The Thesis committee for Lina Maria Gomez
Certifies that this is the approved version of the following thesis:

Ethanol-induced toxicity and neurodegeneration in *C. elegans*

APPROVED BY
SUPERVISING COMMITTEE:

Supervisor:

Jonathan Pierce-Shimomura

S. John Mihic

Ethanol-induced toxicity and neurodegeneration in *C. elegans*

by

Lina Maria Gomez, BS

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For Stella and Eduardo,

my best friends

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Lina Maria Gomez, M.S.Neurosci.

The University of Texas at Austin, 2013

SUPERVISOR: Jonathan Pierce-Shimomura

Alcohol abuse is an enormous problem causing death and disability to over 43 million people worldwide each year (WHO). Chronic alcohol consumption also contributes to abnormal brain morphology and significant brain volume loss indicative of neurodegeneration. Until there are effective treatments to alter maladaptive behavioral patterns in alcohol abuse, more research is needed to prevent alcohol-induced toxicity and degeneration. We used *C. elegans* as a model system to identify genetic modulators of alcohol toxicity and explored whether prolonged alcohol exposure damages the nervous system. In our study, we exposed L4-larval stage worms to varying concentrations of ethanol for three days and found a dose-dependent deficit in crawling. Furthermore, we evaluated degeneration by assessing the health of neurons using fluorescent reporters. Compared to the untreated group, we found that ethanol-exposed worms had a significant neurodegeneration. Previous findings using *C. elegans* have

suggested that the innate immune pathway may protect against neurodegeneration caused by drug toxicity (Schreiber & McIntire, 2012). We find that deletion of either the innate immune gene *nsy-1* (orthologous to the mammalian ASK-1 MAPKKK) or *pmk-1* (orthologous to the mammalian p38 MAPK) caused hypersensitivity to ethanol toxicity. Conversely, boosting innate immune signaling via gain-of-function mutation in *nsy-1* produced resistance to ethanol toxicity and ameliorated ethanol-induced cholinergic degeneration. Our findings indicate that prolonged exposure to ethanol leads to both behavioral impairments and neuronal degeneration in *C. elegans* and that the ASK1/p38 MAP kinase pathway may play a role in ethanol-induced damage to the nervous system.

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INTRODUCTION

Innate immune system function is the first line of defense against all types of exogenous pathogens, toxins and injury. Upon infection, the innate immune system activates signaling cascades that ultimately induce inflammation, immunity and repair. The innate immune system and its role in disease have received significant attention over the past decades. Increasing evidence in animal models indicates that sustained inflammation due to a dysregulated innate immune system results in tissue pathology and production of neurotoxic agents— components that are directly linked to neurodegenerative diseases. Understanding mechanisms of innate immune system activation as it produces inflammatory responses will be vital for providing therapeutic strategies for the prevention and treatment of chronic degenerative diseases.

Due to its well-established role in the pathogenesis of many CNS disorders, understanding the innate immune system inflammatory response as it relates to alcoholism and alcohol-induced brain damage has been an expanding area of interest. Recent discoveries have provided evidence of a direct link between alcohol-induced brain damage and immune system activation, specifically, neuroinflammation. Alcohol exposure results in Toll-like receptors (TLRs) activation, phosphorylation of mitogen-activated protein (MAP) kinase pathways and proinflammatory cytokine production. These exciting discoveries have elucidated mechanisms by which chronic alcohol consumption induces both short and long term damage to the central nervous system.

Advances within the areas of neuroimmunology and alcohol abuse may provide new approaches for the treatment of alcohol-induced neurodegeneration and brain damage.

INNATE IMMUNE SIGNALING PATHWAY

Increasing evidence within the ethanol and neuroimmunology field has suggested a critical role for Toll-like receptors (TLRs) in alcohol-induced brain damage. TLRs are a family of highly conserved membrane glycoproteins that recognize molecules, or pathogen-associated molecular patterns (PAMPs), derived from microbes. TLRs were first cloned in *Drosophila***,** and so far, 11 mammalian homologs have been characterized (Hashimoto et al., 1988). TLRs form a complex with the superfamily of proteins known as interleukin-1 receptors (IL-1R). This complex is activated upon binding of different ligands including bacterial lipopolysaccharide (LPS), heat shock proteins and lipopeptides. Some of the molecules needed to initiate the immune signaling cascade include myeloid differentiation primary-response protein 88 (MyD88), IL-1R-associated kinase (IRAK), transforming growth factor-β (TGF-β)-activated kinase (TAK1), TAK1-binding protein 1 (TAB1), TAB2 and tumour-necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) (See figure 1). Collectively, recruitment of these downstream signaling molecules can activate mitogen-activated protein kinase (MAPK) pathways. Stimulation of the MAPK signaling cassette results in activation of transcription factors (TFs) — AP-1 and NF-ϰB. Ultimately, stimulation of AP-1 and Nf-ϰB increases expression of genes that encode pro-inflammatory mediators such as inflammatory cytokines (Li et al., 2002; Akira and Takeda, 2004, Nguyen et al., 2002).

MAMMALIAN MAP KINASE (MAPK) SIGNAL TRANSDUCTION PATHWAYS

Mammalian MAPKs can also be activated by endogenous stimuli such as growth factors, hormones and cytokines as well as environmental factors including UV irradiation, heat shock and injury. Currently, there are six different groups of MAPKs that have been characterized—extracellular regulated kinases (ERK) 1/2, ERK 7/8, ERK 3/4, ERK5, Jun terminal kinases (JNK 1/2/3) and lastly, p38 (p38 α/β/ϒ/δ). MAPK signaling induces downstream phosphorylation of MAPK kinase kinase (MAP3Ks), MAPK kinase (MAPKK) and MAPK, which act via downstream substrates (i.e. CREB, AP-1, etc.) and eventually induce expression of MAPK-activated protein kinases (MKs). Activation of these pathways has been implicated in many different biological responses and processes including cell proliferation, survival, apoptosis and inflammation (Krishna and Narang, 2008).

The p38 MAPK pathway has been extensively studied, and its critical role in innate immune response has received significant attention over the past decades. Some of the upstream activators of p38 kinases are MKK3, MKK6, ASK1 and TAK1; upon activation, p38 phosphorylates downstream targets that include transcription factors (TF) (i.e. ATF and CREB), which lead to activation of genes that encode proinflammatory proteins. Studies have indicated that activation of p38 initiates production of pro-inflammatory cytokines, enzymes such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Pietersma et al, 1997; Krishna and Narang, 2008). Additionally, activation of the p38 MAPK pathway regulates expression of proinflammatory genes via post-transcriptional and post-translational mechanisms

(Bolling et al., 2003). Although the role of p38 MAPK has been found to be essential in immune function, the exact mechanisms involved in p38 functions remain elusive. Moreover, due to its involvement in various cellular processes (i.e. cell cycle, apoptosis, cell survival, differentiation and development, etc.) its categorization to a specific response has been proven difficult.

