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The Effects of Alcohol on BDNF and CD5 Dependent Pathways

Andrew Jordan Payne

A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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ABSTRACT

The Effects of Alcohol on BDNF and CD5 Dependent Pathways

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Alcohol represents the third leading cause of preventable death in the United States. Yet, despite its prevalent role in impeding human health, there is much to understand about how it elicits its effects on the body and how the body and brain change when an individual becomes physiologically dependent upon alcohol. The work presented herein represents an effort to elucidate the acute and chronic effects of alcohol on the nervous system. We investigate two specific protein pathways and their role in alcohol's effects on the body. The first begins with brain-derived neurotrophic factor (BDNF), which acts on TrkB, and ends with KCC2. We demonstrate that BDNF expression is increased in the VTA during withdrawal from chronic but not acute alcohol exposure and that this increase persists for at least seven days. Concomitantly, we demonstrate that the activation of GABA_A channels on produces less inhibition of VTA GABA neurons in mice treated with chronic intermittent ethanol exposure than in alcohol naïve mice. This effect likewise persisted for at least seven days. We illustrate that BDNF has no apparent direct effect on VTA GABA neuron firing rate. The second pathway begins with the T cell marker CD5 and ends with the anti-inflammatory cytokine, IL-10. We demonstrate that in a genetic CD5 knockout (CD5 KO) mouse model both alcohol consumption as well as the sedative properties of alcohol are reduced. Since CD⁺ B cells secrete more IL-10 than CD5⁻ B cells, we also demonstrate the effects of IL-10 on VTA neurons. We show that IL-10 has direct effects on VTA dopamine (DA) neurons by increasing their firing activity. We relatedly illustrate that IL-10 produces an increase in DA release in the nucleus accumbens (NAc). However, contrary to our hypotheses, we show that IL-10 produces conditioned place aversion rather than conditioned

place preference in a place conditioning paradigm, suggesting that IL-10 might mediate pain-induced secretions of DA. Collectively, these results suggest two potential therapeutic targets to reduce alcohol consumption that need further validation. They also suggest a novel mechanism for the sedative effects of alcohol at moderate and high doses.

Keywords: alcohol, ethanol, addiction, dependence, BDNF, TrkB, KCC2, CD5, IL-10, cytokine, VTA, GABA, NAc

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CHAPTER 1: INTRODUCTION

For thousands of years men and women have consumed alcohol (McGovern et al., 2004). The reasons for this behavior are distinct and varied and can range from the quest for pleasure to a desire to escape one's problems, from a social lubricant to a physiological dependence. Whatever the reason for beginning or continuing to drink, the long-term impact is clear: alcohol destroys. In 2000, alcohol use was the cause of approximately 85,000 annual deaths in the United States, which makes it the third leading cause of preventable death, trailing only behind tobacco use and poor diet and physical inactivity (Mokdad et al., 2005). That is an average of over 230 fatalities per day or roughly 1 life lost every 6 minutes. Economically, the impact of alcohol is tremendous. It has been estimated that alcohol use alone causes a \$249 billion burden to our economy (Sacks et al., 2015). For sake of reference, as of the end of Q1 2020, the annual gross domestic product (GDP) of the United States was \$21.54 trillion, which means that the economic cost of alcohol can be measured on the order of magnitude of a whole percentage point of the country's entire GDP (*Gross Domestic Product, 1st Quarter 2020 (Third Estimate); Corporate Profits, 1st Quarter 2020 (Revised Estimate)*, 2020). Saying that the economic cost of alcohol use is tremendous is an understatement.

In addition to the economic and health impact of alcohol, it takes an immeasurable toll on families and individuals. It destroys marriages and careers, relationships, and lives, and there doesn't seem to be an end in sight. It is for this exact reason that we research alcoholism. The current treatments for alcoholism have a lackluster success rate, and relapse rates can exceed 50% in the first year after treatment (Miller et al., 2001). On top of that, according to the National Institutes of Health, less than 8% of adults who had alcohol use disorder (AUD) received treatment (*Alcohol Facts and Statistics*, 2020). Indeed, we need better treatments for

alcoholism, and perhaps the best way to approach finding better treatment options is to better understand how alcohol works.

In spite of the fact that alcohol has been consumed for millennia and modern researchers have sought to understand alcohol's effects on the brain and body for decades, we are still left with many unanswered questions about the mechanisms by which alcohol produces its effects on the body. With many drugs of abuse, a mechanism of action becomes quite clear, but with alcohol we have observed many small effects throughout the body with nothing we could call a "smoking gun" to tell us how alcohol produces euphoria at low doses, sedation at high doses, and dependence with repeated doses. That is the impetus behind this work. We aim to better understand the mechanisms of action of alcohol acutely and chronically. It is our hope that by doing so we can uncover novel targets for new therapeutics that can improve the success of alcoholism treatment. We have undertaken to accomplish this by investigating two specific molecular pathways.

Brain-derived Neurotrophic factor

The first pathway is initiated by increased secretions of brain-derived neurotrophic factor (BDNF) in the ventral tegmental area (VTA). While BDNF is usually responsible for protective and restorative functions in the brain, we hypothesize that in the case of alcohol dependence, BDNF is at the inception of maladaptive processes that lead to dependence and/or withdrawal. The main receptor for BDNF in the brain is tyrosine receptor kinase B (TrkB). We believe that increases in BDNF expression lead to an increase in TrkB activity in the VTA and that this is the foundation of hyperactive inhibitory pathways that result in decreased dopamine (DA) release during withdrawal. It has been shown in the hippocampus that the activation of TrkB can induce a decrease in the expression of the potassium chloride cotransporter 2 (KCC2). Since the

primary function of KCC2 is to export from the cell the chloride that is ingested through normal inhibitory activity at γ -aminobutyric acid (GABA) synapses, a decrease in expression of KCC2 would naturally result in a rise of intracellular chloride levels, which would significantly disrupt the normal inhibitory activity of GABA type A (GABA_A) channels. This would logically lead to an increase in activity of any neuron where this change in KCC2 expression had occurred. We hypothesize that this is transpiring in GABA neurons in the VTA and that these neurons then become hyperexcitable. We anticipate that this chain of events is thereby inhibiting the activity of VTA DA neurons, making them less active and reducing the release of DA in the nucleus accumbens (NAc). The release of DA in the NAc has long been thought to be correlated with reward and reinforcement. Recent additions to the peer-reviewed literature have refined that view to include learning signals and reward prediction error as well as other factors that we will not discuss here, but suffice it to say that relegating the role of DA in the NAc to reward is a bit too simplistic. However, DA release in the NAc that is initiated in the VTA is still strongly implicated in reinforcement and addiction pathways, and as such is a critical to our understanding of alcoholism.

Neuroimmune interactions

The second pathway is related to neuroimmune interactions. It has been suggested that the nervous system and the immune system are tightly intertwined in various ways. For example, several studies have been conducted to investigate the impact of cytokines of the brain. We will present here the results of one such study conducted in our lab. Although it might seem improbable upon first hearing, the base of evidence supporting connections between the nervous system and the immune system continues to increase.

We hypothesized that the T cell marker, cluster of differentiation 5 (CD5), plays a role in alcohol reward and/or dependence. There is evidence that CD5 is involved in synaptogenesis during development, particularly on GABA neurons (Brask et al., 2004; Fujita et al., 2017; Kim et al., 2002). Additionally, CD5 seems to be a factor in the production and/or release of the anti-inflammatory cytokine interleukin-10 (IL-10). Immune B cells that are positive for CD5 release more IL-10 than B cells that are negative for CD5 (Gary-Gouy et al., 2002). Additionally, the soluble form of CD5 can bind IL-6, which we explored in connection with VTA cellular dynamics as well (Aparicio-Siegmund et al., 2017).

The structure of this work will deviate from the traditional dissertation in that each chapter represents a manuscript prepared for or published in a scientific journal. The format will be consistent with the journal to which the manuscript will be or has been submitted. In total we will highlight four manuscripts: two related to the BDNF pathway and two related to the CD5/IL-10 pathway. The level of contributions for each work are represented by their respective author lists.

Manuscript One

The first manuscript tests the hypothesis that GABA switching occurs with chronic alcohol exposure. We define GABA switching as the phenomenon described above where chloride buildup inside the cell causes the inhibitory GABA_A channels to become less effective and even tend toward becoming excitatory. We utilize cell-attached patch clamp electrophysiology and the GABA_A agonist muscimol to test this hypothesis. We generate what is sometimes called a muscimol sensitivity curve, which is essentially a dose response curve that illustrates the concentrations at which muscimol is effective at inhibiting neuronal activity. We

test these effects in animals that are alcohol naïve and alcohol dependent to assess the chronic effects of alcohol on chloride dynamics.

Manuscript Two

The second manuscript tests the hypothesis that this GABA switching is mediated by the BDNF/TrkB/KCC2 pathway described above. We utilize an enzyme-linked immunosorbent assay (ELISA) to evaluate the expression level of BDNF in the VTA and NAc of animals that are acutely exposed to alcohol, chronically exposed to alcohol, and naïve to alcohol. We use cell-attached patch clamp electrophysiology and the TrkB antagonist ANA-12 to ascertain whether BDNF has any direct effects on VTA GABA neuron activity. We utilize immunohistochemistry (IHC) staining to investigate the expression patterns of KCC2 in animals that are alcohol naïve and alcohol dependent as well as how the TrkB drugs ANA-12 and 7,8-DHF affect this expression pattern. Lastly, we utilize a drinking in the dark binge drinking paradigm to evaluate the impact of ANA-12 and 7,8-DHF on alcohol seeking behavior in naïve and dependent animals.

Manuscript Three

The third manuscript tests the hypothesis that the presence of CD5 impacts alcohol seeking behavior as well as alcohol-induced sedation. For this study, a CD5 knockout mouse (CD5 KO) was used and validated with flow cytometry to ensure that CD5 was not present in the knockout population. We utilized a 24-hour access two bottle choice drinking paradigm to measure alcohol seeking behavior and used an open field behavioral assay as well as a loss of righting reflex assay to measure the sedative properties of alcohol. We also measured the activity of GABA neurons using an *in vivo* single unit measurement technique to determine

whether CD5 had any impact on the baseline activity of GABA neurons in the VTA and measured spontaneous DA release in the NAc.

Manuscript Four

The fourth manuscript tests the hypothesis that the anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokine IL-6 affect the activity of VTA neurons. We use patch clamp electrophysiology to examine the effect that IL-10 and IL-6 have on the firing rate of VTA GABA and DA neurons in a slice preparation. This same preparation allows us to investigate the effects of IL-10 on optogenetically stimulated inhibitory post-synaptic currents (oIPSCs) and miniature IPSCs. We also use *in vivo* microdialysis and *ex vivo* voltammetry to investigate the effect of IL-10 on DA release in the NAc. Lastly, we use a place conditioning paradigm to examine whether IL-10 is, of itself, rewarding when administered directly into the cerebrospinal fluid.

Taken together, we anticipate that the results from these studies will provide evidence of novel mechanisms of the rewarding and sedating effects of alcohol. Future work to validate the involvement of KCC2 in alcohol seeking behavior as well as elucidating CD5 and/or IL-10 as potential drug targets will continue to validate these mechanisms and move toward the potential of drug development.

CHAPTER 2: Ventral Tegmental Area GABA Neurons Are Resistant to GABA(A) Receptor-mediated Inhibition During Ethanol Withdrawal

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**Keywords: ventral tegmental area, GABA(A) receptor, alcohol, VTA GABA neurons,
NMDA receptors, withdrawal, inhibition, adaptation, resistance**

Abstract

The neural mechanisms underlying alcohol dependence are not well understood. GABAergic neurons in the ventral tegmental area (VTA) are a relevant target for ethanol. They are inhibited by ethanol at physiologically relevant levels *in vivo* and display marked hyperexcitability during withdrawal. In the present study, we examined the effects of the GABA(A) receptor agonist muscimol on VTA neurons *ex vivo* following withdrawal from acute and chronic ethanol exposure. We used standard cell-attached mode electrophysiology in the slice preparation to evaluate the effects of muscimol on VTA GABA neuron firing rate following exposure to acute and chronic ethanol in male CD-1 GAD-67 GFP mice. In the acute condition, the effect of muscimol on VTA neurons was evaluated 24 hrs and 7 days after a single *in vivo* dose of saline or ethanol. In the chronic condition, the effect of muscimol on VTA neurons was evaluated 24 hrs and 7 days after either 2 weeks of twice-daily IP ethanol or saline or following exposure to chronic intermittent ethanol (CIE) vapor or air for 3 weeks. VTA GABA neuron firing rate was more sensitive to muscimol than DA neuron firing rate. VTA GABA neurons, but not DA neurons, were resistant to the inhibitory effects of muscimol recorded 24 hours after a single ethanol injection or chronic ethanol exposure. Administration of the NMDA receptor antagonist MK-801 before ethanol injection restored the sensitivity of VTA GABA neurons to muscimol inhibition. Seven days after ethanol exposure, VTA GABA neuron firing rate was again susceptible to muscimol's inhibitory effects in the acute condition, but the resistance persisted in the chronic condition. These findings suggest that VTA GABA neurons exclusively undergo a shift in GABA(A) receptor function following acute and chronic exposure. There appears to be transient GABA(A) receptor-mediated plasticity after a single exposure to ethanol that is mediated by NMDA glutamate receptors. In addition, the resistance to muscimol inhibition in VTA GABA neurons persists in the dependent condition, which may contribute to the

hyperexcitability of VTA GABA neurons and inhibition of VTA DA neurons during withdrawal as well as the motivation to seek alcohol.

Introduction

Alcoholism is a chronic relapsing disorder that has an enormous impact on society. A major goal of research on alcoholism is to characterize the critical neural substrates that are most sensitive to alcohol, adapt in association with chronic consumption and drive subsequent alcohol-seeking behavior. The mesocorticolimbic dopamine (DA) system originating in the ventral tegmental area (VTA) of the midbrain and projecting to the nucleus accumbens (NAc) is known to be involved in reward. The emerging view is that the dysregulated homeostasis that accompanies the development of drug addiction may result from experience-dependent neuroadaptations that hijack normal synaptic transmission in this system (Hyman & Malenka, 2001; Hyman et al., 2006; Kauer & Malenka, 2007; Nugent & Kauer, 2008).

