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The Analgesic-Like Properties of Alcohol in Animal Models of Chronic Pain

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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.

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ANOVA	analysis of variance
AUD	Alcohol Use Disorders
BEC	Blood Ethanol Concentration
CFA	complete Freund's adjuvant
CCI	chronic constriction injury
CDC	Center for Disease Control
CIP	congenital insensitivity to pain
CPA	conditioned place aversion
CPP	conditioned place preference
CRF	corticotropin releasing factor
ETOH	ethanol (alcohol)
IASP	International Association for the Study of Pain
i.g.	intragastric
i.p.	intraperitoneal
i.pl.	intraplantar
LORR	loss of righting reflex
MPE	maximum possible effect
PAMP	pathogen associated molecular profile
i.g.	per oral
SAL	saline
SEM	standard error of the mean
s.c.	subcutaneous
Veh	vehicle
WHO	World Health Organization

Abstract

The Analgesic-Like Properties of Alcohol in Animal Models of Chronic Pain
By Bradley John Neddenriep, B.S.

A Dissertation submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Chronic pain and excessive alcohol consumption are individually problems in our society today. Alcohol Use Disorder (AUD) affects 15.1 million adult Americans each year. Chronic pain affects over 100 million people annually in the United States. However, there is growing evidence suggesting that these two conditions can often be interrelated with chronic pain increasing consumption of alcohol, and excessive alcohol consumption increasing pain that leaves a feedback cycle trapping millions of patients in an ever worsening spiral. Large population-based studies show an association between pain and alcohol abuse, suggesting a link between increased alcohol use and reduced pain. While rodent studies consistently demonstrate antinociception following acute ethanol administration in hot-plate and tail-flick tests. However, little is currently known about the effects of alcohol in chronic pain models. We hypothesize that acute ethanol administration will possess analgesic-like properties in models of chronic pain by engaging opioid receptors in addition to its more commonly studied action at the GABA receptor.

The first aim of this study was to characterize the antinociceptive effects of alcohol in Complete Freund's Adjuvant (CFA) and Chronic Constriction Injury (CCI) mouse models of chronic inflammatory and neuropathic pain models, respectively. The second aim of this study is to investigate the mechanisms behind ethanol's analgesic-like effects including tolerance, receptor activation and correlates with blood alcohol content. Lastly, we investigated whether alcohol maintains its analgesic-like effects in non-reflexive assays in addition to effects in reflexive assays.

Chapter 1: GENERAL INTRODUCTION

1.1 Background and Significance

Chronic pain affects an estimated 116 million American adults and costs the nation up to $635 billion each year (Committee on Advancing Pain Research, Care, and Education; Institute of Medicine, 2011). Chronic pain being defined by the American Pain Society and the International Association for the Study of Pain as pain that “persists beyond the normal tissue healing time, which is assumed to be 3 months” (IASP, 1986). This means that one out of every three adult Americans will experience a painful event that progresses into a chronic pain syndrome. When left untreated, pain can become more complex in its pathophysiology than the pain caused by the original injury or disease. These changes can involve structural and functional alterations in the nervous system such that pain ceases to be symptomatic of the initial cause and becomes an entirely separate condition (Fine, 2011). The burden of chronic pain can be seen not just in the patients’ pain levels, but in comorbid psychological disorders that frequently arise in chronic pain patients. Chronic pain is associated with increased rates of major depressive disorder, suicidal ideation, suicide attempts, anxiety disorder and personality disorder (Ohyayon et al., 2003)(Ratcliffe et al., 2003). In addition to these various comorbidities patients with chronic pain are also noted to be at an increased risk of substance abuse disorders (Apkarian et al, 2013). Though opioids are the most commonly prescribed medication for the treatment of chronic pain (Fine, 2011) the interaction between opioids and chronic pain is already being exhaustively studied due to the growing opioid crisis facing America. More commonly consumed than prescription opioids, however, is alcohol (ethanol). In 2010, it

was found that 138 million American adults, over 50% of all US adults, regularly consumed alcohol, defined as 3-4 drinks per week (CDC, 2010) with an estimated 18 million American adults suffering from clinically defined alcohol abuse disorder (AUD). And unlike the opioid crisis there seems to be a lack of investigation into the interaction between chronic pain and alcohol.

Alcohol (ethanol) abuse is one of the leading causes of preventable death in society responsible for nearly 3.2% of deaths worldwide (WHO, 2004). It occurs regardless of social or ethnic affiliation. It is estimated that at least one person in every family in Western society has suffered, directly or indirectly, from alcoholism. In the United States alone, 4% of the population is affected with the estimated economic burden exceeding $366 billion a year (Chatterjee *et al.* 2010). Alcohol abuse has vast health consequences contributing to a multitude of medical complications including damage to several important organs such as the liver, pancreas, and brain, as well as immune functions. For example, data show that alcohol drinking may lead to increased risk of various psychological disorders such major depressive disorder, suicidal ideation, suicide attempts (Fine, 2011). Alcohol-induced brain damage is a particularly serious problem during pregnancy and adolescence due to the sensitivity of the developing brain to alcohol. Up to 7/1,000 infants are born with fetal alcohol syndrome, one of the most common non-genetic, forms of mental disability (Niccols, 2007). Adolescents, who display as much as a 30% prevalence rate of binge drinking, have higher sensitivities to alcohol-induced brain dysfunction and cognitive impairment of the adult brain, and the onset of adolescent drinking increases the risk for developing alcoholism in the future (Grant & Dawson, 1998; Slawecki *et al.* 2004).

Furthermore, alcohol abuse also has a high comorbidity with chronic pain syndromes (Egli, 2012).

In surveys of population level behavior, it is seen that 25% of chronic pain patients drink heavily and frequently qualifying for AUD (CAPRCE, 2011). This contrasts with a prevalence of 6.2% in the US adult population as a whole (SAMSHA, 2015), meaning that a chronic pain patient is 400% more likely to suffer from AUD than an average US adult. Also, amongst patients reporting alcohol consumption 38% of them do so “to treat pain” (Alford et al 2016). This means that chronic pain patients are more likely to suffer from AUD, and that a large portion of people consuming alcohol are doing so to self-medicate against some type of pain. From this it can be hypothesized that chronic pain is a risk factor for AUD, and that consumption of alcohol can be a self-medication for some alcohol users to ameliorate their pain in much the same manner that opioids are being abused.

Conversely alcohol consumption also seems to be a risk factor for pain, and is even capable of producing neuropathy with prolonged excessive exposure. Among individuals reporting alcohol as their drug of choice 73% report moderate to severe pain to their physicians during regular visits (Kim et al., 2013). This is compared to a prevalence of 28% of patients reporting moderate to severe pain in the US population as a whole (NSDUH, 2015).

Pain is often considered a curse that society is better off without, the French physician, Dr. Albert Schweitzer, proclaimed in 1931 that, “Pain is a more terrible lord of mankind than even death itself.” However, much as death is necessary to allow room for future generations to grow, pain is essential to our survival as individuals and

evolution as a species. Acute nociceptive pain is a normal function of the nervous system that provides important sensory information about the environment, and it is an early warning mechanism that protects against noxious heat, extreme cold, chemical irritants, and mechanical tissue damage. The importance of perceiving pain is most striking in patients with congenital insensitivity to pain (CIP), a rare genetic disorder linked to altered expression of several genes (Nagasako et al., 2003; Oertel and Lötsch, 2008). In particular, a null mutation in the SCN9A gene encoding for the Nav1.7 sodium channel is causally linked to the inability of CIP patients to feel thermal and mechanical pain (Cox et al., 2006). Without acute nociceptive pain, individuals with CIP may continue to engage in harmful behavior that puts them at risk of severe injuries and even death (Protheroe, 1991).

In stark contrast to CIP patients, who feel no pain, chronic pain patients have a heightened sensitivity to pain. The most common symptom of which is spontaneous pain. These opposing pain disorders, CIP and chronic pain, illustrate the paradox of pain; although acute pain is necessary and protective, chronic unremitting pain confers no known physiological advantage and can be so severe that individuals sometimes prefer death. Recently, it has become apparent that chronic pain is not simply long-lasting acute pain and that the underlying mechanisms of these two types of pain are fundamentally distinct: acute pain is a physiological function of the normal nervous system, whereas chronic pain is the manifestation of a pathologically altered nervous system (Woolf and Salter, 2000). An example of this is notable advancements of our understanding pain on the spinal level (Honore et al., 2000). Specifically, increased output of spinal lamina I neurons is causally implicated in the sequelae of chronic pain

(Bester et al., 2000; Coull et al., 2003). Lamina I neurons normally respond only to noxious stimuli, but after peripheral nerve injury or ongoing inflammation, the output of these neurons is transformed from nociceptive-specific to wide-dynamic-range responses (Keller et al., 2007). This functional shift in lamina I neurons may provide a neuronal correlate for allodynia, hyperalgesia, and spontaneous pain. This growing understanding of the spinal cords role in chronic pain is a great example of progress, but it is an improvement in a single area while dozens remain unexplored.

1.2 Molecular and neurobiological overlap of pain and reward.

Although, historically, alcohol was thought to be a relatively unspecific pharmacological agent, studies over the past few decades reveal that this drug has at least a few known primary targets that mediate its more significant effects on brain signaling. While it is true that alcohol can exert a number of significant effects via its metabolic products (i.e. acetylaldehyde), the acute behavioral effects associated with acute alcohol exposure are primarily attributed to the first direct interaction of ethanol with specific molecular targets followed by numerous indirect effects on a variety of neurotransmitter/peptide systems (Vengeliene *et al.* 2008, Spanagel *et al.* 2009). These effects, in turn, result in alterations in gene expression, leading to lasting neurophysiological changes that can trigger alcohol-seeking behavior with repeated exposure. It has been previously hypothesized (Le Magnen et al., 1980; Franklin et al. 1998) that the positive hedonic state produced by addictive drugs is associated with an indifference to pain because neural substrates of analgesia and neural substrates of reinforcement overlap. Some evidence suggests that alcohol's analgesic effects are

mediated by neuroreceptor systems involved in alcohol reinforcement. For example, pharmacological studies partially support the involvement the mu-opioid receptor (MOR) in alcohol's antinociception effects in rodents (Boada et al., 1981; Campbell et al., 2006; Campbell et al., 2007) in addition to its reinforcing effects (Cunningham et al., 1998; Walker and Koob, 2008). Gene knockout studies implicate G protein-coupled inwardly rectifying potassium 2 (GIRK2) channels as a major signal transduction mechanism for analgesic actions of many different drug classes including alcohol as assessed in mice using acute thermal hyperalgesia (Blednov et al., 2003, Ikeda et al., 2000, Mitrovic et al., 2003) and also suggest a role in alcohol reward (Hill et al., 2003) in animal models. Simply put the initial interaction between alcohol and its targets will result in an affective/behavioral change that triggers alterations and rewiring of the neural pathways responsible for pain and addiction.

