± 23	177 ± 45	485 ± 221	1506 ± 343@@@,#
TREM2	100 ± 23	140 ± 40	100 ± 14	172 ± 25
Proteases				
ADAM10	100 ± 33	199 ± 65	700 ± 56**	1429 ± 161@@@@,###
Histone deacetylases				
HDAC1	100 ± 11.7	131 ± 7.0	185 ± 8.8	336 ± 64@@
HDAC2	100 ± 11.7	193 ± 23	354 ± 72*	378 ± 84

Table 3.2 Summary of effects of ethanol and co-culture on gene expression in SH-SY5Y neurons.
(red) *p<0.05, **p<0.1, ***p<0.001, ****p<0.0001 vs. CON-Alone; (blue) @p<0.05, @@p<0.01, @@@p<0.001, @@@@p<0.0001 vs. EtOH-Alone; (green) #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 vs. CON-CC. CON = control, EtOH = ethanol, CC = co-culture. Data was analyzed via two-way ANOVA followed by post-hoc Tukey's as appropriate.

3.4.2 IL-4/IL-13 expression is increased by ethanol in co-cultured BV2 microglia and SH-SY5Y neurons

IL-4 and IL-13 are cytokines which in the periphery modulate inflammatory responses and share receptors that dimerize (Bao & Reinhardt, 2015), resulting in complementary signaling pathways. While IL-4/IL-13 have been discovered in brain (Mori, Maher, & Conti, 2016) and have also been shown to affect dopaminergic neurons (Mori et al., 2017), little is known about this unconventional glial-neuronal signaling pathway. We examined both IL-4 and IL-13 ligands and receptors in our BV2 microglia/SH-SY5Y neuron co-culture model to further study this pathway following ethanol treatment. In the BV2 microglia, we found that ethanol only increased IL-4 (2-fold, $p<0.01$) and IL-4 receptor (IL-4R; 2-fold, $p<0.0001$) expression in cells co-cultured with SH-SY5Y neurons (Figure 3.5A-B). Interestingly, IL-13 was not detectable at all in BV2 microglia alone, but co-culture induced expression that was further increased by ethanol (2.2-fold, $p<0.0001$, Figure 3.5C). The IL-13 receptor (IL-13R) was also increased by co-culture (10-fold, $p<0.05$) as well as ethanol (20-fold, $p<0.01$) in BV2 microglia (Figure 3.5D).

In SH-SY5Y neurons, both IL-4 and IL-13 ligands were not detected (Figure 3.6A,C). However, IL-4R was increased by ethanol in SH-SY5Y alone (2.4-fold, $p<0.01$), as well as further increased by ethanol in cells co-cultured with BV2 microglia (4.8-fold, $p<0.0001$, Figure 3.6B). IL-13 receptor was also increased by co-culture (17-fold, $p<0.0001$), as well as by ethanol in the co-culture setting in SH-SY5Y (41-fold, $p<0.0001$, Figure 3.6D). These data indicate that IL-4 and IL-13 signaling is increased by ethanol in neuronal-microglial co-culture, suggesting a role for IL-4/IL-13 in neuronal-glial ethanol-induced signaling.

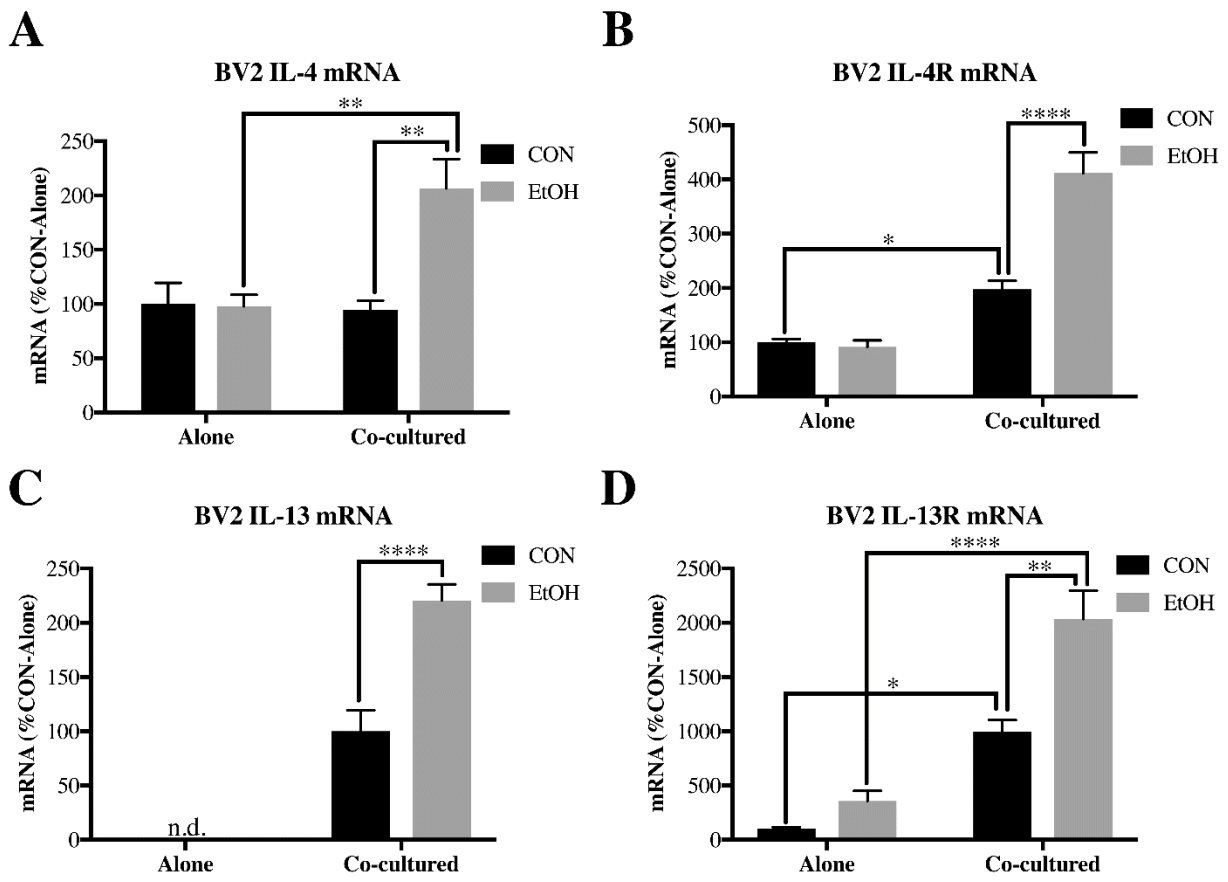

Figure 3.5 IL-4 and IL-13 is increased by ethanol in co-cultured BV2 microglia. BV2 microglia were treated with ethanol (EtOH, 100 mM) for 24 hours either alone or while co-cultured with SH-SY5Y neurons. Cell lysates were examined for mRNA expression. (**A**) IL-4 expression was increased by EtOH in co-cultured BV2 (207 ± 27%). Two-way ANOVA indicated a significant main effect of co-culture ($p<0.05$), EtOH ($p<0.05$), and interaction ($p<0.01$). (**B**) IL-4R expression was increased by co-culture (198 ± 15%), as well as by EtOH in co-cultured BV2 (412 ± 38%). Two-way ANOVA indicated a significant main effect of co-culture ($p<0.0001$), EtOH ($p<0.001$), and interaction ($p<0.0001$). (**C**) IL-13 was not detected in BV2 alone, but was increased by EtOH in co-cultured BV2 (220 ± 15%). (**D**) IL-13R expression was increased by co-culture (997 ± 107%), as well as by EtOH in co-cultured BV2 (2034 ± 261%). Two-way ANOVA indicated a significant main effect of co-culture ($p<0.0001$), EtOH ($p<0.01$), and interaction ($p<0.05$). Data is represented as %CON (control)-Alone ± SEM, n=5-6 per group. n.d. = not detected (>40 cycles). *$p<0.05$, **$p<0.01$, ****$p<0.0001$ vs. indicated group via Tukey's post-hoc.

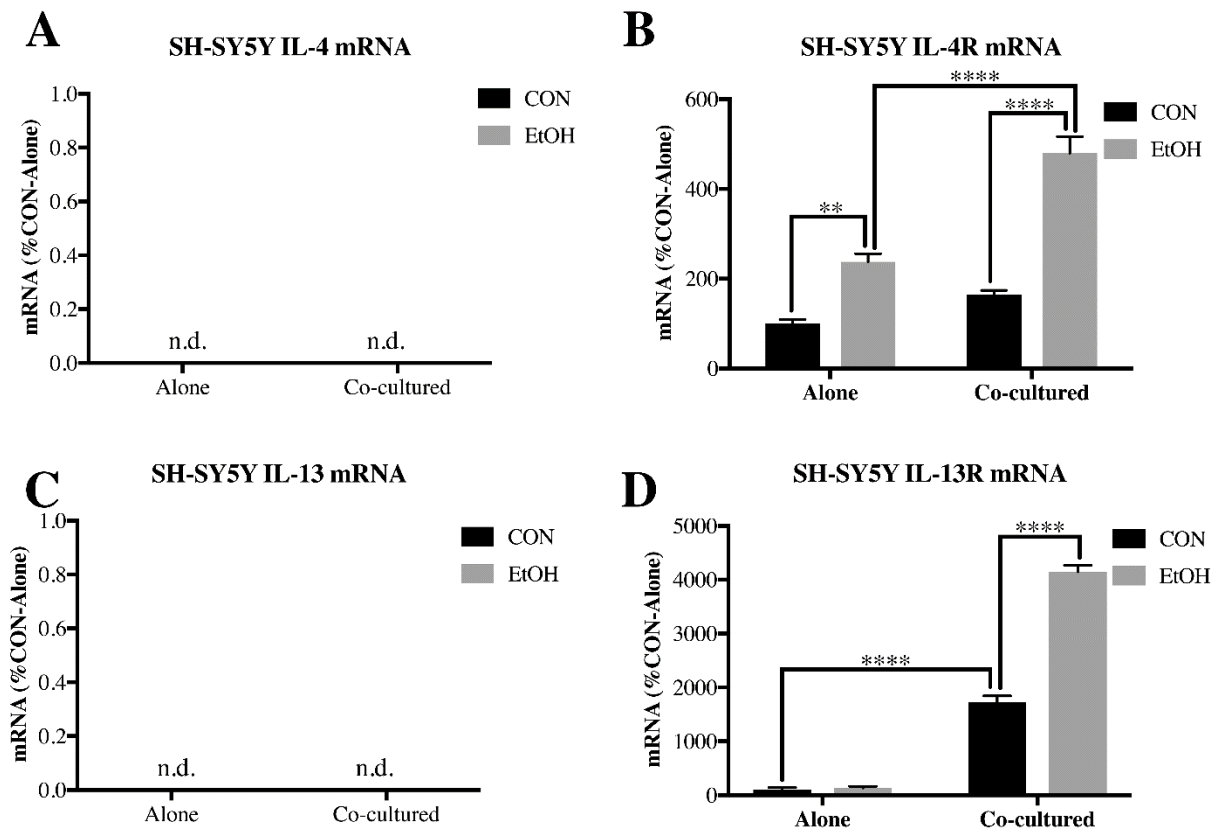

Figure 3.6 IL-4R and IL-13R is increased by ethanol in co-cultured SH-SY5Y neurons. SH-SY5Y neurons were treated with ethanol (EtOH, 100 mM) for 24 hours either alone or while co-cultured with BV2 microglia. Cell lysates were examined for mRNA expression. (**A**) IL-4 expression was not detected in SH-SY5Y. (**B**) IL-4R was increased by EtOH in SH-SY5Y alone (238 ± 18%), as well as by EtOH in co-cultured SH-SY5Y (480 ± 37%). Two-way ANOVA indicates a significant main effect of co-culture ($p<0.0001$), EtOH ($p<0.0001$), and their interaction ($p<0.001$). (**C**) IL-13 expression was not detected in SH-SY5Y. (**D**) IL-13R was increased in co-cultured SH-SY5Y (1723 ± 117%), as well as by EtOH in co-cultured SH-SY5Y (4147 ± 119%). Two-way ANOVA indicated a significant main effect of co-culture ($p<0.0001$), EtOH ($p<0.0001$), and interaction ($p<0.0001$). Data is represented as %CON(control)-Alone ± SEM, n=5-6 per group. n.d. = not detected (>40 cycles). **$p<0.01$, ****$p<0.0001$ vs. indicated group via Tukey's post-hoc.

3.4.3 IL-4 and IL-13 reduce ethanol-induced TNFα and IL-1β in hippocampal-entorhinal slice culture

Previous experiments indicate that IL-4 reduces endotoxin-induced TNFα and IL-1β in human macrophages (Hart et al., 1989; Wong, Costa, Lotze, & Wahl, 1993), however it is unclear how IL-4 and IL-13 may alter ethanol induction of these proinflammatory cytokines in brain. In order to investigate effects of IL-4 and IL-13 in an *ex vivo* setting containing all brain cell types, we treated hippocampal-entorhinal (HEC) brain slices from postnatal day (P) 7 rats with IL-4 and IL-13 recombinant proteins. Both IL-4 and IL-13 reduced TNFα (0.7-fold, $p<0.05$, and 0.48-fold, $p<0.05$, respectively) as well as IL-1β (0.51-fold, $p<0.05$, and 0.2-fold, $p<0.05$, respectively) mRNA expression (Figure 3.7A). We next treated the HEC slices with EtOH either with or without IL-4 and IL-13 proteins. We found that while EtOH increased expression of both TNFα (3.2-fold, $p<0.05$) and IL-1β (1.8-fold, $p<0.05$), both IL-4 and IL-13 reduced this increase (Figure 3.7B). These data indicate that IL-4 and IL-13 are capable of reducing ethanol-induced proinflammatory cytokines TNFα and IL-1β in brain.

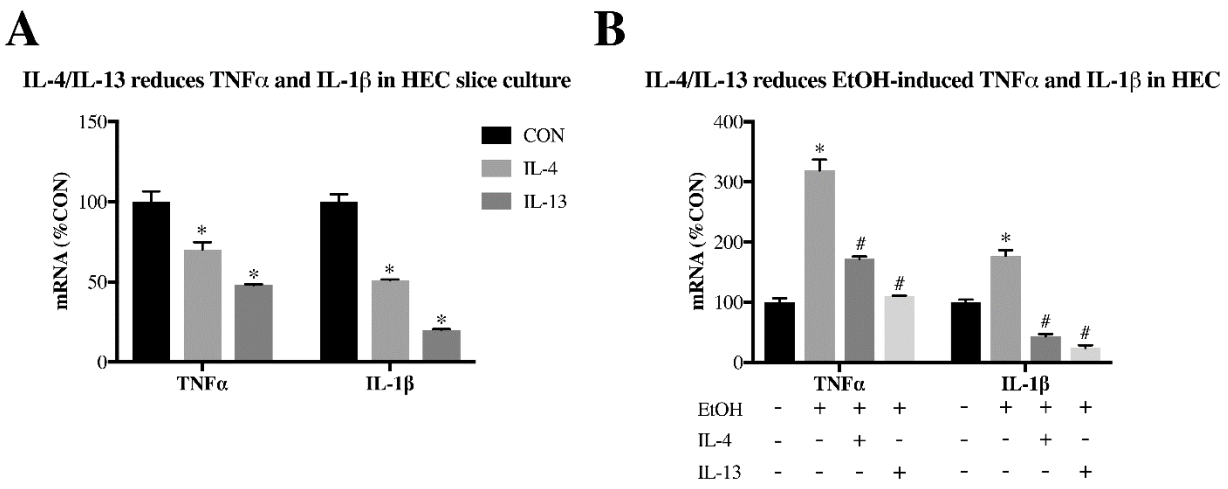

Figure 3.7 IL-4 and IL-13 reduce ethanol-induced TNFα and IL-1β in hippocampal entorhinal (HEC) slice culture. Hippocampal entorhinal (HEC) slice cultures were exposed to either IL-4 (500 ng/mL), IL-13 (1 ug/mL), and/or EtOH (100 mM) for 48 hours. Tissue was processed for mRNA expression. (**A**) TNFα expression was reduced by IL-4 (70 ± 5%) and IL-13 (48 ± 1%). IL-1β expression was reduced by IL-4 (51 ± 1%) and IL-13 (20 ± 1%). (**B**) EtOH increased expression of TNFα (319 ± 18%), which was blocked by both IL-4 (172 ± 4%) and IL-13 (110 ± 1%). EtOH increased expression of IL-1β (177 ± 9.8%), which was blocked by both IL-4 (43 ± 4%) and IL-13 (24 ± 4%). Data is represented as %CON (control), n=5-6 per group. *p<0.05 vs. CON; #p<0.05 vs. EtOH.