The VTA is highly involved in adaptive reward and motivation processing and is composed of DA (65%), γ -aminobutyric acid (GABA; 30%), and glutamate (GLU; 5%) neurons (Dobi et al., 2010). Both *in vivo* (Gessa et al., 1985) and *in vitro* (Brodie & Appel, 1998; Brodie et al., 1990) electrophysiological studies indicate that acute ethanol increases VTA DA neuron firing rate (EC_{50} of 120 mM in the slice) and DA release in limbic structures (Imperato & Di Chiara, 1986), and that withdrawal from chronic ethanol reduces DA firing rate and release in the NAc (Diana et al., 1993). Although the release of DA in the NAc positively reinforces drug use, it has been suggested that the changes in DA are strongly regulated by VTA GABA neurons (Bocklisch et al., 2013; Nugent & Kauer, 2008; Steffensen et al., 2011; Ting & van der Kooy, 2012). GABAergic projections to the VTA come from several regions including the NAc, rostromedial tegmentum, and the ventral pallidum, but another major inhibitory regulation of VTA DA and GABA neurons is by GABAergic interneurons within the VTA (Johnson & North, 1992; Steffensen et al., 1998). In support of this, results from studies in Cre mice [GAD-Cre (Tan et al., 2012) or VGAT-Cre (van Zessen et al., 2012)] expressing channel rhodopsin-2 show that

selective activation of VTA GABA neurons by light stimulation inhibits DA neuron activity, inducing conditioned place aversion (Tan et al., 2012) and disrupting reward consumption (van Zessen et al., 2012). In contrast, silencing the activity of VTA GABA neurons by expressing the proton pump halorhodopsin in VTA GABA neurons disinhibits DA neurons (Bocklisch et al., 2013). We have shown in multiple reports that acute ethanol inhibits the firing rate of VTA GABA neurons in rats with an IC_{50} of 1.0 g/kg (Gallegos et al., 1999; Ludlow et al., 2009; Steffensen et al., 2009; Stobbs et al., 2004), which is one order of magnitude more sensitive than ethanol effects on DA neurons (Brodie & Appel, 1998; Gysling & Wang, 1983; Mereu et al., 1987). VTA GABA neurons are even more sensitive to ethanol in C57BL/6 and CD-1 mice, as they are inhibited with an IC_{50} of 0.25 g/kg (Steffensen et al., 2011). Of most relevance to this study, VTA GABA neurons recorded *in vivo* become hyperexcitable (firing rates averaging >100 Hz) during withdrawal from ethanol and tolerance accrues to ethanol inhibition of VTA GABA neuron firing rate (Gallegos et al., 1999). Thus, VTA GABA neurons undergo pronounced adaptation during withdrawal from chronic ethanol.

We have shown in multiple reports that GABA(A) receptors [GABA(A)Rs] switch their function during opiate dependence (Laviolette et al., 2004; Ting et al., 2013; Vargas-Perez et al., 2014; Vargas-Perez, Kee, et al., 2009). The functional switch results from increased levels of brain-derived neurotrophic factor (BDNF), which activates the high-affinity tyrosine kinase B (TrkB) receptor (Vargas-Perez, Kee, et al., 2009), which is expressed in VTA GABA neurons (Numan et al., 1998). Ethanol withdrawal not only produces adaptations in VTA GABA neurons (Gallegos et al., 1999), but also GABA(A)R subunit composition in the VTA and the hippocampus (Cagetti et al., 2003; Charlton et al., 1997), and it is reasonable to assume that these changes are important for precipitating this switch in the neurobiological substrates mediating ethanol reinforcement. Considerable evidence suggests that activation of GABA(A)R

complexes can produce depolarization in lieu of its more traditional hyperpolarizing response (Coull et al., 2003; Hubner et al., 2001; Kaila et al., 1993; Rivera et al., 1999; Staley et al., 1995). The switching of GABA(A)R functionality occurs during development and under pathological conditions like epilepsy. There is a major gap in our understanding of the neural substrates that adapt with alcohol dependence, and whether or not they are causal or reflective. Thus, the aim of this study was to evaluate GABA(A)R function in VTA GABA neurons during withdrawal from acute and chronic ethanol. We hypothesized that, similar to what we have reported with opiate dependence, VTA GABA neuron GABA(A)R sensitivity to the GABA(A)R agonist muscimol would adapt to chronic ethanol, reflecting a functional shift of the receptors and VTA GABA neurons themselves during withdrawal.

Materials and Methods

Animal subjects

Male glutamate-decarboxylase-67 (GAD-67) green fluorescent protein (GFP) knock-in CD-1 (white albino) mice (Tamamaki et al., 2003) were bred and cared for in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. For each methodology employed, animals were treated in strict accordance with the Brigham Young University Animal Research Committee (IACUC) guidelines, which reviewed and approved the procedures detailed herein. Once weaned at post-natal day 21, all mice were housed in maximum groups of four and given ad libitum access to solid food and water and placed on a reverse light/dark cycle with lights ON from 8 PM to 8 AM. Any mice used in injection experiments were briefly (2-5 min) anesthetized with isoflurane to reduce the stress of the injection and allow for administration of large volumes, and injected intraperitoneally (IP) with a sterile needle. Animals returned to their home cages 30 minutes following an injection.

Chronic intermittent ethanol exposure to establish alcohol dependence

Animals were made dependent on ethanol in one of two methods of chronic intermittent ethanol (CIE) exposure, either by multiple injections or in vapor chambers. In the multiple injections method, mice were injected IP with ethanol (3.0 g/kg) or saline twice-daily for 14 days, which was sufficient to establish dependence, as we have reported previously (Gallegos et al., 1999). However, no attempt was made in injection studies to determine dependence (i.e. increased drinking). In the vapor chamber method, ethanol vapor was used to establish ethanol dependence. We modified the vapor chamber system developed in the lab of Graeme Mason at Yale (Wang et al., 2012). The six automated chamber system consisted of an air-pressurized, feedback-controlled ethanol flask with flow valves to each of three sealed chambers to regulate the flow of air (11 L/min) and concentration of alcohol to three of six chambers placed in a ventilation hood. A breathalyzer (Drager Alcotest 6510) was used in a feedback loop to regulate the concentration of ethanol. In order to avoid overdosing the first week of CIE vapor exposure, mice were exposed to 4, 6, and 8 hrs of vapor before exposing to 16 hrs vapor/day. Control animals were housed in three sealed chambers in the same ventilation hood, but only received air. Blood alcohol levels (BALs) were measured using an enzymatic kit (Sigma-Aldrich, St. Louis, MO). Even at the 5 L/min feedback flowmeter level the air-exposed mice did not show any detectable alcohol above the 1 mg% detection limit of the breathalyzer or the BAL determination method.

Drink-in-the-dark behavioral experiments

To observe a behavioral correlate of alcohol dependence and validate our vapor chamber method, mice were trained and evaluated in a drink-in-the-dark (DID) two-bottle choice alcohol drinking test. Mice were exposed to CIE vapor or air for 3 weeks, as described above. After a withdrawal period of 24 hours, animals were removed from home cages three hours into the dark

cycle and placed individually in cages with the bedding and food removed. They were given two sipper tubes, with one containing tap water and the other containing tap water and ethanol (20% v/v). Mice were allowed to drink from the tubes for 2 hours in the dark and were then returned to their home cages. They repeated this test over 5 consecutive days with no CIE vapor or air exposure during that time. Animals then underwent 3 days of withdrawal and had a one-day challenge DID session on day 9 with identical conditions.

Preparation of brain slices

All brain slice preparations were performed in P18-120 day old GAD-67 GFP CD1 mice in order to visualize GAD-67+ neurons in the VTA by GFP imaging. P18-28 day old mice were used in the naïve experiments (see results below) only. Ethanol was only administered in animals older than day P28. All mice used in ethanol exposure groups were age-matched and were P28-120 days old with a median age at day P55. There was no effect of age on any results in this study. Brains were rapidly extracted under isoflurane anesthesia. Upon extraction, the brain was glued onto a cutting stage. The brain was then sectioned in ice-cold cutting solution (in mM: 194 Sucrose, 30 NaCl, 4.5 KCl, 1 MgCl₂, 26 NaH₂CO₃, 1.2 NaH₂PO₄, 10 Glucose) bubbled with 95 % O₂ / 5 % CO₂. Targeting the VTA, 210 µm thick horizontal slices were sectioned on a vibratome and then placed in an incubation chamber containing artificial cerebral spinal fluid (ACSF; in mM: 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 12 glucose, 1.5 MgSO₄, 2 CaCl₂) bubbled with 95 % O₂ / 5 % CO₂. After a recovery period of at least 30 minutes, brain slices were placed in a recording tissue chamber with ACSF continuously flowing at 35 °C.

Cell-attached recording of spike activity in brain slices

Cell-attached mode studies used electrodes pulled from borosilicate glass capillaries (2.5-6 MΩ) and then filled with a NaCl solution containing (in mM) 124 NaCl, 2 KCl, 1.25

NaH₂PO₄, 26 NaHCO₃, 1.2 MgSO₄, 2 CaCl₂ adjusted to pH 7.4 with KOH. GABA neurons were identified by fluorescence in GAD-67 GFP mice. Fluorescent cells were imaged on a Nikon Eclipse FNI microscope with a 40x/0.80 n.a. objective lens. The filter cube for GFP detection was a Nikon C-FL ENDOW GFP 96343 cube (Bandpass: 450-490 nm; Barrier: 500-550 nm; dichroic: 495 nm). Excitation was performed with a Sutter Lambda TLED transmitted light source at 506 nm. Cells were then imaged using differential interference contrast imaging in order to facilitate cell attached recordings. Neurons that did not fluoresce and were characterized by relatively slow, regular firing activity were assumed to be DA neurons. Positive pressure was applied to the electrode when approaching the neuron. By applying suction to the electrode, a seal (10 MΩ – 1 GΩ) was created between the cell membrane and the recording pipette. Spontaneous spike activity was then recorded in cell-attached mode with an Axon Instruments Multiclamp 700B amplifier, sampled at 10 kHz using an Axon 1440A digitizer, and collected and analyzed using pClamp10 software. Neurons were not clamped throughout these experiments although recorded in voltage clamp mode (voltage-clamp was set to 0 mV). Firing rate recordings in this study were performed in cell-attached mode in order to avoid dialysing the contents of the cells and disrupting the cytoplasmic milieu (e.g. the Cl⁻ ion gradient), which we have shown previously is perturbed with opiate dependence (Ting et al., 2013; Vargas-Perez et al., 2014). A stable baseline recording of firing activity was obtained for 5-10 min before adding drugs. Neurons that did not achieve a stable baseline firing rate during this time were rejected from the study. Muscimol (0.01, 0.1, 1.0, 10.0 μM) in ACSF was perfused in successive doses for 3-5 minutes at each dose until the neuron stopped firing. ACSF was then applied for 10 minutes. On a given experimental day, 2-3 horizontal slices containing the VTA were sectioned from a mouse. VTA neurons were recorded and analyzed with at least 4 mice/group.

Drug preparation and administration

Muscimol (Sigma-Aldrich) was solubilized in ACSF and superfused on brain slices at 0.01, 0.1, 1.0, and 10.0 μM . Drugs used for injections were solubilized in sterile 0.9 % saline and injected IP: Ethanol (16% v/v solution; 3.0 or 4.0 g/kg) and MK-801 (0.5 mg/kg; Sigma-Aldrich, St. Louis, MO).

Statistical analyses

For neuronal firing rate, results are presented as percent of baseline firing rate \pm standard error of the mean (SEM). Statistical significance required ≥ 95 % level of confidence ($p \leq 0.05$). Firing rate was analyzed for two min (baseline) before muscimol perfusion and for two minutes at the end (peak effect) of the drug application, or the last two minutes before any drug was applied. For comparison between groups, a mixed model ANOVA was used. Values were Greenhouse-Geisser corrected for sphericity. Data was found to be reasonably distributed using the Wilks-Shapiro test of normality. Using the criterion of median plus or minus 3 interquartile range (IQR) outlying data points were identified. After determining that the outliers were not due to data input error they were bounded to the median ± 3 IQR for analysis purposes. A priori hypothesis testing was accomplished with Bonferroni correction post-hoc tests. Analysis software included Minianalysis (Synaptosoft, Decatur, GA), Clampfit (Molecular Devices, Sunnyvale, CA), Microsoft Excel, STATA (StataCorp, College Station, TX), and Igor Pro (Wavemetrics, Oswego, OR). Significance levels were indicated on graphs with asterisks *, **, ***, corresponding to significance levels $p < 0.05$, 0.01 and 0.001, respectively. Figures were constructed with Igor Pro software.