It is believed to be the indirect effects of alcohol that ultimately cause the interaction in the neural circuits responsible for pain and alcohol reward because of the changes in neuronal plasticity. Initial nociceptive sensitivity is often associated with hyperalgesic priming, a form of peripheral sensitization involving neuronal plasticity in primary afferent nociceptors (Reichling and Levine, 2009). In contrast, central sensitization mechanisms represent an augmented response to pathological pain states involving the propagated recruitment of central neurons in the nociceptive response, leading to a broadening of nociceptive field and amplification of pain processes (Woolf and Salter, 2000, Latremoliere and Woolf, 2009). Central sensitization of pain corresponds to the functional enhancement of nociceptive circuitry along the ascending neuraxis, including the dorsal horn of the spinal cord (Woolf, 1983), the rostroventral

medulla (Porreca et al., 2002) and various limbic centers such as the central amygdala and prefrontal cortex (discussed below). In turn, functional gain in reinforcement-related limbic centers associated with a recruitment of stress (Koob and Le Moal, 2008) or immune system (Crews et al., 2011) factors may modify the central processing of nociceptive stimuli, resulting in aberrant plasticity linking pain and various affective disorders associated with the compulsive seeking of pain relief. Indeed, both pathological pain (Ji et al., 2003) and addiction (Nestler, 2001, Hyman, 2005) have been conceptualized as disorders of dysregulated neural plasticity involving mechanisms commonly ascribed to learning and memory processes. Consequently, functional enhancement of shared central circuitry following a history of excessive drinking or chronic pain may facilitate negative reinforcement, whereby compulsive drinking serves as a pain-reduction process.

The complexities of all these interactions are compounded by the fact that ethanol causes both acute and lasting changes in gene expression upon repeated exposure to the drug. There are many changes in signal transduction caused by ethanol exposure, but a primary pathway worth highlighting is the cAMP-PKA pathway that involves the activation of cAMP response binding element (CREB). This transcription factor leads to the expression of numerous ethanol-responsive genes (Diamond *et al.* 1997, Ron *et al.* 2005). These genes control significant physiological functions including neurotransmission, cell structure, signal transduction, metabolism and more (Lonze & Ginty, 2002). Among these CREB-targeted genes, some of the most notable regarding alcohol dependence include corticotropin releasing factor (CRF), prodynorphin, brain-derived neurotrophic factor, and neuropeptide Y, though

there are numerous others (Heilig & Koob 2007, Blendov *et al.* 2006, Martinez *et al.* 2005). Additionally, there are also CREB-independent genes as well as epigenetic effects induced by ethanol (Aragon *et al.* 1991, Egger *et al.* 2004, Saxonov *et al.* 2006). All these aforementioned changes may occur in a regionally-specific manner or in the brain as a whole. Thus, while the complexities of ethanol's actions on the brain are vast, they also overlap heavily with the complex pain circuitry in humans leading to hypothesis that a pathological feedback loop between chronic pain and alcohol consumption can enhance both pain and AUD in human patients.

Figure 1. Representation of the neurocircuitry of pain and addiction, and how they both overlap heavily in human brains. (Adapted from Egli et al 2012).

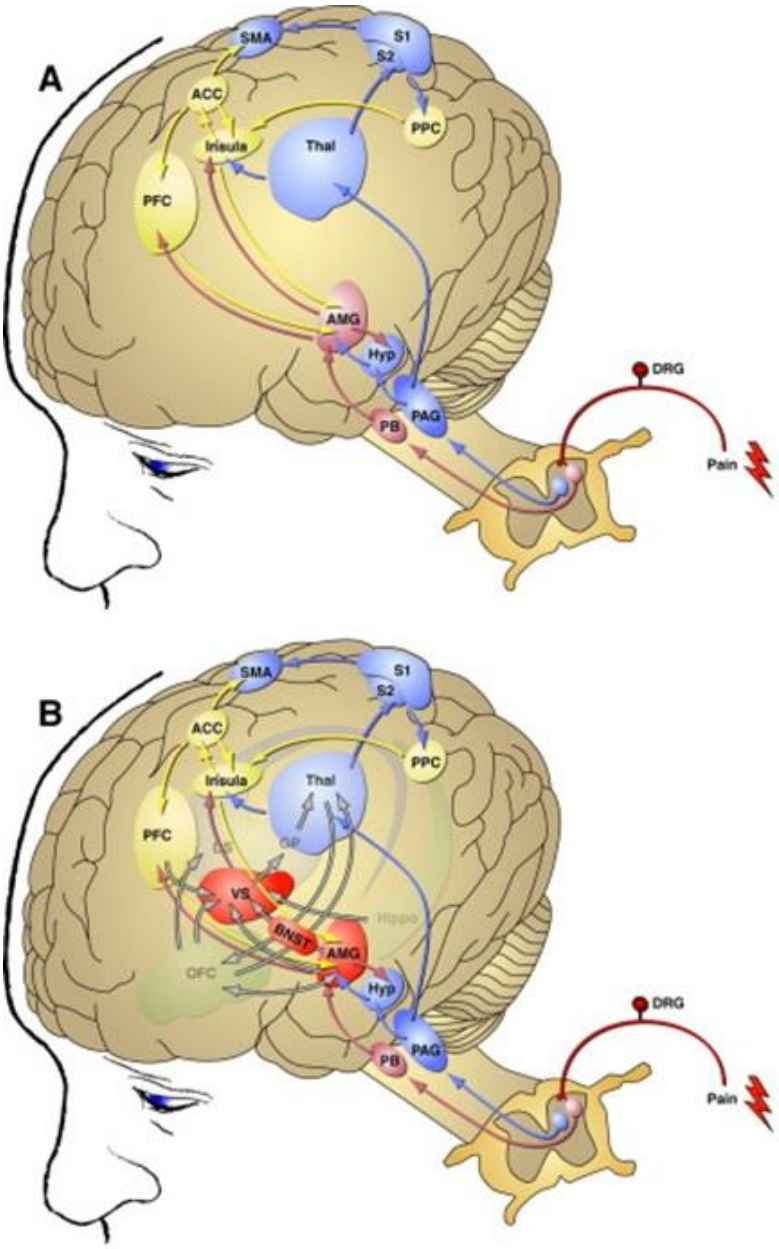

Figure 1. Intersection of neural substrates mediating nociception and alcohol dependence. (A) Ascending pathways mediating the supraspinal processing of pain. Blue structures are involved in the "fast" processing of pain via the spinothalamic tract and arrive indirectly at the amygdala. Pink structures are involved in the "fast" processing of pain via the spinoparabrachial-amygdala pathway that arrives directly at the amygdala. Yellow structures are involved in the "slower" cognitive/affective processing of pain. (B) Pathways for the supraspinal processing of pain superimposed on key elements of addiction circuitry implicated in negative emotional states. Addiction circuitry is composed of structures involved in the three stages of the addiction cycle: binge/intoxication (ventral striatum, dorsal striatum, and thalamus), withdrawal/negative affect (ventral striatum, bed nucleus of the stria terminalis, central nucleus of the

amygdala; red structures), preoccupation/anticipation (prefrontal cortex, orbitofrontal cortex, and hippocampus). Note the significant neuroanatomical intersection of the supraspinal regulation of pain and addiction in the amygdala.

1.3 Animal models of pain

Preclinical animal models confer several advantages in that they: 1) allow the detailed study of chronic pain at the cellular and molecular level; 2) facilitate identification, design, and testing of candidate pain-relieving compounds; and 3) inform us on the safety of these novel compounds before testing and use in the human population. However, as a major difficulty, animals cannot self-report, which makes assessing the extent and severity of pain a major challenge inherent to all animal pain models. The underlying assumption in each model is that an animal's behavior in response to noxious stimuli can be reliably and objectively evaluated and that this objective measurement reflects some dimension of the human experience of pain. These animal models can be broadly grouped as evoked, spontaneous or affective/emotional (Burma et al, 2016).

1.3.1 Evoked assessment of pain.

Evoked tests involve application of a noxious stimulus to a subject and measuring the time it takes to react in a manner that would avoid the noxious stimulus. Commonly employed tests such as the tail-flick or tail-immersion test are believed to reflect spinal-mediated nociceptive responses (Le Bars et al., 2001), whereas the hot–cold plate test engages both spinal and supraspinal nociceptive mechanisms (Gårdmark et al., 1998; Le Bars et al., 2001). Several techniques have been developed to assess both mechanical allodynia/hyperalgesia (Frey, 1896; Randall and Selitto, 1957) and

thermal hypersensitivity; by radiant heat (Hargreaves et al., 1988), acetone test (Yoon et al., 1994), hot–cold plate (Woolfe and Macdonald, 1944), or water bath (D'Amour and Smith, 1941). Measurements of mechanical allodynia are particularly reliable for detecting altered nociceptive thresholds in nerve-injured or chronically inflamed animals (Muley et al., 2016; Reitz et al., 2016).

1.3.2 Spontaneous Measurements of Pain

Although spontaneous pain is a debilitating feature of chronic neuropathic pain, its study has been stifled by a critical lack of assessment tools. Recently, the grimace scale has emerged as a highly reliable and reproducible assay for spontaneous pain (Langford et al., 2010). The grimace scale, developed for use in mice, rats, rabbits, cats, and horses, grades orofacial features that correlate with pain and vary depending on the pain severity (such as tightening or closing of the eyes and presence of nose and cheek bulge), which is similar to pain assessment scales used in human infants and nonverbal adults (Keating et al., 2012; Leach et al., 2012; Matsumiya et al., 2012; Costa et al., 2014; Holden et al., 2014). Although the grimace scale is valuable for assessing acute responses to pain, its utility for monitoring the progression and chronicity of pain is less reliable because animals, just like humans (Craig et al., 1991), learn to mask behaviors demonstrating pain or weakness (Matsumiya et al., 2012). Other non-reflexive measurements of pain include weight bearing (Schött et al., 1994; Bove et al., 2003), home cage monitoring for abnormal behaviors (such as altered locomotor activity nesting or grooming; Houghton et al., 1997; Goulding et al., 2008; Richardson, 2015; Negus et al, 2016), and open-field tests (Bailey and Crawley, 2009; Parent et al., 2012). Animals can also be subjected to free-choice tests (place conditioning, place-escape

tests; Sufka, 1994; Davoody et al., 2011; Fuchs and McNabb, 2012), which correlate ongoing pain with reward processes. In combination with pharmacological interventions, place conditioning is used to assess an animal's motivation to seek pain relief and indirectly assess the effectiveness of the treatment based on the animal's preference or aversion to the environment that has been paired with the treatment. Although measures of spontaneous pain are less direct and less conducive to high-throughput testing, there are numerous advantages to measuring non-reflexive or spontaneous pain in preclinical studies.