3.4.4 IL-10 and TGFβ are increased by ethanol in co-cultured BV2 microglia and SH-SY5Y neurons

IL-10 and TGFβ are both cytokines that are activated downstream of IL-4 and IL-13 signaling (Mitchell et al., 2017; Wen et al., 2002; Zhou et al., 2012), and play roles in learning and memory as well as having neuroprotective roles (Caraci et al., 2015; Lobo-Silva et al., 2016) that may be impacted by alcohol pathology. To determine whether these genes are impacted by neuronal-glial ethanol induced signaling, we examined expression of IL-10 and TGFβ in our co-culture model. In BV2 microglia, both IL-10 (1.8-fold, $p<0.01$) and TGFβ (3.2-fold, $p<0.05$) were increased by ethanol when co-cultured with SH-SY5Y neurons (Figure 3.8A-B). A main effect of co-culture on IL-10 expression was found in SH-SY5Y neurons, and ethanol significantly induced TGFβ in co-cultured SH-SY5Y neurons (Figure 3.8C-D). Overall, these data indicate that downstream IL-4/IL-13 genes TGFβ and IL-10 are induced in neuronal-glial cultures, further indicating a role for IL-4/IL-13 pathway induction in these cells.

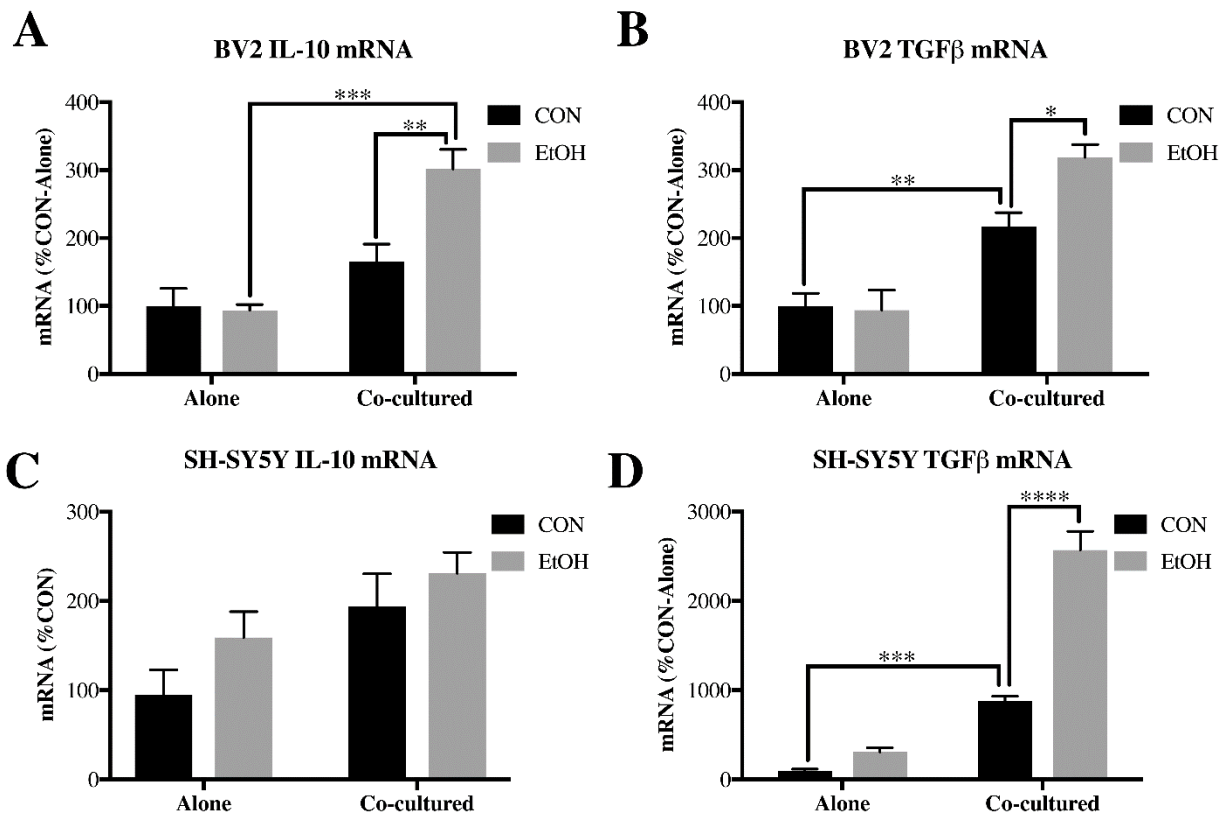

Figure 3.8 IL-10 and TGFβ are increased by ethanol in co-cultured BV2 microglia and SH-SY5Y neurons. BV2 microglia and SH-SY5Y neurons were treated with ethanol (EtOH, 100 mM) for 24 hours either alone or while co-cultured. Cell lysates were examined for mRNA expression. (**A**) IL-10 expression was increased by EtOH in co-cultured BV2 (182 ± 17%). Two-way ANOVA indicated a significant main effect of co-culture ($p<0.0001$), EtOH ($p<0.05$), and interaction ($p<0.05$). (**B**) TGFβ was increased by co-culture (217 ± 20%), and by EtOH in co-cultured BV2 (319 ± 19%). Two-way ANOVA indicated a significant main effect of co-culture ($p<0.0001$), EtOH ($p<0.05$), and interaction ($p<0.05$). (**C**) IL-10 expression was found to be significantly increased in co-cultured SH-SY5Y (main effect of co-culture, $p<0.05$) via two-way ANOVA. (**D**) TGFβ expression was increased by co-culture (880 ± 53%), and by EtOH in co-cultured SH-SY5Y (2571 ± 221%). Two-way ANOVA indicated a main effect of co-culture ($p<0.0001$), EtOH ($p<0.0001$), and interaction ($p<0.0001$). Data is represented as %CON(control)-Alone, n=5-6 per group. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

3.5 Discussion

This study demonstrates for the first time that neuronal-microglial co-culture alters ethanol-induced innate immune signaling molecules. TNFα and IL-1β, cytokines which are upregulated by ethanol in BV2 microglia alone, were unchanged when co-cultured with SH-SY5Y neurons. Similarly, TLR3 and TLR7, which are increased by ethanol in SH-SY5Y neurons alone, are unchanged when co-cultured with BV2 microglia. This was accompanied by a lack of ethanol induction of HMGB1 in the media of the co-cultured cells. Previous studies have indicated that HMGB1 facilitates the activation of multiple TLRs, including TLR3, TLR4, and TLR7 (Park et al., 2004; Yanai et al., 2009). Therefore, the lack of HMGB1 induction in co-cultured microglia and neurons may play a role in the lack of TLR and NFκB induced genes, such as TNFα and IL-1β (see diagram in Figure 3.9A). Our findings were surprising in that previous studies find that ethanol induces HMGB1 in hippocampal entorhinal brain slice culture as well as *in vivo* in brain (Crews et al., 2013) as well as various innate immune signaling molecules and TLRs (Qin & Crews, 2012; Qin et al., 2008). Our results therefore suggest a unique and specific mechanism of inhibiting HMGB1 and other TLR/cytokine expression that exists as a paracrine signaling mechanism between neurons and microglia. Astrocytes, as well as direct cell-to-cell contact, may modulate this response in brain, leading to increased HMGB1 and cytokines observed in slice culture and *in vivo*. Indeed, certain microglial-neuronal signaling molecules, such as fractalkine, are neuroprotective when in soluble form but membrane forms are actually pro-inflammatory (Morganti et al., 2012). However, further research is needed to explore the unique modulatory effects of neuronal-microglial co-cultures.

IL-4 and IL-13 are both described as anti-inflammatory cytokines in the periphery, and in microglia these cytokines have been found to promote an M2 phenotype (Hamzei Taj et al.,

2018; Liu et al., 2016). However, the impact of ethanol on IL-4/IL-13 signaling has been poorly understood. We observed for the first time that ethanol induced both IL-4/IL-13 and their receptors on BV2 microglia only when in the presence of SH-SY5Y neurons. Interestingly, we also observed that IL-13 was not detectable in BV2 alone, paralleling previous studies that found that endotoxin induces IL-13 in primary microglia co-cultured with neurons, but not alone or with astrocytes (Shin et al., 2004). Interesting new research also finds that IL-4 and IL-13 may play roles in learning and memory (Brombacher et al., 2017; Gadani et al., 2012), suggesting a role for neurons in IL-4/IL13 signaling. Furthermore, a novel role for neuronal IL-13Rα1 was recently discovered, in which stimulation of this receptor led to dopaminergic cell loss (Mori et al., 2017). In our study, we confirmed that neuronal IL-4/IL-13 signaling is enhanced following ethanol treatment in neuronal-microglial co-cultures, with an increased induction of IL-4R/IL-13R by ethanol in co-cultured SH-SY5Y neurons. Our data indicates that neuronal-microglial signaling induces IL-4/IL-13 signaling in response to ethanol, a novel discovery which may have relevance in both neuroprotection and learning, although further studies are needed to extend these findings.

IL-4 and IL-13 have been demonstrated to reduce endotoxin-induced IL-1β and TNFα in peripheral macrophages (Hart et al., 1989; Wong et al., 1993), suggesting that in brain these cytokines may inhibit the expression of pro-inflammatory cytokines. Indeed, we confirmed using hippocampal-entorhinal slice culture that both IL-4 and IL-13 not only decrease expression of IL-1β and TNFα, but also inhibit ethanol-induction of these cytokines. In brain, excessive expression of IL-1β and TNFα has been linked to disruption of long-term potentiation (LTP) (Bellinger, Madamba, & Siggins, 1993; Tancredi et al., 1992), an important mechanism that regulates learning and memory formation. Pro-inflammatory cytokines are associated with the

progression of neurodegenerative disorders and in high concentrations may exacerbate cell death (Smith, Das, Ray, & Banik, 2012) . Therefore, our findings suggest that IL-4/IL-13 signaling may underlie the lack of induction of these cytokines in neuronal-microglial co-culture, and may play a role in preventing neuronal dysregulation.

We also found that downstream of IL-4/IL-13 signaling, ethanol induces both TGFβ and IL-10 in co-cultured BV2 microglia, and TGFβ is induced in co-cultured SH-SY5Y neurons. Although the exact mechanism of this activity is unknown, IL-4/IL-13 signaling in peripheral macrophages activates transcription factor STAT6 (Oh, Geba, & Molfino, 2010). IL-4 has also been shown to activate STAT6 in BV2 microglia (Li et al., 2014). This suggests that IL-4/IL-13 may be signaling in a STAT-6 dependent manner to promote expression of TGFβ and IL-10 (see Figure 3.9B), however further studies are needed to elucidate these responses.

Overall, our findings indicate a novel role for IL-4/IL-13 in ethanol-induced signaling between microglia and neurons. Interestingly, we also observed an overall effect of co-culture alone on BV2 microglia and SH-SY5Y gene expression, indicating that signaling molecules between the two cell types even in basal settings influence their activity. Further research is needed to elucidate these signaling mechanisms, however. We further show that ethanol induction of TLR agonist HMGB1 is blocked by co-cultured neurons and microglia. These data reveal that neuronal-microglial signaling is altered by ethanol, and indicate that more research is needed to further explore these cell-to-cell signaling mechanisms and how they play a role in alcohol pathology.

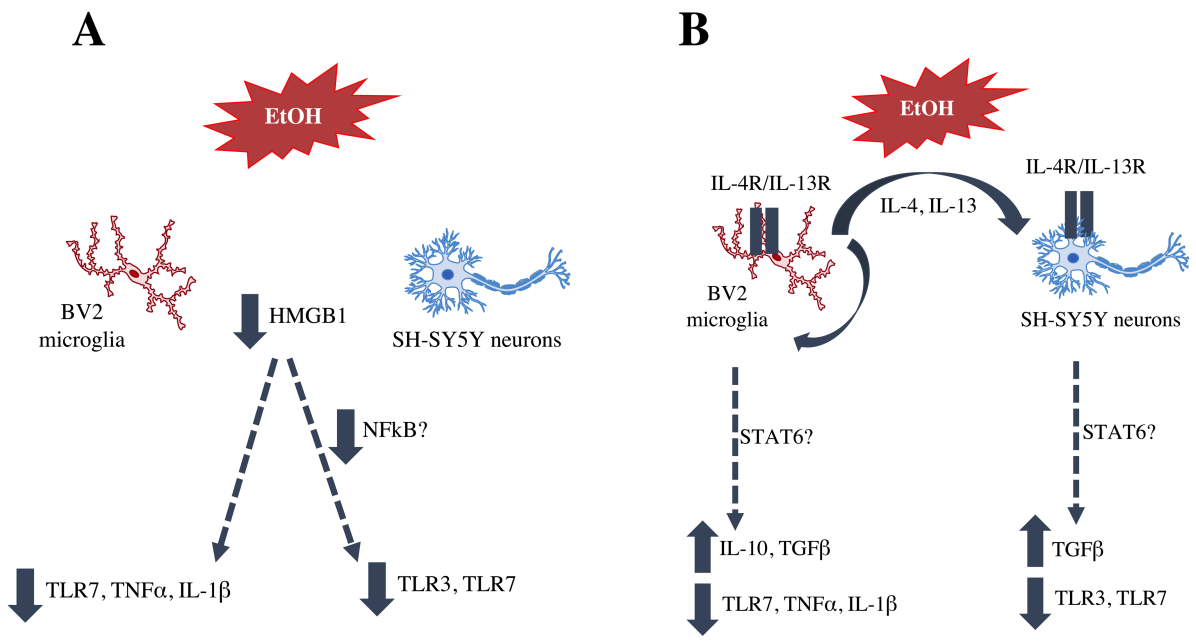

Figure 3.9 Ethanol increases IL-4/IL-13 signaling between microglia and neurons. (**A**) Schematic illustrating a lack of HMGB1 release in co-cultured neurons and microglia that is correlated with a decrease in IL-1β, TNFα and TLRs, possibly via altered NFκB activity. (**B**) Schematic illustrating ethanol-induced IL-4/IL-13 signaling between BV2 microglia and SH-SY5Y neurons. Ethanol increases IL-4 and IL-13 in BV2 microglia, as well as IL-4R and IL-13R in SH-SY5Y neurons. Downstream targets of IL-4/IL-13, such as IL-10 and TGFβ, are also increased in co-cultured cells, and IL-1β, TNFα and TLRs are decreased, possibly via a STAT6-mediated mechanism.