Results

Sensitivity of VTA neuron firing rate to the GABA(A) receptor agonist muscimol

We have previously shown that VTA GABA neurons are sensitive to the GABA(A)R agonist muscimol at sub-micromolar concentrations (Ting et al., 2013; Vargas-Perez et al., 2014). We present data on firing rate of putative VTA DA neurons along with VTA GABA neurons in GAD-67 GFP mice. Neurons in the VTA of GAD-67 GFP mice that did not exhibit fluorescence, but were characterized by relatively slow, regular firing activity, were presumed to be DA neurons. The firing rate of VTA GABA neurons in ethanol-naïve animals was significantly faster than putative VTA DA neurons ($F_{(1, 35)} = 18.91, p = 0.0001$; GABA neurons = 12.9 ± 2.0 Hz vs DA neurons = 4.8 ± 0.9 Hz; n=14, 7). We evaluated the effects of muscimol on VTA neuron firing rate at concentrations ranging from 0.01 – 10.0 μ M. Muscimol dose-dependently reduced the firing rate of all neurons tested ($F_{(4, 55)} = 36.56, p < 0.0001$). As reported previously (Ting et al., 2013; Vargas-Perez et al., 2014), superfusion of muscimol inhibited VTA GABA neuron firing rate with an $IC_{50} < 0.1 \mu$ M (**Fig. 1A**). However, muscimol inhibited VTA DA neuron firing rate with an $IC_{50} > 1.0 \mu$ M (**Fig. 1B**). In ethanol naïve mice, VTA GABA neurons were significantly more sensitive than VTA DA neurons to muscimol in the 0.1 – 10 μ M range (**Fig. 1C**; $F_{(4, 55)} = 3.76, p = 0.0090$). A priori hypothesis testing with Bonferroni correction revealed that GABA neuron firing rate was more depressed than DA neuron firing rate by 0.1 μ M muscimol ($F_{(1, 90)} = 15.36, p = 0.001$) and 1.0 μ M muscimol ($F_{(1, 90)} = 22.42, p < 0.001$; 0.01 μ M: n=10, 7; 0.1 μ M: n=10, 7; 1.0 μ M: n=13, 6; 10.0 μ M: n=10, 5).

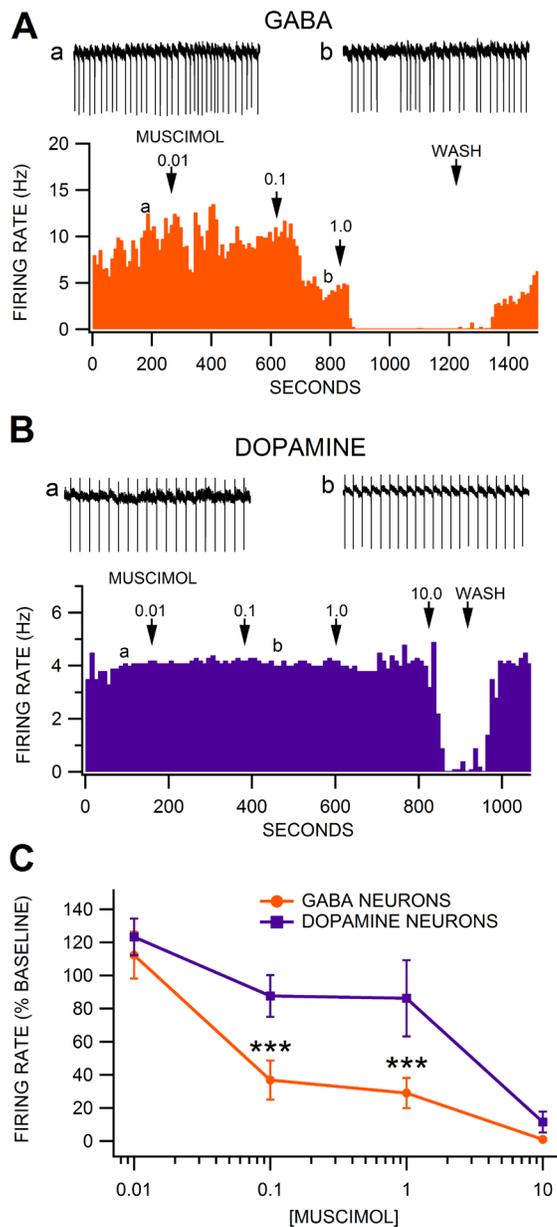


Figure 2-1: Comparison of the sensitivity of VTA GABA vs DA neurons to muscimol inhibition of firing rate. (A) This representative VTA GABA neuron had a baseline firing rate of approximately 10 Hz. The GABA(A)R agonist muscimol mildly inhibited the firing rate of this neuron at 0.01 μM , moderately inhibited it at 0.1 μM , and abolished its activity at 1.0 μM . Insets a,b are 5 sec traces of GABA neuron spike activity recorded at the times indicated on the graph. (B) The ratemeter shows the activity of a representative VTA DA neuron, which had a baseline firing rate of approximately 4 Hz. Muscimol had little or no effect on this DA neuron until the firing rate was suppressed at 10.0 μM . Insets a,b are 5 sec traces of DA neuron spike activity recorded at the times indicated on the graph. (C) The firing rate of VTA GABA neurons was significantly more sensitive than VTA DA neurons to muscimol at 0.1 and 1.0 μM . Asterisks *** represent significance levels $p < 0.001$.

Muscimol inhibition of VTA neuron firing rate after a single injection of ethanol

Firing rate was recorded in VTA GABA and DA neurons 24 hours after acute saline or ethanol administration (non-dependent condition; **Fig. 2A,B**). GAD-67 GFP mice were administered saline or 4.0 g/kg ethanol IP, which produced sedation for 1-2 hr with complete recovery in all mice. In a subset of mice, the NMDA antagonist MK-801 (0.5 mg/kg) was administered IP 15 minutes before ethanol or saline. There was no significant difference in baseline firing rate between any of the acute treatment groups ($F_{(3, 37)} = 1.786, p = 0.167$; **Fig. 2A-C**). Muscimol depressed VTA GABA firing rate in a dose dependent manner ($F_{(4, 119)} = 60.76, p < 0.0001$; **Fig. 2A-C**). Acute withdrawal from a single exposure of ethanol altered the response of VTA GABA neurons to muscimol ($F_{(15, 119)} = 3.69, p = 0.001$; **Fig. 2B-C**). Posthoc analysis with Bonferroni correction revealed that muscimol suppressed VTA GABA neuron firing rate more in 7 day ethanol withdrawn mice than in 24 hour withdrawn mice ($t_{(166)} = 3.81, p = 0.008$; **Fig. 2C**) or MK-801 pretreated control mice ($t_{(166)} = -3.79, p = 0.008$). VTA GABA neuron firing rate in 24 hour withdrawn ethanol mice was less sensitive to muscimol depression of firing rate than saline mice at 0.1 μM muscimol ($t_{(166)} = 3.95, p = 0.004$). At 1.0 μM muscimol, MK-801 pretreated control mice were less sensitive to muscimol inhibition of GABA neuron firing rate than MK-801 pretreated ethanol withdrawn mice ($t_{(166)} = 4.01, p = 0.004$; n=8 each), 7 day withdrawn ethanol-injected mice ($t_{(166)} = -4.08, p = 0.004$; n=8 each), or saline-injected mice ($t_{(166)} = -4.39, p < 0.004$; n=8 each). Specifically at 0.1 μM muscimol, VTA GABA neuron firing was affected more by muscimol in 7 day withdrawn mice from a single ethanol injection than in MK-801 (0.5 mg/kg IP administered 15 min prior to ethanol or saline) pretreated 24 hour withdrawn mice ($t_{(166)} = -4.04, p = 0.004$; n=9 each), MK-801 pretreated control mice ($t_{(166)} = -4.48, p < 0.004$), or 24 hour withdrawn ethanol mice ($t_{(166)} = 6.30, p < 0.004$; **Fig. 2C**).

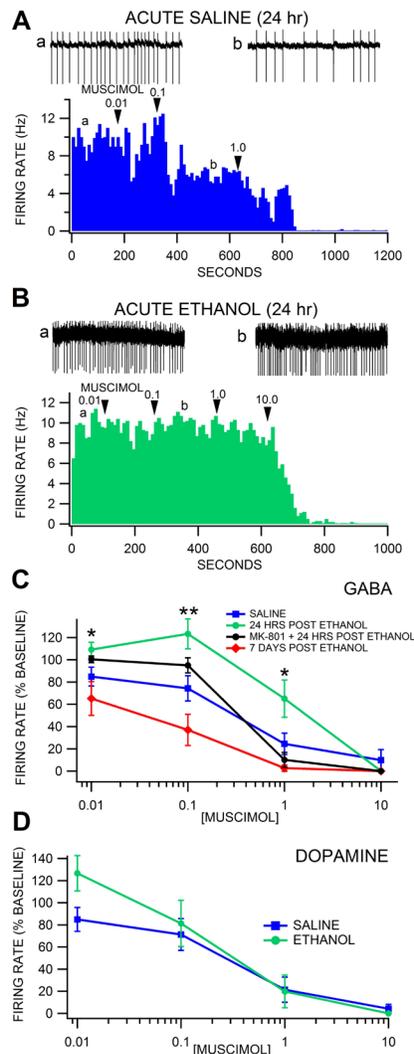


Figure 2-2: Decreased sensitivity of VTA GABA neurons to muscimol 24 hrs after a single injection of ethanol. (A) The ratemeter shows the firing rate of a VTA GABA neuron (traces in a, b recorded at times indicated on graph) recorded in a brain slice from a mouse injected with saline once, 24 hours before recording. This representative neuron had a baseline firing rate of approximately 9.3 Hz. Muscimol (0.01-10.0 μ M) inhibited the firing rate of this VTA GABA neuron. All insets are representative 5 sec traces of GABA neuron spike activity recorded before (inset a) and after (inset b) 0.1 μ M muscimol. (B) The ratemeter shows the firing rate of a GABA neuron, which had a baseline firing rate of approximately 9.6 Hz. Insets a,b show representative 5 sec traces before and after application of muscimol in a mouse that was treated with 4.0 g/kg ethanol once, 24 hr before recording. This mouse was resistant to muscimol's inhibitory effects up to 1.0 μ M. (C) Muscimol significantly inhibited the firing rate of VTA GABA neurons in saline-injected animals. Muscimol's inhibitory effect was significantly reduced 24 hr after a single injection of ethanol, but sensitivity was restored by administration of MK-801. Sensitivity to muscimol was restored in mice tested 7 days post ethanol injection. (D) Dopamine neurons were equally sensitive to muscimol 24 hr after an ethanol injection in ethanol-exposed vs air-exposed controls. Asterisks *, ** represent significance levels $p < 0.05$ and $p < 0.01$ between ethanol and saline groups.

Firing rate was also recorded in putative VTA DA neurons (GAD-67 GFP-negative cells) 24 hours after acute saline or ethanol administration. Muscimol suppressed DA neuron firing rate in a dose dependent manner ($F_{(4, 28)} = 18.19, p = 0.0003$; **Fig. 2D**). A single *in vivo* ethanol exposure did not affect DA neuron firing rate ($F_{(1, 9)} = 1.41, p = 0.2655$). There was no main effect of withdrawal from a single *in vivo* exposure to ethanol on sensitivity to muscimol suppression of DA neuron firing rate ($F_{(3, 28)} = 1.52, p = 0.2491$).

Muscimol inhibition of VTA neuron firing rate after chronic ethanol

We have reported previously that VTA GABA neurons recorded in freely-behaving rats evince hyperexcitability and tolerance to acute ethanol following 2 weeks of twice-daily injections of ethanol (Gallegos et al., 1999). The hyperexcitability of VTA GABA neurons was optimal 16-24 hrs after withdrawal from the last injection. We used similar methods for establishing alcohol dependence in GAD-67 GFP mice. Similar to what we reported in rats, we observed that most ethanol-injected mice exhibited more agitation, less grooming, rigid tail and piloerection than the saline-injected mice 24 hours after 2 weeks of twice-daily injections of 3.0 g/kg ethanol. Unlike what we found previously in rats *in vivo*, there was no difference in baseline VTA GABA neuron firing rate in the slice preparation *ex vivo* in ethanol-treated mice compared to saline-treated mice ($F_{(1,12)}=0.1, p=0.76$; Ethanol = 8.8 ± 1.6 Hz vs Saline = 7.9 ± 0.9 Hz; n=8, 5). Muscimol depressed VTA GABA firing rate in a dose-dependent manner ($F_{(4, 37)} = 65.66, p < 0.0001$; **Fig. 3**). Chronic ethanol injections significantly affected the response of VTA GABA neurons to muscimol ($F_{(4,37)} = 3.82, p = 0.0466$) recorded 24 hrs after the last ethanol injection. A priori hypothesis testing with Bonferroni correction revealed that VTA GABA neuron firing rate differed significantly between chronic saline vs ethanol injected mice at 0.1 μ M muscimol in

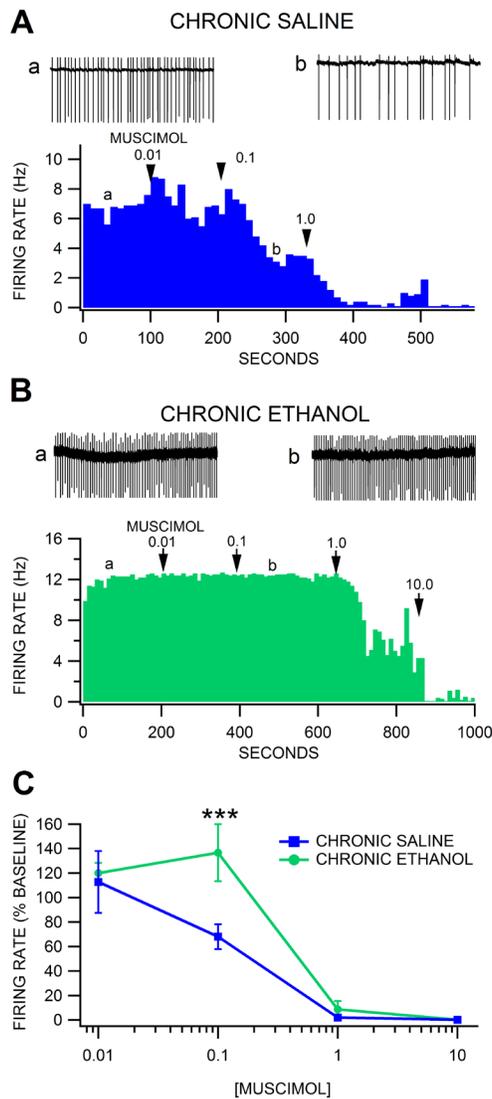


Figure 2-3: Decreased sensitivity of VTA GABA neurons to muscimol after chronic ethanol injections. (A) The ratemeter shows the firing rate of a VTA GABA neuron (traces in a,b recorded at times indicated on graph) recorded in a brain slice from a mouse injected with saline twice-daily for 14 days. This representative neuron had a baseline firing rate of approximately 7 Hz. Muscimol markedly inhibited the firing rate of this VTA GABA neuron, with suppression of activity at the 1.0 μM concentration. All insets are representative 5 sec traces of GABA neuron spike activity recorded before (inset a) and after (inset b) 0.1 μM muscimol. (B) The ratemeter shows the firing rate of a GABA neuron in a mouse that was treated with 3.0 g/kg ethanol twice daily for 14 days. The baseline firing rate was approximately 12 Hz. This mouse was resistant to muscimol's inhibitory effects until it was inhibited by 1.0 μM muscimol. (C) Muscimol inhibited the firing rate of VTA GABA neurons in chronic saline-treated animals, which was significantly reduced in chronic ethanol-treated mice. Asterisks *** represent significance levels $p < 0.001$.

chronic ethanol-treated mice (**Fig. 3B,C**; n=7), evincing relative resistance to muscimol inhibition of VTA GABA neuron firing rate ($F_{(1,48)} = 18.97, p = 0.0004$; **Fig. 3A,C**; n=5).