1.3.3 Assessment of Affective and Emotional Aspects of Pain

In addition to measurements of spontaneous or evoked pain, there has been a push toward evaluating the affective and emotional consequences of pain. The emotional toll of chronic pain has often been overlooked in preclinical studies, not necessarily because of ignorance but likely because there has been a lack of reliable experimental models. Studies investigating the interrelationship between affect and chronic pain have used the forced-swim, open-field, or elevated plus-maze tests, which evaluate anxiety-like behaviors in animals with persistent pain (Bailey and Crawley, 2009; Yalcin et al., 2014). Home-cage monitoring can reveal more subtle changes in behavior, such as locomotor activity (Buvanendran et al., 2008), nesting (Negus et al., 2016), burrowing, and voluntary wheel running (Cobos et al., 2012), that can provide additional insights into the wellbeing of an animal (Richardson, 2015). Despite the availability of behavioral assays to monitor quality of life or levels of stress and anxiety,

animal models of chronic pain are limited by their ability to recapitulate fully the comorbid diseases known to associate with the human chronic pain condition.

Given these reports we have determined that our measurements of pain should include all these aspects of pain. Mechanical threshold will allow us to determine mechanical hypersensitivity and antinociception in a manner widely accepted in the field. However, this approach only reveals the sensory dimension of pain, and as such we will be including conditioned place preference and voluntary wheel running as measures of the more spontaneous and affective/emotional dimension of pain.

The overall goal of this thesis is to use animal models to explore the interaction of alcohol and chronic pain in the hope of contributing new findings that could lead to the discovery of novel therapeutic targets that may aid in curbing excessive alcohol drinking and improvements in the treatment and management of chronic pain.

Chapter 2: Characterization of the analgesic-like effects of ethanol in acute and chronic pain mouse models.

2.1 Introduction

Chronic pain is estimated to cost over 635 billion dollars annually (CAPRCE, 2011). Chronic pain is also highly comorbid with major depression, suicidal ideation and suicide attempts (Fine, 2011) Excessive alcohol consumption was estimated to cost 249 billion dollars annually in 2010 (NCCDPHP, CDC, 2010). AUD is highly comorbid with personality disorders including major depression and suicidal ideation (Darvishi et al, 2015). Combined these two problems account for nearly 900 billion dollars annually and are two major risk factors for suicide and major depression which accounts for 93 billion and 210 billion dollars respectively leaving 1.2 trillion dollars of potential loss from these issues (Sheperd et al., 2015; Health Care Bulletin: 2015). This trillion-dollar problem is not just two separate issues, there is significant evidence that AUD and chronic pain are highly comorbid, including growing evidence that they share neurobiological circuitry that will react to priming from either source (Egli et al., 2012). As a result, it is important to understand the interaction between ethanol and chronic pain to better address AUD and chronic pain in our society.

Alcohol has mixed effects based on tissue concentration ranging from grossly stimulatory effects at lower doses to highly sedative effects at higher doses (Hendler et al. 2013). This range in effects can make translation into behavioral models more difficult because of the inverted-U shape of its dose effect curve. Alcohol is also most

commonly consumed by drinking in human individuals, and the pharmacokinetics of ethanol administered orally are different from alternate routes of administration with slower more prolonged rises of BEC reported in oral administration (Iwaniec and Turner 2013). This means a complete time course and dose response curve is necessary to fully understand ethanol's effect in behavioral models.

Until recently investigations into alcohol's antinociceptive effect has been limited to more acute pain in both humans and animals. Besides anecdotal and historical suggestions of alcohol being analgesic, it was shown that alcohol was antinociceptive against acute thermal hyperalgesia in humans (Perinno et al, 2008). In rats it has also been shown to be antinociceptive to thermal stimuli based on intraperitoneal administration before a hotplate test (Campbell, 2006; Gatch, 2009). However, current studies have been limited in their translation due to several factors. These studies used intraperitoneal injections of ethanol that show different pharmacokinetics than intragastric administration (Livy et al., 2003). These studies have been done in both inbred and outbred strains of mice, but studies have used male mice instead of examining both sexes for potential sex differences (Poine et al., 1991; Adalsteinsson et al., 2006). Initial investigations into the mechanism of action behind this acute antinociceptive effect have implicated opioid receptors, specifically mu opioid receptor activation (Mogil et al., 1993; Campbell et al., 2006). This provides us with an ample background to expand upon, but still leaves many questions regarding chronic pain and ethanol unanswered.

Another common phenomenon seen in drugs of abuse, including ethanol, is behavioral tolerance. In general, drug tolerance is characterized by a reduction of the drug's effect on a behavioral parameter, either via altered metabolism of the drug or via altered functionality, where the effects of the drug decrease in spite of unaltered concentration. Alcohol administration in rodents produces many well-known behavioral effects including anxiolysis, impaired motor coordination, impaired cognitive function, sedation, and hypnosis. There are multiple classes of tolerance described for alcohol behavioral effects and they are defined by the timeframe and pattern of exposure. First, acute and rapid functional tolerance can occur within minutes of the first drink (Kalant, 1998). Chronic tolerance is the decrease in sensitivity that develops as a result of repeated exposures to a drug and is measured in days or weeks rather than minutes (Kalant, 1998). Much remains to be established regarding development of tolerance to alcohol-induced antinociception in rodents. Tolerance to ethanol's antinociceptive effects of alcohol in the tail-flick test was reported in rats after chronic exposure to the drug in liquid diet (Gatch, 1999). The goal of the present study was to investigate the development of rapid and chronic tolerance to the antinociceptive effects of ethanol in mouse chronic pain models.

The aim of our studies was to characterize the effects of ethanol as an antinociceptive agent in mouse models of pain. Given the previous reported data on ethanol's anti-nociceptive effect in acute settings and the difference in acute/chronic pain, we chose a series of tests to model amelioration of chronic pain behaviors in mice of both sexes after acute oral and chronic administration (gavage) of ethanol. After replicating some acute data for validity, we tested a range of ethanol doses against

mice inflamed with Complete Freund's Adjuvant (CFA) or Chronic Constriction Injury (CCI) to determine its effect on their mechanical withdrawal threshold as measured by Von Frey Filament. CFA is inflammatory agent made from heat killed mycobacteria. The suspension mimics the pathogen associated molecular profile (PAMP) recognized by our immune system. This induces both paw inflammation and mechanical hyperalgesia that last at least for 2 weeks (Sheehan et al 2019). CCI is a surgical technique that induces peripheral neuropathy by constricting the trunk of the sciatic nerve with an immunogenic material (Bennett and Xie, 1986). This neuropathic model of pain induces mechanical hyperalgesia for over 8 weeks. (Bagdas et al., 2015). While previous reports suggest that alcohol has antinociceptive effects in acute noxious stimuli, its effects in chronic pain models are currently unknown.

We then attempted to correlate the antinociceptive effects of alcohol in chronic pain models with the blood ethanol concentration of mice dosed with behaviorally active doses of the drug. Additionally, studies of acute pain have shown that tolerance develops to the antinociceptive effects of ethanol much as it develops to other behavioral measures such as loss of righting reflex (LORR) (Werner et al., 2009; Radcliffe et al., 2013) Given this phenomenon occurring in studies of acute pain we investigated the development of tolerance to antinociceptive effects of ethanol in chronic pain models. Furthermore, we determined the effect of alcohol on total activity by locomotor boxes after acute ethanol gavage to assess the impact of ethanol concentrations used in our studies on overall animal activity.

2.2 Methods

2.2.1 Animals

Male and female adult C57BL/6J mice (25-30 g; 8-10 weeks) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a 21°C humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care (AALAC)-approved animal care facility. They were housed in groups of five and had free access to food and water. The rooms were on a 12-h light/dark cycle (lights on at 6:00 a.m.). Mice were 8–10 weeks of age and weighed approximately 20–25 g at the start of all the experiments. All experiments were performed during the light cycle (between 6:00 a.m. and 6:00 p.m.) and the study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. All studies were carried out in accordance with the National Institute of Health guide for the Care and Use of Laboratory animals.

2.2.2 Chemicals

For antinociceptive activity ethanol was dissolved in water and prepared as a 20% (v/v) solution which were delivered by oral gavage (i.g.) for all experiments. Ethanol doses (.5 – 1.25 g/kg) were chosen based on effective doses obtained in dose response curves conducted before each study, which were consistent with those found in literature (Alanka *et al.*1992, Browman *et al.*2000). To induce peripheral inflammation, mice were injected with intraplantar (i.pl.) complete Freund's adjuvant (CFA; Sigma-Aldrich, MO, USA), using a 1710 TLL Hamilton microsyringe (Hamilton Company, NV, USA) with a 30½-gauge needle. To assess changes in paw volume and mechanical threshold, mice were injected in the left hindpaw with 20 µl of CFA undiluted (100%

pure). Control animals received i.pl. injections of sterile mineral oil (Sigma-Aldrich, MO, USA).

2.2.3 Chronic constriction injury of the sciatic nerve

Mice were anesthetized with 4% isoflurane and maintained with 1.5-2% isoflurane in oxygen using a face mask and a vaporizer (VetEquip Inc, Pleasanton, CA). An incision was made just below the hip bone, parallel to the sciatic nerve. The left common sciatic nerve was exposed at the level proximal to the sciatic trifurcation and a nerve segment 3-5 mm long was separated from surrounding connective tissue. Two loose ligatures with 5-0 silk suture were made around the nerve with a 1.0-1.5 mm interval between each of them. Muscles were closed, and the wound was sutured. This procedure results in CCI of the ligated nerve and mechanical hypersensitivity continues at least 2 months (Bagdas et al., 2015). For sham surgery, same protocol was used without ligating of sciatic nerve. All animals were randomly assigned to CCI or sham surgeries. Animals were used between 2 to 3 weeks post-surgery and tested for their mechanical thresholds a day before stretching or CPA. While all sham mice showed similar mechanical thresholds compared to their baseline values, CCI mice showed a robust reduction on their left paw mechanical thresholds.

2.2.4 Von Frey testing

Mechanical withdrawal thresholds were determined by von Frey filaments as previously described (Bagdas et al., 2015). Mechanical withdrawal thresholds were determined, with slight modifications, as described in previous studies (Chaplan et al., 1994). Mice were placed in a Plexiglas cage on a mesh metal flooring and allowed to

acclimatize for 30 minutes before testing. Withdrawal thresholds were measured by applying a series of calibrated von-Frey filaments (Stoelting, Wood Dale, IL; logarithmically incremental force from 2.83 to 5.88 expressed in dsLog 10 of [10 pound force in milligram]) to the hind paw. Using a modified up-down method,5 in the absence of a paw withdrawal response (paw withdrawn, licking, or shaking) to the initially selected filament, a thicker filament corresponding to a stronger stimulus was presented. Once a paw withdrawal occurred, the next weaker stimulus was chosen. Each hair was presented vertically against the paw, with sufficient force to cause slight bending, and held for 2 to 3 seconds. A stimulation of the same intensity was applied 3 times at intervals of a few seconds. Mechanical hypersensitivity values are reported as %MPE= (Test Force – Post Injury Force) / (Baseline Force – Post Injury Force)

2.2.5 Hot Plate assay

Mice were placed into a 10-cm wide glass cylinder on a hot plate (Thermojust Apparatus, Columbus, OH) as a measure of antinociception. The hot plate was a rectangular heated surface surrounded by Plexiglas and maintained at 55°C. The device was connected to a manually operated timer that recorded the amount of time the mouse spent on the heated surface before showing signs of nociception (e.g., jumping, paw licks). Two control latencies at least 10 min apart were determined for each mouse. The normal latency (reaction time) of 8 to 12 s was assessed with a saline injection.