INNATE IMMUNITY IN *C. ELEGANS*

Although the mammalian immune system consists of innate and adaptive layers of protection, the nematode worm *C. elegans* relies solely on innate immune system activation against pathogenic attack. This is one of the advantages of using *C. elegans* as a model organism for the study of innate immunity—it allows for a clear dissection and characterization of key innate immunity components independent from adaptive immune system interaction. Similarly, *C. elegans* is a powerful tool that provides a genetically tractable model system that can be used to identify genes responsible for innate immune response at the molecular and cellular levels. One approach that has been fundamental in the identification of innate immune genes is through the use of genetic screens. Here, worms are exposed to a strong mutagen, which results in random gene mutations in the subsequent generation of worms. Genetic manipulations available in *C. elegans* offer complementary tools for studying the contribution of individual genes to innate immune function.

Although the innate immune system is conserved across species, there are certain differences between *C. elegans* and mammalian immune systems. Firstly, there are no

known homologs of the transcription factor NF-κB or adaptor protein MYD88. Secondly, the *C. elegans* gene *tol-1*, orthologous to the mammalian *Tlr* genes, does not appear to regulate innate immune response upon PAMP exposure. Thirdly, there are no known cytokines produced by the *C. elegans* innate immune system upon microbe infection. Although some of important components of mammalian innate immune system are not encoded in the *C. elegans* genome, there are, however, evolutionarily conserved signal transduction pathways including p38 MAPK, β-catenin, and FOXO, which are activated during immune response (Troemel et al., 2006; Pukkila-Worley et al., 2012) Furthermore, due to the lack of homology to NF-kB and MYD88 mediated immune response, *C. elegans* is a powerful genetic model, which has proven useful in identifying and elucidating TLR-independent components of innate immunity.

RECENT STUDIES IN *C. ELEGANS* INNATE IMMUNITY

Genetic screens have yielded important discoveries in innate immunity *C. elegans*. For instance, the mammalian p38 mitogen-activated protein (MAP) kinase pathway has been identified to be homologous with the *C. elegans* NSY-1/SEK-1/PMK-1 pathway. NSY-1 is orthologous to the mammalian ASK-1 gene; SEK-1 mammalian ortholog is MKK3/MKK6 and PMK-1 is orthologous to the mammalian p38 MAP kinase. Activation of *nsy-1* requires activation of the upstream gene *tir-1*, orthologous to the mammalian SARM. Additionally, recent findings have indicated that the *nsy-1/sek-1/pmk-1* pathway is activated upon exposure to pathogen infection, osmotic stress and biogenic amines (Hisamoto et al., 2001; Kim et al., 2002; Schreiber and Mcintire, 2012).

nsy-1, sek-1, pmk-1 null mutants exhibited higher susceptibility to killing by a wide variety of infections including Gram-negative pathogens *P. aeruginosa* (Kim et al., 2002; Troemel et al., 2006), *Salmonella enterica* (Aballay et al., 2003), *Yersinia pestis* (Bolz et al., 2010) and *Serratia marcescens* (Shivers et al., 2010); as well as the Gram-positive pathogens *Enterococcus faecalis* (Shivers et al., 2010) and *Staphylococcus aureus* (Sifri et al., 2003). Similarly, it was discovered that *sek-1* and *pmk*-1 are required for defense against oxidative agents as well as the crystal toxin produced by *Bacillus thuringensis*, respectively (Inoue et al. 2005;Troemel et al., 2006;). Together, these findings have provided important insight into conserved aspects of innate immune pathways. Combining findings in *C. elegans* and rodent models will shed light to the molecular components of innate immune response and their involvement in human disease.

LPS: ITS ROLE IN NEURODEGENERATION AND ALCOHOLISM

Lipopolysaccharide (LPS) is an endotoxin present in the outer membranes of Gram-negative bacteria. LPS binding to TLRs activates a signaling cascade, which ultimately results in an immune response. Studies have demonstrated that systemic or central administration of LPS induces the expression of proinflammatory cytokine mRNAs and proteins such as IL-1β AND TNF- α in the brain (Van Dam et al., 1992; Laye et al., 1994; Quan et al., 1999). Although LPS is one of several other molecules recognized by TLRs— the interaction between LPS and TLRs and their role in neuroinflammation/neurodegeneration remain elusive.

Recent studies demonstrated that shortly after systemic LPS and alcohol administration induced increases in TNF-α production (Qin et al., 2008**).** This was one of the first studies to suggest a link between systemic LPS and cytokine-mediated neuroinflammation. Shortly after this discovery, Qin and colleagues extended their previous study by administering 3 mg/kg of LPS and measuring its immediate (1hr post LPS administration) and chronic (10 days ethanol treatment) effect on cytokine production. They found that LPS quickly generated significant increases in TNFα, MCP-1, and IL-1β mRNA and protein levels in mouse brain, serum and liver.

A recent study by Blednov and colleagues (2011) described the effect of LPS administration on alcohol consumption. Different strains of mice were tested for alcohol preference, motivation and consumption following LPS administration. They found that two injections of LPS produced persistent increases in alcohol consumption, and that this effect was dependent on genetic background and gender (Blednov et al., 2011). Their results are consistent with previous findings-- mice lacking functional chemokines, cytokines, or TLR4 show reduced ethanol consumption in comparison to wild type. The results of this study strongly suggest that there is a neuroimmune regulation of ethanol consumption and that innate immune signaling may indirectly promote alcohol drinking in mice and possibly humans.

TLR4 IS ACTIVATED BY ETHANOL

It was recently discovered that acute or chronic ethanol exposure activated glial cells by stimulating the TLR4 and IL-1R signal transduction pathways in cultured

astrocytes (Blanco et al., 2005). Subsequent studies by Silvia Alfonso-Loeches et al. demonstrated that TLR4 is essential for ethanol-induced neuroinflammatory signaling since TLR4-deficient mice did not show activation of astrocytes, pro-inflammatory cytokines as well as MAPK and NFkB signaling pathways after chronic ethanol exposure (Alfonso-Loeches et al., 2010). This was the first study to provide evidence that glia activation following ethanol exposure is associated with increased levels of pro-inflammatory molecules IL-1, caspase-3, iNOS and COX-2 in wild type mice.

Further corroborating the role of TLR4 signaling in ethanol-induced effects, a study by Wu and colleagues (2012) found that inhibiting the TLR4/MyD88 signaling cascade using either naloxone (an opioid and TLR4 antagonist) or TLR4 or MyD88-knockout mice reduced sedation and motor impairment in mice that were acutely exposed to ethanol. In this particular study, naïve mice with null mutations in the *Tlr4* and *Myd88* genes were injected with a single, high or moderate, alcohol dose and were assessed for alcohol-induced sedation and motor dysfunction. Furthermore, *Tlr4* and *Myd88* knockout mice were assessed for alcohol-induced behavioral changes following naloxone treatment before alcohol exposure. They found that mice null in *Tlr4* and *Myd88* recover more quickly from alcohol-induced motor impairment and sedation. They also found that low and high alcohol doses activate proinflammatory-signaling molecules within the CNS. However, they did not observe p38, JNK or ERK phosphorylation following acute ethanol exposure in hippocampal cells *in vitro*. Their data contradict previous findings by Alfonso-Loeches et al., indicating that the mechanisms underlying chronic and acute

ethanol-induced neuroinflammation may be constitutively different. Their data suggest that other inflammatory mediators may also be involved in the alcohol-induced inflammatory damage in the brain.