The effects of alcohol dependence on the sensitivity of VTA neurons to muscimol was also evaluated with CIE vapor exposure (200 mg% for 16 hours/day) and drink-in-the-dark (DID) ethanol consumption in GAD-67 GFP mice. Mice exposed to ethanol vapors increase their consumption of alcohol when dependent (Dhaher et al., 2008; Lopez & Becker, 2005). To validate the utility of our modified vapor chamber system in producing alcohol dependence with CIE exposure in GAD-67 GFP mice, we calibrated three feedback flowmeter settings (1, 3, and 5 L/min) in a 3 L alcohol pressurized flask with breathalyzer values and blood alcohol levels (BALs; Sigma Enzymatic method). We obtained breathalyzer values of 52.1 ± 4.2 , 130.2 ± 4.8 and 225.6 ± 4.1 mg% (n=6) ethanol and BALs of 46.1 ± 3.7 , 119.7 ± 2.9 , and 234.9 ± 5.5 mg% (n=6) ethanol at feedback flowmeter flask settings 1, 3, and 5 L/min, respectively. Based on these calibrations, a separate cohort of mice was exposed to feedback flowmeter settings corresponding to 200 mg% breathalyzer values for 16 hours (1000-0200 hours)/day. We evaluated ethanol consumption in the DID procedure following three weeks of continuous CIE or air in order to avoid any alcohol exposure in the air-exposed mice. Ethanol vapor-exposed GAD-67 GFP mice consumed significantly more ethanol than air-exposed mice 24 hr after withdrawal from three weeks of CIE ($F_{(1,11)}=6.9, p=0.02$; n=6,8), as well as 9 days after withdrawal from CIE ($F_{(1,5)}=5.94, p=0.02$; n=3 each; **Fig. 4**). In addition, similar to chronic ethanol injections, we observed that most ethanol-exposed mice exhibited more agitation, less grooming, rigid tail and piloerection than the saline-injected mice 24 hours after 3 weeks of ethanol vapor exposure. Thus, three weeks of CIE in our vapor chambers at the 200 mg%

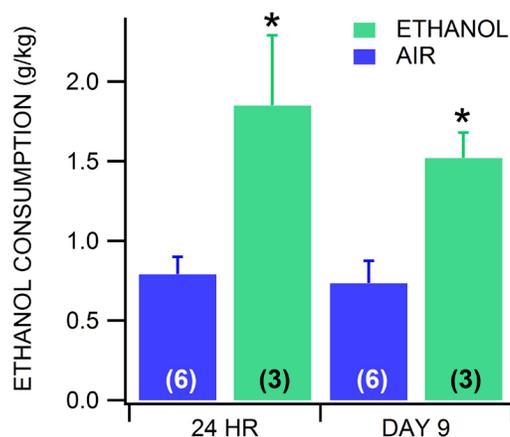


Figure 2-4: Drink-in-the-dark model of alcohol dependence. In CD-1 GAD-67 GFP mice exposed to three continuous weeks of CIE vapor (n=3) or air (n=6), ethanol consumption in the DID test was significantly increased in mice receiving ethanol vapors vs air-exposed mice. Dependent mice displayed increased drinking 24 hours after withdrawal from CIE vapor, and again on day 9 after last CIE vapor exposure. On day 9, mice were in 72 hour withdrawal following the last drinking test performed. Asterisks * mark statistical significance ($p < 0.05$) between air and CIE vapor groups.

breathalyzer level was used to evaluate the sensitivity of VTA neurons to muscimol in separate cohorts.

Firing rate was evaluated in VTA neurons 24 hours after three weeks of CIE to alcohol vapor vs air controls. Similar to chronic ethanol injections, there was no difference in baseline VTA GABA neuron firing rate in the slice preparation *ex vivo* in CIE vapor-exposed mice compared to air-exposed mice ($F_{(1,44)}=0.02$, $p=0.89$; Ethanol = 9.6 ± 1.1 Hz vs Air = 9.4 ± 1.0 Hz n=26, 19). Muscimol depressed VTA GABA neuron firing rate in a dose-dependent manner ($F_{(4,137)} = 27.03$, $p < 0.0001$). Treatment condition altered the response of VTA GABA neurons to muscimol ($F_{(8, 137)} = 4.71$, $p = 0.0014$). A priori hypothesis testing with Bonferroni correction revealed that VTA GABA neuron firing rate was higher in chronic ethanol animals (Fig. 5B,C) compared to chronic air animals (Fig. 5A,C) when the slices were perfused with $0.1 \mu\text{M}$

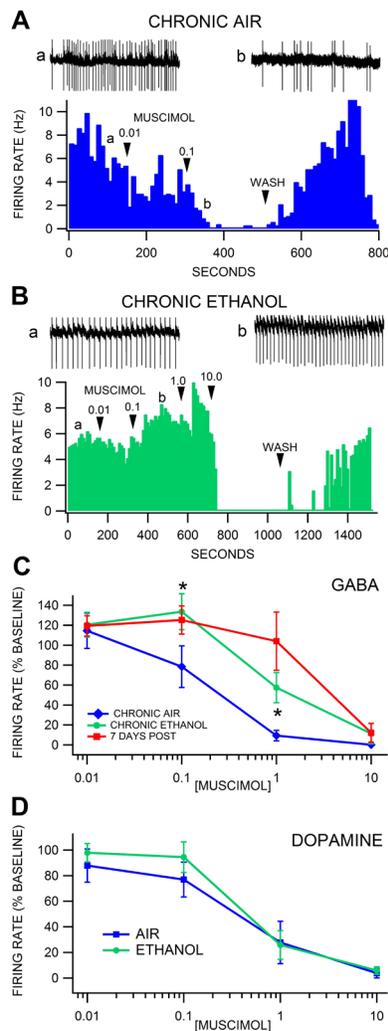


Figure 2-5: Decreased sensitivity of VTA GABA, but not DA neurons, to muscimol after chronic intermittent ethanol vapor exposure. (A) The ratemeter shows the firing rate of a VTA GABA neuron (traces a, b recorded at times indicated on graph) recorded in a brain slice from a mouse exposed to air for 3 weeks. This representative VTA GABA neuron was characterized by irregular activity but with a baseline firing rate of approximately 7 Hz. The firing rate of this neuron was inhibited by 0.01 μM and suppressed by 0.1 μM muscimol. All insets are representative 5 sec traces of GABA neuron spike activity recorded before (inset a) and after (inset b) 0.1 μM muscimol. (B) The ratemeter shows the firing rate of a GABA neuron in a mouse that was exposed to CIE vapor for 3 weeks. The baseline firing rate was approximately 5 Hz. This VTA GABA neuron was more resistant to muscimol's inhibitory effects up to 1.0 μM than its air-exposed control. In fact, the firing rate of this neuron was mildly enhanced by muscimol at the 0.1 and 1.0 μM concentrations. (C) Muscimol significantly inhibited the firing rate of VTA GABA neurons in animals exposed to air for three weeks, which was significantly reduced in mice exposed to CIE vapors for 3 weeks. In addition, VTA GABA neuron firing rate was resistant to muscimol inhibition after 7 days of withdrawal. (D) Dopamine neurons were equally sensitive to muscimol in ethanol vapor-exposed mice vs air-exposed controls. Asterisks *,** represent significance levels $p < 0.05$ and $p < 0.01$, respectively between ethanol and air groups.

muscimol ($F_{(1, 202)} = 9.93, p = 0.019; n=15, 13$) and 1.0 μM muscimol ($F_{(1, 202)} = 9.92, p = 0.019; n=19, 14$). Additionally, VTA GABA neurons in ethanol-dependent mice after 7 days of withdrawal discharged at a higher rate than in chronic air mice during perfusion with 1.0 μM muscimol ($F_{(1, 202)} = 26.27, p < 0.001; n=15, 7$).

Firing rate was also evaluated in VTA DA neurons 24 hours after CIE vapor exposure vs air exposure. Dopamine neuron firing rate was unaffected by chronic ethanol exposure ($F_{(1, 21)} = 0.15, p = 0.7003$). Muscimol decreased DA neuron firing rate in a dose dependent manner ($F_{(4, 56)} = 34.63, p < 0.0001$). Chronic ethanol exposure did not affect muscimol inhibition of DA neuron firing rate ($F_{(4, 56)} = 0.56, p = 0.4854$; **Fig. 5D**; $n=14, 9$).

Discussion

The overall aim of this study was to investigate the electrophysiological components of the mechanism underlying the hyperexcitability of VTA GABA neurons following withdrawal from chronic ethanol. Mainly, we have reported previously that VTA GABA neuron firing rate often exceeds 100 Hz for 4-8 hrs during withdrawal from chronic ethanol in rats (Gallegos et al., 1999). This is 4-5X the baseline level of VTA GABA neuron firing rate in naïve rats. In addition, tolerance accrues to ethanol inhibition of VTA GABA neurons firing rate following 11 days of chronic ethanol exposure. The focus of this study was to determine the role of GABA(A)Rs in VTA GABA neuron hyperexcitability produced by acute and chronic ethanol exposure in GAD-67 GFP mice, similar to what we have described with opiate dependence (Ting et al., 2013; Vargas-Perez et al., 2014). VTA GABA neurons are resistant to the GABA(A)R agonist muscimol following withdrawal from exposure to acute and chronic ethanol, which was not exhibited in putative VTA DA neurons.

Putative DA neurons in the VTA were not as sensitive to muscimol as VTA GABA neurons. Muscimol binds to the orthosteric site (i.e. where GABA binds) on the $\alpha 1$ subunit of the

GABA(A)R. A possible explanation for the decreased sensitivity is that the $\alpha 1$ subunit is selectively expressed in GABA neurons (Fritschy & Mohler, 1995), but not in DA neurons, in the VTA (Okada et al., 2004; Tan et al., 2010), and has been implicated in addiction to some benzodiazepines (Tan et al., 2010). Interestingly, consistent with what has been reported by us and others (Tan et al., 2010), VTA GABA neurons exhibit much more spontaneous and spike-related GABA inhibitory input than DA neurons. This phenomenon occurs despite the fact that GABA neurons have faster firing rates than DA neurons both *in vivo* and *in vitro*, which is operational even with pronounced afferent GABAergic drive. The effects of acute and chronic ethanol on muscimol effects on putative DA neurons were studied along with GABA neurons. There are ambiguities associated with their identification in GAD-67 GFP mice, as 5% of the neurons in the VTA are neither GABA nor DA neurons (Margolis et al., 2012), and some GABA neurons express GAD-65 (Merrill et al., 2015). Thus, while VTA GABA neurons in GAD-67 GFP mice can be unequivocally identified as GAD-67+ neurons, caution must be taken in the identification of DA neurons in this study. However, since DA neurons represent 65% of and GABA neurons 30% of VTA neurons (Margolis et al., 2012), it is highly likely that the GFP-negative neurons were DA neurons.

We show that VTA GABA neurons are less sensitive to the inhibitory effects of muscimol 24 hours after a single *in vivo* injection. We were originally surprised by this finding, because we had hypothesized that chronic ethanol exposure would be required to shift the sensitivity of VTA GABA neurons to muscimol, as we have demonstrated with opiate dependence. However, this short-term plasticity was blocked by prior administration of the NMDAR antagonist MK-801 (0.5 mg/kg), suggesting that NMDAR activation is required for acute ethanol's effect on VTA GABA neuron muscimol sensitivity. Because the mechanism is GLU-dependent, at least with acute ethanol exposure, there must be some connection between

NMDAR activation and the altered function of GABA(A)Rs on VTA GABA neurons. In support of this, GLU plasticity on VTA DA neurons has been shown to be induced by GABA(A)R agonists (Vashchinkina et al., 2014; Vashchinkina et al., 2012). Future studies could address interactions between GLU and GABA plasticity, or a link between acute NMDAR activation and an increase in intracellular chloride concentration, which would shift the reversal potential of the GABA(A)R. Alternatively, blocking NMDARs may result in some upstream hindrance to learning the novel rewarding stimulus with an end result of decreased plasticity in the VTA due to ethanol. Some have reported a link between BDNF signaling and NMDA receptor activation with alcohol and amphetamine exposure (Fuller et al., 2015; Kolb et al., 2005). We remain interested in BDNF as an intermediate signaling molecule in the establishment of alcohol dependence, as we have displayed its involvement with opiate dependence (Vargas-Perez, Kee, et al., 2009). Future experiments could look at NMDA activation or BDNF signaling during the chronic exposure to ethanol.