2.2.6 Complete Freund's Adjuvant

Mice were injected i.pl. with 20 µL of CFA (100 %undiluted) and a vehicle of mineral oil was used to approximate the original chemical properties of pure CFA as purchased.

The left hind paw was injected after placing the animals in a restraint tube. Animals were tested for their mechanical hypersensitivity 24 hours after administration of CFA.

2.2.7 Blood Ethanol Concentration (BEC) Analysis

All BEC analysis was performed by Justin Poklis at VCU NIDA P30 Center. Blood was collected following i.g. injection of 20% EtOH 1.25 g/kg using venous puncture of the mouse facial vein and the blood collected in heparinized (Elkins-Sinn, Inc., Cherry Hill, NJ) tubes. The blood was then prepared so that the ethanol could be extracted and quantified via gas chromatography/mass spectrometry (GC/MS) Calibrators were prepared from blank whole blood. Fifty µl of deuterated ethanol (1 ng/µl, Radian Corporation, Austin TX) were added to 250 µl of calibrator blood and samples were allowed to stand overnight. The following day, 2.5 ml of cold acetonitrile (Fisher Scientific, Raleigh, NC) was added drop-wise, the mixture was vortexed, centrifuged (Marathon 6 K Centrifuge, Fisher Scientific) at 4000 rpm for 10 min, and then stored in the freezer (−20°C) overnight, allowing three layers to form. The acetonitrile layer was removed, 2 ml of 0.2 N NaOH was added, and the mixture was vortexed. Next, 4 ml of 9:1 hexane:ethyl acetate (Fisher Scientific, Raleigh, NC) was added and the vials were then vortexed and spun at 30 rpm for 30 min. After mixing, the vials were centrifuged (4000 rpm) for 10 min. Once again, the organic layer was removed and evaporated to dryness while heated to 40°C. Upon drying, 50 µl of derivatizing agent (Regisil plus 10% TMCS, Regis Technologies, Morton Grove, Il) was added and vortexed. The vials were heated at 40°C for 1 h. Each sample was injected into a GC/MS (Hewlett Packard 6890, Palo Alto, CA) with a split/splitless injection port and a Hewlett Packard 7683 autosampler for quantitative analysis. The mass selective detector (MSD) was a Hewlett Packard model 5973. The initial oven temperature was 190°C and

the final temperature was 230°C. The injection-port temperature was 230°C and the transfer temperature was 280°C. An HP-1 column, 12 m×0.2 mm, 0.33 μm film thickness was used.

2.2.8 Tolerance studies

Chronic tolerance was established by a regimen of daily gavage with 1.25 g/kg or vehicle. Animals selected as the “tolerance” group were given an initial dose of 1.25 g/kg ethanol before assessing their mechanical hypersensitivity. This group was then given daily gavage of ethanol for either 4 or 10 days. After the 4 or 10 days of repeated injection the animals were given a challenge dose of 1.25 g/kg and their mechanical hypersensitivity was reassessed. Animals in the vehicle group went through a similar paradigm as the “tolerance” mice and except 10 doses of vehicle were given between initial testing and challenge testing. Mice were male and female C57BL/6J that had received a CCI injury as previously reported in the above methods.

2.2.9 Antagonism studies

Antagonism studies were performed using the previously described method for determining mechanical threshold in animals following CCI surgery using von Frey filaments. All antagonists were given as an intraperitoneal injection before being placed on an experimental mesh. Following antagonist pretreatment animals were then given an oral gavage of 20% ethanol at 1.25 g/kg. Naloxone was given as a 30-minute pretreatment time at doses of 2 and 4 mg/kg. Naltrindole, a delta selective antagonist, was given as a 30-minute pretreatment at a dose of 10 mg/kg. Nor-BNI, a kappa

selective antagonist, was given as a 2-hour pretreatment at a dose of 10 mg/kg. Mechanical hypersensitivity was then assessed over a 2-hour period post gavage. Results from minute 30 are reported below and correspond to the antinociceptive time course of ethanol as shown in previous chapters. Antinociceptive effects are shown by increased mechanical threshold.

2.2.10 Locomotor controls

Locomotor effects induced by acute ethanol was assessed in Omnitech photocell activity cages (28 × 16.5 cm) (Columbus, OH) using a single day procedure. Each apparatus consisted of two banks of eight cells with locomotor activity recorded as the interruptions of the photocell beams for the duration of the test. Mice were allowed to acclimate to the room at least 1 hr before the beginning of testing. Animals were injected with either saline or ethanol 0.5, 1,25 g/kg (i.g.) and run for 2 hours. Locomotor activity scores were defined as the number of interruptions of the photobeam cells measured for 120 minutes. Data were expressed as mean ± SEM of the number of photocell interruptions.

2.2.11 Statistical Analysis

For all studies with both sexes 2-way repeated ANOVA was used to assess the primary variables of sex and treatment over time. For BEC we used a one-way ANOVA to determine ethanol levels over time. Data were analyzed using standard one way analysis of variance (ANOVA) with genotype as the independent variable. Each analysis was followed by Holm-Sidak post-hoc tests to further analyze significant data with the alpha level set at 0.05. %MPE calculations for Von Frey studies were determined with %MPE =

(Test value-Post Injury value)/(Baseline value- Post Injury value). %MPE calculations for Hot Plate studies were determined with %MPE = (Test Value-Baseline escape latency)/(20 seconds-Baseline escape latency).

2.3 Results

2.3.1 Ethanol induced acute antinociception in the hot-plate test

Treatment with (1-2) g/kg ethanol was able to produce a significant increase in the time to response on the hotplate assay. (Figure 2a) [$F(3,56) = 5.143$ $p = .0033$]. Subsequent treatment with 2 g/kg ethanol revealed that the peak antinociceptive response occurred 30 minutes post injection. This data was statistically significant by [$F(3,56) = 4.323$ $p = .0082$]. Our results with the hot-plate test after i.g. administration are in line with previous studies (Mogil et al., 1993; Campbell et al., 2007) demonstrating the antinociceptive effects of ethanol in the hot plate assay after i.p. administration in mice and rats. However, the antinociceptive effect of ethanol in chronic pain models is currently unknown.

FIGURE 2.

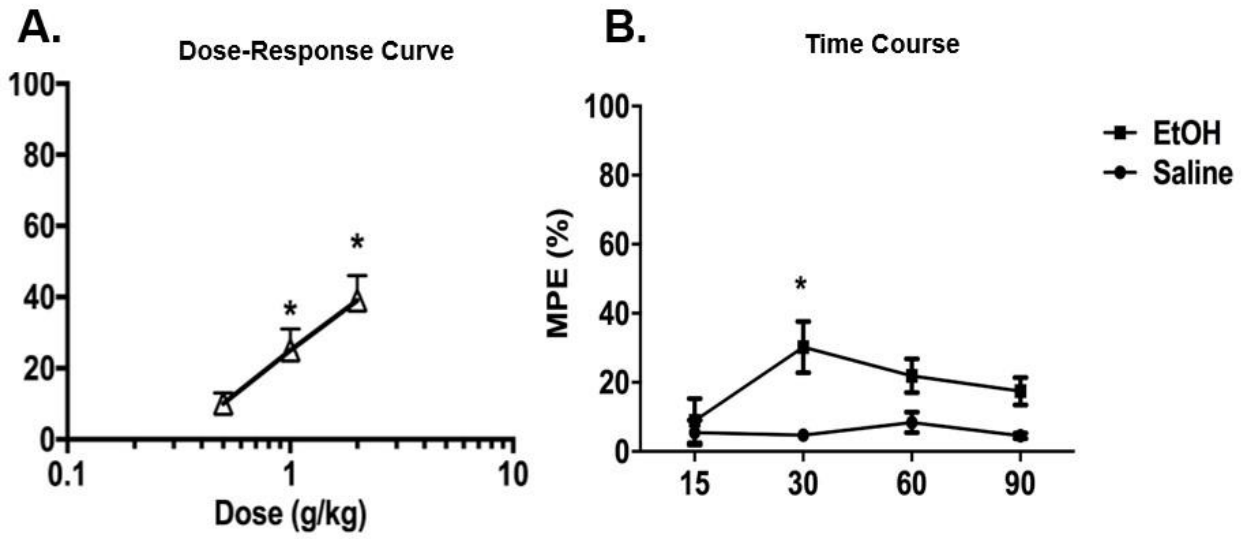

Figure 2. Antinociceptive Effects of Alcohol in the Hot-Plate Test. **A)** Dose response curve of EtOH (0.5-2 g/kg, i.g.) in mice. *P<.05 vs .5 g/kg i.g. EtOH. **B)** Time course of antinociceptive effects of 2 g/kg EtOH in the hot plate assay *P<.01 vs saline at a given time point. n=8-10 per group.

2.3.2 Ethanol induced antinociception in CCI models of neuropathy

Treatment with p.o ethanol (0.5-1.25) induced a dose-dependent antinociceptive effect in neuropathic CCI male mice [F treatment x dose (5,140) = 53.63 $p<0.0001$] and female mice [F treatment x dose (5,140) = 42.67 $p<0.0001$]. Ethanol was able to fully restore mechanical withdrawal thresholds at the highest dose of 1.25 g/kg while lower doses had a proportionately lower antinociceptive response. There were no significant differences in sex with regard to dose or time course as assessed by comparison of %MPE reversal of mechanical hypersensitivity. Peak effects were observed at 30 minutes post gavage while a significant but reduced effect was observed at 60 minutes post gavage.

Figure 3.