Although the mechanism by which ethanol interacts and promotes TLR4 and IL-1R recruitment remains unknown, a study by Blanco et al., found that stimulation and signaling of TLR4/IL-1R complex in glia occurs exclusively through *lipid rafts*, or cholesterol-enriched plasma membrane microdomains. Additionally, they suggest that ethanol is a TLR4 and IL-1R agonist and that it may interact with other proinflammatory signaling molecules including IRAK and ERK (Blanco et al., 2008).

NF-κB AND ITS ROLE IN NEURODEGENERATION AND ALCOHOLISM

NF-κB is a family of transcription factors (TFs) known for their regulation of genes involved in immune response, synaptic plasticity, cell death/cell survival, and inflammation (Crews et al., 2006; Rulten et al., 2006; Ward et al., 1996; Zou and Crews, 2006, 2010). In its inactive form, the NF-κB family of dimers is composed of five members: p65, p50, REL-B, cytoplasmic REL, and p52. NF-κB heterodimer (p65/p50) is kept inactive by IκB—an inhibitor protein that keeps NF-κB from translocating to the nucleus (Akira and Takeda, 2004). NF-κB can be activated by a variety of extracellular stimuli such as growth factors, inflammatory mediators and glutamatergic excitotoxicity. (Yakovleva et al., 2011). Additionally, it was recently demonstrated that chronic or acute ethanol exposure activates NF-κB TFs in the CNS resulting in downstream activation of TNF-α and proinflammatory cytokines (Crews et al., 2006). A different study performed

DNA microarray analysis in ethanol treated mice in order to identify ethanol-regulated genes. Upon either acute or chronic ethanol administration, they found that ethanol differentially regulated the NF-κB pathway in a dose-dependent manner: acute ethanol treatment resulted in increased NF-κB protein expression, whereas chronic ethanol treatment led to significant inhibition of NF-κB transcription (Rulten et al., 2006). These findings provide evidence that at the mammalian transcripitional level, ethanol may be acting via the NF-κB pathway.

CYTOKINES AND ETHANOL

It was recently discovered that there is reciprocal communication between the brain and the immune system, mediated by signaling molecules known as cytokines. Cytokines are widely expressed in glia (astrocytes and microglia) and their receptors are ubiquitously expressed in all cell types in the CNS. During physiological conditions, cytokines regulate inflammation by producing pro or anti-inflammatory responses. For instance, pro-inflammatory cytokines such as tumor-necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-6 levels are elevated during brain injury as well as in response to infections and endotoxins. Increased cytokine production has been associated with a variety of pathologies such as neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and multiple sclerosis, mood disorders (depression) as well as alcoholism (Glass et al., 2010).

The effect of alcohol on cytokine production and innate immune activation is complex and variable. For instance, during the last decade, it has been established that

alcohol consumption causes increased levels in both systemic and brain levels of cytokines. Specifically, chronic alcohol intake in humans was associated with elevated levels of TNF- α, IL-1 and IL-6 (McClain et al., 2004; McClain and Cohen, 1989 and Crews et al., 2006). Conversely, low to moderate alcohol consumption has been shown to reduce the risk of developing type-2 diabetes and coronary heart disease (Hendriks and Van Tol, 2005**;** Collins et al., 2009, Sinforiani et al., 2011**).** It is proposed that the mechanism by which alcohol protects from these diseases is by inducing anti-inflammatory signals via anti-inflammatory cytokines such as IL-4 and IL-10. *In vitro* studies demonstrated that moderate alcohol exposure increased production of IL-10 in monocytes. Additionally, they found that alcohol treatment in macrophages inhibited the production of proinflammatory cytokines via the NF-kB pathway (Mandrekar et al., 2001). Both of these findings suggest that light-to-moderate alcohol intake exerts anti-inflammatory effects by both suppressing pro-inflammatory signals as well as by increasing anti-inflammatory cytokine production. Collectively, this evidence suggests that ethanol induces different effects on cytokine production and immune response depending on the dosage (acute or chronic exposure) and on the history of alcohol consumption (see figure 2).

INCREASED MCP-1 IN THE ALCOHOLIC BRAIN

Another important cytokine that has been implicated in alcohol-induced neuroinflammation and ethanol consumption is monocyte chemoattractant protein-1 (MCP-1 or also known as CCL2) (Blednov et al., 2005). For instance, a study in human postmortem brains of alcoholics found elevated MCP-1 protein concentration in the

ventral tegmental area (VTA), substantia nigra, hippocampus and amygdala in comparison to non-alcoholic brains (He et al., 2008). This evidence identifies another key component in the neuroimmune involvement in alcohol-induced brain damage.

ALCOHOL-INDUCED CNS DAMAGE

Chronic alcohol exposure results in a dysregulation of the innate immune system by altering cytokine expression and immune response, all which may ultimately lead to long-term damage to the central nervous system. For example, individuals who consumed alcohol chronically had abnormal brain morphology, significant volume loss and reduced neurogenesis (Crews and Nixon, 2009), all of which are indicative of neurodegeneration. It has been noted that alcohol-induced neurodegeneration occurs during intoxication, and that the frequency and recency of binge-drinking episodes best predicts the extent of alcohol-induced brain damage.

Severe alcohol abuse impairs learning and memory abilities and can sometimes develop into more severe forms of mental dysfunction such as alcoholic-related amnesia and/or dementia. The most common form of alcoholic amnesia is Korsakoff's syndrome, being the second leading cause of adult dementia in the U.S. (Eckard and Martin, 1986) -- characterized by severe memory loss, impaired executive function and apathy (Kessels and Kopelman, 2012). Although memory deficits are accompanied by this condition, intelligence and other cognitive functions remain intact. Imaging studies have found significant changes in morphology and physiology in the brains of alcoholics. These changes have been associated with the memory loss and cognitive impairments seen in

individuals with Korsakoff's syndrome and alcoholic-related dementia. Typically, there is significant volume loss and weight reduction in the brains of alcoholics (Crews and Nixon, 2009). Specifically, postmortem studies have shown shrinkage of both gray and white matter across different brain regions as well as reduced ventricle size (Sullivan and Pfefferbaum, 2005). Even though alcohol-induced damage has been observed throughout the entire brain, the frontal cortex appears to be particularly vulnerable (Kubota et al., 2001). Disrupted frontal lobe function contributes to impaired judgment, reduced affect, decreased motivation, distractibility, and impulsivity (Crews and Nixon, 2009); behaviors that are typically observed in alcoholics. Other affected brain regions include the hypothalamus, medial septal nucleus, insula, cerebellum and hippocampal area (Fadda and Rossetti, 1998).

In addition to inducing abnormal brain morphology and volume reduction, chronic alcohol consumption also inhibits neurogenesis. For instance, a study by Herrera et al., (2003) found that ethanol selectively reduced the number of newly formed neurons in the dentate gyrus of ethanol dependent animals. A different study found that chronic, but not acute, ethanol administration resulted in significantly decreased neurogenesis in the dentate gyrus of animals that were pretreated with both ethanol and LPS (Qin and Crews, 2008). This, and other studies have found that chronic alcohol exposure results in reduced neurogenesis, and that this effect occurs via LPS induced neuroinflammation—elucidating the mechanism by which chronic alcohol consumption contributes to neurodegeneration via an innate immune response.