An increase in GABA release onto VTA DA neurons in brain slices 24 hr after a single *in vivo* exposure to ethanol (2 g/kg) has been reported in C57BL/6 and DBA/2 mice (Melis et al., 2002; Wanat et al., 2009), suggesting some plasticity in VTA GABA neuron inhibition of VTA DA neurons that is mediated by NMDARs. Regardless, this state appears to be temporary, as we show here that the sensitivity of VTA GABA neurons to muscimol is restored 7 days after a single exposure to ethanol. Most importantly, DA neuron sensitivity to muscimol was unaffected by a single exposure to ethanol. While there are two studies demonstrating down-regulation of the $\alpha 1$ subunit of the GABA(A)R in the VTA with 12 weeks of chronic ethanol (Charlton et al., 1997; Ortiz et al., 1995), we know of only one study identifying the specific GABA(A)R subunits expressed in VTA GABA neurons (Tan et al., 2010). A reduction in the levels of the GABA(A)R $\alpha 1$ subunit has been documented in the VTA with chronic ethanol (Charlton et al.,

1997; Ortiz et al., 1995; Papadeas et al., 2001). The possibility exists that this reduction is the underlying mechanism for the decreased sensitivity of VTA GABA neurons to muscimol and the lack of sensitivity of VTA DA neurons in mice exposed to chronic ethanol.

Two models of chronic exposure to ethanol were used in this study: twice daily injections of ethanol and CIE in vapor chambers. As mentioned above, we have shown in previous studies that VTA GABA neurons recorded in freely-behaving rats evince hyperexcitability and tolerance to acute ethanol following 2 weeks of twice-daily injections of ethanol (Gallegos et al., 1999). The hyperexcitability of VTA GABA neurons was optimal 24 hrs after withdrawal from the last injection. Here we show that VTA GABA neurons recorded during withdrawal from twice-daily injections of ethanol resulted in lowered sensitivity to muscimol compared to saline-injected control mice. In effect, lowered sensitivity of GABA(A)Rs on VTA GABA neurons might explain why VTA GABA neurons become hyperexcitable during withdrawal, as they could be experiencing less inhibition from other local circuit GABA neurons or projection GABA neurons from the NAc, ventral pallidum, etc. While chronic injection studies are supportive, it is difficult to determine dependence in rats and mice. The CIE vapor chamber approach enables determinations of dependence, mainly increased alcohol drinking, and eliminates the risk of infection from repeated injections. We show the validity of our alcohol vapor chamber system for establishing alcohol dependence using increased alcohol consumption. Similar results have been shown using DID as a measure of alcohol dependence in other labs (Dhaher et al., 2008; Lopez & Becker, 2005). We compared drinking between ethanol-exposed and naïve air-exposed mice in the DID procedure after withdrawal from 3 weeks of 16 hrs ON, 8 hrs OFF CIE and found that ethanol consumption increased significantly at 24 hours and 9 days after withdrawal. After chronic exposure to ethanol vapors, VTA GABA neuron firing rate is also resistant to the inhibitory effects of muscimol, but importantly, this resistance persists after 7 days of withdrawal

from chronic ethanol but not acute ethanol exposure. None of these effects were seen in putative VTA DA neurons.

The fact that alterations in muscimol sensitivity are seen only in VTA GABA neurons and not VTA DA neurons strengthens the claim that alcohol's effects on DA in the brain are mediated through VTA GABA neurons. Rather than DA elevation due to alcohol being caused by a direct action on DA neurons, ethanol could disinhibit DA neurons through acting on VTA GABA neurons. We have previously shown that VTA GABA neurons are hyperexcitable during withdrawal from chronic alcohol (Gallegos et al., 1999), which might explain the downregulation of DA neural activity and DA release that is characteristic of alcohol dependence. This decrease of DA activity during withdrawal could contribute to the increased hedonic drive to seek alcohol in order to alleviate the negative consequences of withdrawal. However, it is important to note that VTA GABA neuron baseline firing rate measured in the *ex vivo* slice did not differ between ethanol-exposed and control groups. This finding runs counter to our hypothesis. Thus, we cannot rule out changes in circuit responses to explain increased muscimol resistance in ethanol exposed mice. The discrepancy between *in vivo* and *ex vivo* recordings may be due to the loss of GLUergic and GABAergic inputs due to slicing. Indeed, GLU transmission may be a critical player, as our MK-801 experiments would suggest: mainly, that MK-801 reverses ethanol-induced muscimol resistance 24 hr after a single intoxicating dose of ethanol. Regardless, as hypothesized, changes in muscimol sensitivity would still suggest that there could be a decrease in number of GABA(A)Rs, the affinity of GABA(A)Rs for muscimol, a change in subunit composition of the GABA(A)R (e.g. $\alpha 1$ subunit), enhanced GABA(A)R desensitization, or a shift in function of the GABA(A)R, as we have reported in a series of papers with opiate dependence (Laviolette et al., 2004; Ting et al., 2013; Vargas-Perez et al., 2014; Vargas-Perez, Kee, et al., 2009). Future studies with perforated patch recordings in dissociated

neurons from alcohol-dependent mice and/or chloride imaging will address this issue, particularly with regard to the chloride gradient and the reversal potential of GABA(A)Rs. We are currently developing a procedure to perform perforated-patch clamp recordings in mature animals to address this question.

In conclusion, this study examined the inhibitory effects of the GABA(A)R agonist muscimol on VTA neurons following alcohol exposure. VTA GABA neurons display resistance to muscimol's inhibitory effects following both acute and chronic exposure to ethanol, that persists following chronic exposure, which has implications for the state of the GABA(A)Rs on VTA GABA neurons along the continuum of alcohol dependence. The changes described in this study contribute to altered function of VTA neurons during withdrawal from alcohol that may contribute to the motivation for alcohol-seeking behaviors in dependent individuals.

Acknowledgements

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CHAPTER 3: BDNF levels increase in withdrawal from chronic alcohol exposure: a potential mechanism for alcohol withdrawal

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Abstract

Brain-derived neurotrophic factor (BDNF) is implicated in varied physiological processes. Its main receptor in the central nervous system is tyrosine receptor kinase B (TrkB), and the main ligand for TrkB in the central nervous system is BDNF. It has been shown previously that activating TrkB can cause a downregulation of the chloride-exporting potassium chloride cotransporter 2 (KCC2), presumably resulting in a decreased chloride gradient. We have shown previously that opioid dependence produces a functional switch in GABA(A) receptors on GABA neurons in the ventral tegmental area (VTA) during opioid dependence that is mediated by BDNF and TrkB. It is hypothesized that this switch, caused by an increase in BDNF levels, elicits hyperexcitable GABA neurons in the VTA due to a reduced efficacy of inhibitory currents. Here we investigate this mechanism of adaptation in the context of chronic alcohol exposure. We demonstrate that BDNF levels are elevated in the VTA and the nucleus accumbens (NAc) during withdrawal from chronic ethanol (EtOH) exposure. We also observe that blocking TrkB activity decreases EtOH seeking behavior in non-dependent animals. Further, we investigate the expression patterns of KCC2 in connection with chronic EtOH administration. Additional work is underway to validate this mechanism and further elucidate its putative role in alcohol dependence.

Introduction

Alcohol addiction has caused immeasurable grief to individuals, families, and communities. Furthermore, it has staggering financial and health consequences. Nearly 17 million Americans have an alcohol use disorder (AUD), and approximately 1.3 million adults received treatment for an AUD (Health, 2016; SAMHSA, 2016). The estimated economic costs of AUD to the US in 2015 was \$249 billion, and almost three-quarters of the total cost was related to binge drinking (Sacks et al., 2015; SAMHSA, 2016). In 2000 there were roughly 85,000 alcohol-related deaths in the U.S. making alcohol consumption the number three preventable cause of death (Mokdad et al., 2004). The current therapies for alcoholism have a low success rate, and more effective treatments are in significant need. At present, there is much left to understand about the molecular actions of alcohol and the neural mechanisms that lead to alcohol addiction as well as how interventions might be devised to mitigate it.

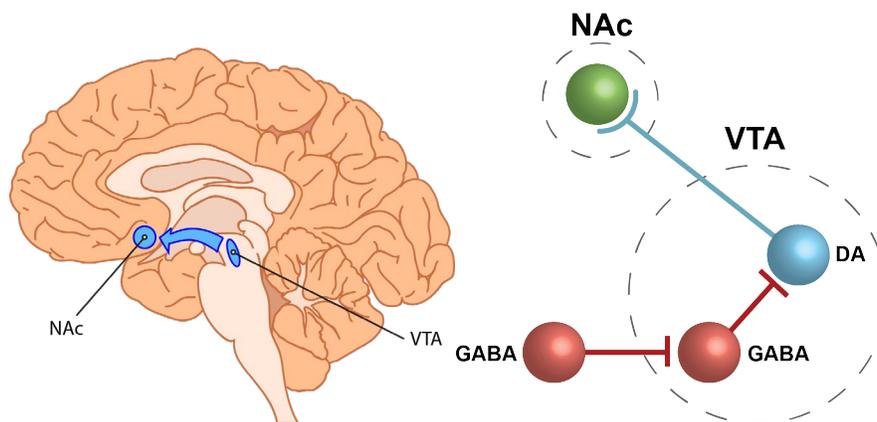


Figure 3-1: The circuit of interest: (top) DA neurons originate in the VTA and project to the NAc, (bottom) Illustration of some of the synapses of interest. VTA GABA neurons receive inhibitory input from other brain regions.

A commonly accepted theory of addiction is that it has its genesis in alterations to neural dopamine (DA) transmission. The DA circuit of greatest interest is the mesocorticolimbic or mesolimbic DA system, which is critical for reward and reinforcement circuitry. This system

begins in the ventral tegmental area (VTA) in the midbrain and projects to areas of the cortex and limbic system, including the nucleus accumbens (NAc) (see **Fig. 1**). The connection from the VTA to NAc is highly implicated in reward and addiction. While DA release in the NAc is not necessarily a direct measure of reward, it is critical to reinforcement of behaviors.

There are three types of neurons in the VTA: DAergic, inhibitory γ -aminobutyric acid (GABA)ergic, and excitatory glutamatergic neurons, which comprise 65%, 30% and 5% of VTA neurons, respectively (Dobi et al., 2010). Under normal circumstances, GABA connections in the VTA regulate DA release in response to normal synaptic GABA release by allowing inward flux of chloride (Cl^-) ions following activation of GABA_ARs. We have shown previously that these GABA neurons are particularly sensitive to EtOH (EtOH) and change their activity levels after extended exposure, becoming hyperexcitable during withdrawal from chronic EtOH exposure, exhibiting firing rates that often exceed 100 Hz for hours (Gallegos et al., 1999). It is thought that some of this change is mediated by brain-derived neurotrophic factor (BDNF) (Vargas-Perez et al., 2014; Vargas-Perez, Ting, et al., 2009). VTA GABA neurons also receive inhibitory input from other brain regions, which is the main synapse of focus in this study.

Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor (BDNF) is a peptide in the neurotrophin family, a group of four known compounds with similar molecular weight and structure (Hallbook, 1999). The first neurotrophin, nerve growth factor (NGF), was discovered in the 1950's due to its ability to facilitate neuron survival (Levi-Montalcini & Hamburger, 1951). Roughly 30 years later the second neurotrophin, BDNF, was isolated from pig brain tissue (Barde et al., 1982). Since then, considerable evidence has mounted that BDNF, like NGF, also imbues cells with enhanced survival in a variety of areas in the nervous system [reviewed in (Huang & Reichardt, 2001)].

BDNF finds widespread expression in discrete areas of the nervous system, including structures such as the hippocampus and various thalamic and brainstem nuclei (Conner et al., 1997). Importantly, it plays a role in proper memory function and neural plasticity, both of which are factors in addiction (Bekinschtein, Cammarota, Izquierdo, et al., 2008; Bekinschtein, Cammarota, Katche, et al., 2008; Yamada & Nabeshima, 2003). Additionally, it has been known to play a role in neural development and in neurogenesis (Huang & Reichardt, 2001; Zigova et al., 1998). There is also some evidence that BDNF could be linked to a variety of diseases, including but not limited to depression and Alzheimer's disease (Dwivedi, 2013; Zhang et al., 2012). There are two main receptors for BDNF: the tyrosine receptor kinase B (TrkB) and the p75 receptor (Patapoutian & Reichardt, 2001). The latter will not be discussed here, but could also be worth investigating with a similar approach to the one described here. TrkB is the main receptor for BDNF and BDNF is the main ligand for TrkB, although a second ligand, NT-4, does exist [reviewed in (Hempstead, 2015; Huang & Reichardt, 2001)]. This receptor is gaining interest as a therapeutic target for conditions such as Alzheimer's disease, obesity, and depression, as mentioned above in connection with its primary ligand. It is worth noting, however, that using TrkB as a therapeutic target must be done with care, as it has been observed that mice that overexpress BDNF exhibit deficits in memory and can later experience epileptiform activity (Cunha et al., 2009; Isgor et al., 2015; Scharfman, 1997).

Potassium-Chloride Cotransporter 2 (KCC2)

One of the downstream effects of activating TrkB can be the downregulation of the potassium chloride cotransporter 2 (KCC2), as has been observed in the hippocampus (Rivera et al., 2002). KCC2 is a neuron-specific membrane protein expressed only in the central nervous system (Sivakumaran et al., 2015; Wu et al., 2016). One of its main roles is that of removing Cl⁻ ions from the intracellular space, thus helping to maintain a normal Cl⁻ gradient (Gagnon et al.,

2013; Payne, 1997; Sivakumaran et al., 2015). It is closely related to $\text{Na}^+ \text{K}^+ \text{Cl}^-$ cotransporters 1 and 2 (NKCC1 and NKCC2). These are not neuron specific but can have the effect of importing Cl^- into the cell (Arroyo et al., 2013). Both groups of transporters affect the Cl^- gradient, which is critical to the proper functioning of inhibitory synapses, and dysregulating it could cause certain cells to become hyperexcitable. This is evidenced by the selective inhibition of KCC2, which yields epileptiform activity (Sivakumaran et al., 2015). It is also worth noting that the complete removal of the KCC2 gene causes death at birth due to hypoxia (Hubner et al., 2001). Furthermore, it is thought that BDNF signaling from microglia may be a cause for neuropathic pain; a condition of hypersensitive pain pathways (Coull et al., 2005). For this reason KCC2 has recently been evaluated as a pharmacological target for CNS disorders such as neuropathic pain and morphine-induced hyperalgesia (Gagnon et al., 2013). We have shown previously that intra-VTA administration of furosemide, a non-selective KCC2 inhibitor, produces effects similar to those found in chronic opiate dependence (Ting et al., 2013).