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CHAPTER 4: ETHANOL INDUCES INTERFERON EXPRESSION IN NEURONS AND ASTROCYTES: ROLE OF ASTROCYTE-TO-NEURON-SIGNALING

4.1 Summary

Rationale: Alcohol use disorder (AUD) involves dysregulation of innate immune signaling in brain. Toll-like receptor 3 (TLR3), an innate immune receptor that is upregulated in post-mortem human alcoholics, leads to induction of interferon (IFN) signaling. IFNs have been linked to depressive-like symptoms and therefore may play a role in addiction pathology. Astrocyte-neuronal signaling may contribute to maladaptation of neuronal circuits.

Objectives: In this manuscript, we examine ethanol (EtOH) induction of interferon signaling in neuronal, astrocyte, and microglial cell lines and assess astrocyte-neuronal interactions.

Methods: U373 astrocytes, SH-SY5Y neurons, and BV2 microglia were treated with EtOH and analyzed for autocrine/paracrine interferon signaling.

Results: EtOH induced TLR3, IFNβ and IFNγ in SH-SY5Y neurons and U373 astrocytes, but not in BV2 microglia. The interferon response gene TRAIL was also strongly upregulated by TLR3 agonist Poly(I:C) and EtOH in U373 astrocytes. TRAIL blockage via neutralizing antibody prevented induction of interferons in SH-SY5Y neurons but not in U373 astrocytes. Blocking TRAIL in conditioned media from EtOH-treated astrocytes prevented induction of interferons in SH-SY5Y neurons. Finally, an *in vivo* model of chronic 10-day binge EtOH exposure in C57BL6/J mice, as well as single acute treatment with Poly(I:C), showed increased TRAIL +IR cells in both orbitofrontal and entorhinal cortex.

Conclusions: This study establishes a role of astrocyte to neuron TRAIL release in ethanol-induced interferon responses. This may contribute to alcohol associated negative affect and suggest potential therapeutic benefit of TRAIL inhibition in AUD

4.2 Introduction

Alcohol use disorder (AUD) features prominent dysregulation of innate immune signaling in brain (Crews, Lawrimore, Walter, & Coleman, 2017; Mayfield, Ferguson, & Harris, 2013). AUD is also associated with progressively increasing negative-affect or depressive symptoms that are thought to promote the development and worsening of the disease (Boden & Fergusson, 2011). Innate immune signaling, particularly interferons (IFNs), have been associated with the development of depression in humans and *in vivo* (Borsini et al., 2017; Callaghan et al., 2018; Fritz, Klawonn, Jaarola, & Engblom, 2018; Mina et al., 2015; Pinto & Andrade, 2016). IFNγ was recently found to be upregulated in postmortem human alcoholic cortex (Johnson et al., 2015), and chronic ethanol (EtOH) induces IFNγ *in vivo* (Duncan et al., 2016; Pascual, Balino, Aragon, & Guerri, 2015). Negative-affect is a key aspect in the cycle of addiction, which is thought to drive self-administration (Koob, 2015; Koob & Volkow, 2010; Volkow, Koob, & McLellan, 2016). The induction of IFNs by ethanol, as well as the association of IFNs with negative-affect suggests a potential contribution for IFNs in the negative affective stage of addiction pathology. However, little is known about the mechanism of ethanol-induction of IFNs.

Toll-like receptors (TLRs), which are an important part of the innate immune system that respond to viral and bacterial components, also recognize endogenous agonists to promote sterile inflammation. TLRs are upregulated in post-mortem human alcoholic brain [for review see (Crews et al., 2017)] as well as in rodent models (Lippai et al., 2013). Endosomal TLRs initiate

interferon responses ultimately by induction of interferon regulatory factor (IRF) transcription factors. TLR3 is an endosomal TRIF-dependent TLR that results in IRF3 activation and interferon gene induction. TLR3 has been linked to alcohol drinking behavior in rodents (Jang, Lee, Park, Han, & Kim, 2016), and 10 day binge ethanol treatment in mice not only upregulates TLR3 but also potentiates cytokine responses following systemic treatment with TLR3 agonist Poly(I:C) (Qin & Crews, 2012). TLR3 is induced by ethanol in the prefrontal cortex and nucleus accumbens following alcohol self-administration (McCarthy, Warden, Bridges, Blednov, & Harris, 2017). We have shown previously that TLR3 is upregulated in postmortem human alcoholic brain, as well as in SH-SY5Y neurons *in vitro* (Lawrimore & Crews, 2017). Thus, it is critical to understand the role of TLR3 and IFN induction in response to ethanol.

In peripheral macrophages, it has been well established that TLR3 activation leads to induction of type I (e.g., IFNβ) and type II (IFNγ) interferons (Sen & Sarkar, 2005) as well as a host of interferon response genes (IRGs) (Levy & Darnell, 2002). These include antiviral proteins such as TNF-related apoptosis-inducing ligand (TRAIL/TNFSF10/APO2L/CD253). TRAIL can cause either cell death in TRAIL cell death-sensitive cells, or interferon and other cytokine induction. This has been observed in multiple cell types (Croft & Siegel, 2017; Henry & Martin, 2017; Kumar-Sinha, Varambally, Sreekumar, & Chinnaiyan, 2002; Wilson, Dixit, & Ashkenazi, 2009). For instance, TRAIL does not induce cell death in T-cells (LeBlanc & Ashkenazi, 2003), though neuronal lines can be sensitized to TRAIL-mediated cell death by NGF (Ruggeri, Cappabianca, Farina, Gneo, & Mackay, 2016). However, interferon and TRAIL signaling, as well as how ethanol impacts these pathways, is still poorly understood among different brain cell types. We hypothesized that TRAIL signaling would regulate interferon induction in neurons.

Multiple studies have reported that ethanol sensitizes microglia, inducing a hyper-ramified morphology associated with increases in cytokine secretion. Although microglia are brain specific innate immune monocytes, we have previously found that neurons also play a role in ethanol-induced innate immune signaling that is unique from that of microglia (Lawrimore & Crews, 2017). Astrocytes also have immunological properties and have been shown to play an important role in ethanol-induced inflammation (Adermark & Bowers, 2016), although the effect of ethanol is poorly understood. Innate immune signaling in brain shares the autocrine (cell-to-self signaling) and paracrine (cell-to-cell signaling) mechanisms found among systemic innate immune cells. For example, neuronal ligand CX3CL1 (fractalkine) signals to microglia through CX3CR1 altering immune signaling, synaptic plasticity (Sheridan et al., 2014) as well as amyloid β-complement processing in Alzheimer's disease models (Lian et al., 2015). Although it is known that ethanol can alter astrocyte activation, little is known about astrocyte innate immune signaling and how it might impact neurons through paracrine signaling pathways in brain.

In this manuscript, we utilized neuronal, microglial, and astrocytic cell lines (SH-SY5Y, BV2, and U373 respectively) to examine ethanol-induced interferon signaling in each cell type. We also examine astrocyte to neuronal signaling in the context of neuronal interferon induction. Interestingly, although BV2 microglia do respond to ethanol (Lawrimore & Crews, 2017), the response did not include induction of TLR3 or interferons. In contrast, in SH-5Y5Y neurons and U373 astrocytes, ethanol treatment resulted in a strong induction of TLR3, IFNβ and IFNγ in both cell types. TRAIL was also strongly upregulated by both ethanol and TLR3 agonist Poly(I:C) in U373 astrocytes. TRAIL neutralizing antibody prevented ethanol induction of IFN in SH-SY5Y neurons, suggesting TRAIL released by ethanol stimulates autocrine induction of

interferon in neurons. Similarly, TRAIL neutralizing antibody blocked IFN induction in neurons by ethanol-conditioned U373-astrocyte media consistent with astrocyte TRAIL-mediated paracrine induction of IFN in neurons. Furthermore, mice treated systemically with TLR3 agonist Poly(I:C) or ethanol show increases in cortical TRAIL positive immunoreactive cells. These studies link ethanol to novel IFN-TRAIL innate immune signaling between neurons and astrocytes which may contribute to the pathology of AUD.

4.3 Materials and Methods

4.3.1 Cell lines and treatment reagents

BV2 were acquired from ICLC (Genoa, Italy, #ATL03001). BV2 were cultured using Dubecco's modified Eagle serum (DMEM, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 1X GlutaMAX (Life Technologies), and 1X antibiotic-antimycotic (Life Technologies). 16 hours prior to treatments, media was changed to 2% FBS.

SH-SY5Y was acquired from ATCC (Manassas, Virginia, #CRL-2266). SH-SY5Y were cultured using DMEM/F-12 + GlutaMAX (Life Technologies), 10% FBS, and 1X antibiotic-antimycotic. Prior to treatments, SH-SY5Y were differentiated using 10 uM retinoic acid (RA, Sigma-Aldrich, St. Louis, MO, #R2625) for 4 days in Neurobasal media (Life Technologies) containing 2% B27 supplement (Life Technologies), 0.5 mM GlutaMAX and 1X antibiotic-antimycotic. Media was refreshed 16 hours prior to treatments.

U373-MG was acquired from UNC Cell Culture Facility. U373 were cultured using Alpha MEM (Life Technologies), 10% FBS, and 1X antibiotic-antimycotic. 16 hours prior to treatments, media was changed to 2% FBS.

All cell types were maintained in a humidified 5% CO_2 incubator at 37°C. Cells were treated at 90% confluency. Reagents used included EtOH (timepoint and concentration dependent on experiment; see results), Poly(I:C) (50 ug/mL, Amersham/GE Healthcare, Pittsburgh, PA, #27-4732-01), LPS (100 ng/mL, Sigma-Aldrich, #L2630), Imiquimod (1 ug/mL, Invivogen, #tlrl-imq), TRAIL (200 ng/mL, Millipore, #GF092), and TRAIL neutralizing antibody (2 ug/mL, BD Biosciences, #550912).

4.3.2 Conditioned media transfer

SH-SY5Y were cultured and differentiated with retinoic-acid as described in the previous section. U373 were cultured then treated with EtOH (100 mM, 24hr). Following treatment, media was collected and the ethanol was allowed to evaporate overnight as previously described (Walter & Crews, 2017). This conditioned media was then transferred to the SH-SY5Y cultures. Immediately following media transfer, the TRAIL neutralizing antibody was added to the appropriate groups. Cell lysates were harvested 24 hours later for mRNA analysis using RT-PCR.

4.3.3 Mouse treatment

Male C57BL/6 mice (8 weeks old) were treated with either EtOH [5 g/kg, 25% w/v, intragastric (i.g.)] or water (i.g.) daily for 10 days, or were treated with a single dose of LPS [0.5 mg/kg, intraperitoneal (i.p.)], Poly(I:C) (13 mg/kg, i.p.), or saline (i.p.) as previously described (Qin & Crews, 2012; Qin et al., 2008). Mice were sacrificed 24 hours following the last dose. Following each treatment, mice were anesthetized using sodium pentobarbital, then transcardially perfused with 0.1 M PBS, followed by 4.0% paraformaldehyde in PBS. Brains were removed and sent to Neuroscience Associates (Knoxville, TN) for histological sectioning. Mice were housed in a temperature- (20°C) and humidity-controlled vivarium on a 12 h/12 h

light/dark cycle, and provided *ad libitum* access to food and water. Experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill, and conducted in accordance with National Institutes of Health regulations for the care and use of animals in research.

4.3.4 Real-time (RT)-PCR

Total RNA was extracted from cell lysates using TRIzol (Invitrogen, Carlsbad, CA). RNA concentration was determined using a Nanodrop (Thermo Scientific, Waltham, MA) and was reverse-transcribed to cDNA. The SYBR green PCR master mix (Life Technologies) was used for real-time PCR analysis. The relative differences in expression between groups were expressed using cycle time (Ct) values normalized with β-actin, and relative differences between control and treatment groups were calculated and expressed as relative increases setting control as 100%. Primers used are listed in Table 4.1.

Gene	Species	Forward (5'-3')	Reverse (5'-3')
Caspase 8	Human	GGCACAGAGATTAAGTCCATTGAT	GGACACACAGACTCGAATGC
CD14	Human	AGA GGC AGC CGA AGA GTT CAC	GCG CTC CAT GGT CGA TAA GT
DR3	Human	AGAGATACTGACTGTGGGACC	CCCAGAACACACCTACTCTGC
DR4	Human	ACCTTCAAGTTTGTCGTCGTC	CCAAAGGGCTATGTTCCCATT
DR5	Human	GCCCCACAACAAAAGAGGTC	AGGTCATTCCAGTGAGTGCTA
FADD	Human	GAAAACGCGCTCTTGTCGAT	GCCCGAGGCATAGGAACTTG
Fas	Human	GTCTCCTGCGATGTTTGGC	TTCAAGGAAAGCTGATACCTATTTC
HMGB1	Human	GGA GAT CCT AAG AAG CCG AGA	CAT GGT CTT CCA CCT CTC TGA
IFNAR1	Human	ATTTACACCATTTCGCAAAGCTC	TCCAAAGCCCACATAACACTATC
IFNAR2	Human	TCATGGTGTATATCAGCCTCGT	AGTTGGTACAATGGAGTGGTTTT
IFNβ	Human	CACAACAGGTAGTAGGCGACA	AGAAGCACAACAGGAGAGCA
IFNγ	Human	TCAGCTCTGCATCGTTTTGG	GTTCCATTATCCGCTACATCTGAA
IFNγR1	Human	TCTTTGGGTCAGAGTTAAAGCCA	TTCCATCTCGGCATACAGCAA
IFNγR2	Human	TCACCGTCCTAGAAGGATTCAG	AAACTCTGGTGGTTCAAAAGACA
IKKβ	Human	CTGGCCTTTGAGTGCATCAC	CGCTAACAACAATGTCCACCT
IL-10	Human	GACTTTAAGGGTTACCTGGGTTG	TCACATGCGCCTTGATGTCTG
IL-1β	Human	ATG ATG GCT TAT TAC AGT GGC AA	GTCGGAGATTCGTAGCTGGA
IL-6	Human	ACT CAC CTC TTC AGA ACG AAT TG	CCA TCT TTG GAA GGT TCA GGT TG
MCP1	Human	CTCTCGCCTCCAGCATGAAA	AGGGTGTCTGGGGAAAGCTA
MD-2	Human	GAA GCT CAG AAG CAG TAT TGG GTC	GGT TGG TGT AGG ATG ACA AAC TCC
NGFR	Human	CCTACGGCTACTACCAGGATG	CACACGGTGTTCTGCTTGT
RAGE	Human	CTA CCG AGT CCG TGT CTA CCA	CAT CCA AGT GCC AGC TAA GAG
S100B	Human	CTTTCCAGCCGTGTTGTAGC	CTGCATGGATGAGGAACGCA
TLR3	Human	TTG CCT TGT ATC TAC TTT TGG GG	TCA ACA CTG TTA TGT TTG TGG GT
TLR4	Human	CTC TGG GGA GGC ACA TCT TC	CCC AGG TGA GCT GTA GCA TT
TLR7	Human	GATAACAATGTCACAGCCGTCC	GTTCCTGGAGTTTGTTGATGTTC
TNFα	Human	CCC AGG CAG TCA GAT CAT CTT CT	ATG AGG TAC AGG CCC TCT GAT
TRAIL	Human	TGCGTGCTGATCGTGATCTTC	GCTCGTTGGTAAAGTACACGTA
β-Actin	Human	GAT GCA GAA GGA GAT CAC TGC	ATA CTC CTG CTT GCT GAT CCA
IFNβ	Mouse	AGCTCCAAGAAAGGACGAACA	GCCCTGTAGGTGAGGTTGAT
IFNγ	Mouse	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
TLR3	Mouse	GTG AGA TAC AAC GTA GCT GAC TG	TCC TGC ATC CAA GAT AGC AAG T
β-Actin	Mouse	GTA TGA CTC CAC TCA CGG CAA A	GGT CTC GCT CCT GGA AGA TG

Table 4.1 Primer sequences for RT-PCR.