MECHANISM UNDERLYING ALCOHOL-INDUCED CNS DAMAGE

Currently, the mechanism of alcohol-induced neurodegeneration and how the innate immune system is influencing this damage remains unclear. In a binge ethanol model of alcohol dependence (Zou and Crews, 2006), it was demonstrated that alcohol induced oxidative stress and proinflammatory signals all of which are indicative of neurotoxicity and neuronal death. Similarly, ethanol reduced BDNF and CREB-regulated gene expression at similar times when neurodegeneration was most noted. Another study suggested oxidative damage to have an important role in alcohol-induced neuronal damage.

It has been proposed that NADPH oxidase (NOX) plays in role in alcohol-induced neurodegeneration and neurotoxicity. Activation of NOX in microglia results in the formation of reactive oxygen species (ROS) in the brain ultimately leading to neurotoxicity (Qin et al., 2004). Previous studies found significant increases in proinflammatory cytokine, chemokine and ROS levels following chronic alcohol exposure. Qin and Crews expanded these findings by establishing a critical role of the NOX-ROS complex in alcohol-induced neurodegeneration. Data from this study suggested that chronic ethanol consumption leads to long lasting increases in both NOX production and proinflammatory oxidative stress in the brains of mice and humans. Conversely, treatment with diphenyliodonium (DPI), a known NOX inhibitor, resulted in decreased ROS production, which in turn stopped alcohol-induced neurodegeneration in ethanol treated mice. Results from this study further support the theory of neuroinflammation and oxidative stress as key mediators in alcohol-induced

neurodegeneration and neuronal death (Qin and Crews, 2012). Although this evidence elucidates a pathway by which atrophy and degeneration occur following chronic alcohol consumption, the exact mechanism that underlie CNS degeneration as a result of chronic alcohol consumption remain elusive.

PROTECTIVE ROLES OF THE INNATE IMMUNE SYSTEM

There have been discrepancies in deciphering the role of inflammatory response in neurodegenerative diseases, mood disorders and alcoholism. While an inflammatory response due to injury or infection is necessary for repair and protection of neurons as well as the maintenance of normal functioning, sustained inflammation can cause degeneration and damage to the CNS. Although a growing number of studies have demonstrated that proinflammatory molecules have detrimental effects in the CNS, there is evidence of beneficial roles of the innate immune response.

For instance, in a study by Arnett et al. (2001), mice lacking TNF- α and TNF- α receptors had a significant reduction in neuronal remyelination in their model of Multiple Sclerosis (MS). They found that TNF receptor 2 (TNFR2), and not TNFR1 was critical for oligodendrocyte regeneration, and that TNF- α is involved in nerve remyelination and reparation (Arnett et. al 2001). In addition to its involvement in degenerative processes, TNF- α has been shown to play a role in ischemic injury as well as epileptic seizures. Heldmann et al., (2005) investigated the effect of TNF- α inhibition on the survival of hippocampal neurons in rats that had endured an ischemic injury. They found that rats treated with anti-TNF antibodies had reduced hippocampal and striatal neurogenesis in

comparison to animals given control protein. Theses results suggest that TNF- α signaling may be necessary for neuronal repair and survival after an ischemic insult (Heldmann et al., 2005). In a different study by Bruce and colleagues (1996), mice genetically deficient in TNF receptors that received an ischemic or excitotoxic brain injury exhibited increased neurodegeneration and oxidative stress, indicating that TNF may have neuroprotective properties by activating antioxidant pathways (Bruce et al., 1996).

Another proinflammatory cytokine that has been linked to CNS repair is interleukin-1β (IL-1 β). Its role in the remyelination process was demonstrated in a study by Mason et al., (2001). Following curprizone (a strong demyelinating agent that results in impairment and death of oligodendrocytes) treatment, IL-1β knockout mice showed decreased remyelination as well as insulin-like growth factor-1 (IGF-1) levels in comparison to wild-type animals. Together, these findings indicate that receptors and cytokines that are involved in the innate immune response (such as TNF and IL-1 β) have neuroprotective properties against injury and toxin-induced demyelination. More research is needed to elucidate innate immune response and its role in normal brain functions as well as in neurodegeneration; currently this mechanism remains unknown, and it appears that the immune system can have dual roles in the CNS.

Chapter Two: ethanol-induced toxicity and neurodegeneration in *C. elegans*

INTRODUCTION

Excessive alcohol consumption results in damage to various organs particularly the brain. Neuropathologies consequent to chronic alcohol consumption include reduced brain volume, abnormal brain morphology and decreased neurogenesis (Crews and Nixon, 2009; Fadda and Rossetti, 1998). Conversely, lower to moderate alcohol consumption can exert protective actions by reducing the risk of developing type 2 diabetes, cerebrovascular and coronary heart disease by promoting anti inflammatory and anti oxidative processes (Hendriks and Van Tol, 2005; Collins et al., 2009, Sinforiani et al., 2009). These findings suggest that alcohol consumption has beneficial and deleterious health effects dependent on frequency and dosage of drinking.

Emerging evidence indicates a role for innate immune system activation in neurodegenerative diseases as well as alcohol abuse disorders. For instance, chronic alcohol exposure caused increases in brain levels of inflammatory mediators, upregulation of innate immune gene expression and activation of innate immune signaling pathways, all which have been implicated in CNS atrophy and degeneration (Alfonso-Loeches et al. 2010; Blanco et al., 2005; Arlinde et al., 2004; Zou and Crews, 2010).

Despite increasing evidence supporting a role for innate immunity in alcohol-

induced brain damage, the mechanisms of alcohol-induced neurodegeneration and how the innate immune system is influencing this damage remain unclear. We used the genetically tractable organism *Caenorhabditis elegans* to explore molecular and cellular mechanisms of alcohol-induced neurodegeneration. Firstly, its short-life span, transparent body and well-characterized nervous system allow for a simpler approach to studying neurodegeneration. Secondly, its lack of an adaptive immune system facilitates the study of innate immunity independent of adaptive immune system activation. Lastly, it has been demonstrated that *C. elegans* exhibits behavioral intoxication at physiologically relevant ethanol doses, and that these effects are mediated by conserved genes and targets in the nervous systems of invertebrates and mammals (Morgan and Sedensky, 1995; Bettinger and McIntire, 2004; Davies et al., 2003, 2004; Davis et al., 2008; Graham et al., 2009; Kapfhamer et al., 2008; Lee et al., 2009; Mitchell et al., 2007; Speca et al., 2010).

In this study, we developed an *in vivo* model of chronic alcohol exposure to investigate whether high doses of ethanol could result in neuronal degeneration and toxicity in *C. elegans*. Because a novel model of Alzheimer's disease (AD) developed by Crisp et al. (in submission, 2013) demonstrated that a specific subset of neurons (VC-class cholinergic neurons 4 and 5) were particularly susceptible to amyloid precursor protein (APP)-induced degeneration, we explored whether prolonged alcohol exposure could also lead to cholinergic neurodegeneration. We discovered that alcohol-treated WT worms exhibited notable deficits in crawling as well as increased cholinergic degeneration. Additionally, we found that mutations in the *nsy-1* gene bidirectionally

influenced locomotion rates following chronic alcohol exposure and that boosting activity of *nsy-1* resulted in enhanced resistance from alcohol-induced neurodegeneration and toxicity.