Combined Model

To combine these mechanisms (see **Fig. 2**), our model suggests that BDNF acts on TrkB to down-regulate KCC2 in the VTA. Because KCC2 normally removes Cl^- from the intracellular space, its down-regulation leaves an excess of Cl^- inside the cell. This results in a shift in the excitability of the cell. This hyperexcitability is manifest in what is known as the Cl^- reversal potential, or rather, the membrane potential at which the normal direction of Cl^- ion flux reverses. This shift occurs in GABA_A receptors in response to opiates, which we call GABA switching (Vargas-Perez, Ting, et al., 2009).

We hypothesize that GABA switching caused by activation of this BDNF/TrkB/KCC2 pathway is playing an important role in the development of EtOH dependence, as it is involved in opiate dependence. In order to test this hypothesis, we have transected this pathway at two

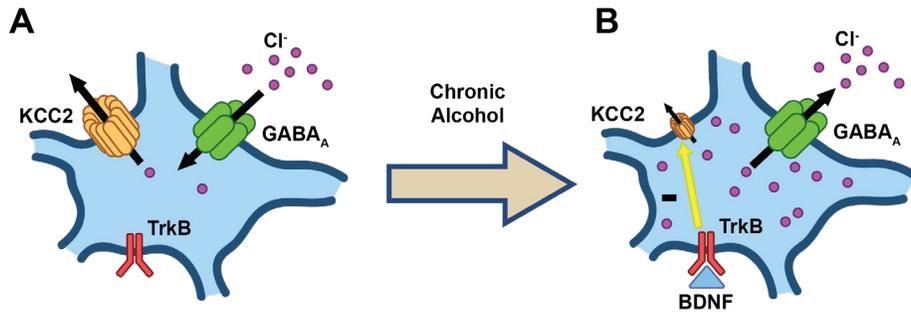


Figure 3-2: A representation of the current working model for alcohol dependence. A) In an alcohol-naïve state (Cl⁻) ions flow inward through GABA_A channels in response to GABA release, causing an inhibitory hyperpolarization of the cell and KCC2 then removes excess Cl⁻ from the intracellular space. B) In a dependent state, BDNF activates TrkB, which downregulates KCC2, yielding higher residual Cl⁻ inside the cell and allowing Cl⁻ to flow outward through the GABA_A channel and cause an excitatory depolarization of the cell.

different points: TrkB and KCC2. We activated and blocked TrkB using an agonist and antagonist and only activated KCC2 due to the extreme consequences of selective inhibition of KCC2. We established dependence through a chronic intermittent EtOH (CIE) methodology or through chronic injections.

Methods

Animals

All animals used in this study were treated in compliance with the Institutional Animal Care and Use Committee (IACUC) at Brigham Young University (BYU). Male mice older than postnatal day 28 were group housed except as experimentally required during drinking paradigms and were given *ad libitum* access to standard mouse chow and water. Animal housing was maintained on an inverted light cycle with lights turning off at 10:00 and turning on at 22:00 each day. Any activities conducted during the animals' dark cycle were performed using red

light to minimize interruption of circadian rhythms (Barbini et al., 1996; Roedel et al., 2006; Van den Hoofdakker & Beersma, 1988). Strains used include a C57BL/6J wild type strain and a GAD67 GFP+ strain on a CD-1 background.

Drug Administration

Chronic injections of EtOH or saline as well as acute injections of ANA-12 (Tocris and Cayman Chemical), 7,8-dihydroxyflavone (Sigma), and vehicle were accomplished via intraperitoneal injection using a sterile 27-gauge needle. Chronic injections of EtOH and saline were done twice daily for a minimum of 12 days. For drinking studies, acute injections were administered 3 hr prior to the commencement of the drinking session to ensure that the 4 hr timepoint was during the session (Cazorla et al., 2011). For immunohistochemistry (IHC) studies, acute injections were given 4 hr prior to cardiac perfusion.

ELISA

Measures of brain-derived neurotrophic factor (BDNF) and its precursor, proBDNF, were obtained using an enzyme-linked immunosorbent assay (ELISA) kit (Biosensis Cat. No. BEK-2211-1P & BEK-2217-1P respectively). The standard Biosensis sample preparation for brain tissue extracts using RIPA buffer was used. In short, tissue samples were extracted and snap frozen in liquid nitrogen then stored at -70°C until time of use. They were then homogenized via sonication in ice cold RIPA buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1.0% Triton X-100, and 0.5% sodium deoxycholate with a pH between 7.5-8.0. Samples were kept on ice for 30 min, and homogenization and ice-cold incubation were repeated. The homogenate was centrifuged at 10,000-20,000 x g at a temperature of 4 °C for 30 min and the supernatant fluid was transferred into new centrifuge tubes and stored at -70 °C. The samples were then centrifuged under the same conditions prior to being used in the ELISA.

Alcohol Consumption

Alcohol consumption was measured using a drinking in the dark (DID) model of binge drinking as previously described or a similar two-bottle choice paradigm (Anderson et al., 2016). In the DID paradigm, C57 mice were single housed during the duration of the experiment to ensure that consumption rates could be individually measured for each mouse. During each drinking session, the water bottle was removed and replaced by a bottle containing 20% EtOH, diluted from a 190 proof stock (Decon Laboratories, Inc.). At the conclusion of the session, the EtOH bottle was removed and the water bottle was returned to the cage. The EtOH bottle was weighed before and after each session, and three control bottles were placed in empty cages to control for evaporation, drips during the session, and loss during the inversion of the bottle. Ethanol solutions were made fresh each day. Drinking sessions were scheduled 4 days per week with the first three sessions lasting 2 hr and the final session lasting 4 hrs. The drinking sessions began at three hours into the dark cycle, for optimal activity. Drinking sessions were administered for two weeks prior to chronic EtOH exposure and two weeks following chronic EtOH exposure. In the two-bottle choice paradigm, the mice were placed in separate cages at the beginning of the session and presented with two bottles: one containing water and one containing 20% EtOH. Consumption was measured by volumetric graduations, and testing was performed 5 days per week, 2 hr per session. They were returned to their home cages after the session. All other details of the procedure are the same as in the DID paradigm.

Chronic Intermittent EtOH

To produce dependence, mice were exposed to EtOH vapors using a chronic intermittent EtOH (CIE) paradigm (Becker & Lopez, 2004; Griffin et al., 2009). In short, air was bubbled through an EtOH bath and then supplied to half of the cages for 16 hours per day. The EtOH used was 200 proof to prevent accumulation of water in the flask (Decon Laboratories, Inc.).

This exposure began at 10:00 each morning and concluded at 02:00 the following morning. Mice received EtOH in this manner for four consecutive days each week and were then given air with no EtOH for the following 3 days. The schedule was controlled automatically by a timer and electrically operated solenoid valve. This schedule was kept for 6 total weeks of exposure, after which DID was repeated. The other half of the cages received clean air during the entire CIE schedule.

Immunohistochemistry

Brains used in IHC studies were preserved using cardiac perfusion of phosphate buffered saline (PBS) and then 4% paraformaldehyde (Alfa Aesar). Brains were then extracted and stored overnight in 4% paraformaldehyde (PFA). The brain was then moved to a 10% sucrose solution and incubated at 4 °C until it dropped to the bottom of the vial. It was then moved to a 20% sucrose solution and then a 30% sucrose solution, incubating at 4 °C each time until the brain sank to the bottom of the vial. The brains were then fixed to a microtome (American Optical Company, model 860) and frozen using dry ice. Slices containing the VTA or NAc were prepared at a thickness of 40 µm and stored in cryoprotectant. Slices were incubated in primary antibodies for KCC2 (Rabbit anti-KCC2 polyclonal antibody, ThermoFisher Cat No. PA5-78544, 1:500 dilution) and FOX3/NeuN (Mouse anti-FOX3 monoclonal antibody, EnCor Biotechnology Cat. No. MCA-1B7, 1:1000 dilution) as well as secondary antibodies (Donkey Anti-Rabbit IgG H&L AlexaFluor 647, 1:1000 dilution, and Goat Anti-Mouse IgG H&L AlexaFluor 405, 1:1000 dilution, Abcam Cat. Nos. ab150075 & ab175660 respectively). Primary antibodies incubated for 18 hrs at 4 °C and secondary antibodies incubated for 2 hrs at room temperature, all while being gently agitated using an orbitron rotator (Boekel Scientific). Next, slices were mounted to microscope slides using Vectashield (Vector Laboratories). A

confocal microscope was used to generate a Z stack for each image, and image analysis was accomplished using a custom workflow (available upon request).

RESULTS

BDNF and Pro-BDNF Expression in EtOH Withdrawal

Prior to investigating any effects of EtOH on TrkB or KCC2, it was imperative to understand whether BDNF expression is altered in chronic alcohol exposure. Since an increase in BDNF expression is the beginning of the mechanism proposed in this model, this was a critical point to validate prior to proceeding. We conducted an ELISA to evaluate the expression of mature BDNF as well as its precursor pro-BDNF in mice that had been exposed to at least three weeks of CIE or clean air in the vapor chambers. This was done at 24 hours of withdrawal and 7 days of withdrawal to assess how long the effects would last. We also made these same

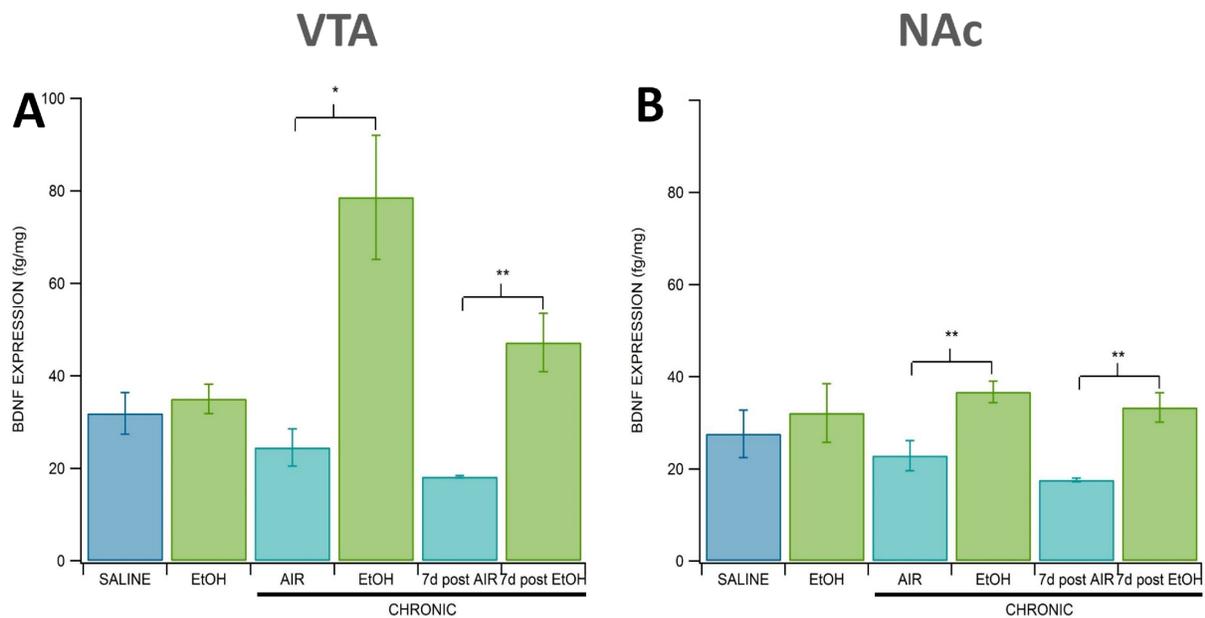


Figure 3-3: BDNF expression in the VTA (Left) and NAc (Right) of mice exposed to acute vs. chronic ethanol, including time points at 1 day of withdrawal and at 7 days of withdrawal. Acute exposure was administered by injection of saline vs. EtOH and chronic exposure was administered using the CIE methodology. The air group received no alcohol in the air supply.

measurements on animals that had received only a single intraperitoneal injection of EtOH or saline to compare the acute and chronic effects of alcohol on BDNF expression. We found that there were no acute alterations to mature BDNF or pro-BDNF at 8hr, 16hr, or 24hr of withdrawal from a single intraperitoneal injection (2.5g/kg) of alcohol. However, mature BDNF was significantly increased in withdrawal from chronic alcohol exposure (**Fig. 3**). Twenty four hours after the last exposure to EtOH, BDNF expression in the VTA and NAc was significantly increased, which effect persisted at 7 days of withdrawal. In the NAc, this same pattern occurred but with a much more muted amplitude.

Effects of BDNF on VTA GABA Neuron Firing Rate

With an understanding of the increased expression of BDNF in the VTA in withdrawal from chronic alcohol exposure, and in light of the fact that VTA GABA neurons are hyperexcitable under these same circumstances, we desired to know if BDNF was having direct effects on GABA neurons in the VTA, causing this hyperexcitability (Steffensen et al., 2009). We measured this using patch clamp electrophysiology in an acute brain slice preparation. We

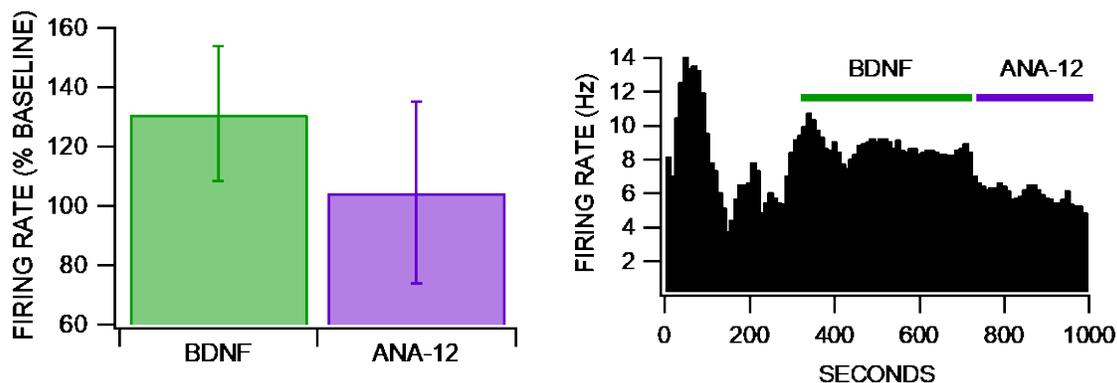


Figure 3-4: Firing rate of VTA GABA neurons appears not to be impacted by acute superfusion of BDNF. (Left) Mean firing rate as a % of baseline with acute superfusion of BDNF first and the TrkB antagonist ANA-12 next. (Right) A representative trace illustrating the pattern of firing rate as it progresses throughout the experiment.

found that with acute application of BDNF via superfusion, no change in VTA GABA neuron activity was significant, although a slight trend toward an increase exists, which then disappears with the TrkB antagonist ANA-12 (**Fig. 4**). This was measured on the 5 min and 30 min timescale.