4.3.5 Enzyme-linked immunosorbent assays (ELISAs)

Following treatments, media was collected and spun down at 500xg for 10 minutes to remove cell debris. Media was analyzed using human TRAIL/TNFSF10 DuoSet ELISA (R&D Systems) per manufacturer's instructions.

4.3.6 MTT Assay

SH-SY5Y were plated on a 96 well plate and differentiated as described in the previous section. Cells were treated with varying concentrations of TRAIL (1-1000 ng/mL) as well as TRAIL neutralizing antibody (2 ug/mL) that has been demonstrated to block TRAIL-induced apoptosis (Cantarella et al., 2003) in the appropriate groups. 24 hours following treatment, the MTT assay (Abcam) was used to assess cell death per manufacturer's instructions.

4.3.7 Immunohistochemistry

Neuroscience Associates sectioned brains into coronal slices (35 μm) and were returned to the lab for immunohistochemical staining as previously described (Vetreno & Crews, 2012; Vetreno, Qin, & Crews, 2013). Briefly, sections were washed with PBS followed by a hydrogen peroxide (0.6%) rinse, then antigen retrieval using Citra solution at 70 degrees C for 1 hour. Sections were then blocked using rabbit serum, followed by an overnight incubation in goat anti-TRAIL (1:50, R&D #AF1121). The next day, sections were incubated with rabbit anti-goat IgG (1:200) at room temperature for 1 hour. Sections were then incubated in ABC solution at room temperature for 1 hour, followed by visualization using nickel-enhanced 3,3'-diaminobenzidinne (DAB). Sections were quantified for immunopositive cells using Nikon software, setting threshold for cell size and intensity that was applied unbiased to all sections.

4.3.8 Statistical analysis

Where appropriate, t-tests or one-way ANOVAs with post-hoc Tukey's/Dunnet's test were used. A p-value of less than 0.05 was considered significant. All data analysis was conducted using Prism (Graphpad, La Jolla, CA).

4.4 Results

4.4.1 Ethanol induces TLR3 and IFNs in U373 astrocytes and SH-SY5Y neurons, but not BV2 microglia

Previous studies have found ten days of binge ethanol (5 g/kg/day) increases expression of TLR3 in mouse (Qin & Crews, 2012), as well as in adolescent-intermittent ethanol-treated rats (Vetreno & Crews, 2012). TLR3 expression is also increased in post-mortem human alcoholic brain (Crews, Qin, Sheedy, Vetreno, & Zou, 2013). Furthermore, ethanol (100 mM, 24 hr) upregulates TLR3 in SH-SY5Y neurons, but not in BV2 microglia (Lawrimore & Crews, 2017). To determine ethanol-induced cell type-specific responses in this pathway, expression of TLR3, IFNβ and IFNγ was examined following ethanol treatment (100 mM, 24 hr) in U373 astrocytes, SH-SY5Y neurons, and BV2 microglia. EtOH increased expression of TLR3 (2.2-fold, $p<0.05$), IFNβ (2.9-fold, $p<0.05$), and IFNγ (2.6-fold, $p<0.01$) in U373 astrocytes (Figure 4.1A-B). Furthermore, in SH-SY5Y, EtOH increased expression of TLR3 (5.4-fold, $p<0.01$), IFNβ (2.7-fold, $p<0.001$), and IFNγ (5-fold, $p<0.001$) (Figure 4.1C-D). However, BV2 microglia did not show induction of TLR3 or IFNs in response to ethanol (Figure 4.1E-F). U373 astrocytes were found to be quite sensitive to the effects of ethanol. We found that 15 mM of ethanol, which is below the legal driving limit in the United States, upregulated IFNβ (3-fold, $p<0.05$) and TLR3 (2.6-fold, $p<0.01$) at 24 hours (Figure 4.2). Furthermore, multiple IFN signaling genes, cytokines, and TNF superfamily genes were upregulated in a concentration and time-dependent manner in U373 astrocytes (see Tables 4.2 and 4.3). Also, we found that multiple markers of

"A1" astrocyte activation (Liddelow et al., 2017) such as AMIGO2, GFAP, and FKBP5 were all upregulated by EtOH (100 mM, 24hr; data not shown). In SH-SY5Y, we further found that EtOH upregulated IFN receptors and multiple components of the TNF superfamily signaling genes (Table 4.4). Thus, ethanol induces TLR3 and IFN expression in U373 astrocytes and SH-SY5Y neurons, but not BV2 microglia.

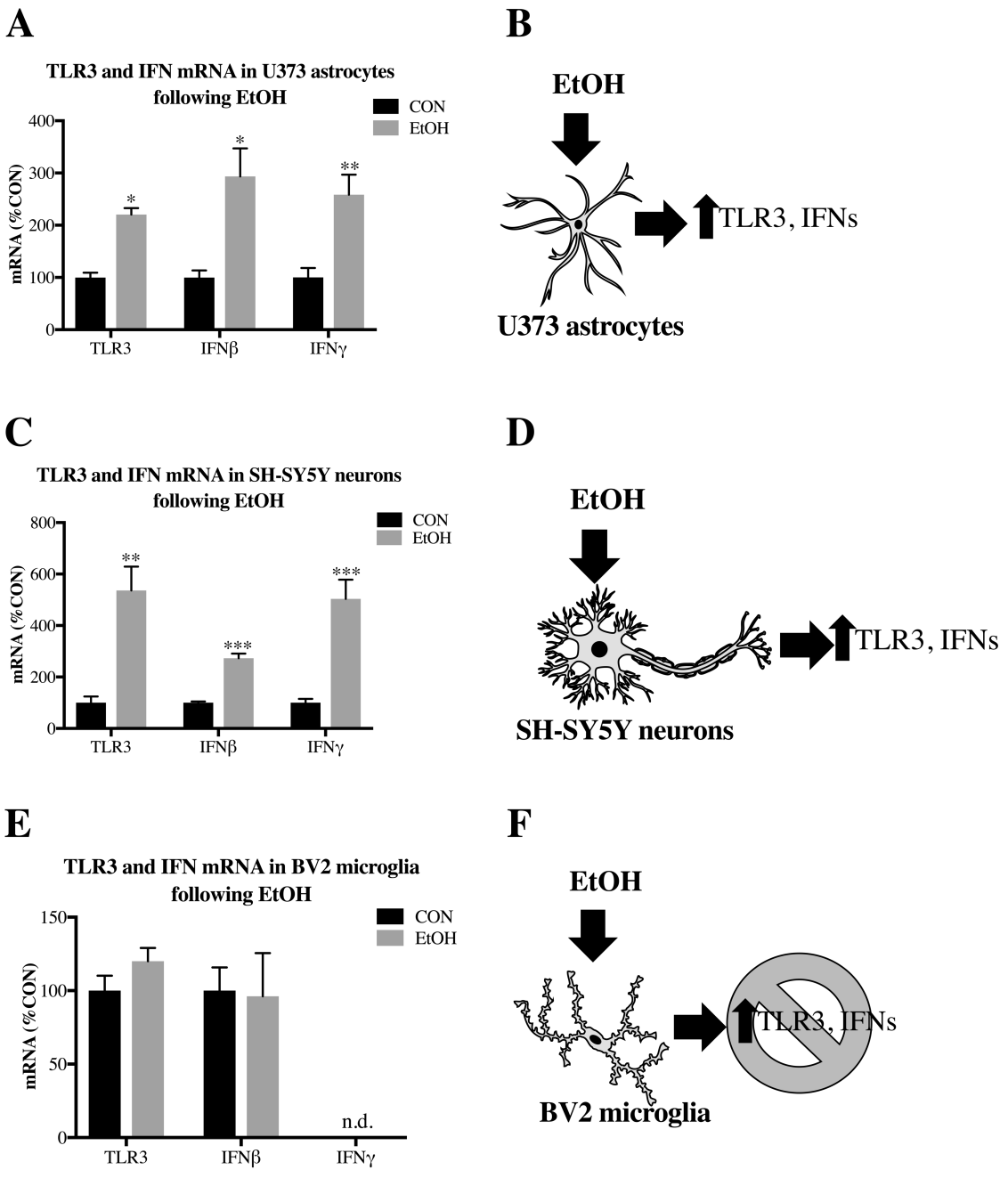

Figure 4.1 Ethanol increases TLR3, IFNs in U373 astrocytes and SH-SY5Y neurons, but not BV2 microglia. U373 astrocytes, SH-SY5Y neurons, and BV2 microglia were treated with ethanol (EtOH, 100 mM) for 24 hours. Cell lysates were collected and mRNA expression was measured using RT-PCR. Data is expressed as %CON. (**A-B**) EtOH increased expression of TLR3 (221 ± 12%), IFNβ (293 ± 54%), and IFNγ (258 ± 38%) in U373. (**C-D**) EtOH increased expression of TLR3 (536 ± 93%), IFNβ (273 ± 18%), and IFNγ (504 ± 75%) in SH-SY5Y. **e-f** EtOH did not increase either TLR3, IFNβ, or IFNγ in BV2. n=5-6 per group; *p<0.05, **p<0.01, ***p<0.001 vs. CON.

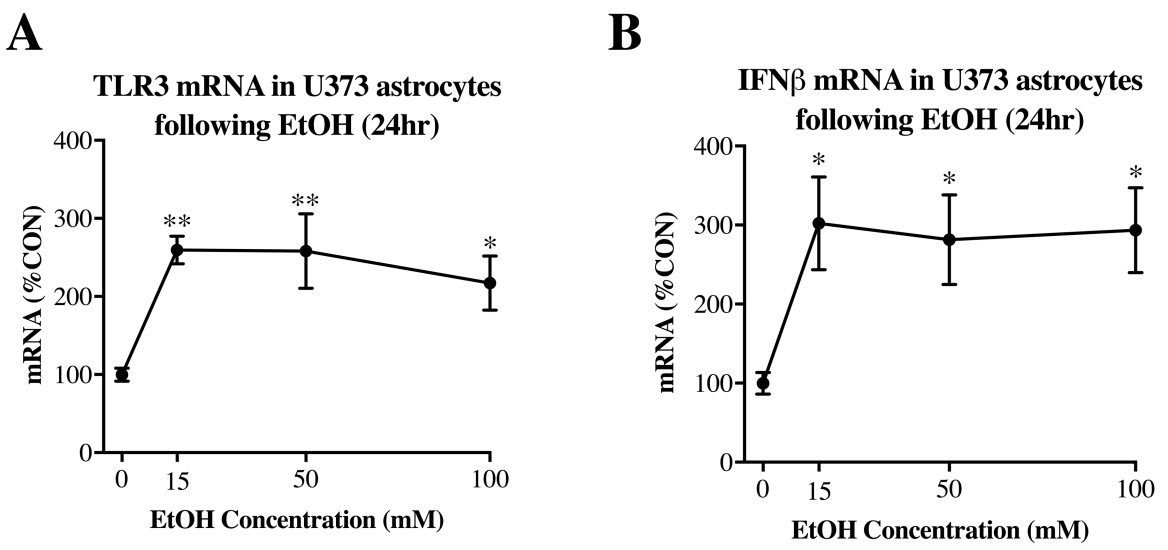

Figure 4.2 Ethanol increases IFNβ and TLR3 at low concentrations in U373 astrocytes. U373 astrocytes were treated with varying concentrations of EtOH (15, 50, or 100 mM) for 24 hours. Cell lysates were collected and mRNA expression was measured using RT-PCR. Data is expressed as %CON. (**A**) EtOH increased expression of IFNβ mRNA at 15 mM (302 ± 59%), 50 mM (282 ± 57%), and 100 mM (293 ± 54%). (**B**) EtOH increased expression of TLR3 mRNA at 15 mM (259 ± 18%), 50 mM (258 ± 48%), and 100 mM (217 ± 35%). n=5-6 per group; *p<0.05, **p<0.01 vs. CON.

U373 astrocyte EtOH concentration curve (24hr)				
Gene ID	**CON**	**15mM**	**50mM**	**100mM**
IFN signaling genes				
IFNAR1	100 ± 16	149 ± 14	192 ± 51	117 ±18
IFNAR2	100 ± 14	157 ± 19	191 ± 30	**158 ± 16***
IFNβ	100 ± 14	**302 ± 59***	**282 ± 57***	**293 ± 54***
IFNγ	100 ± 18	75 ± 6.7	156 ± 48	**258 ± 38****
IFNγR1	100 ± 12	**170 ± 16***	**178 ± 31***	111 ± 14
IFNγR2	100 ± 7.9	**165 ± 14***	**154 ± 23***	137 ± 11
NGFR	100 ± 6.9	**759 ± 134****	**996 ± 124*****	**2663 ± 193******
TRAIL	100 ± 7.7	**173 ± 16***	**207 ± 40****	**166 ± 22***
Cytokines and TLR signaling genes				
CD14	100 ± 9.5	143 ± 11	133 ± 22	94 ± 14
HMGB1	100 ± 16	122 ± 14	118 ± 25	88 ± 11
IL-10	100 ± 13	136 ± 9.8	101 ± 23	131 ± 23
IL-1β	100 ± 10	**172 ± 18***	136 ± 22	**162 ± 17***
IL-6	100 ± 5.8	140 ± 10	**146 ± 18***	**149 ± 11***
MCP1	100 ± 8.4	100 ± 7.5	89 ± 8.7	79 ± 3.3
MD2	100 ± 8.5	135 ± 6.7	127 ± 15	138 ± 14
RAGE	100 ± 15	**209 ± 22****	**191 ± 27***	163 ± 23
S100B	100 ± 12	115 ± 12	94 ± 13	74 ± 7.5
TLR3	100 ± 8.5	**259 ± 18****	**258 ± 48****	**217 ± 35***
TLR4	100 ± 15	156 ± 28	171 ± 44	122 ± 17
TLR7	100 ± 11	155 ± 38	168 ± 46	138 ± 32
TNFα	100 ± 12	100 ± 30	107 ± 28	132 ± 10
TNF superfamily signaling genes				
Caspase 8	100 ± 30	124 ± 34	144 ± 35	91 ± 19
TNFRSF25 (DR3)	100 ± 9.8	**166 ± 8.9****	**170 ± 14****	**167 ± 14****
TNFRSF10A (DR4)	100 ± 11	93 ± 22	144 ± 23	**201 ± 31***
TNFRSF10B (DR5)	100 ± 6.8	102 ± 6.3	119 ± 19	108 ± 9.8
FADD	100 ± 6.0	**118 ± 3.0***	114 ± 2.9	**125 ± 5.8****
Fas	100 ± 29	108 ± 30	130 ± 39	65 ± 12

Table 4.2 Summary of ethanol concentration curve in U373 astrocytes. U373 astrocytes were treated with EtOH at varying concentrations for 24 hours. Cell lysates were collected for mRNA analysis using RT-PCR. Data is expressed as %CON. n=5-6 per group; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. CON.