RESULTS

There is a dose-dependent deficit in crawling after prolonged exposure to ethanol

We first examined the behavioral effects of ethanol in *C. elegans*. To determine appropriate ethanol doses to study chronic (72 hours) ethanol exposure, we treated L4-larval stage worms with 100mM, 200mM and 400mM ethanol. Previous work by Davies et al. 2003, showed that internal concentrations of ethanol for worms exposed to 400 mM and 500 mM ethanol were about 22 mM and 29 mM, respectively. These concentrations are equivalent to human blood alcohol concentrations of 0.1% (Davies et al., 2003).

We examined crawling behavior by quantifying the number of head bends over a 20 second period following chronic ethanol exposure. We found that treatment with 200mM and 400mM ethanol strongly inhibited crawling behavior as indicated in figure 3 (p-value = 0.00 for 200mM and 400mM ethanol at $\alpha = 0.05$). Our results are consistent with previous reports that showed dose-dependent depression in locomotion in animals treated with ethanol (Davies et al., 2003, 2004; Alaimo et al., 2012; Morgan and Sedensky, 1995).

nsy-1 activity bidirectionally influences ethanol-induced toxicity

Once we confirmed alcohol-induced behavioral defects using our *in* vivo assay of chronic ethanol exposure and found that 400mM ethanol produced the most dramatic

effect on crawling behavior in 'wild type' worms, we next examined ethanol-induced crawling impairments in animals carrying mutations in the *nsy-1* gene (orthologous to the mammalian apoptosis signal-regulating kinase 1 (ASK1)). Our rationale for testing these strains arose from a study by Schreiber and McIntire, 2012, in which a screen using dopamine-induced lethality identified a mutation in the *nsy-1* gene that resulted in strong resistance to toxic effects of exogenous dopamine as well as various types of amphetamines. Furthermore, *nsy-1* gene both prevented degeneration and maintained neuronal function in a subset of cholinergic and dopaminergic neurons in Alzheimer's disease (AD) and Parkinson's disease models, respectively. (Schreiber & McIntire, 2012; Crisp et al., in submission).

We hypothesized a protective role for innate immunity; specifically, that boosting innate immune signaling, through gain-of-function mutation in *nsy-1* enhances resistance to ethanol-induced toxicity and neurodegeneration. We found that *nsy-1(gf)* mutants had significantly higher number of head bends (3.8 head bends/20 sec) compared to wild type animals (2.2. head bends/20 sec) after chronic ethanol treatment (p-value = 0.00) (Figure 4). This suggests that increasing *nsy-1* activity may be protective of crawling deficits associated with ethanol intoxication.

We next explored whether *nsy-1(lf)* mutants showed opposite phenotypes to *nsy-1(gf)* strain. We found that *nsy-1(lf)* worms were hypersensitive to ethanol by showing significantly reduced number of head bends in comparison to control 'wild type' (p-value = 0.005) and *nsy-1(gf)* (p-value = 0.00). Together, our findings suggest that there is an

inverse relationship between innate immunity and ethanol toxicity, and that *nsy-1* activity correlates with the concentration of exogenous ethanol.

It was previously discovered that *nsy-1* interacts with downstream gene *pmk-1* (orthologous to mammalian p38 MAP kinase) in response to pathogenic bacterial attack, and that *pmk-1(lf)* mutant worms showed enhanced susceptibility to killing upon infection (Troemel et al., 2006). We thus tested whether *pmk-1(lf)* worms were hypersensitive to ethanol toxicity and found significantly impaired crawling behavior in comparison to *nsy-1(gf)* mutants (p-value = 0.00). These findings further support the idea that immuno-compromised mutants are more vulnerable to the effects of toxic agents and bacterial infection, and for the first time, we show that innate immunity in *C. elegans* is modulated by prolonged ethanol exposure.

The *nsy-1* and *pmk-1* loss-of-function mutant animals we tested had lower average number of head bends (5.6 and 5.9 head bends/ 20 sec, respectively) than the wild-type animals (6.8 head bends/20 sec) in the absence of ethanol treatment (figure 5). To account for these genotype-specific crawling differences, we calculated the effect of ethanol relative to the untreated crawling rates for each strain. We found *nsy-1(lf)* and *pmk-1(lf)* mutants showed significantly reduced number of head bends in comparison to *nsy-1(gf)* worms indicating that the crawling deficits are mediated by ethanol and not by the specific genotype (p-value = 0.00 for *nsy-1(lf)* and *pmk-1(lf))* (figure 5).

To further explore alcohol-induced toxicity, we assessed lethality following

chronic ethanol exposure. Because earlier results indicated that ethanol treated *nsy-1 (lf)* and *pmk-1 (lf)* mutant worms had dramatic locomotor impairments, we predicted increased lethality rates in these strains. We observed ~ 29% dead *pmk-1 (lf)* worms in comparison to only ~8% dead wild-type animals. Intriguingly, *nsy-1 (lf)* mutants did not display enhanced ethanol-induced lethality as was observed in *pmk-1(lf)* mutants, with only ~ 1% dead worms (figure 6).

Chronic ethanol exposure results in degeneration of cholinergic neurons

Analysis and characterization of cholinergic neuronal degeneration in our laboratory prompted us to study the effect of chronic alcohol exposure on the health of these neurons (VC4 and 5). Using fluorescent reporters (see methods), we first investigated whether alcohol treatment induced cholinergic degeneration in wild type strains. We discovered that this specific subset of cholinergic neurons was dying following ethanol treatment (Figure 7). Compared to untreated 'wild type' worms, ethanol treated worms had significantly higher percentage of VC4 and VC5 degeneration (~45%). To our knowledge, this is the first demonstration of cholinergic degeneration following prolonged alcohol exposure in *C. elegans*.

To test whether increasing *nsy-1* activity produced resistance against alcohol-induced neuoredegeneration, we compared wild-type versus *nsy-1(gf)* mutant worms and found significant resistance in these mutants to damage to cholinergic neuron somas and processes (figure 8). This suggests that boosting innate immunity through *nsy-1* ameliorates alcohol-induced toxicity as well as neurodegeneration.

DISCUSSION

We have discovered behavioral effects of prolonged ethanol exposure on *C. elegans*, suggesting this genetically tractable organism is useful for studying conserved mechanisms of alcohol-induced effects. Our study complemented previous findings by demonstrating that *nsy-1(gf)* mutants are resistant against the toxic effects of prolonged exposure to ethanol in addition to amphetamines and dopamine (Schreiber & McIntire, 2012). Moreover, our results provide evidence that genetic modulation of *nsy-1* activity (by either increasing or decreasing *nsy-1* signaling) bidirectionally influences ethanol-induced toxicity. For instance, we found that *nsy-1* gain of function mutants had increased crawling rates compared to wild type after chronic treatment to ethanol. Conversely, *nsy-1(null)* mutants showed significantly inhibited locomotion rates compared to wild type and *nsy-1(gf)* worms. This indicates that alcohol exposure may trigger activation of the conserved p38 MAP kinase pathway—an innate immune signaling cassette that is activated in response to pathogenic attack and osmotic and oxidative stress (Krishna and Narang, 2008; Inoue et al., 2005). We propose that increased *nsy-1* activity increases protein NSY-1/ASK1 synthesis, which in turn may promote cell survival and immune-like function yielding protection against toxic agents including ethanol.