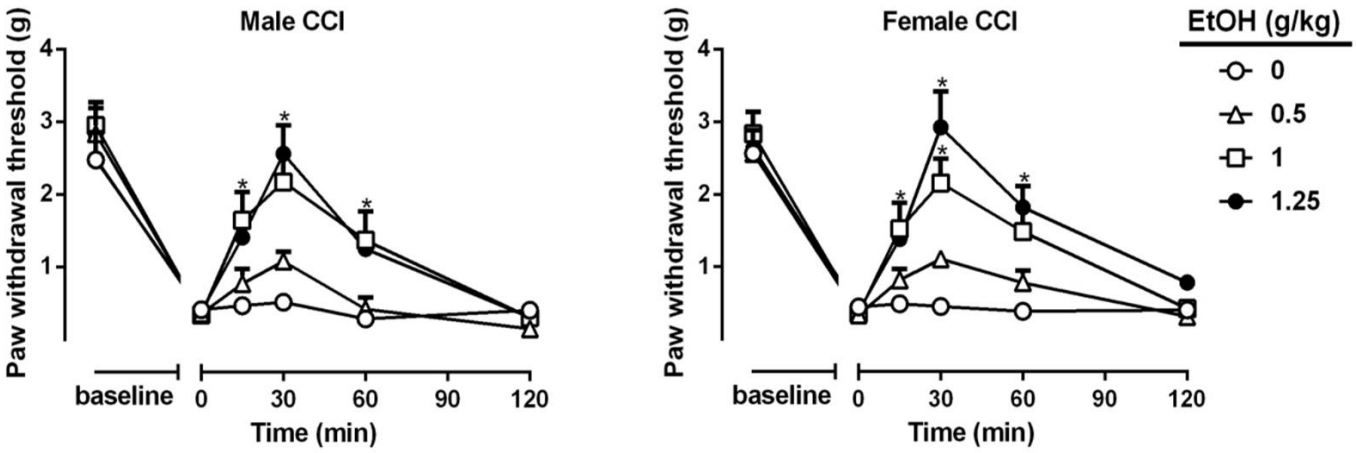

Figure 3. Antinociceptive Effects of Alcohol in the CCI model. Mechanical thresholds in male and female C57BL/6J mice before and after CCI surgery. Mechanical hypersensitivity time course after i.g. EtOH (0.5-1.25 g/kg) and their antinociceptive effect in male and female CCI animals. *P<.05 vs veh at a given timepoint. n=8 per treatment group.

2.3.3 Ethanol induced antinociception in CFA models of inflammation

Treatment with p.o ethanol (.5-1.25) induced a dose-dependent antinociceptive effect in CFA inflamed male [F treatment x dose (5,140) = 45.88 $p<0.0001$] and female mice [F treatment x dose (5,140) = 57.90 $p<0.0001$]. Ethanol was able to fully reverse mechanical hypersensitivity in the CFA-treated mice at a dose of 1.25 g/kg while lower doses had a proportionately lower antinociceptive response. There were no significant variations in sex with regard to dose or time course as determined by %MPE reversal of mechanical hypersensitivity. Peak effects were observed at 30 minutes post gavage while a significant but reduced effect was observed at both 15 and 60 minutes post gavage.

FIGURE 4.

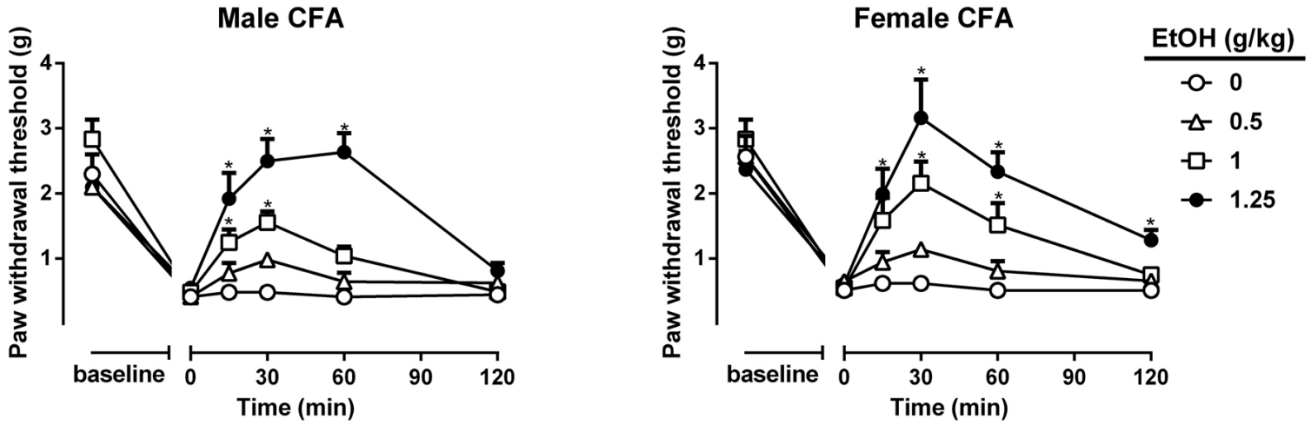

Figure 4. Antinociceptive Effects of Alcohol in the CFA model. Mechanical thresholds in male and female C57BL/6J mice before and after CFA injection. Mechanical hypersensitivity time course after i.g. EtOH (.5-1.25 g/kg) and their antinociceptive effect in male and female CFA animals. *P<.05 vs veh at a given timepoint. n=8 per treatment group

2.3.4 BEC correlation with antinociceptive response

Following injection of 1.25 g/kg, the maximally effective observed dose, there was a rapid increase in blood ethanol levels followed by a slow reduction in concentrations. This follows previously observed data on BEC reported for both mice and rats using i.g. administration (Livy et al., 2003). The maximum antinociceptive effect based on previously mentioned mechanical hypersensitivity occurs at 30 minutes

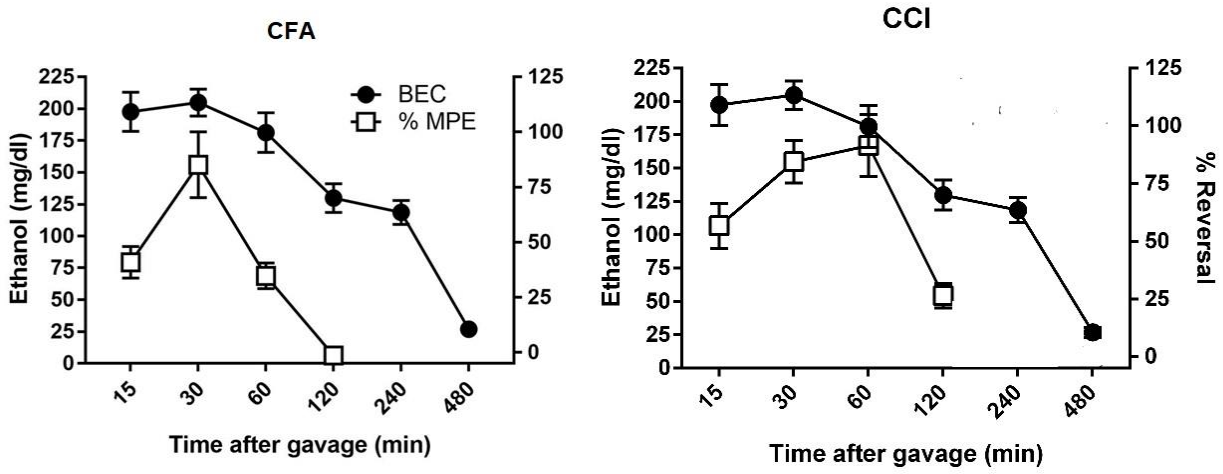

Figure 5: PD-PK relationship of the antinociceptive effects of alcohol in mice.

Subjects were male and female C57BL/6J mice. Left axis showing BEC over time. Right axis showing the reversal of mechanical hypersensitivity. Combined to show a time course of %MPE and blood ethanol content of C57BL/6J mice dosed with 1.25 g/kg EtOH i.g. % MPE = Test Value-Baseline)/(Pre CCI Values). n=8 for time points 60, 120, 240 and 480 min and n= 12 for time points 15 and 30 per treatment group.

2.3.5 Antinociceptive tolerance induced by repeated ethanol exposure

Repeated treatment with 1.25 g/kg ethanol for four days is not capable of inducing a producing a significant amount of tolerance as assessed determined by a change in the final assessed mechanical threshold compared with the initial threshold following 1.25 g/kg i.g. ethanol (Figure 5a) [$F(2,26) = 2.846$ $p = 0.1155$]. Repeated treatment with 1.25 g/kg ethanol for ten days is capable of producing a significant amount of tolerance as assessed by mechanical allodynia following a final challenge dose of 1.25 g/kg (Figure 5b). [$F(2,26) = 13.65$ $p<0.0001$] Tolerance is indicated at the 30-minute time point by having a reduced mechanical threshold in the repeated ethanol mice, while mice repeatedly gavaged with water instead of ethanol still show a high mechanical threshold after treatment with ethanol indicative of antinociception. This data shows that, similar to other behavioral effects, the antinociceptive effects of ethanol can be reduced after repeated or chronic exposure to ethanol (Werner et al., 2008). Future experiments to determine potential mechanisms by which ethanol acts as an antinociceptive agent.

FIGURE 6.

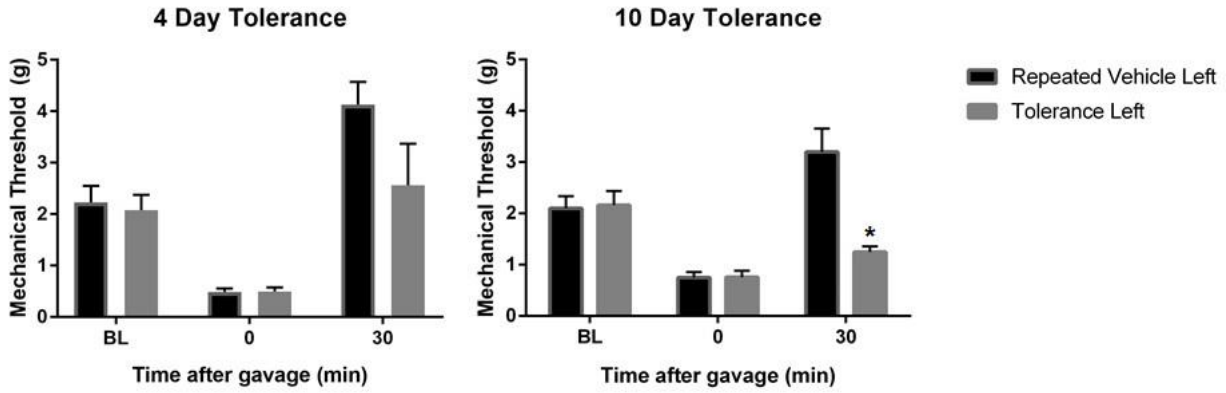

Figure 6. Tolerance to the antinociceptive effects of ethanol following repeated exposure

5a) Mechanical hypersensitivity in C57BL/6J mice following 4 days of gavage using vehicle or 1.25 g/kg ethanol. BL indicates pre-surgical mechanical thresholds while min 0 indicates the threshold following CCI surgery to induce neuropathy. Min 30 indicates the mechanical threshold 30 minutes after a pretreatment with 1.25 g/kg ethanol by oral gavage on challenge day following repeated gavage. n=8-10 per group. 5b) Mechanical hypersensitivity in C57BL/6J mice following 10 days of gavage using vehicle or 1.25 g/kg ethanol. BL indicates pre-surgical mechanical thresholds while min 0 indicates the threshold following CCI surgery to induce neuropathy. Min 30 indicates the mechanical threshold 30 minutes after a pretreatment with 1.25 g/kg ethanol by oral gavage on challenge day following repeated gavage. n=8-10 per group. * $p<0.05$.