Alcohol-induced Changes in KCC2 expression

After determining that there were not clear effects of acute BDNF superfusion on VTA GABA neuron firing rate, we wanted to determine what chronic changes were occurring in the BDNF pathway in response to the increase in BDNF observed in **Fig. 3**. To accomplish this, we evaluated the expression of KCC2 in an alcohol naïve state and in alcohol withdrawal.

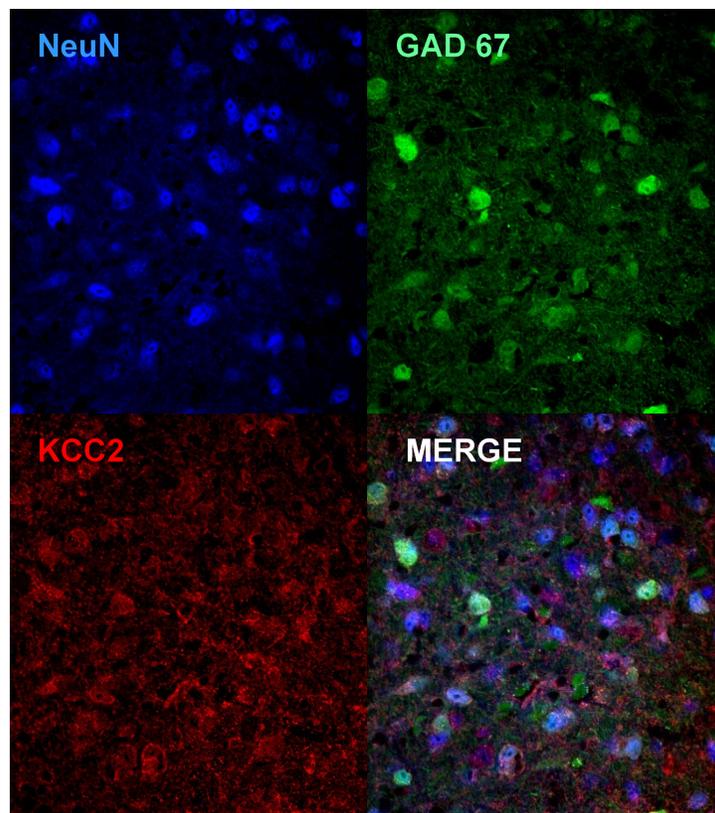


Figure 3-5: Fluorescence imaging of brain slices with a transgenic GAD67-GFP marker in the green channel, a NeuN neuronal stain in the blue channel, and a KCC2 stain in the red channel.

We measured KCC2 expression using immunohistochemistry (IHC) and fluorescence microscopy with a transgenic GAD67-GFP marker in the green channel, a NeuN neuronal stain in the blue channel, and a KCC2 stain in the red channel (**Fig. 5**). We observed that chronic alcohol increased the expression of KCC2 in both the NAc and the VTA without any drugs applied (**Fig. 6**). When a TrkB agonist (7,8-DHF) was given IP 4hrs prior to fixing the brain, the effect was maintained, and expression increased further in both the chronic saline and chronic alcohol conditions. When a TrkB antagonist (ANA-12) was given IP 4hrs prior to sacrificing the animal, the effect was reversed, and a decrease in KCC2 expression was observed in response to chronic EtOH exposure. This was an unexpected result and will be discussed in more detail hereafter.

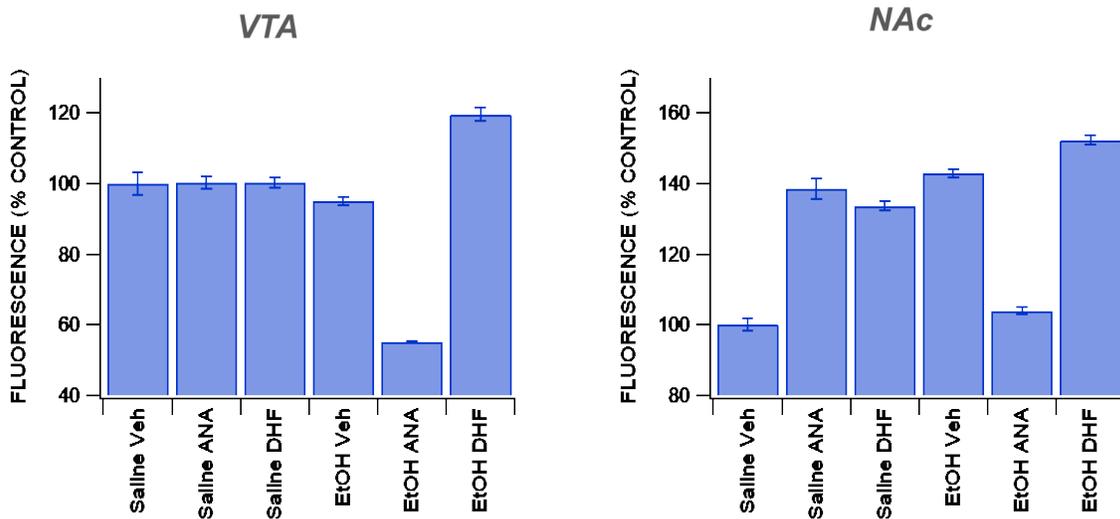


Figure 3-6: Immunohistochemistry group means in the ventral tegmental area (VTA) and nucleus accumbens (NAc). Mice were chronically injected with either saline or EtOH and given an acute injection of ANA-12, 7,8-DHF, or vehicle 4 hours before cardiac perfusion.

Role of TrkB in EtOH seeking behavior

Prior to measuring EtOH seeking behavior, we wanted to validate that the CIE methodology we employed caused an increase in drinking, which we use as an indirect measure of dependence. To accomplish this, we first measured baseline drinking behavior using a two-bottle choice paradigm and then administered one week of CIE. We then repeated this cycle another three times for weeks 3-8, and on the fourth cycle we omitted the CIE in week 9 and allowed the animals to withdraw then measured drinking for one more week to see if the increase in drinking persisted after a week of withdrawal. We observed that after the second week of CIE, there was a significant difference in drinking behavior between the EtOH vapor mice and the air mice, which continued to amplify through week 8 (Fig. 7). After one week of withdrawal in week 9, the drinking in the EtOH exposed mice began to reduce slightly but was still significantly higher than the air exposed mice, suggesting persistent dependence.

With this tool validated, it was critical to ascertain whether this BDNF pathway impacts EtOH seeking behavior. To assess this, we used a modified drink-in-the-dark (DID) paradigm in

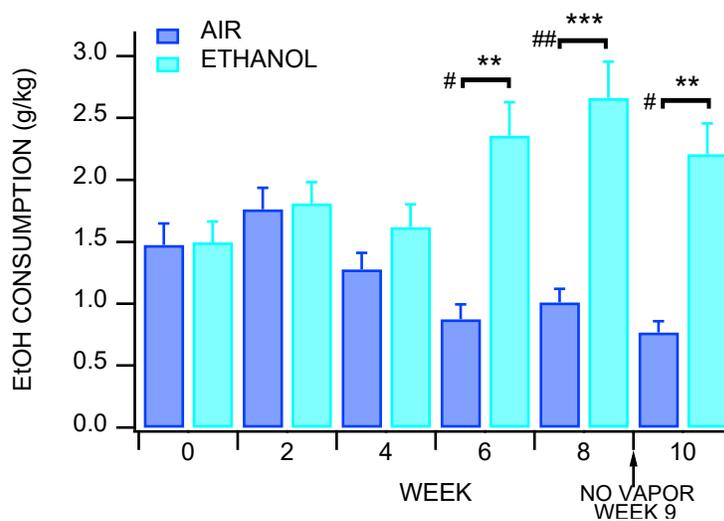


Figure 3-7: Drinking behavior as measured by two bottle choice, showing an increase in drinking behavior in the EtOH exposed mice with no increase in the air exposed mice.

conjunction with CIE. First, we measured baseline drinking behavior before any chronic alcohol administration. Following a 6-week CIE period, we retested drinking for a week to assess whether CIE produced an increase in drinking behavior. We then measured drinking for another week with an acute injection of a TrkB drug or vehicle 3.5 hours prior to each session.

We observed that drinking behavior did increase after CIE for the mice exposed to EtOH, as expected (**Fig. 8**). As predicted, the air control mice showed an increase in drinking behavior when given a TrkB agonist and a decrease in drinking behavior when given a TrkB antagonist. However, there was no significant difference between the mice that received EtOH during CIE when either drug was given.

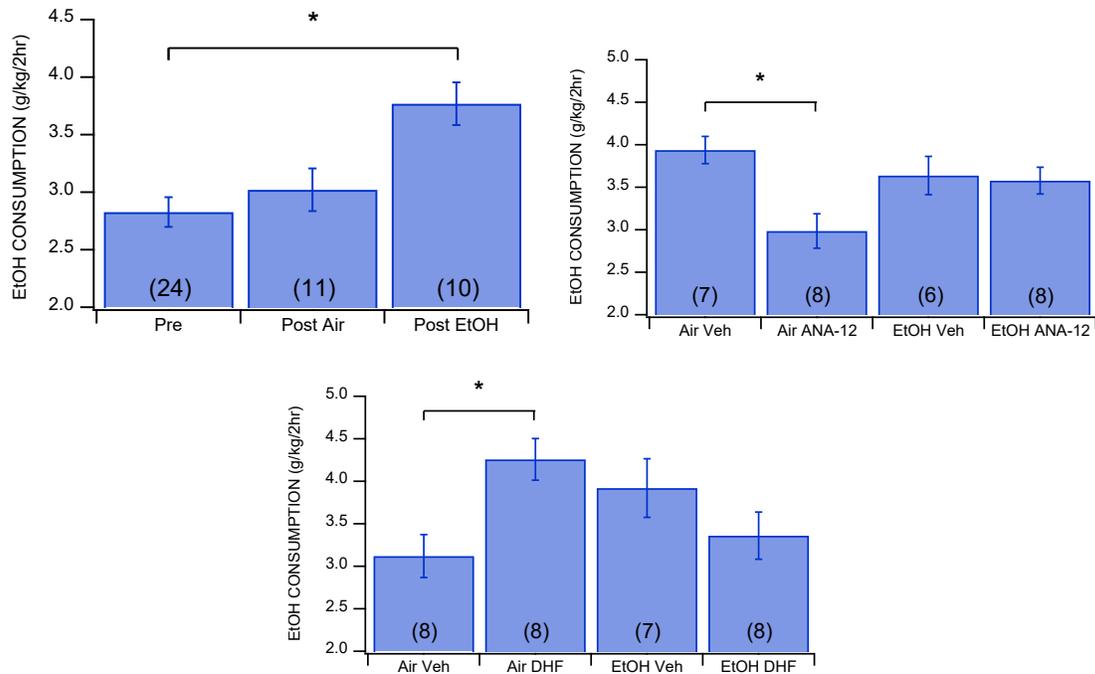


Figure 3-8: Alcohol consumption in response to CIE and TrkB drugs. (Top left) Alcohol consumption before and after CIE for both the air-exposed mice and the EtOH-exposed mice. (Top right) Consumption after CIE with an acute injection of ANA-12 or vehicle. (Bottom) Consumption after CIE with an acute injection of 7,8-DHF or vehicle.

Discussion

BDNF Expression

Consistent with our hypothesis, we found that BDNF expression increased in the VTA in withdrawal from CIE exposure but not following acute EtOH exposure. This effect persisted for at least 7 days, suggesting that the increase was related to adaptations that occurred as a result of CIE exposure. This same trend was observed in the NAc but was less pronounced.

It has been shown that BDNF has preservative functions in the brain (Acheson et al., 1995). Consequently, one might expect it to have protective effects as well. Our data suggest that BDNF is likely having an adverse effect in this context, contrary to its typical functions. Since EtOH is a solvent harmful to living tissues (used as a sanitizing agent at high concentrations), it seems reasonable to postulate that the BDNF secretions we report are generated in response to perceived damage or dysregulation caused by EtOH and are intended to be protective but result in deleterious effects. Additionally, we have reported a connection between BDNF and opiate dependence (Vargas-Perez et al., 2014; Vargas-Perez, Ting, et al., 2009). So, while the peptide may have positive effects in some cases, there is evidence it can also be detrimental. At this point, it is unclear whether this increase in BDNF is the result of microglia or synaptic release. This is worthy of future investigation.

VTA GABA Neuron Activity

Since it is apparent that BDNF is over-expressed in EtOH withdrawal, we wanted to determine if it was having direct effects on naïve VTA GABA neurons, given their robust adaptations to chronic EtOH. We found that there were no obvious changes in activity in GABA neuron activity in naïve mice with the slight exception of a subtle trend toward an increase in

firing rate, which is reversed by the TrkB antagonist ANA-12. With a larger sample size it could be verified whether this is a real effect or noise.