We show that alcohol induces degeneration of cholinergic neurons and that increasing *nsy-1* activity ameliorates this degeneration. Further experiments will reveal whether alcohol exposure triggers degeneration in other classes of neurons, and whether this is a patterned degenerative process. Additionally, we will need to investigate ethanol

toxicity using lower ethanol concentrations at various time points. By doing so, we will determine whether there is a time-dependent effect of ethanol on crawling, neurodegeneration and lethality in *nsy-1* and *pmk-1* mutants. Furthermore, it will be important to determine p38/MAP kinase pathway's activity biochemically and the mechanism of ethanol-induced toxicity and neurodegeneration—is neuronal death occurring via a necrotic or apoptotic process?

Deciphering the role of innate immunity in the degenerating central nervous system (CNS), identifying the mechanism underlying alcohol-induced neurodegeneration and lastly, understanding how alcohol modulates immunity would be vital in providing therapeutic approaches for the treatment and prevention of neuronal damage.

CONCLUSION

Our results suggest that the level of p38 MAP kinase innate immune signaling bidirectionally influences alcohol-induced toxicity in *C. elegans*. To our knowledge, this study provides the first example of neurodegeneration following prolonged alcohol in *C. elegans*. We believe this is the first evidence demonstrating that boosting innate immune signaling via *nsy-1* produces enhanced resistance to alcohol-induced toxicity and neurodegeneration. More research is needed for a better understanding of the mechanisms of p38 MAP kinase-activity dependent induction of immune response and its role in alcohol-induced CNS damage. We hope that the results presented in this study may provide insight into the genetic modulators and signaling pathways associated with

alcohol-induced neurodegeneration and toxicity, and that the knowledge learned from worms can be tested in species closer in homology to humans, such as rats and mice.

MATERIALS AND METHODS

Nematode culture and strains: Strains used were wild-type *C. elegans*, and mutant strains BZ981 *nsy-1 (eg691)*; AU3 *nsy-1(ag3)*; and KU25 *pmk- 1(km25)*. For visualizing VC neurons, Ptph-1::GFP (strain LX959) was used. Strains were grown on nematode growth media (NGM) agar plates seeded with OP50 bacteria at 20°C as previously described (Brenner et al., 1974). Animals cultured on plates contaminated with fungi or other bacteria were excluded from this study.

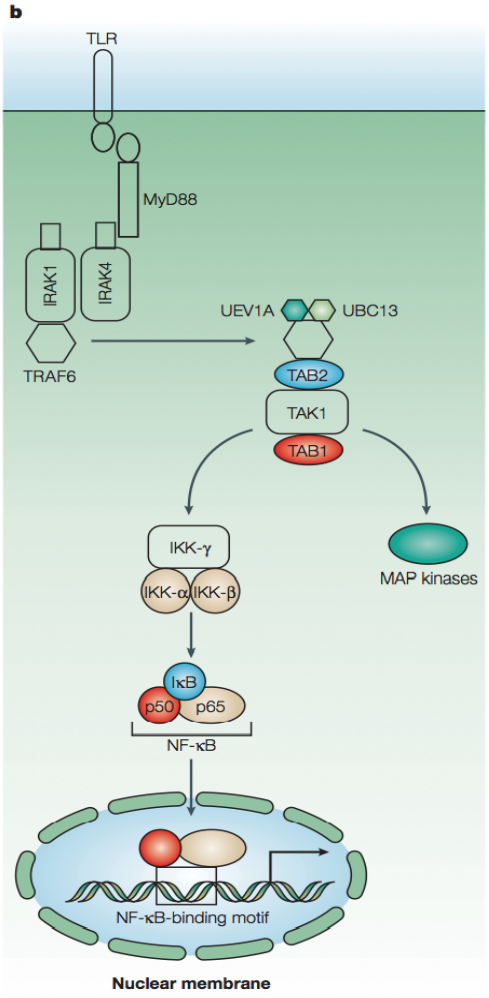
*Behavioral Tests***:** For all behavior assays, a suspension of E. coli (50 microliters) was placed in the center of plates and allowed to air-dry. Each assay was conducted on worms beginning at L4-larval stage onward over several trials. 15 minutes prior to the assay, cold 100% ethanol was added to the dried assay treatment plates to a final concentration of 400 mM ethanol unless otherwise noted.

Crawling and survival assays: 15 worms were placed on ethanol plates; then plates were sealed and stored at room temperature (20°C) and scored for crawling and survival at 72 hours of ethanol exposure. For locomotion, worms were scored for number of head bends in a 20 second window using one-way (single strain concentration effects) or two-way (multiple strains comparing both genotype and concentration) ANOVA. Only worms crawling on the bacterial lawn were scored. Significance is indicated for $p < 0.05$ with

Bonferroni correction. For survival, the percentage of dead animals after 72 hours of ethanol exposure was compared using Chi-Square Test.

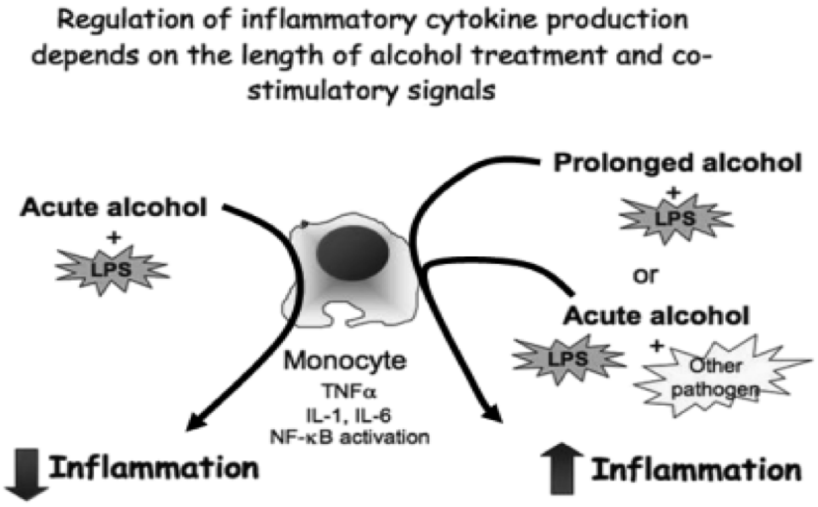
*Quantification of Neurodegeneration***:** Age-synchronized animals were paralyzed on 2% agar pads containing 0.7 mM sodium azide. Neurons that had dimly-lit somas with missing/broken projections or absent GFP labeling in the appropriate neuronal location were scored as degenerating. All animals were evaluated within 10 minutes of azide treatment. All animals were treated at L4-larval stage onward with the sterilization drug 5-fluoro-2'-deoxyuridne (FUDR, 0.12mM final) (Sigma). The percentage of VC4 and VC5 neurons that succumbed to alcohol-induced degeneration was compared using one-way (single strain concentration effects) or two-way (multiple strains comparing both genotype and concentration) ANOVA.