2.3.6 Antagonism of ethanol induced antinociception in neuropathic mice

To determine possible mechanisms involved in the antinociceptive effect of ethanol on chronic pain we used a pharmacological approach to investigate the role of opioid receptor system. This was determined due to previous evidence showing the role of the mu opioid system in the antinociceptive effect of ethanol in models of acute pain (Campbell et al., 2007). The mu selective antagonist naloxone at a dose of 2 mg/kg was capable of partially blocking the effect of ethanol. A higher dose of 4 mg/kg of naloxone was capable of fully blocking the effect of ethanol (Figure 6b) [$F(2,26) = 12.44$ $p = 0.0087$]. While naloxone has highest affinity for the mu receptor (K_i 1.1nm) it also has notable affinities for both other opioid receptors, kappa (K_i 12nm) and delta (K_i 16nm) (Gouardères et al., 1985). This suggests that the increased effect from the higher 4 mg/kg dose of naloxone may be due to additional effects at the delta and kappa receptors and prompted us to investigate using selective antagonists for both kappa and delta opioid receptors. We found that pretreatment with i.p. nor-BNI, a kappa selective antagonist, 10 mg/kg but not naltrindole, a delta selective antagonist,10 mg/kg is capable of blocking the antinociceptive effects of ethanol 1.25 g/kg in neuropathic mice following CCI surgery (Figure 6a) [$F(2,26) = 8.32$ $p = 0.032$]. Together these data suggest that both the kappa and mu opioid receptors are necessary for the antinociceptive effects of mice in chronic pain.

FIGURE 7.

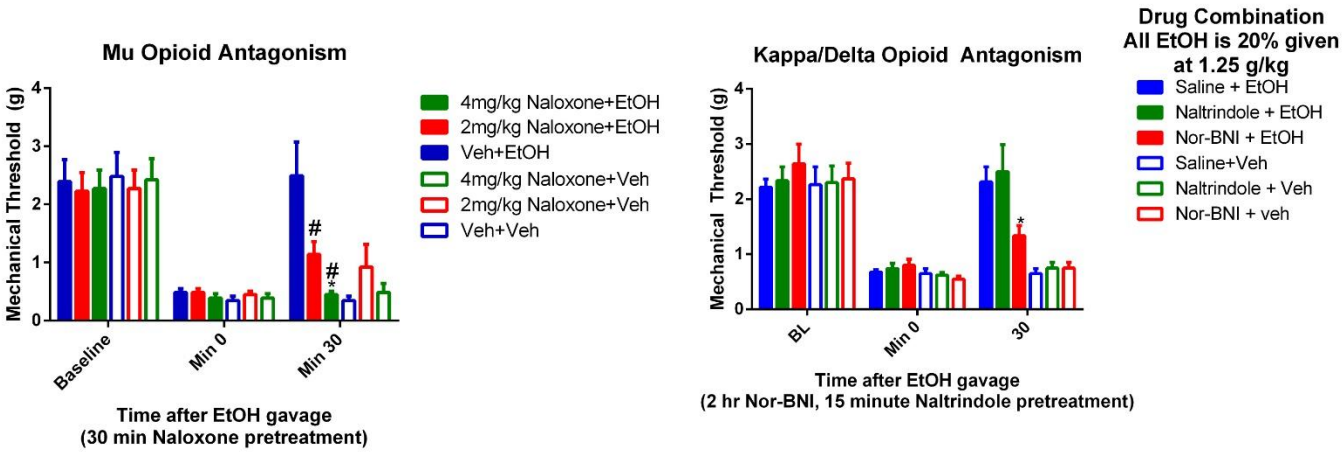

Figure 7. Antagonism of ethanol induced antinociception in CCI mice using mechanical threshold. 7a. Min 0 indicates the threshold for animals after CCI that have received only naloxone at 2 mg/kg. Min 30 indicates 30 min after i.g. EtOH in CCI animals pretreated with naloxone. *P<.05 vs veh+veh; #P<.05 vs veh+EtOH. Veh, Vehicle; EtOH, ethanol. n=8/group. **7b.** Withdrawal threshold for male C57BL/6J mice after CCI surgery. BL indicates the baseline threshold for animals after CCI. Min 30 indicates 30 min after i.g. EtOH in CCI animals pretreated with the antagonists. *P<.05 vs Saline + EtOH. n=8/group.

2.3.7 Impact of ethanol on locomotion

To determine whether analgesic-like effects of alcohol in our assays of pain were not due to motor impairment, we assessed general activity following low (0.5 g/kg, i.g.) and high dose (1.25 g/kg, i.g.) of ethanol in male C57BL/6J mice. There was no significant changes in locomotion between animals treated with saline and those treated with either dose of ethanol. [$F(2,21) = 0.1533$ $p = .8588$] (Figure 8). This suggests the doses of ethanol used to attain behaviorally effective results in our assays of pain are not confounded by motor impairment of animals.

FIGURE 8.

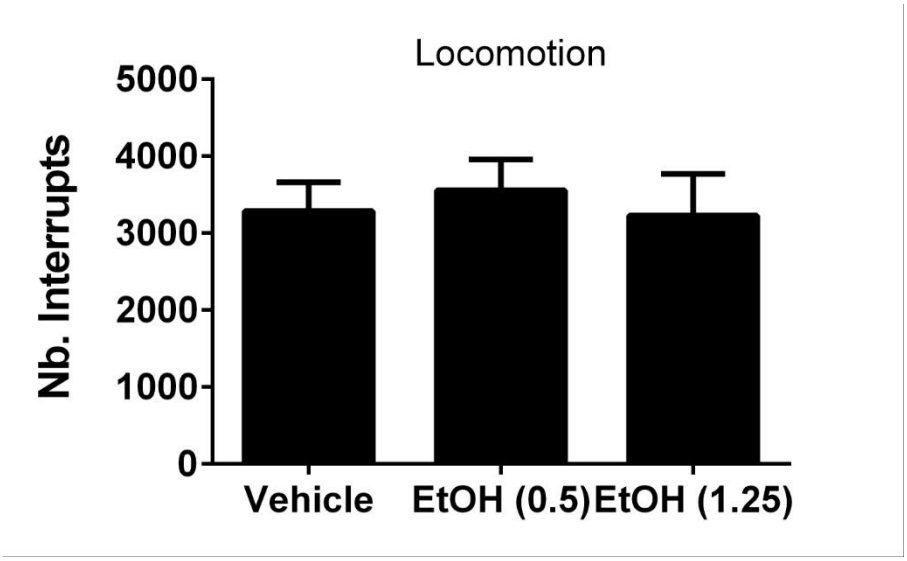

Figure 8. Impact of ethanol on locomotion

General activity assessed by beam breaks over a 120-minute period. Male and female C57BL/6J mice were used. No significant differences are observed between any treatment given. All treatments were administered by oral gavage with a pretreatment time of 5 minutes. n=6 per group

2.4 Summary

The initial goal of our experiments was to determine the antinociceptive effect of ethanol in acute thermal pain tests after i.g. administration in mice. While ethanol induced a dose-dependent antinociceptive effect in the hot-plate test after acute i.g. administration, it was not fully efficacious. These results led to further investigation into the antinociceptive effects of ethanol in inflammatory and neuropathic models of chronic pain. To explore this, we employed two models of chronic pain based on CCI induced neuropathy and CFA induced inflammation in C57BL/6J mice. It was found that ethanol was antinociceptive in a dose-dependent manner and at a dose of 1.25 g/kg it fully reversed mechanical hypersensitivity in both models of chronic pain. A time dependent effect was also observed in animals after ethanol gavage. There was a peak antinociceptive response at 30 minutes post gavage. This peak response correlated to a peak in BEC following gavage with 1.25 g/kg ethanol. Interestingly, the % MPE declines rapidly after the 30-minute timepoint, while BEC levels remain relatively high. This effect could be a result of acute functional tolerance as previously hypothesized (Erwin and Deitrich, 1996), or due to a threshold/ceiling effect of ethanol in antinociceptive behavior. Further investigation will be required to determine the cause of this effect but there is a clear association between BEC and antinociceptive effect These data could be a result of acute functional tolerance or a threshold effect for ethanol as an antinociceptive agent and deserve future consideration. An additional goal of this study was to characterize tolerance to the anti-nociceptive effects of ethanol observed in

behavioral models of pain. To test this hypothesis C57BL/6J mice were exposed to a regimen of repeated ethanol or vehicle exposure by oral gavage followed by challenge testing with a previously antinociceptive dose of ethanol. Tolerance to ethanol's antinociceptive effect was not observed after 4 days of repeated gavage of 1.25 g/kg ethanol, but after 10 days of oral gavage of the drug. This corresponds to previous reports that prolonged or chronic exposure to ethanol can create tolerance to its behavioral effects in mice such as anxiolysis, impaired motor coordination, impaired cognitive function, sedation and LORR (Werner et al., 2009; Radcliffe et al., 2013; Ozburn et al., 2013). Lastly, these studies characterized the mechanism of action mediating ethanol's antinociceptive effects in models of chronic pain. Similar to reports from studies of acute pain, we found that naloxone, a mu receptor antagonist, was able to block the effect of ethanol on mechanical hypersensitivity in the chronic model of CCI induced neuropathy. A higher dose of 4 mg/kg was more effective in blocking the effect of ethanol. The similarity in receptor shape between mu, kappa, and delta opioid receptors means that antagonists to a single receptor often have some degree of affinity at other receptors. To test the role of kappa and delta opioid receptors we used high doses of the delta selective agonist, naltrindole, and the kappa selective antagonist, nor-BNI. Only nor-BNI had an effect at 10 mg/kg suggesting that the kappa and mu systems together may be contributing to the antinociceptive effect of ethanol in chronic pain models. Further investigation into the opioid system in addition to other neurochemical systems will be needed to fully elucidate the mechanisms of action for ethanol in pain.

Chapter 3: The Effects of Alcohol on non-reflexive Assays in chronic pain models.

3.1 Introduction

Pain is a subjective human experience with many aspects that can be difficult to model in animals. This is partially due to the concept of pain evolving from one dimensional to a multi-dimensional entity involving sensory, cognitive, motivational, and affective qualities (Kumar and Elavarasi, 2016). While reflexive measures have been adequate to model the sensory dimension of pain, they have fallen short in capturing the other dimensions (De la Puente et al., 2018). As a result, new assays have been developed to better assess these affective dimensions of pain including voluntary wheel running (Cobos et al., 2012) and conditioning paradigms such as conditioned place preference/avoidance (Navratilova et al., 2013).

The Conditioned place preference (CCP) assay has been a uniquely valuable tool in assessing affective dimensions of pain by coupling animal choices and preferences with the location of a given drug treatment. This means that animals treated with a drug that experience a pleasant sensation, such as pain relief, will associate that treatment with the location or chamber in which they received the analgesic drug. Though it is susceptible to confounding interpretations from drugs that: establish preference in naïve animals such as impair memory formation and cognitive function, or alter motor function, it has been a unique tool to probe affective pain in animals that are incapable of expressing a verbal preference.