U373 astrocyte EtOH timecourse (15mM)				
Gene ID	**0hr**	**1hr**	**6hr**	**24hr**
IFN signaling genes				
IFNβ	100 ± 20	120 ± 44	67 ± 12	**269 ± 60***
IFNγ	100 ± 17	142 ± 29	122 ± 39	133 ± 22
IFNγR1	100 ± 13	138 ± 27	132 ± 33	**328 ± 73****
IFNγR2	100 ± 14	105 ± 16	119 ± 28	**226 ± 43***
TRAIL	100 ± 12	157 ± 31	169 ± 31	**470 ± 118*****
Cytokines and receptors				
IL-1β	100 ± 12	128 ± 23	187 ± 50	**447 ± 98*****
IL-6	100 ± 11	108 ± 16	84 ± 13	**171 ± 30***
RAGE	100 ± 17	139 ± 29	115 ± 17	**324 ± 60*****

Table 4.3 Summary of ethanol time course in U373 astrocytes. U373 astrocytes were treated with EtOH (15 mM) at varying timepoints. Cell lysates were collected for mRNA analysis using RT-PCR. Data is expressed as %CON. n=5-6 per group; *p<0.05, **p<0.01, ***p<0.001 vs. CON.

SH-SY5Y neuron EtOH treatment		
Gene ID	**CON**	**EtOH**
IFN signaling genes		
IFNβ	100 ± 5.1	**273 ± 18*****
IFNγ	100 ± 15	**504 ± 75*****
IFNAR1	100 ± 9.7	**345 ± 42*****
IFNAR2	100 ± 8.5	**297 ± 21******
IFNγR1	100 ± 15	**461 ± 84****
IFNγR2	100 ± 12	**328 ± 29******
NGFR	100 ± 12	**5127 ± 471******
TRAIL	100 ± 12	**432 ± 92****
TNF superfamily siganling genes		
Caspase 8	100 ± 21	**251 ± 30****
TNFRSF25 (DR3)	100 ± 33	178 ± 33
TNFRSF10A (DR4)	100 ± 11	**164 ± 12****
TNFRSF10B (DR5)	100 ± 9.7	**137 ± 7.5***
FADD	100 ± 7.1	124 ± 12

Table 4.4 Summary of ethanol effects on IFN and TNF superfamily in SH-SY5 neurons. SH-SY5Y neurons were treated with EtOH (100 mM, 24 hours). Cell lysates were collected for mRNA analysis using RT-PCR. Data is expressed as %CON. n=5-6 per group; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. CON.

4.4.2 Stimulation with TLR3 agonist Poly(I:C) increases IFN expression and IFN response gene TRAIL in U373 astrocytes

Astrocytes show clear induction of TLR3 and IFNs in response to ethanol. We previously assessed the impact of TLR3 activation in SH-SY5Y neurons and BV2 microglia (Lawrimore & Crews, 2017). In order to now investigate TLR3 signaling in U373 astrocytes, cells were treated with TLR3 agonist Poly(I:C) (50 ug/mL, 24hr). Poly(I:C) strongly upregulated both IFNβ (63-fold, $p<0.0001$), IFNγ (2.4-fold, $p<0.05$), and interferon regulated gene TNFSF10, also known as TRAIL (121-fold, $p<0.0001$) (Figure 4.3A). Furthermore, Poly(I:C) increased TRAIL secretion into the media (1.6-fold, $p<0.001$) (Figure 4.3B). In contrast, stimulation of TLR4 with LPS showed weaker responses, and stimulation of TLR7 with imiquimod showed no significant induction in the immune genes assessed (see Table 4.5). The concentrations of LPS (Shin, Lee, Lee, Jin, & Lee, 2014) and imiquimod (Gay et al., 2013) tested cause significant immune gene induction in microglia. Thus, U373 astrocytes show induction of IFNs and TRAIL in response to TLR3.

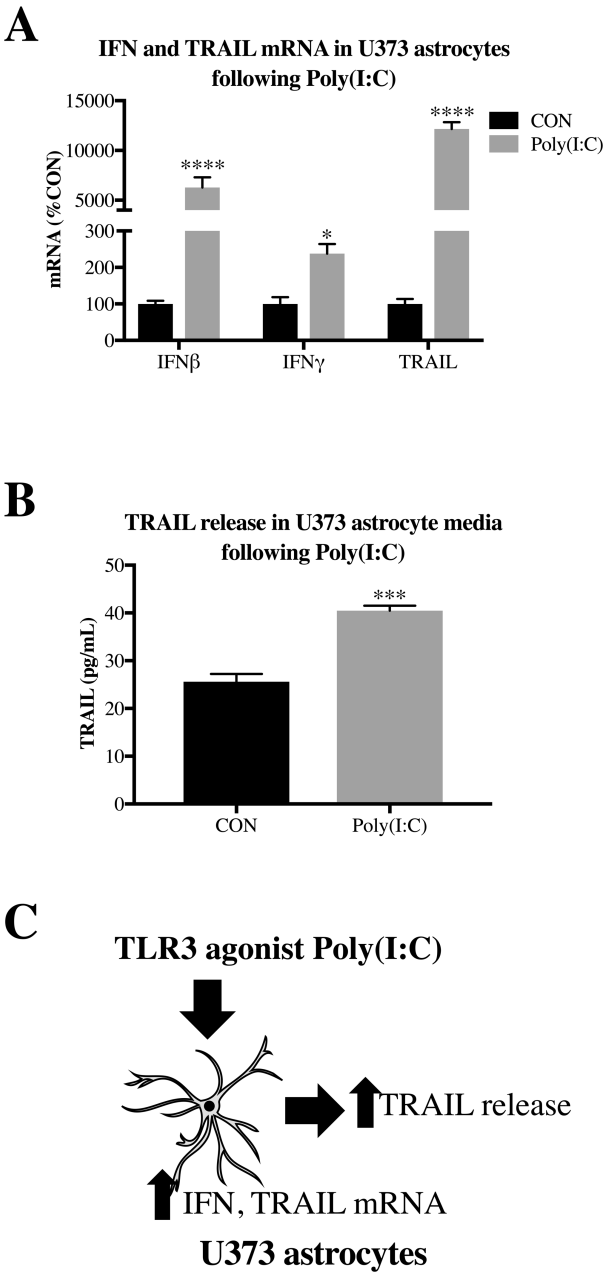

Figure 4.3 Stimulation with TLR3 agonist Poly(I:C) increases IFN expression and IFN response gene TRAIL in U373 astrocytes. U373 astrocytes were treated with TLR3 agonist Poly(I:C) (50 ug/mL) for 24 hours. Cell lysates were collected for mRNA analysis using RT-PCR, and cell media was collected for analysis of released TRAIL using ELISA. (**A**) Poly(I:C) increased expression of IFNβ (6283 ± 1008%), IFNγ (238 ± 26%), and TRAIL (12151 ± 689%). (**B**) Poly(I:C) increased release of TRAIL (158 ± 4.1%) in the media. (**C**) Schematic summarizing Poly(I:C) effects on interferon/TRAIL signaling in U373 astrocytes. n=5-6 per group; ***p<0.001, ****p<0.0001 vs. CON.

U373 astrocyte TLR agonist treatment (24hr)				
Gene ID	**CON**	**Poly(I:C)**	**LPS**	**Imiquimod**
IFN signaling genes				
IFNAR1	100 ± 22	71 ± 5.2	51 ± 16	72 ± 21
IFNAR2	100 ± 10	**174 ± 5.5****	**58 ± 7.5****	**68 ± 5.1***
IFNβ	100 ± 8.9	**6284 ± 1008*****	**236 ± 16***	138 ±24
IFNγ	100 ± 18	**238 ± 26***	142 ± 45	79 ± 20
IFNγR1	100 ± 18	**367 ± 32****	72 ± 18	83 ± 17
IFNγR2	100 ± 12	**169 ± 4.5****	**56 ± 6.9****	**69 ± 6.2***
NGFR	100 ± 12	96 ± 8.4	79 ± 11	88 ± 11
TRAIL	100 ± 14	**12152 ± 689****	**345 ± 64****	81 ± 14
Cytokines and TLR signaling genes				
IKKβ	100 ± 6.9	130 ± 6.1	86 ± 11.3	87 ± 9.8
IL-1β	100 ± 11.5	**2911 ± 133****	**252 ± 24*****	103 ± 14
IL-6	100 ± 7.0	**63999 ± 3037****	**150 ± 14***	89 ± 9.1
MCP1	100 ± 21	**28297 ± 800****	**1241 ± 81****	156 ± 41
TLR3	100 ± 22	**2839 ± 307****	**401 ± 106***	134 ± 41
TLR7	100 ± 11	118 ± 7.0	71 ± 13	81 ± 14
TNFα	100 ± 8.7	**28331 ± 1864****	**583 ± 72****	93 ± 10

Table 4.5 Summary of TLR3 agonist Poly(I:C), TLR4 agonist LPS, and TLR7 agonist imiquimod treatment in U373 astrocytes. U373 astrocytes were treated with TLR3 agonist Poly(I:C) (50 ug/mL), TLR4 agonist LPS (100 ng/mL), or TLR7 agonist imiquimod (1 ug/mL) for 24 hours. Cell lysates were collected for mRNA analysis using RT-PCR. Data is expressed as %CON. n=5-6 per group; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. CON.

4.4.3 Ethanol increases TRAIL expression and release in U373 astrocytes

TRAIL is a type II transmembrane protein that is cleaved and released where it acts on multiple receptors. TRAIL is linked to IFN signaling, being both induced by IFNs as well as induction of IFNs, dependent upon cell type (Kumar-Sinha et al., 2002). Since we found that ethanol increases TLR3 in U373, and that TLR3 stimulation increases TRAIL in U373, we next examined whether ethanol could also induce TRAIL in U373 astrocytes. We treated U373 astrocytes with ethanol and examined both TRAIL mRNA and release in the media. We found that ethanol increased TRAIL mRNA expression at 15 mM (1.7-fold $p<0.05$), 50 mM (2-fold, $p<0.01$), and 100 mM (1.7-fold, $p<0.05$) (Figure 4.4A), as well as released TRAIL as low as 15 mM (1.7-fold, $p<0.05$) (Figure 4.4B). EtOH rapidly released TRAIL protein into the media (Figure 4.4E) that was accompanied by a delayed slow increase in mRNA even at 15 mM (Figure 4. 4D). Thus, ethanol promotes induction of TRAIL release and expression in U373 astrocytes.

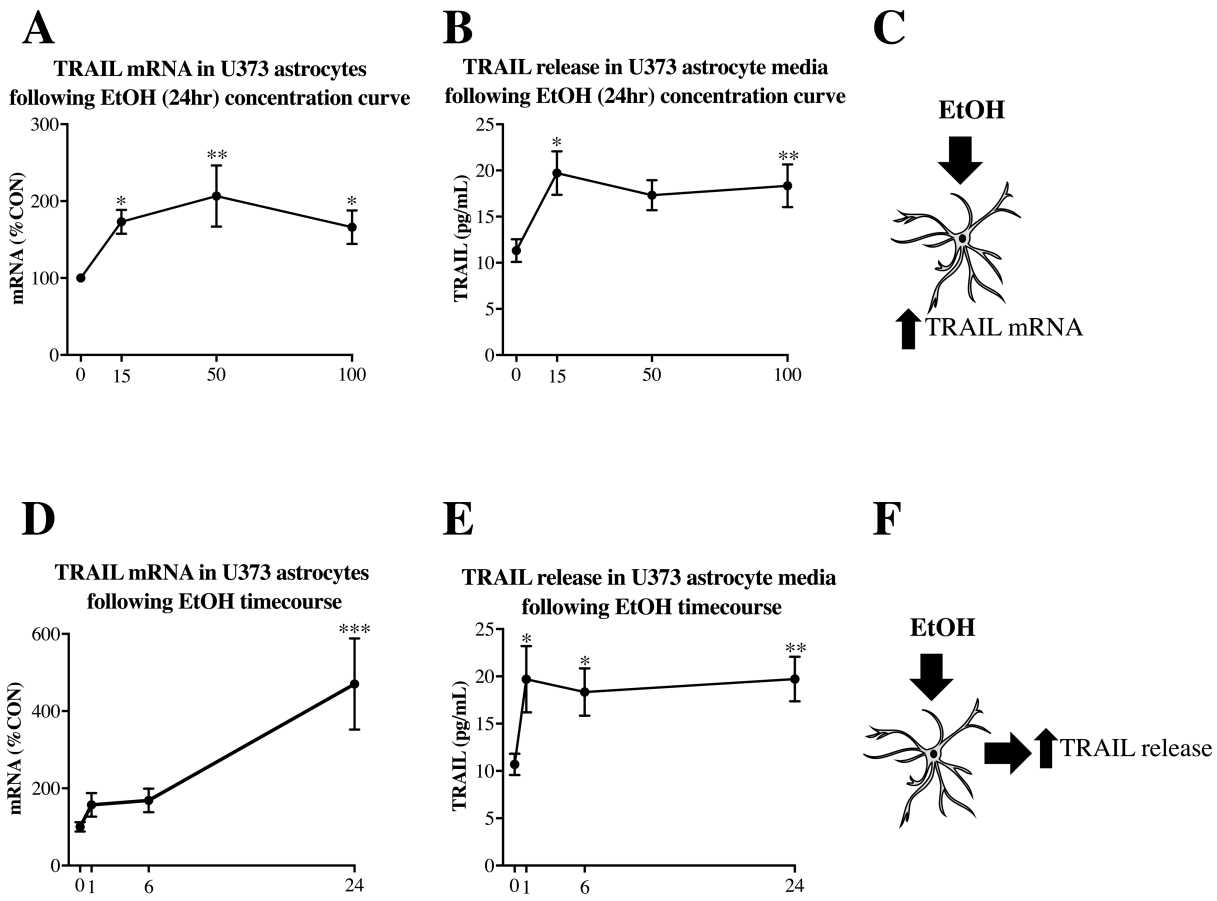

Figure 4.4 Ethanol increases TRAIL expression and release in U373 astrocytes. U373 astrocytes were treated with EtOH at varying concentrations and timepoints. Cell lysates were collected for mRNA analysis using RT-PCR, and cell media was collected for analysis of released TRAIL using an ELISA. RT-PCR data is expressed as %CON. (**A**) EtOH upregulated TRAIL mRNA at 15 mM (173 ± 17%), 50 mM (207 ± 41%), 100 mM (166 ± 23%). (**B**) EtOH increased TRAIL release in the media at 15 mM (174 ± 28%), and 100 mM (162 ± 27%). (**C**) Schematic illustrating EtOH increases TRAIL mRNA in U373 astrocytes. (**D**) EtOH increased TRAIL mRNA at 24 (469 ± 132%) hours. (**E**) EtOH increased TRAIL release in the media at 1 (174 ± 36%), 6 (162 ± 28%), and 24 (174 ± 28%) hours. (**F**) Schematic illustrating EtOH increases TRAIL release in U373 astrocytes. n=5-6 per group; *p<0.05, **p<0.01, ***p<0.001 vs. CON.