Figure 1: TLR signaling in mammals



Akira, S. and Takeda, K.2004.Toll Like Receptor Signaling. *Nat Rev Immunol*. **4**:499 -511.

Figure 2: Ethanol effect on production of proinflammatory molecules



Crews et al., 2006. Cytokines and alcohol. *Alcohol Clin Exp Res*. **30**: 720–730.

Figure 3: Dose-dependent deficit in crawling after prolonged exposure to ethanol. Average number of head bends determined over a 20 second period following 72 h exposure to ethanol is shown for wild type (N = 25 trials of 15 animals per trial). For all figures (except figure 3), data shown are 95% mean CI; (*) indicates significance at $\alpha = 0.05$ (**) $\alpha = 0.01$ with multiple comparison Bonferroni correction.

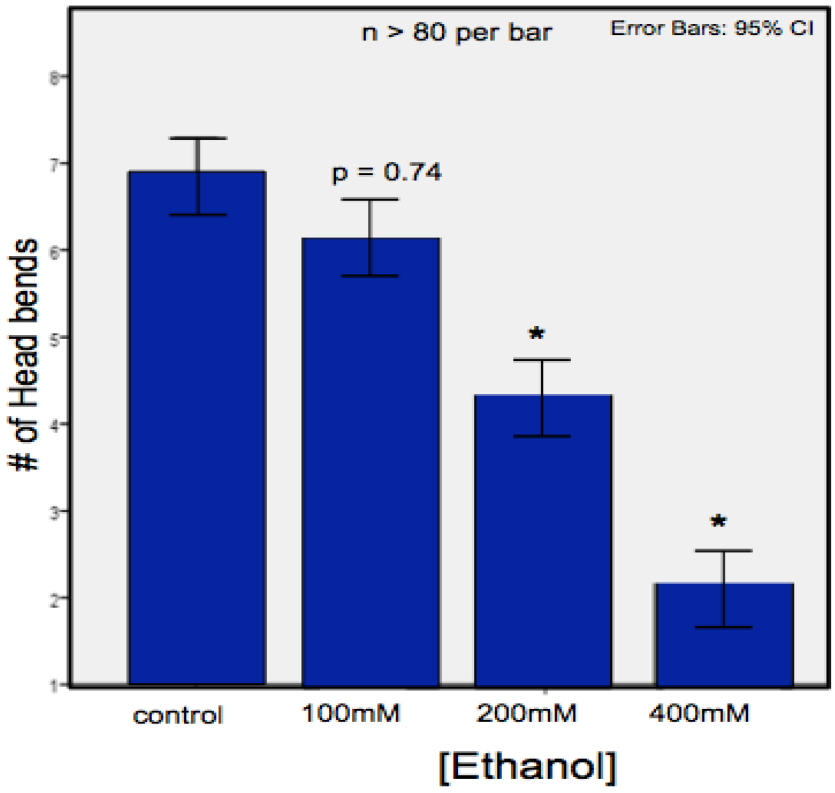


Figure 4: Crawling deficits following prolonged exposure to ethanol in innate immune mutants. Boosting innate immune signaling through gain of function mutation in *nsy-1* resulted in enhanced resistance to ethanol-induced toxicity compared to wild type (p-value = 0.00). Loss of function alleles in *nsy-1* and *pmk-1* resulted in hypersensitivity to ethanol compared to wild-type and *nsy-1(gf)*, respectively).

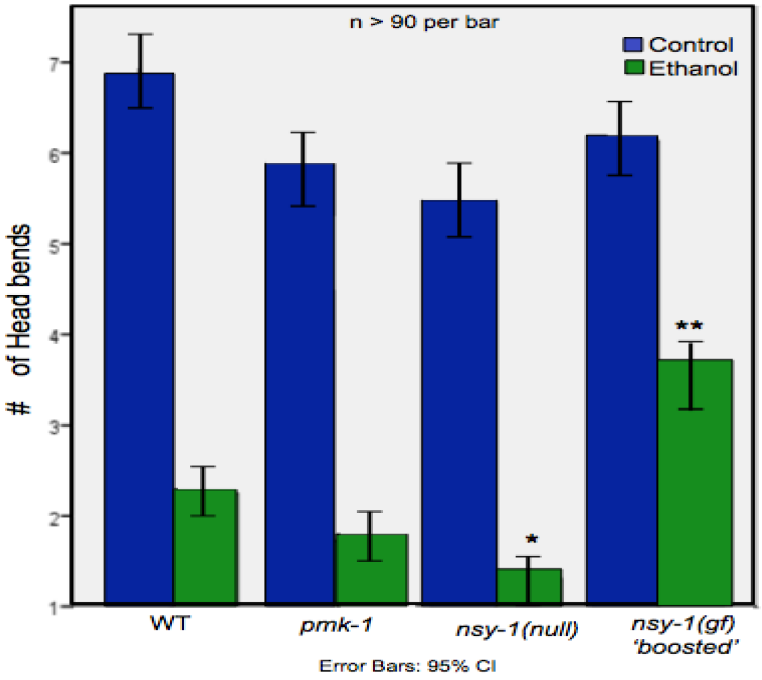


Figure 5: normalized to untreated. Increasing activity through *nsy-1* gene resulted in enhanced resistance to ethanol-induced toxicity compared to wild type. Loss of function alleles in *nsy-1* and *pmk-1* resulted in hypersensitivity to ethanol compared to wild-type and *nsy-1(gf)*.