Voluntary wheel running is another assay that can be used to assess non-reflexive aspects of pain in animals. Wheel running is a voluntary behavior that mice naturally engage in when given the opportunity (Goh et al., 2015). A noxious stimulus, such as CFA, is capable of reducing the amount of running performed by an animal, while, at the same time not decreasing the animal's ability to move. This suggests that the reduced running is due to a choice to engage in less behavior because the activity is "painful", and the animals are attempting to avoid this pain. Recent studies have validated this model using morphine and non-steroidal anti-inflammatory drugs, such as ketoprofen, were to restore distance run in animals injected with CFA to the levels of their vehicle counterparts (Cobos et al. 2012).

The aim of these studies was to further characterize the effects of ethanol as an antinociceptive agent in non-reflexive assays in mice. For that, we tested ethanol in a place conditioning paradigm and voluntary wheel running after CCI surgery or CFA injection, respectively.

3.2 Methods

3.2.1 Animals

Male and female adult C57BL/6J mice (25-30 g; 8-10 weeks) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a 21°C humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care (AALAC)-approved animal care facility. They were housed in groups of six and had free access to food and water. The rooms were on a 12-h light/dark cycle (lights on at 6:00 a.m.). Mice were 8–10 weeks of age and weighed approximately 20–25 g at the start of all the experiments. All experiments were performed during the light cycle (between 6:00 a.m. and 6:00 p.m.) and the study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. All studies were carried out in accordance with the National Institute of Health guide for the Care and Use of Laboratory animals.

3.2.2 Chemicals

Ethanol was dissolved in DI water and prepared as a 20% (v/v) solution which were delivered by oral gavage (i.g. or i.g.) for all experiments. An ethanol dose of 1.25 g/kg was chosen based on effective doses obtained in previously mentioned studies. To induce peripheral inflammation, mice were injected with intraplantar (i.pl.) complete Freund's adjuvant (CFA; Sigma-Aldrich, MO, USA), using a 1710 TLL Hamilton microsyringe (Hamilton Company, NV, USA) with a 30½-gauge needle. To assess changes in distance run mice were injected in the left hindpaw with 20 µl of CFA

undiluted (100% pure). Control animals received i.pl. injections of sterile mineral oil (Sigma-Aldrich, MO, USA).

3.2.3 Conditioned place preference

An unbiased eight-day CPP paradigm was performed. A three-chamber design CPP apparatus (ENV3013; Med Associates, St Albans, VT) was used to determine possible place preference in ethanol treated mice. On day 1, animals were allowed to freely move in all chambers (two conditioning chambers with a central acclimation chamber) for a 15-min duration and the baseline time spent for each chamber was recorded. Each CPP box consisted of three chambers: two outer chambers (20 × 20 × 20 cm each; white mesh & wall or black rod & wall) and a small grey chamber in the middle connected to each outer chamber with a door. White and black chambers were used to condition animals to test drug or vehicle. Based on the time spent in each conditioning chamber, animals were divided into equal group of mice whenever is possible. Mice were confined in differed chambers after vehicle or alcohol administration (1.25 g/kg, i.g.) for 20 min for a six-day conditioning period (days 2–7). These conditioning sessions were included two sessions as morning and afternoon for each day; animals were confined in one chamber (e.g. white) in the morning and in other chamber (e.g. black) in the afternoon. While control groups received saline in both morning and afternoon sessions, the drug group received ethanol in one session and saline in other session. Pretreatment time for ethanol was 5 minutes. The drug-paired chamber was determined by randomization. Morning and afternoon sessions were 4 h apart from each other. All sessions were conducted by the same experimenter. On day

8, mice were given access to move freely in all chambers for a 15-min duration without any drug administration. The preference score was found by determining the difference between time spent in the drug paired side on day 8 versus the time in drug paired side on day 1. A significant positive response in time spent in the drug-paired chamber was interpreted as a CPP.

3.2.4 Voluntary wheel running

Voluntary wheel running was assessed in polycarbonate wheels (diameter 21.5 cm; width 5 cm) with a steel rod axle. They were placed in this wheel directly from their home cage and testing was initiated immediately after placing the animals in the test wheel. The wheel could be turned in one direction. Multiple activity cages were contained within a testing room. Rotations completed was assessed by electronic counter over a 2-hour period. Rotations completed was converted to a distance traveled by the following formula: distance traveled = (rotations completed) x (wheel circumference). All mice were naïve before initial baseline testing and no habituation or training was performed in the wheels. Wheels were free rotating and allowed mice to stop and start running at will. After the baseline values were taken, the mice were i.pl. injected in the left hindpaw with 20 uL CFA to induce peripheral inflammation, or with mineral oil as a control. The effects of i.g. alcohol on CFA-induced decrease in voluntary wheel running was assessed 3 days after CFA injection. Before being placed in their wheel mice were given an oral gavage of 1.25 g/kg and returned to their home cages for 5 minutes. A noxious stimulus should reduce the distance traveled in 2-hours while an

anti-nociceptive effect is determined by restoring distance traveled to the distance traveled by control treated (non-inflammatory) mice.

3.3 Results

3.3.3 Ethanol induced antinociception in CCI models of neuropathy

In this experiment, we used the CPP test in a model of peripheral neuropathy to evaluate the ability of ethanol to induce preference in CCI injured mice, which would be interpreted as pain relief in mice experiencing ongoing, spontaneous pain (Navratilova et al., 2016). Administration of ethanol at a dose of 1.25 g/kg i.g. shows a significant CPP in CCI Injured mice [F CCI x EtOH (1, 36) = 8.956; P = 0.005], but not in vehicle-treated mice (Figure 7). This testing was performed while CCI injured mice still showed mechanical hypersensitivity suggesting that ethanol is antinociceptive in the CCI model of neuropathic pain.

FIGURE 9.

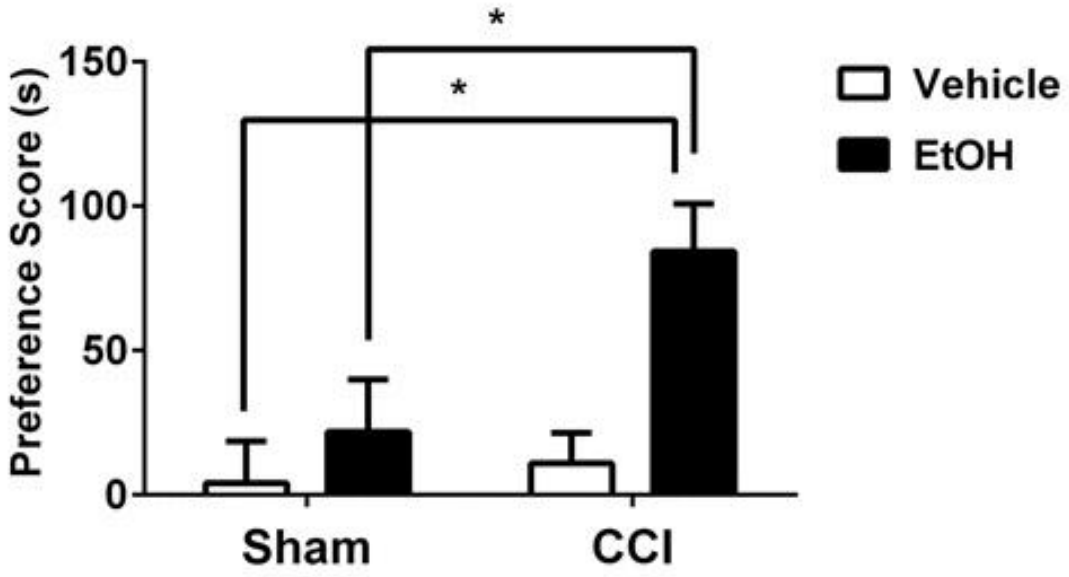

Figure 9: Antinociceptive effects of ethanol in CCI animals assessed by CPP.

EtOH induced place preference in CCI but not sham mice. C57BL/6J male mice conditioned with 1.25 g/kg or vehicle i.g. daily for 6 days as either sham or CCI animals. There is no preference established in sham animals at doses that are antinociceptive. CCI animals conditioned with EtOH display preference for the EtOH paired chamber. Preference score is calculated [(time spent drug side)-(time spent on veh side)=preference]. *P<.05 CCI EtOH vs sham. n=12 per treatment group.

3.3.4 Effects of ethanol on CFA-induced decrease in voluntary wheel running

3 days after i.pl. injection with undiluted CFA there was a significant reduction in distance traveled for CFA mice compared to their vehicle counterparts without any ethanol treatment [F CFA x VEH (1,56) = 13.65 p<.00001]. This demonstrates that CFA induced inflammation is capable of reducing voluntary wheel running when untreated. Animals treated with ethanol 1.25 g/kg following CFA injection are not significantly different than animals treated with ethanol 1.25 g/kg following a mineral oil injection [F CFA x VEH (1,56) = 13.65 p<.00001]. Together these data suggest that ethanol does not increase wheel running by itself, but when animals are in an inflamed state there is a significant increase in voluntary running that can be interpreted as an antinociceptive effect. This antinociceptive effect in an affective assay matches the previously reported data from the affective assay of conditioning, CPP.

FIGURE 10

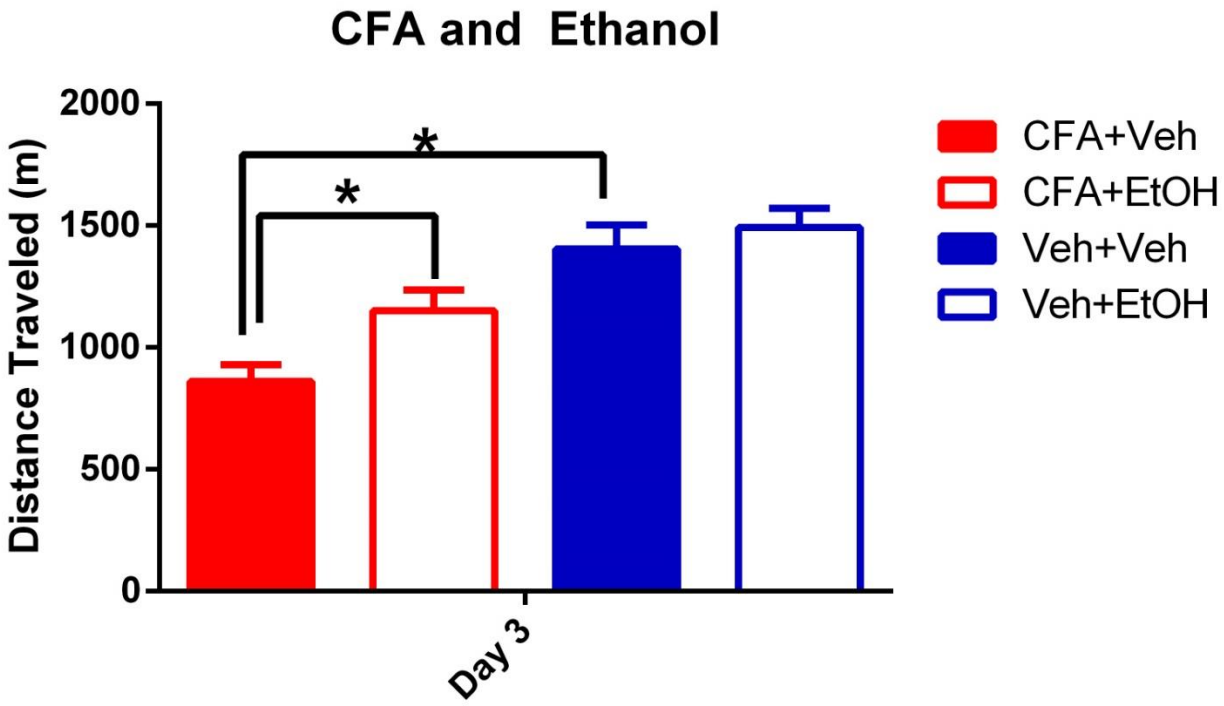

Figure 10: Ethanol induced antinociception assessed by voluntary wheel running

Distance traveled on day 3 was reduced in C57BL/6J male mice treated with CFA and vehicle compared to non-inflamed animals. *p<.05. No significant difference in distance traveled was seen between non-inflamed animals treated with ethanol and non-inflamed animals treated with vehicle. Inflamed animals treated with ethanol ran significantly further than inflamed animals treated with vehicle *p<.05. Distance traveled by inflamed animals treated with ethanol were not significantly different from non-inflamed animals treated with ethanol. n=12 per treatment group.