4.4.4 Ethanol induces interferons in human SH-SY5Y neurons via TRAIL, but not in U373 astrocytes.

Little is known about TRAIL signaling in brain, although in breast carcinoma cells (Kumar-Sinha et al., 2002), TRAIL induces IFNs. To determine if ethanol induced TRAIL is linked to IFN induction, we used a TRAIL neutralizing antibody (αTRAIL) to block TRAIL. The TRAIL neutralizing antibody was added to the media 30 minutes prior to ethanol treatment of U373 astrocytes. While ethanol increased expression of IFNβ (2.6-fold, $p<0.05$) and IFNγ (5.6-fold, $p<0.001$), these increases were not blocked by the TRAIL neutralizing antibody (Figure 4.5A-C). These data suggest that ethanol-induced interferon expression in astrocytes is not dependent on autocrine TRAIL signaling in U373 astrocytes and is more likely to be related to TLR3 or other ethanol induced signaling. We found that both EtOH and TRAIL induced IFNs in SH-SY5Y neurons (Figure 4.6A-F). Addition of the TRAIL neutralizing antibody 30 minutes prior to ethanol treatment of SH-SY5Y neurons did prevent ethanol-induced IFNβ and IFNγ gene induction (Figure 4.6A-C). Similar to the effects of ethanol, the TRAIL neutralizing antibody blocked IFNβ and IFNγ induction in response to recombinant TRAIL (200 ng/mL) (Figure 4.6D-F). Since TRAIL signaling has been shown to have neurodegenerative effects under certain conditions (Aktas et al., 2005; Cantarella et al., 2003), we examined whether TRAIL induces cell death in SH-SY5Y. TRAIL did not induce cell death at any of the concentrations examined (Figure 4.6G). Ethanol similarly does not induce cell death in SH-SY5Y neurons (Lawrimore & Crews, 2017). Thus, these data suggest that EtOH induces IFN expression via TRAIL signaling in SH-SY5Y neurons in the absence of cell death.

Since astrocytes secrete TRAIL in response to ethanol, but do not show IFN responses in response to TRAIL, we hypothesized that neurons might respond to TRAIL from astrocytes in a paracrine fashion. We treated U373 astrocytes with ethanol for 24 hours then collected the

media. To examine ethanol-induced signaling molecules separate from the direct effects of ethanol, we allowed the ethanol to evaporate overnight (Walter & Crews, 2017). U373-astrocyte-derived ethanol conditioned media (EtOH-CM) was collected and then transferred to SH-SY5Y neurons with or without the addition of the TRAIL neutralizing antibody. We found that EtOH-CM induced both IFNβ (2.2-fold, $p<0.05$) and IFNγ (1.8-fold, $p<0.05$) in SH-SY5Y neurons. Interestingly, this induction of IFN was abolished by the TRAIL neutralizing antibody (Figure 4.7B-C). These data suggest that ethanol releases TRAIL from U373 human astrocytes that induce interferon expression in SH-SY5Y neurons. Thus, ethanol causes both autocrine and paracrine TRAIL-mediated IFN induction in neurons. Ethanol can directly cause IFN induction in neurons. Further, astrocytes communicate with neurons via TRAIL release in response to ethanol, leading to IFN induction.

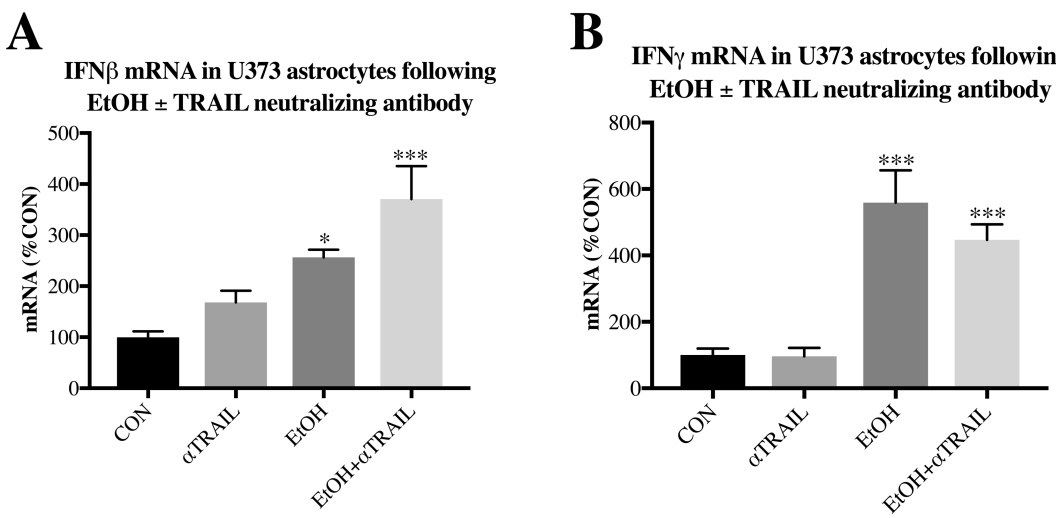

Figure 4.5 Ethanol induced interferon expression in U373 astrocytes is not blocked by TRAIL neutralizing antibody. U373 astrocytes were treated with EtOH (100 mM). 30 minutes prior to EtOH, TRAIL neutralizing antibody (αTRAIL; 2 ug/mL) was added to the specified groups. 24 hours later, cell lysates were collected for mRNA analysis using RT-PCR. (**A**) IFNβ was increased by EtOH (257 ± 15%) which was not blocked by αTRAIL (370 ± 65%). (**B**) IFNγ was increased by EtOH (559 ± 98%) which was not blocked by αTRAIL (447 ± 47%). (**C**) Schematic illustrating that EtOH increases TRAIL U373 astrocytes, and that TRAIL neutralization does not affect EtOH-induced IFN induction. n=5-6 per group; *p<0.05, ***p<0.001 vs. CON.

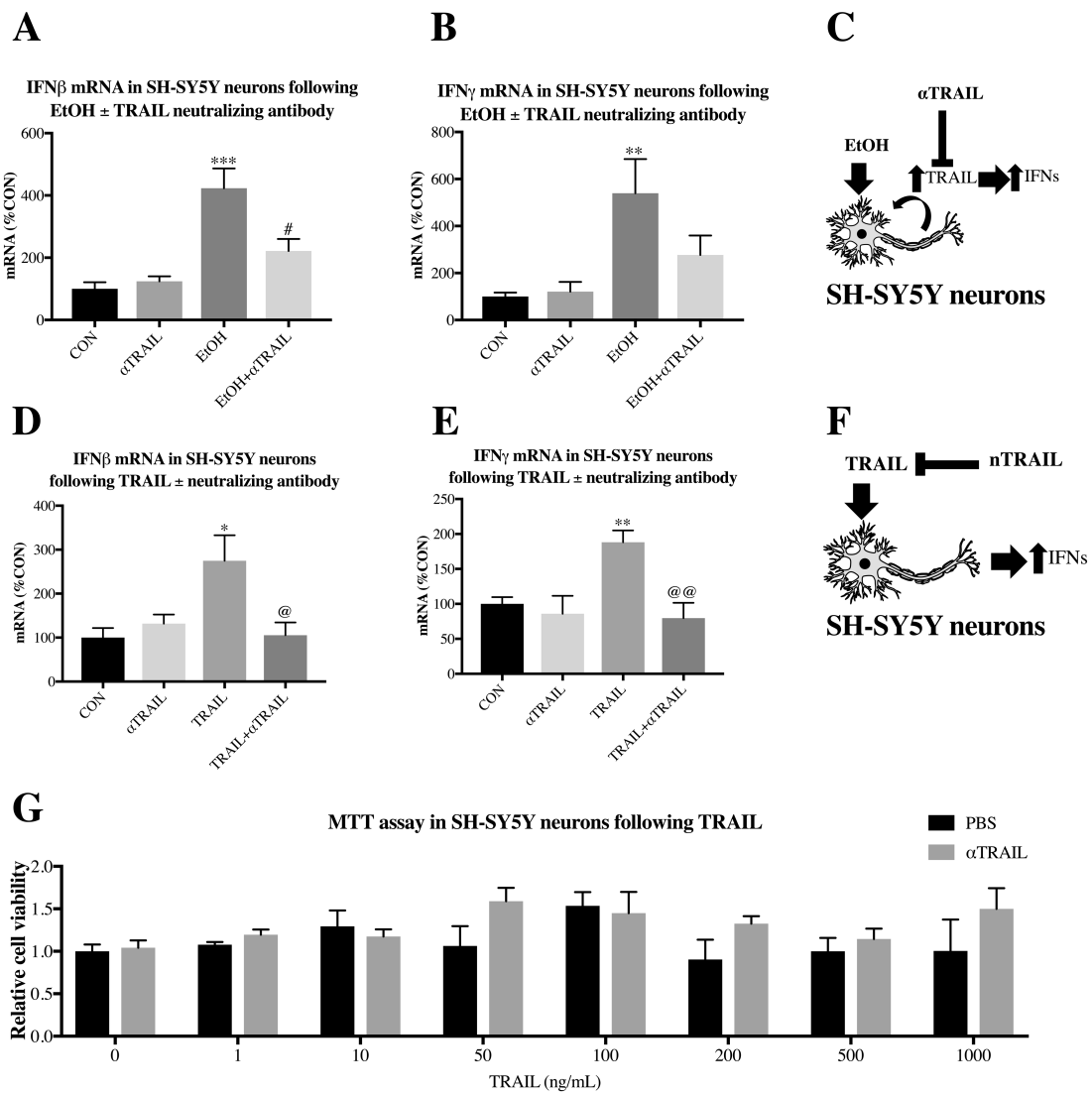

Figure 4.6 Ethanol and TRAIL-induced interferons is blocked by TRAIL neutralizing antibody in SH-SY5Y neurons. (**A-C**) SH-SY5Y neurons were treated with EtOH (100 mM). 30 minutes prior to EtOH, TRAIL neutralizing antibody (αTRAIL; 2 ug/mL) was added to the specified groups. 24 hours later, cell lysates were collected for mRNA analysis using RT-PCR. (**A**) IFNβ was increased by EtOH (433 ± 64%) which was blocked by αTRAIL (221 ± 39%). (**B**) IFNγ was increased by EtOH (539 ± 146%) which was blocked by αTRAIL (278 ± 82%). (**C**) Schematic illustrating EtOH increases IFNs in SH-SY5Y that is blocked by TRAIL neutralizing antibody. (**D-F**) SH-SY5Y were treated with TRAIL (200 ng/mL). 30 minutes prior to TRAIL, TRAIL neutralizing antibody (αTRAIL; 2 ug/mL) was added to the specified groups. 24 hours later, cell lysates were collected for mRNA analysis using RT-PCR. (**D**) IFNβ was increased by TRAIL (275 ± 58%) and was blocked by αTRAIL (106 ± 29%). (**E**) IFNγ was increased by TRAIL (188 ± 17%) and was blocked by αTRAIL (80 ± 22%). (**F**) Schematic illustrating that TRAIL increases IFNs in SH-SY5Y that is blocked by TRAIL neutralizing antibody. n=5-6 per group; *p<0.05, **p<0.01, ***p<0.001 vs. CON; #p<0.05 vs. EtOH; @p<0.05, @@p<0.01 vs. TRAIL.

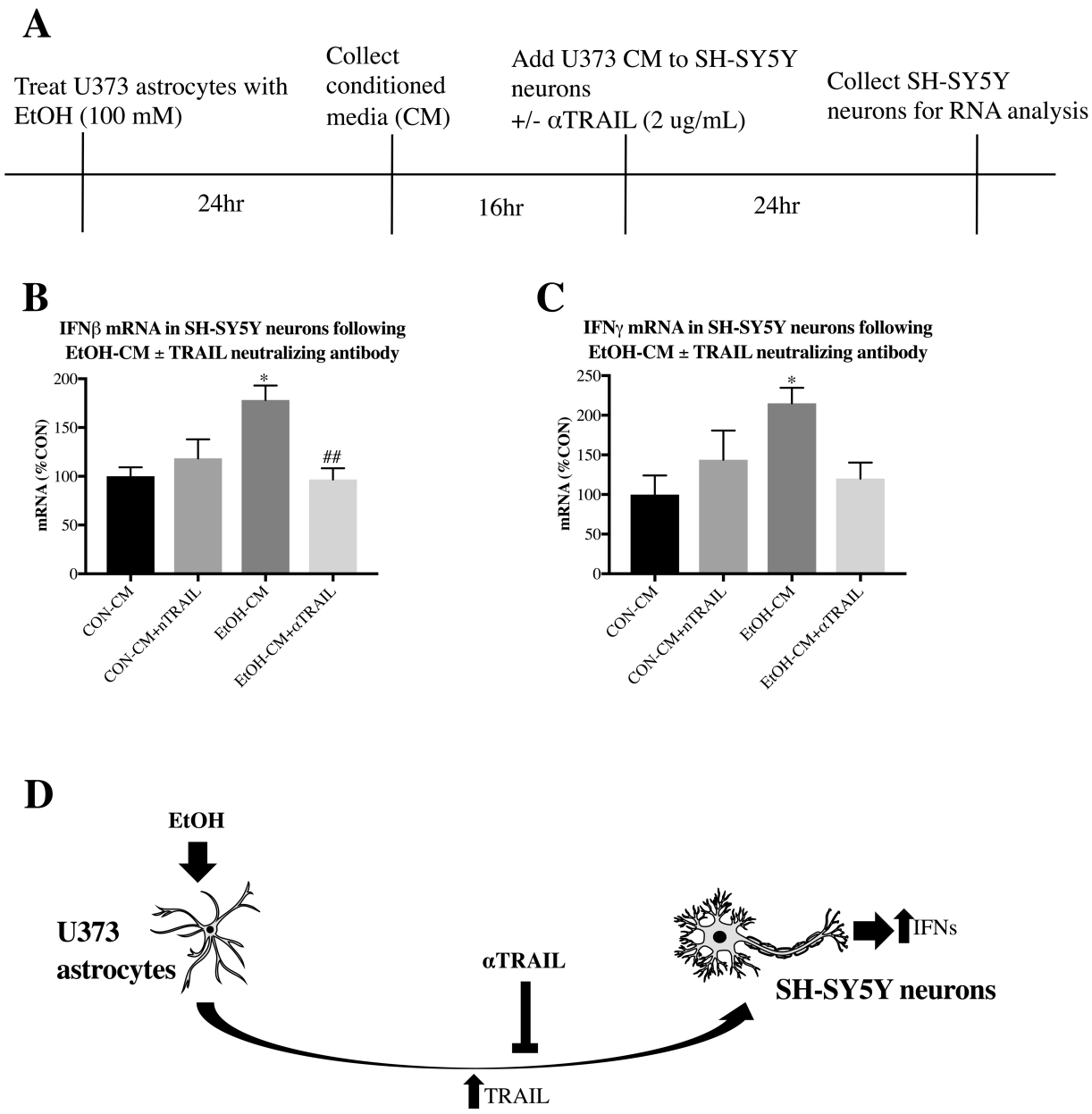

Figure 4. 7 Ethanol-induced TRAIL from U373 astrocytes induces interferons in SH-SY5Y neurons. (**A**) Schematic diagraming treatment outline. U373 astrocytes were treated with EtOH (100 mM, 24 hr) followed by media removal. EtOH was allowed to evaporate from the media before transferring to SH-SY5Y. TRAIL neutralizing antibody (αTRAIL) was added immediately following media transfer to the specified groups. 24 hours later, cell lysates were collected for mRNA analysis using RT-PCR. (**B**) IFNβ was increased by ethanol-conditioned media (EtOH-CM; 215 ± 19%), which was blocked by αTRAIL (120 ±20%). (**C**) IFNγ was increased by EtOH-CM (178 ±15%), which was blocked by αTRAIL (97 ± 11%). (**D**) Schematic outlining signaling pathway between EtOH treated U373 and induction of interferons in SH-SY5Y. n=5-6 per group; *p<0.05 vs. CON; #p<0.05 vs. EtOH-CM.