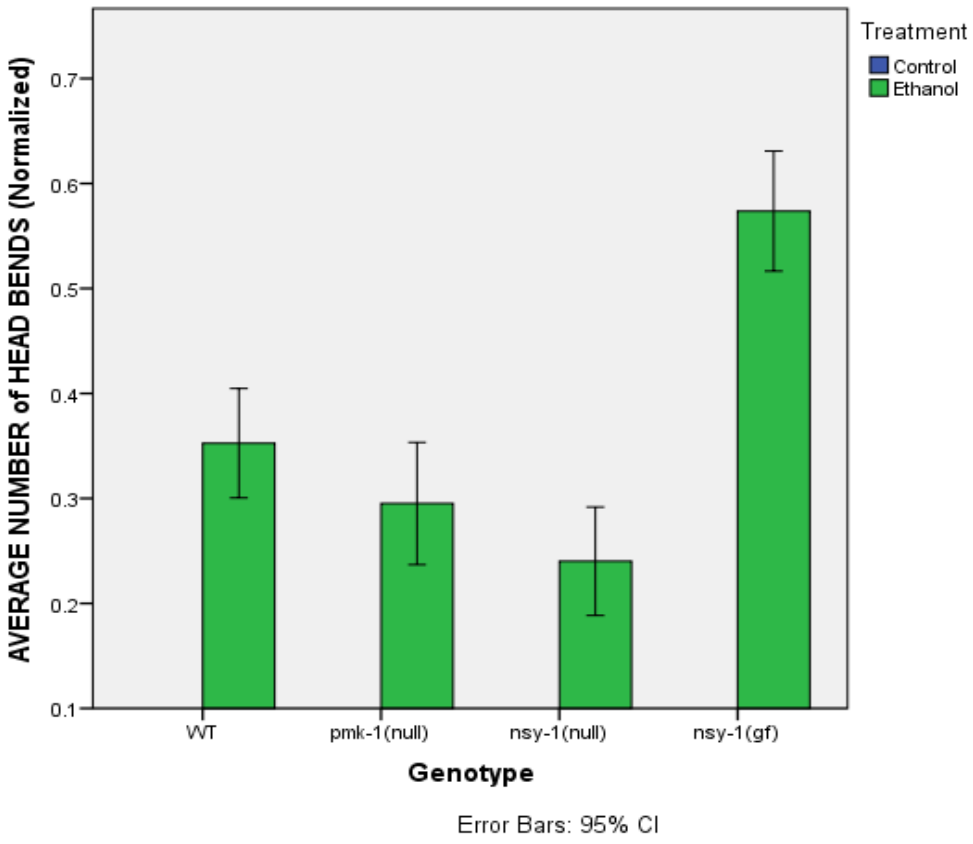


Figure 6: Lethal effect of prolonged exposure to ethanol. *pmk-1(lf)* mutants exhibited higher lethality (29.3%) to ethanol in comparison to wild type animals (8.3%). Lethality is less than 1% for untreated strains. Statistical analysis was conducted using Chi Square Test.

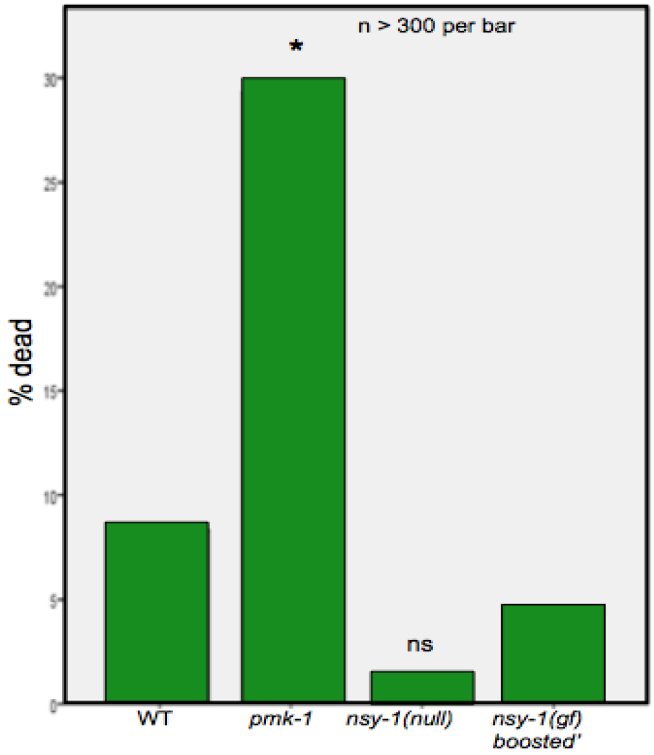


Figure 7: Chronic ethanol exposure results in degeneration of cholinergic neurons in wild type worms. On 400mM, wild type exhibited 47% degeneration. Untreated wild-type animals had 19% degeneration.

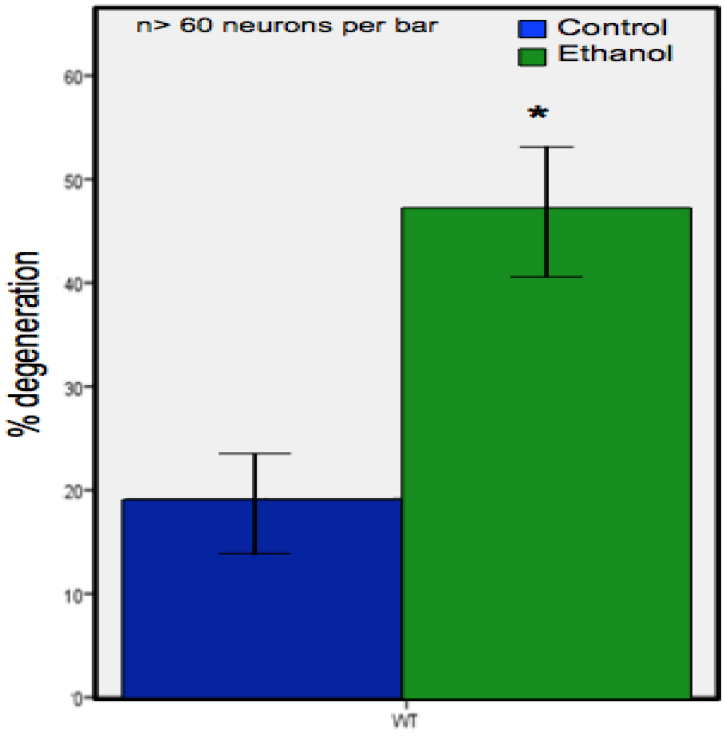
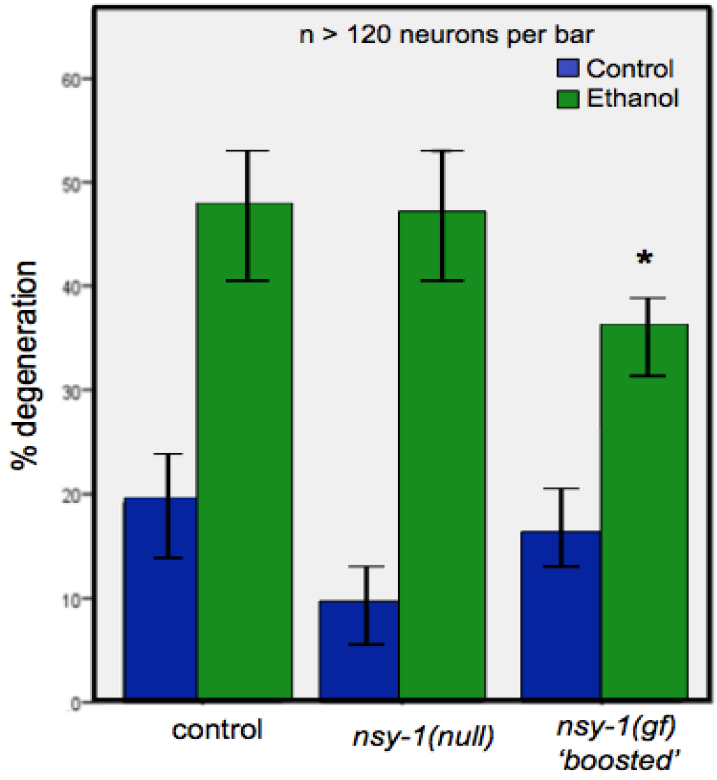


Figure 8: Boosting innate immune signaling protects against alcohol-induced cholinergic degeneration. *nsy-1(gf)* mutants show lower % degeneration (35%) compared to wild type (47%) and *nsy-1(lf)* (47%).



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