3.4 Summary

This study investigated the effects of ethanol in non-reflexive assays of pain. This was tested by using conditioned place preference and voluntary wheel running in C57BL/6J mice. Following CCI surgery, animals conditioned with 1.25 g/kg ethanol for 6 days did significantly prefer the ethanol paired chamber compared to the vehicle paired chamber in the CPP test. Importantly, there was also no preference shown in animals that underwent sham surgery and were conditioned with 6 days of ethanol. This suggests that ethanol had an analgesic-like effect by establishing preference in a model of neuropathic pain while creating no preference in non-neuropathic animals using equal doses of 1.25 g/kg ethanol. To test ethanol's effect on voluntary wheel running C57BL/6J mice were given an intraplantar injection of CFA to decrease running, following this noxious stimulus, animals were dosed with 1.25 g/kg ethanol and allowed to run freely. Ethanol was capable of significantly increasing the distance run in CFA animals while not altering distance traveled in vehicle treated animals. This suggests that ethanol is has analgesic-like properties in affective assays of inflammatory pain in addition to its effects in neuropathic pain.

Chapter 4: General Discussion-

The analgesic-like effect of ethanol in chronic pain and its implication in AUD.

4.1 Introduction

Prior to our studies there have been a few reports describing the antinociceptive effect of ethanol in rodent models of acute thermal pain (Mogil et al., 1993). These studies found that doses of 1-2.5 g/kg ethanol i.p. were capable of producing significant antinociception in the tail-flick and hot-plate tests using adult male rats and mice (Mogil et al., 1993; Gatch 1999; Campbell et al., 2007). However, doses of 2-3 g/kg ethanol i.p. have been shown to impair rotarod performance in male C57BL/6J mice (Stromberg 1988). Repeated administration of i.p. ethanol in rats also produces tolerance to the anti-nociceptive effects of ethanol after 8 days of ethanol exposure in liquid diet (Gatch 2002). As mentioned above, all of these studies of ethanol induced antinociception have been using i.p. administration. However, human intake of ethanol is primarily intragastric prompting us to use an i.g. gavage of ethanol in our studies. Given the excessive use of alcohol in chronic pain patients, it was important to complement these results in acute pain assays by studying the effect of alcohol in chronic pain models. Investigation has also been done into the mechanism behind the antinociceptive effect of ethanol for these acute models (Campbell et al., 2007). The mu opioid receptor system was implicated by this work and given the dysregulation in the natural opioid system following chronic pain (Schrepf et al., 2016) it is important to investigate the role of the opioid system in chronic pain models as well. Additionally, previous studies of ethanol and acute pain were limited to male animals

but given recent pushes to represent female animals equally in research our work included female animals for all antinociceptive testing in chronic pain.

Lastly, previous studies have been done with reflexive measures of pain. These measures have proven very useful for opioid development and have been widely used in the field of pain, but there has been new focus on the multiple dimensions of pain and how best to assess these varying dimensions. While reflexive measures capture the immediate sensory dimension of pain, affective assays are being developed to assess the spontaneous and emotional aspect of pain (Burma et al., 2016). Given the increased emotional component to chronic pain compared with an acute pain event, it is even more important to include affective assays in any studies of antinociception for chronic pain. As such we have included two affective measures of pain to further characterize the behavioral effect of ethanol on chronic pain models. Voluntary wheel running has been used previously to investigate analgesics in CFA inflamed mice (Cobos et al., 2016) and was used to assess the analgesic-like effect of ethanol in reversing CFA-induced reduction in wheel running in inflamed and non-inflamed mice of both sexes. We additionally used the conditioning model of CPP to determine whether ethanol is capable of producing a preference. In animals with chronic pain you can create a preference by associating analgesic treatment with a given chamber to create a model of “pain-relief” in mice by examining their choice in response to treatment. These affective assays are essential in chronic pain models because of the added emotional burden and allow for a greater characterization of chronic pain in rodents.

4.2 Results

We found that ethanol was antinociceptive in assays of inflammatory and neuropathic pain as assessed by reflexive von Frey filament testing. Ethanol was maximally effective at a dose of 1.25 g/kg without producing any sedative effects. This suggests that ethanol may be more efficacious in models of chronic pain than acute pain when comparing their %MPE in hot plate (40% MPE) and mechanical threshold testing. Though there have been previous reports of sex difference in the effects of ethanol on mice, such as increased anxiety behaviors following chronic ethanol intake in males only (Jury et al., 2017), and more rapid increases in intake in female mice compared with males (Sneddon et al., 2019) no major sex differences were found across our studies in C57BL/6J mice. Additionally, peak BEC was observed at 30 minutes post injection, which corresponds to peak antinocieption also at 30 minutes post injection in CCI-injured mice and CFA-injected mice. BEC levels in mice given 1.25 g/kg of alcohol reached a maximum of 215 mg/dl. According to CDC reports of safe drinking, this is equivalent to 10 drinks over a 2-hour timespan, or 3 times the safe legal drinking limit in most states (CDC 2016). However, the antinociceptive effect drops off rapidly over 2 hours while the ethanol concentration remains relatively stable up to 4 hours. This suggests that animals may be rapidly acclimating to the ethanol and are showing reduced behavioral responses to a constant BEC. This phenomenon has been previously described as acute functional tolerance and has been observed in various other behavioral measures of ethanol's sedative and hypnotic effects (Radcliffe et al., 2013; Ponomarev et al., 2004).

Tolerance to this antinociceptive effect in our CCI model can also develop after chronic exposure to the drug. In animals treated with a 10-day regimen of alcohol injection (i.g.), it was found that their mechanical thresholds were less affected by ethanol compared with an

initial assessment before their 10-days of tolerance induction. This effect appeared to be time dependent with a shorter 4-day treatment regimen demonstrating a non-significant reduction in the effect of ethanol, similar to recent studies showing 4 days of ethanol exposure to be insufficient in producing tolerance to the antinociceptive effects of in rats (Gatch, 2002)

Consistent with previous reports (Campbell et al., 2007), we were also able to determine that both the mu and kappa opioid receptors are necessary for ethanol's antinociceptive effects in chronic pain. However, our results show that delta opioid receptors were determined did not play a significant role, since the delta selective antagonist naltrindole was unable to alter the antinociceptive effect of ethanol. Other non-opiate receptors such as the NMDA receptor and GABA receptors are likely contributing to this effect potentially through anxiolytic mechanisms (Mogil, 1993).

We were also able to show that ethanol has analgesic-like effects in two affective assays of chronic pain. Voluntary wheel running can be used to model motivation and CFA-treated animals have been shown to decrease their voluntary wheel running (Cobos et al., 2013). We were able to show that ethanol reverses CFA-induced reduction in wheel running in, while not significantly altering voluntary wheel running in non-inflamed animals. Additionally, ethanol administration in mice induced a significant place preference for a treatment chamber when given to CCI injured animals but not their sham counterparts in the CPP test. This suggests that CCI injury is inducing a neuropathic state that is often used as a model for neuropathic pain (Bagdas et al., 2016) and this negative state can be reversed with ethanol to create a preference that can be quantified by experimenters.

These results provide a new insight into ethanol as it relates to chronic pain while confirming and expanding on knowledge gained from previous reports of the antinociceptive effect of ethanol in acute pain models.

4.3 Significance in AUD

Given our results that ethanol possesses analgesic-like properties in chronic inflammatory and neuropathic pain models in mice, it is important to consider how this can translate to human patients suffering chronic pain. In surveys of population level behavior, it is seen that 25% of chronic pain patients drink heavily and frequently qualifying for AUD (CAPRCE, 2011). This contrasts with a prevalence of 6.2% in the US adult population as a whole (SAMSHA, 2015), meaning that a chronic pain patient is 400% more likely to suffer from AUD than an average US adult. Also, amongst patients reporting alcohol consumption 38% of them do so “to treat pain” (Alford et al 2016). This means that chronic pain patients are more likely to suffer from AUD, and that a large portion of people consuming alcohol are doing so to self-medicate against a chronic pain state. While experimental studies in humans have produced mixed results in terms of alcohol analgesic effects (Thompson et al., 2017), we have shown the first direct evidence that ethanol does possess analgesic-like properties in chronic pain. One implication of this is that the painkilling properties of alcohol could contribute to the increased usage of alcohol observed in patients with persistent pain. Furthermore, the accessibility and relative inexpensiveness of alcohol is likely to encourage its use as an analgesic in preference to more difficult-to-obtain drugs or interventions. This effectiveness could explain alcohol misuse in those with persistent pain

despite its potential substantial threats to long-term health including a risk to develop further chronic pain conditions (Kim et al., 2013; NSDUH, 2015).

4.4 Future directions

While our studies were able to show that alcohol can exert analgesic-like effects in rodent models of chronic pain, the clinical observation that people suffering from chronic pain conditions may escalate their alcohol intake, is not well studied. A rigorous examination of alcohol intake in rodents following various chronic pain manipulations would provide valuable insight into the interaction between AUD and Chronic pain in humans. Additionally, the emotional and cognitive dimensions of pain should be further explored with new and innovative affective assays of pain. The above studies were also limited to CFA induced inflammation and CCI induced neuropathy, it would be valuable to investigate the effects of ethanol in more clinically relevant acute and chronic pain models such as mono-iodoacetate induce arthritis or chemotherapy induced peripheral neuropathy. Lastly, it has been shown that the behavioral effects of ethanol can vary based on genetic backgrounds (Adkins et al., 2017), so it would be useful to do studies across strains and substrains to investigate the genetic aspects to the interaction between alcohol and chronic pain.

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