4.4.5 Ethanol and Poly(I:C) induce TRAIL in mouse brain

To determine if ethanol and/or the TLR3 agonist Poly(I:C) alters brain TRAIL expression *in vivo*, we used a mouse model of chronic ethanol binge as well as a single acute Poly(I:C) treatment. C57BL/6 mice underwent a 10 day EtOH binge (5 g/kg, daily i.g.) or a single dose of TLR3 agonist Poly(I:C) (13 mg/kg, i.p.) and sacrificed 24 hours later to assess TRAIL expression in brain We found both ethanol and Poly IC increased the number of TRAIL immunopositive reactive cells (TRAIL +IR) by approximately 20% in orbitofrontal cortex and entorhinal cortex relative to controls; EtOH ($p<0.05$) or Poly(I:C) ($p<0.05$) (Figure 4.8A-B). We also observed that the TLR4 agonist LPS (0.5 mg/kg, i.p.) caused a similar induction of TRAIL (data not shown), suggesting TRAIL upregulation is sensitive to several immune stimuli. Thus, TRAIL is induced by ethanol and Poly(I:C) in mouse brain.

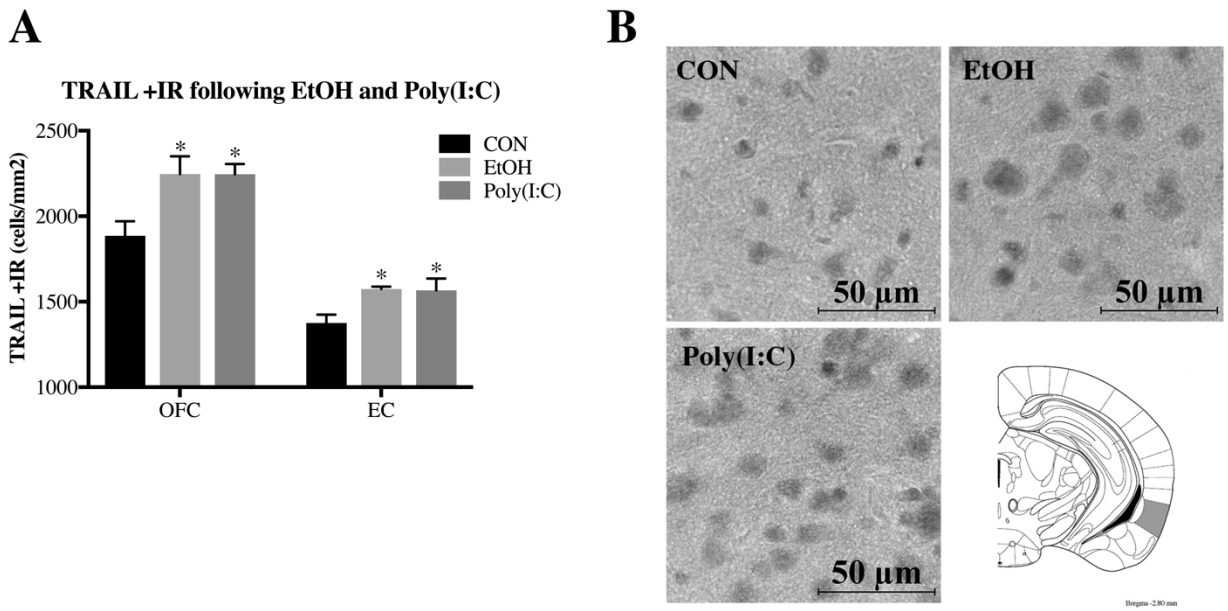

Figure 4. 8 Ethanol and Poly(I:C) increase TRAIL +IR in mouse orbitofrontal and entorhinal cortex. C57BL6/J male mice (8 weeks old) were treated with either EtOH (once daily, 10 days, 5 g/kg, i.g.), Poly(I:C) (single dose, 13 mg/kg, i.p.), water (once daily, 10 days, i.g.) or saline (i.p.). Since no significant differences were seen between water (i.g.) and saline (i.p.) controls, these were grouped together into a single control [CON] group. Brains were processed for immunohistochemical staining by Neurosciences Associates and stained for TRAIL positive immunoreactive (+IR) cells. (**A**) The number of TRAIL +IR cells in both orbitofrontal and entorhinal cortex was increased by EtOH ($p<0.05$) and Poly(I:C) ($p<0.05$). (**B**) Representative image of TRAIL +IR cells in entorhinal cortex, imaged at 100X. Positive cells are indicated by darker staining. *$p<0.05$ vs. CON, n=5-6 per group.

4.5 Discussion

In this manuscript, we found that ethanol induces TLR3, IFNs and TRAIL in both U373 astrocytes and SH-SY5Y neurons, but not in BV2 microglia. Our studies find that TRAIL contributes to both autocrine and paracrine forms of neuroimmune signaling in brain, as diagramed in Figure 4.9. In SH-SY5Y neurons, ethanol directly induced IFN gene expression that was prevented by TRAIL neutralizing antibody. Also, astrocytes signal in a paracrine fashion to neurons via TRAIL to induce neuronal IFNs. Several other studies have identified paracrine glial-neuronal signaling pathways in brain, such as fractalkine which may modulate synaptic plasticity (Sheridan et al., 2014) as well as dysregulated amyloid β-complement signaling in Alzheimer's disease models (Lian et al., 2015). Our study finds a novel paracrine signaling mechanism from astrocytes to neurons involving TRAIL-mediated IFN induction in neurons due to alcohol. The relationship between TRAIL and IFNs has been identified previously in other cell types (Huang et al., 2009; Peteranderl & Herold, 2017). In peripheral immune cells, TRAIL is often considered an interferon response gene (IRG), one of dozens of genes induced by IFN receptor signaling (Huang et al., 2009; Levy & Darnell, 2002; Peteranderl & Herold, 2017; Sato et al., 2001). We found that TRAIL induces IFNs in SH-SY5Y neurons. This has also been observed previously in breast carcinoma cells, showing a reciprocal interaction between these signaling systems (Kumar-Sinha et al., 2002). TRAIL was originally discovered to be involved in the initiation of cell death pathways (Pan et al., 1997). However, TRAIL has also been found to result in cytokine induction through NFκBkB and IRF mediated pathways (Henry & Martin, 2017; Kumar-Sinha et al., 2002).

In our studies TRAIL did not cause detectable cell death in SH-SY5Y at concentrations of 200 ng/mL (24h treatment). These findings are consistent with previous studies in SH-SY5Y

neurons finding a 16 hour treatment with TRAIL (500 ng/mL) did not result in cell death (Ruggeri et al., 2016). Some studies find that a longer 48-hour treatment of TRAIL (100 ng/mL) reduces SH-SY5Y neuronal viability by approximately 30% (Cantarella et al., 2003) and induction of cell death by TRAIL in resected human brain tissue (Nitsch et al., 2000). Others have found that TRAIL induces cell death in SH-SY5Y neurons after NGF treatment (Ruggeri et al., 2016). This suggests that cell death pathways activated by TRAIL in neurons can vary and may be slower to initiate than interferon signaling in SH-SY5Y. Nevertheless, in our setting we observe neuronal induction of IFNs by TRAIL in the absence of clear cell death. While implications of this *in vivo* are unclear, it is possible that persistent or ongoing upregulation of TRAIL signaling is needed to initiate neuronal cell death, as is observed in multiple sclerosis models (Aktas et al., 2005).

We found important differences between TRAIL-IFN signaling in response to ethanol between neurons and astrocytes. TRAIL antagonism prevented ethanol induction of IFNs in neurons but not astrocytes. Previously, we also found that TLR3 agonist Poly(I:C) induces a strong cytokine response in SH-SY5Y neurons (Lawrimore & Crews, 2017), however our current data suggests TRAIL, rather than TLR3 may be required for ethanol-induction of IFN in neurons. In astrocytes, however, we suspect that TLR3 signaling may be required for ethanol-induced IFN-TRAIL signaling. TLR3 stimulation via Poly(I:C) in U373 astrocytes strongly upregulated expression of IFNs as well TRAIL. TLR3 is upstream of interferon signaling and is prominently expressed in astrocytes (Farina et al., 2005; McCarthy, Bridges, Blednov, & Harris, 2017). We demonstrated here that Poly(I:C) strongly upregulates cytokines as well as interferons in U373 astrocytes (see Table 4), consistent with studies conducted in primary human astrocytes (Farina et al., 2005; Serramia, Munoz-Fernandez, & Alvarez, 2015). The subdued

effect of TLR4 and TLR7 stimulation in U373 astrocytes we observed is similar to previous studies that found little proinflammatory stimulation by TLR agonists other than Poly(I:C) in primary astrocytes (Farina et al., 2005). This is consistent with strong TLR3 responsivity in astrocytes. Importantly, we uniquely demonstrated that Poly(I:C) induces a strong upregulation of TRAIL as well in U373 astrocytes, with TRAIL antagonism having no effect on IFN induction, supporting the hypothesis that TLR3 is upstream of IFNs and TRAIL in astrocytes. Further, our finding of increased media TRAIL at one-hour post-treatment suggests that cleavage of membrane bound TRAIL might also be occurring, as TRAIL has been shown to be cleaved and released by cysteine proteases (Mariani & Krammer, 1998). Future studies are required to definitively dissect the mechanism of TRAIL secretion in astrocytes. While we found that Poly(I:C) induces TRAIL in U373 astrocytes, previous studies indicate that Poly(I:C) also upregulates other interferon stimulated genes in astrocytes such as ISG54 and ISG56 (Imaizumi et al., 2014), suggesting that further research into TLR3-TRAIL-IFN mediated responses in astrocytes is warranted, especially given the ability of astrocytes to secrete factors that alter neurons (Liddelow et al., 2017).

While in the periphery interferons are known for their anti-viral activity, in brain they may play different roles. In particular, exogenous interferons have been well-documented in their ability to cause depression in clinical settings and in rodents (Borsini et al., 2017; Callaghan et al., 2018; Fritz et al., 2018; Lian et al., 2015; Mina et al., 2015; Pinto & Andrade, 2016). Negative-affect and depressive phenotypes are critical components in the cycle of addiction (Koob, 2015; Koob & Volkow, 2010; Volkow et al., 2016). Both the negative-affect/depressive phenotypes found with exogenous IFNs and the development of negative-affect in rodent models of alcohol addiction follow similar patterns of progressive onset (Breese et al., 2008; Breese,

Overstreet, & Knapp, 2005; Raison et al., 2009; Wills, Knapp, Overstreet, & Breese, 2009; Zheng et al., 2014). Further, IFNγ was found to be upregulated in postmortem human alcoholic cortex (Johnson et al., 2015), and chronic ethanol *in vivo* can induce IFNγ (Duncan et al., 2016) (Pascual et al., 2015). IFNγ has also been suggested to play a role in modulating social behavior in mice (Filiano et al., 2016). IFNγ knockout mice lack LPS-induced conditioned place aversion, indicating a role for IFNγ in negative-affect behaviors (Fritz et al., 2018). Due to the link between interferons and depression, interferons may represent a target for the negative-affect stage of alcohol use disorders (Coleman & Crews, 2018). Blocking interferon induction by TRAIL in neurons could be a potential cell-specific target for alcohol use disorders and other neurologic disorders(Cantarella et al., 2015). Further research is needed to investigate the possible therapeutic potential of TRAIL inhibition as well as the mechanisms underlying cell-to-cell TRAIL signaling in brain.

Although microglia are generally associated with neuroimmune signaling and primary microglia have been reported to release IFNβ (McDonough et al., 2017), we find IFNs and TLR3 receptor induction by ethanol is primarily in astrocytes and neurons. Astrocytes play a vital role in modulating neuronal plasticity in brain (Haydon & Nedergaard, 2014) and are becoming a prominent focus of alcohol research (Adermark & Bowers, 2016). Previous studies have indicated that the astrocyte secretome, such as multiple extracellular matrix proteins, is changed following chronic ethanol exposure during early postnatal life (Trindade, Hampton, Manhaes, & Medina, 2016), and that ethanol upregulates immune associated factors (e.g., COX-2, iNOS) in primary cultured rodent astrocytes (Valles, Blanco, Pascual, & Guerri, 2004). Thus, though microglia undoubtedly play important roles in neuroimmune responses to ethanol, the contribution of astrocytes should be further investigated.

It is also important to note that our *in vitro* studies utilized immortalized cell lines. SH-SY5Y neurons are a well-utilized model for studying Parkinson's disease (Korecka et al., 2013), BV2 microglia demonstrate a similar response to primary microglia following LPS stimulation (Henn et al., 2009), and U373 express astrocyte markers and have similar cytokine responses as primary astrocytes (Imaizumi et al., 2013; Rosenberger et al., 2016). Further, similar to our findings in U373 astrocytes, primary human astrocytes have also been found to express interferon response gene TRAIL following treatment with proinflammatory cytokines (Choi et al., 1999). Also, our findings *in vivo* were consistent with our *in vitro* observations. To investigate the impact of ethanol on TRAIL *in vivo* in brain, we used our 10-day binge model of ethanol intoxication in mice (Qin & Crews, 2012; Qin et al., 2008) that induces brain neuroimmune gene responses and determined TRAIL+IHC. TRAIL+IR was increased in both orbitofrontal and entorhinal cortex by ethanol treatment, regions that are affected by addiction pathology (Crews & Boettiger, 2009; Schoenbaum & Shaham, 2008). Thus, ethanol induces TRAIL in our selected *in vitro* models as well as in our *in vivo* binge ethanol model of AUD induced cortical pathology. In conclusion, we identify a novel signaling system whereby ethanol induces IFNs in neurons via neuronal and astrocytic secretion of TRAIL.

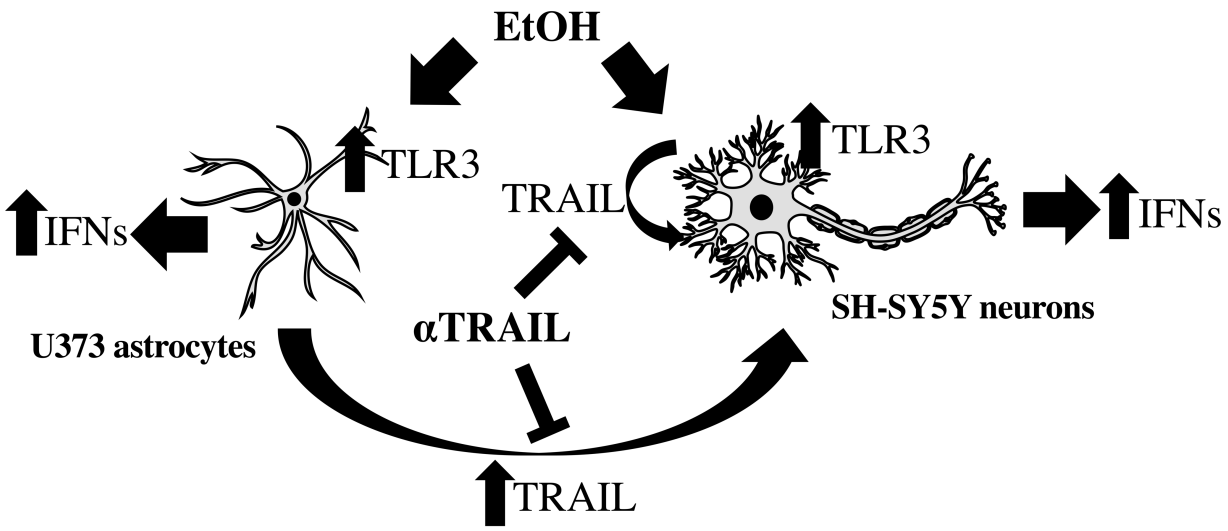

Figure 4.9 Summary of TRAIL-IFN interaction between U373 astrocytes and SH-SY5Y neurons. EtOH increases TLR3 and IFN mRNA expression in both U373 astrocytes and SH-SY5Y neurons. EtOH also increases release of TRAIL from U373 astrocytes, which further causes an increase of IFN expression in SH-SY5Y. Blocking TRAIL signaling via a neutralizing antibody (αTRAIL) negates both EtOH-induced and TRAIL-induced IFN expression in SH-SY5Y.

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CHAPTER 5: DISCUSSION

5.1 Summary

In this dissertation, we have found a novel role for neurons in ethanol induced innate-immune signaling, discovered that microglial-neuronal co-culture influences ethanol-induced innate immune genes, and also found a role for ethanol-induced TRAIL in astrocyte-to-neuron signaling. Multiple components of the innate immune signaling pathway are upregulated in post-mortem human alcoholic brain (see Table 1.1) (Coleman, Zou, & Crews, 2017; Coleman, Zou, Qin, & Crews, 2018; Crews, Qin, Sheedy, Vetreno, & Zou, 2013; He & Crews, 2008; Johnson et al., 2015; Vetreno, Qin, & Crews, 2013; Zou & Crews, 2012). Neuroimmune signaling has previously been linked to both addiction pathology (Coleman & Crews, 2018; Crews, Lawrimore, Walter, & Coleman, 2017; Crews & Vetreno, 2011, 2016; Jacobsen, Hutchinson, & Mustafa, 2016; Mayfield, Ferguson, & Harris, 2013; Montesinos, Alfonso-Loeches, & Guerri, 2016) as well as neurodegeneration (Crews & Vetreno, 2014; Heneka, Kummer, & Latz, 2014). However, the neuronal contribution to innate immune signaling, as well as how glial-neuronal signaling plays a role in ethanol induced responses, are poorly understood. This dissertation sought to better determine the roles of neurons and glia in these responses via in vitro models of neuronal and glial cell lines.

5.2 Ethanol, TLR3, and TLR4 agonists have unique innate immune responses in SH-SY5Y neurons and BV2 microglia

In Chapter 2, we first characterized basal levels of innate immune signaling molecules (ISMs) in both SH-SY5Y neurons and BV2 microglia, where we determined that SH-SY5Y

neurons express various components of the innate immune signaling pathway, albeit in lower levels compared to BV2 microglia. Interestingly, however, SH-SY5Y displayed a robust response to both TLR3 agonist Poly(I:C) as well as ethanol. In particular, ethanol induced release of TLR agonist HMGB1, increased activation of transcription factor NFκB, as well as increased expression of multiple toll-like receptors (TLRs) such as TLR3 and TLR7. These effects were seen as low as 15 mM, which is below the legal driving limit, suggesting a unique sensitivity to the effects of alcohol in neurons vs. microglia.

While glia have previously been implicated in ethanol-induced innate immune signaling, (Alfonso-Loeches, Pascual-Lucas, Blanco, Sanchez-Vera, & Guerri, 2010; Blanco, Valles, Pascual, & Guerri, 2005; Fernandez-Lizarbe, Montesinos, & Guerri, 2013; Fernandez-Lizarbe, Pascual, & Guerri, 2009), a role for neurons in these pathways have previously not been demonstrated. These studies therefore represent a novel role for neurons in ethanol-induced innate immune signaling, and represent a possible target for therapeutic strategies.

5.3 Neuronal-microglial co-culture alters ethanol-induced innate immune signaling: a role for IL-4/IL-13

In Chapter 3, we used a Transwell co-culture system with BV2 microglia and SH-SY5Y neurons to model neuronal-microglial signaling. Here we found that simply co-culturing these two cell-types together altered expression of innate immune signaling genes in both cell types. Furthermore, we found that co-culture prevented ethanol-induced HMGB1 release, while increasing IL-4 and IL-13 signaling, which correlated with a decrease in expression of several NFκB regulated genes.

While IL-4 and IL-13 function as anti-inflammatory cytokines in the periphery, these cytokines have been found to have roles in modulating long-term potentiation (LTP) and learning and memory in brain (Brombacher et al., 2017; Derecki et al., 2010; Gadani, Cronk,

Norris, & Kipnis, 2012; Maher, Nolan, & Lynch, 2005). Furthermore, the IL-13 receptor (IL-13R1α) has been found on dopaminergic neurons and also implicated in Parkinson's disease models (Mori et al., 2017). Our studies expanded upon these studies by finding a novel role for IL-4/IL-13 signaling between microglia in neurons in ethanol-induced innate immune responses. However, additional research is needed to determine the mechanism underlying co-culture influencing gene expression, as well as whether the IL-4/IL-13 signaling pathway may function as a potential therapeutic target for alcohol-related pathologies.

5.4 Ethanol induces interferon expression in neurons and astrocytes: role of astrocyte-to-neuron signaling

In Chapter 4, we discovered that ethanol upregulates interferon (IFN) expression in SH-SY5Y neurons and U373 astrocytes, but not in BV2 microglia. We further found that IFN response gene TRAIL was released by ethanol-treated astrocytes, and increased IFN expression in SH-SY5Y neurons. This study found a novel role for TRAIL as an ethanol-induced astrocyte-to-neuron signaling molecule.

IFNs are cytokines which are implicated in various neuropsychiatric symptoms, including depression (Borsini et al., 2018; Callaghan et al., 2018; Fritz, Klawonn, Jaarola, & Engblom, 2018; Mina et al., 2015; Pinto & Andrade, 2016). IFNγ is upregulated in post-mortem human alcoholic brain (Johnson et al., 2015) as well as *in vivo* in mouse ethanol models (Duncan et al., 2016; Pascual, Balino, Aragon, & Guerri, 2015), further implicating IFNs in alcohol pathology. We also found that IFN stimulated gene TRAIL can further induce IFNs in SH-SY5Y, suggesting a novel role for TRAIL signaling in neurons. While we did not observe ethanol or TRAIL-mediated cell death in SH-SY5Y neurons, it possible that alternative alcohol exposure models (e.g., multiple ethanol binges) would replicate neurodegenerative models of alcohol

exposure. Further research is needed on the possible therapeutic qualities of targeting TRAIL activity in brain.

5.5 Conclusions and future directions

These studies clearly demonstrate a novel role for neurons in ethanol-induced innate immune signaling and neuronal-glial signaling (see Figure 5.1 for summary schematic). However, the conclusions of these studies are largely from the use of cell lines and *in vitro* assays. As such, it is important to verify these findings using either (i) multiple strains of neuronal/glial cell lines, (ii) primary cell cultures, and/or (iii) *in vivo* models. In addition, while the retinoic-acid differentiated SH-SY5Y cells used in these studies primarily display a dopaminergic phenotype, there may be differential responses to ethanol and/or TLR agonists in different neuronal subtypes. Different brain regions contain varying populations of unique neuronal subtypes (e.g., dopaminergic, cholinergic, GABAergic, glutamatergic), varying densities and phenotype of glial cells (Cragnolini, Montenegro, Friedman, & Masco, 2018; De Biase & Bonci, 2018), and receive unique inputs from other brain regions. All of these factors contribute to region-specific responses. Therefore, using either brain slice cultures and/or *in vivo* models are essential in determining region-specific innate immune signaling and represent a key future direction for this body of work. Moreover, recent studies have indicated that differential epigenetic modulation of genes may underlie regional variation in microglia activity (Ayata et al., 2018), suggesting the utilization of epigenetic mechanisms as a possible approach in understanding region-specific glia effects.

In the peripheral immune system, the term "inflammation" commonly refers to tissue swelling and neutrophil infiltration (Selders, Fetz, Radic, & Bowlin, 2017). However, so-called "pro-inflammatory" and "anti-inflammatory" innate immune molecules also function outside of

traditional inflammation modulatory roles in the brain. While families of cytokines and other immune molecules that in the periphery function as pro- or anti-inflammatory are often labeled as such, it is important to note that this terminology is an inaccurate classification to describe their action in brain. Cytokines function as neurotransmitter modulators, alter neuronal excitability, and play roles in brain development, (Boulanger, 2009; Galic, Riazi, & Pittman, 2012) without causing traditional signs of inflammation. Further research needed to identify the diverse actions of cytokines and other immune molecules in brain.

Another important area that requires further research is the hypothesis of "trained" innate immunity, where the innate immune system appears to be sensitized to future insults (Tchessalova, Posillico, & Tronson, 2018). We have previously found that mice treated with ethanol display increased responsivity to both TLR3 and TLR4 ligands (Qin & Crews, 2012; Qin et al., 2008), demonstrating that this trained immunity is activated by alcohol. Whether alcohol "priming" the innate immune system can be detected using *in vitro* experiments utilizing different cell types while controlling for cell-to-cell-communication remains an open question.

Multiple soluble and membrane-found factors are responsible for neuronal-microglial signaling (Szepesi, Manouchehrian, Bachiller, & Deierborg, 2018). Further work is needed to study not only HMGB1, IL-4/IL-13, and TRAIL, which are all molecules that may play neuronal-glial roles, but other innate immune signaling molecules which may function in similar capacities. Especially in the context of how co-culture modifies cell phenotype, it is important to assess possible changes in secreted proteins which may modulate these effects (e.g., microglial-neuronal signaling molecules such as fractalkine or CD200). In addition, how microglia and astrocytes influence innate immune signaling of the other has yet to be determined.

Furthermore, it is important to note the differential effects of acute vs. chronic ethanol, intoxication vs. withdrawal, as well as amount of ethanol on innate immune gene regulation. For example, in the brain of ethanol-treated mice, during intoxication (1 hr after 6g/kg gavage of ethanol), cytokines such as TNFα are actually downregulated, but are elevated at 24 hours, during withdrawal, and these acute effects are seen only with higher amounts of ethanol (4.5 g/kg) (Walter & Crews, 2017). Epigenetic modulators, such as HDACs, are also decreased with acute ethanol in the amygdala of rats (1 g/kg, 1 hr after injection) but increased with chronic exposure (16 days of Lieber-DeCarli ethanol diet), representing another possible modulator of innate immune gene induction (Pandey, Kyzar, & Zhang, 2017; Pandey, Ugale, Zhang, Tang, & Prakash, 2008). Our treatment of 100 mM ethanol for 24 hours most likely resembles an acute binge, although further characterization of epigenetic modifications, effects of repeat "binges" as well as chronic vs. withdrawal ethanol are needed to elucidate these effects.

In terms of alcohol use disorder, these studies indicate an importance for utilization of cell-specific targeting approaches. While innate immune signaling was previously established in peripheral macrophages, these studies indicate that different cell types in brain, as well as signaling among different cell types, are differentially affected by both ethanol and TLR ligands. While speculative, our findings that ethanol upregulates cytokines and TLRs in microglia and neurons alone, but are prevented from being induced by ethanol when these cells are co-cultured, may suggest that in the alcoholic brain, there could be a "disconnection" that occurs between cells causing a resemblance to our single-cell type studies. These studies also suggest possible novel therapeutic approaches, such as TRAIL, for AUDs or other neurodegenerative disorders, however close attention must be paid to individual cell-type responses to these agonists.

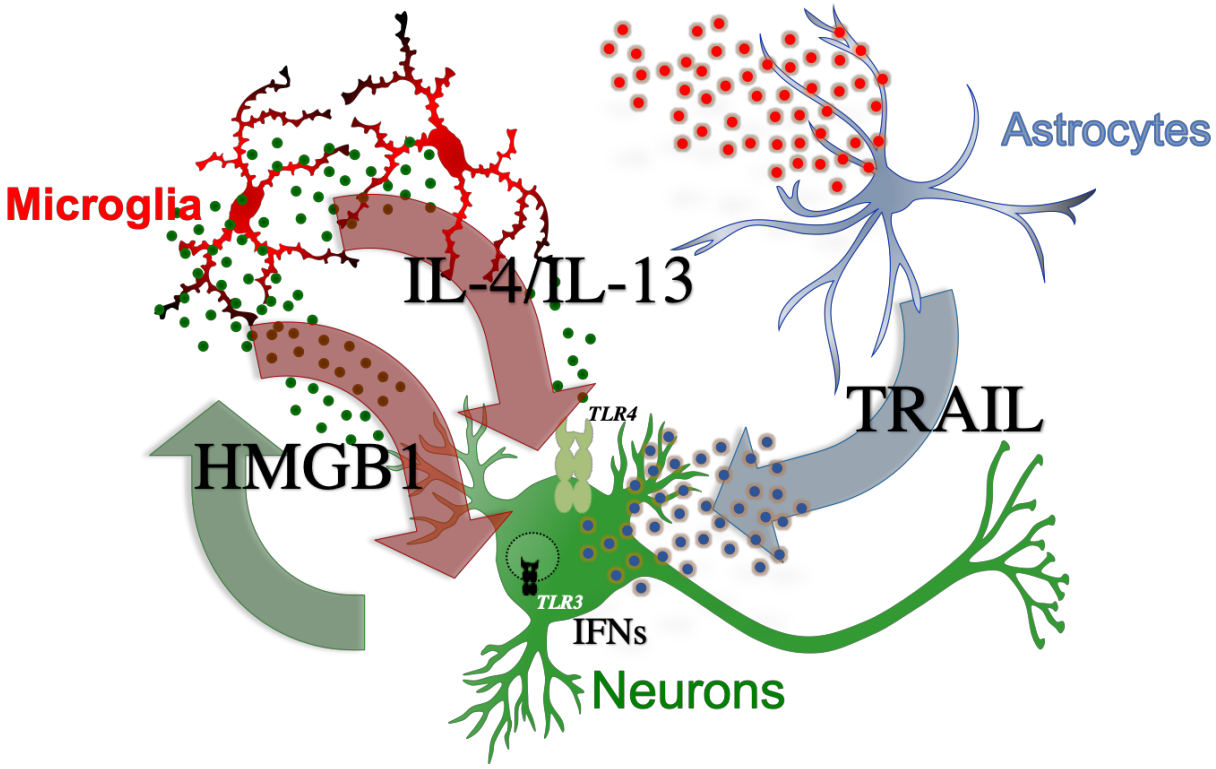

Figure 5.1 Ethanol influences neuronal-glial innate immune signaling. Both neurons and microglia secrete HMGB1 in response to ethanol (Chapter 2). Co-culture enhances ethanol-induced IL-4/IL-132 signaling in microglia and neurons (Chapter 3). Ethanol induces TRAIL from astrocytes, which induces interferon expression in neurons (Chapter 4).

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