Changes in KCC2 Expression During Withdrawal from Chronic EtOH

The KCC2 IHC yielded unexpected results. In the animals that received chronic saline injections, there were no differences in KCC2 expression in the VTA when a TrkB agonist or antagonist was given. Consistent with our expectations, we observe a decrease in KCC2 expression in the VTA in withdrawal from CIE exposure and without exposure to any additional drugs. We hypothesized that ANA-12 would block this effect, but it reduced expression. We hypothesized that 7,8-DHF would decrease expression, but we observed an increase in expression compared to our vehicle condition. Interestingly, the TrkB agonist and antagonist had opposite effects, which gives added credibility to these results, but the expression patterns shifted in the opposite direction of what we expected. Similar results were noted in the NAc with the notable exception being that in the chronic saline-injected animals we did see an increase in KCC2 expression with both the TrkB agonist and antagonist.

These results are difficult to interpret within the theoretical construct of our current model. However, additional experiments are underway to validate these results.

Role of TrkB Receptors in EtOH Consumption

Prior to using our CIE methodology, it was essential to validate that this procedure produced evidence of dependence. We measured drinking behavior alternating with CIE every other week, and we observed that the mice who received EtOH during the CIE weeks increased their drinking while those that received only clean air during the CIE weeks did not. This confirms that our CIE apparatus and methodology produces an increase in drinking for mice exposed to EtOH vapors.

When we injected ANA-12 after 6 weeks of CIE, we discovered that it reduced drinking behavior. This was consistent with our hypothesis, but the effect was only observed in the air-exposed animals. The EtOH dependent animals were unaffected by the ANA-12 injection. This could be due to adaptation of TrkB receptors because of repeated withdrawal sessions during the weeks of CIE. As we noted above, BDNF expression increases substantially in the VTA in withdrawal from CIE exposure, and during CIE the mice were withdrawn from alcohol every weekend for three days. It is therefore possible that during this time the increased exposure to BDNF secretions caused a downregulation of TrkB, which would have reduced the impact of ANA-12 in our drinking paradigm.

When we injected 7,8-DHF under the same circumstances, the result was the functional opposite of the ANA-12 injections. We observed an increase in drinking behavior in the air-exposed group, but again, we saw no difference in the dependent group. This also supports the idea that perhaps there is TrkB adaptation occurring in CIE exposure that is blunting the effects of the TrkB drugs.

Conclusion

In summary, it is apparent that the BDNF/TrkB/KCC2 pathway is involved in EtOH consumption, but there is yet much to uncover about the specifics of this involvement. The fact that the TrkB antagonist did not decrease drinking in EtOH-dependent animals (as assessed by increased drinking) reduces the therapeutic potential of using TrkB as a drug target for AUD. However, there is still much potential for KCC2 to be used as a drug target, and it seems probable that blocking KCC2 will result in very few side effects, since activating KCC2 will reduce intracellular chloride levels, and those levels are already low in healthy individuals.

Thus, it would represent a use-dependent therapeutic approach. Future work is needed to further elucidate the potential of KCC2 as a novel target for AUD therapeutics.

CHAPTER 4: CD5 knockout mice exhibit reduced sensitivity to the sedative
effects of alcohol and reduced alcohol seeking behavior

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Abstract

Cluster of differentiation 5 (CD5) is expressed in both T and B cells and has been found to display an altered expression profile following chronic ethanol use and during ethanol (EtOH) withdrawal. Specifically, the number of CD5+ B cells is reduced during withdrawal while the number of T cells is increased. Given the apparent sensitivity of these cells to EtOH, and recent research suggesting that some of EtOH's effects are accounted for by neuroimmune interactions, we assessed EtOH drinking behavior and sedation in CD5 knockout (KO) mice. We found that CD5 KO mice display decreased EtOH consumption compared with wild-type (WT) controls in a 24-hour access two-bottle choice drinking paradigm. Additionally, CD5 KO mice displayed considerable resistance to the sedating effects of EtOH. We found no difference in dopamine release dynamics in the nucleus accumbens nor in the ethanol-induced inhibition of GABA neurons in the ventral tegmental area, both of which are neuronal substrates implicated in the rewarding properties of EtOH. These results suggest that CD5 KO mice are less sensitive to some of the effects of EtOH and that peripheral neuroimmune mechanisms involving CD5 may be a factor in EtOH consumption and sedation.

Introduction

Unlike most drugs of abuse, the mechanisms of action of ethyl alcohol (EtOH) on the brain remain largely elusive. It has been proposed that interactions between the nervous system and the immune system could mediate some of the acute effects of EtOH on the brain, and the evidence base to support this hypothesis continues to grow. Relatedly, it has been observed that some immune factors are sensitive to chronic EtOH administration (Margoles et al., 2016; Zhang et al., 2017). One such marker is cluster of differentiation 5 (CD5).

T cells are key players in the adaptive immune response. Sometimes called Ly-1 (or Lym-1) in mice and Leu 1 in humans, CD5 is a 67kDa trans-membrane glycoprotein that is expressed on T cells and B cells with a higher expression on T cells (Huang et al., 1987; Youinou et al., 1999). It has been reported that CD5 has numerous effects on the immune system as well as effects on related systems. It is known to regulate development, intracellular signaling, and cytokine production (Henderson et al., 2015; Li et al., 2019). Additionally, it has been shown to be a positive and negative regulator of T cell receptor signaling, a negative regulator of B cell receptor signaling, and a promoter of mature T cell survival [Reviewed in (Burgueno-Bucio et al., 2019)]. It has also been found that CD5 can regulate basal NF- κ B signaling in T cells (Matson et al., 2020). Because of its high level of structural conservation between the murine and human forms, it is sometimes assumed to be of critical importance (Huang et al., 1987). B cells that are CD5⁺ play a crucial role in regulating myelinization of neurons during development while T cells contribute to synaptogenesis during development (Tanabe & Yamashita, 2018; Zarif et al., 2018; Ziv et al., 2006). Specifically, T cells encourage the creation of inhibitory synapses to GABA neurons while discouraging the creation of excitatory synapses through interferon γ (Brask et al., 2004; Fujita et al., 2017; Kim et al., 2002). Furthermore, it's been reported that B cells that are CD5⁺ produce more interleukin-10 (IL-10) than CD5⁻ B cells

and it has been observed that the soluble form of CD5 can bind interleukin-6 (IL-6) (Aparicio-Siegmund et al., 2017; Gary-Gouy et al., 2002). This in conjunction with our recent observations that IL-10 and other cytokines may be influential in the rewarding and reinforcing properties of EtOH or other drugs of abuse makes CD5 particularly interesting (data not yet published).

Neurons in the ventral tegmental area (VTA) have been shown to be sensitive to the effects of EtOH (Steffensen et al., 2009; Stobbs et al., 2004). In particular, GABA neurons are known to be inhibited by an acute dose of EtOH, at doses of EtOH that are physiologically relevant to reward, while dopamine (DA) neurons are excited by acute EtOH, albeit at high levels of EtOH (Brodie et al., 1990; Gessa et al., 1985). These effects are seen both *in vivo* and *in vitro*, albeit with greater sensitivity *in vivo*. Additionally, in chronic EtOH exposure, VTA GABA neurons become hyperexcitable during withdrawal from EtOH (Gallegos et al., 1999). Interferon γ is known to both enhance and inhibit GABAergic tone and is also known to have increased expression following EtOH consumption, with T cells serving as one of the main sources for interferon γ in the VTA (Duncan et al., 2016; Filiano et al., 2016; Janach et al., 2020; Johnson et al., 2015; Laso et al., 1999; Muller et al., 1993; Pascual et al., 2015; Zhu et al., 2011).

The purpose of this study was to evaluate the impact of CD5 expression on EtOH effects in a mouse model. It was hypothesized that CD5 would play a role in the acute effects of EtOH and that the brain in a CD5-deficient animal would be found in a hyper-DAergic state. The results of this study add to the understanding of the acute effects of EtOH on the brain, an area which is in much need of additional insight and novel substrates to consider for treatment of AUD.

Methods

Animal Subjects

This study was conducted using male and female CD5 knockout (CD5 KO) mice on a C57BL/6J background. As a genetically similar control, male and female C57BL/6J mice were used. The data from the male and female cohorts were compared for similarity and conjoined if there were no significant sex differences. All mice were bred, housed, and handled in compliance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Procedures and protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Brigham Young University (BYU) in Provo, Utah. Mice were only used for this study after postnatal day 28. For the drinking paradigm, the mice were not used until their body weight had stabilized at its full adult value. All animals were housed in maximum groups of 5 in individually-ventilated cages in a temperature and humidity-controlled environment with the exception of the mice in the drinking paradigm, which were housed singly for the duration of their participation in the test. The animal housing rooms were maintained on a 12 hr reverse light cycle. For activities that needed to be performed during the dark cycle, red light filters were used to minimize the impact on circadian rhythms. Animals were given *ad-libitum* access to water and solid food continuously except during the brief periods during which behavioral measures were conducted.

Flow Cytometry

For CD5 expression analysis in the wild type and CD5 knockout mice, single cell suspensions of CD4⁺ T cells from CD5KO and WT C57/BL6 mice spleens were purified using a positive selection CD4⁺ T cell isolation kit (Miltenyi Biotec). Purified CD4⁺ T cells were then stained with anti-CD5-PE (12±0051; eBioscience) and anti-CD4-APC (17±0041; eBioscience) and CD5 surface expression was analyzed via flow cytometer (BD Accurri C6).

Locomotor Activity

Locomotor activity was measured using an open field behavioral paradigm. Prior to the test, an intraperitoneal (IP) injection of saline or EtOH (0.5 - 2.0 g/kg body weight) was administered using a sterile needle. For all IP EtOH injections, a 16% (w/v) EtOH solution filtered at 0.2 μm was utilized. For all saline injections, a 0.9% NaCl solution filtered at 0.2 μm was utilized. Animals were injected without the use of anesthesia to prevent the introduction of confounding variables. Immediately following the injection, the mouse was placed into an open field where its movement in the chamber was recorded for 30 min using an infrared (IR) video surveillance camera (Night Owl NI-103A-I-TD camera, VITEK VTQ-B01 B/W Quad Processor, Pinnacle Studio Software). Total distance traveled during the session was then calculated using Noldus Ethovision (Wageningen, the Netherlands) by tracing the path followed by the mouse during this window of time. Saline was administered in the first session to establish a baseline locomotor activity for each animal. Following two days of recovery, the EtOH sessions were administered, starting at the lowest dose and progressing to the highest dose with two days between each session. The percent baseline calculation was accomplished by dividing the distance traveled during the respective EtOH trial by the distance traveled during the saline trial.

Loss of Righting Reflex

The loss of righting reflex (LORR) was measured following a sedating IP injection of EtOH (4.0 g/kg). Following the injection, the mouse was placed on its back in a behavioral chamber and observed and recorded through IR camera surveillance. After the mouse had righted itself, it was removed and returned to its home cage. The video files were then scanned manually to identify the time that the animal righted itself. This time was recorded, and the duration of LORR was calculated by subtracting the time of injection from the time of righting.

Two Bottle Choice Drinking

To measure EtOH consumption, a 24-hr access two bottle choice drinking paradigm was used. Briefly, animals were separated into individual cages and given *ad-libitum* access to food and two bottles, one containing water and the other containing 20% (w/v) EtOH for a period of 24 hr (190 proof EtOH, Decon Laboratories, Inc. Cat. No. UN1170). At the end of the 24-hr window, the bottle containing the EtOH was removed and replaced by a second water bottle. On the next day, one of the water bottles was again replaced by a bottle containing EtOH. This pattern was repeated such that the animals had access to EtOH for a 24-hr period every other day for 3 days per week. This pattern persisted for a total of three weeks of intermittent EtOH access. Each time the EtOH bottle was added to the cage, the position was alternated to control for intrinsic side preference. EtOH consumption was calculated on a pure EtOH per kg of body weight.

Fast Scan Cyclic Voltammetry

To measure DA release dynamics, fast scan cyclic voltammetry (FSCV) was utilized using a modification of methods previously published (Yorgason et al., 2020). Briefly, the mouse was anesthetized by brief exposure to isoflurane and then quickly decapitated. The brain was then rapidly extracted and placed in artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl₂, 2.4 CaCl₂, 1.2 NaH₂PO₄, 21.4 NaHCO₃, 11.1 D-Glucose (NaCl, Fisher Chemical Cat. No. S271-3, KCl, Macron Fine Chemicals Cat. No. 6858-04, MgCl₂, VWR Cat. No. 0288-500G, CaCl₂, Fisher Chemical Cat. No. C614-500, NaH₂PO₄, Fisher BioReagents Cat. No. BP329-500, NaHCO₃, Macron Fine Chemicals Cat. No. 7412-06, D-Glucose, Fisher Chemical Cat. No. D16-3). The glutamate antagonist Ketamine (0.1 mg/mL) was also added to the cutting solution to reduce excitotoxicity and thereby improve slice health (Mylan, Cat. No. NDC 67457-001-00). The brain was sectioned at room temperature using a

Leica vibratome (model VT 1200 S) to a thickness of 300 μm and incubated in ACSF maintained at 35 $^{\circ}\text{C}$ until the end of the experiment. Recordings were made using carbon fiber electrodes (7 μm diameter carbon fiber, Thornel T-650, Cytec). A strand of carbon fiber was aspirated into a borosilicate glass capillary tube and then pulled on a vertical Narishige pipette puller. The carbon fiber was then cut so that 100-150 μm was left protruding out the tip of the electrode. When a DA signal with an appropriate voltammogram in the NAc was located, baseline spontaneous DA currents were recorded. The rate of occurrence of these spontaneous currents was then compared between groups. Demon Voltammetry Analysis Software (Wake Forest Innovations) was used to collect and analyze the FSCV data (Yorgason et al., 2011).

Single-Unit Recordings

Single-unit recordings were performed *in vivo* under 1-2% isoflurane anesthesia (Fluriso, VetOne, Cat. No. ADC 13985-528-60). Following brief anesthesia, the mouse was placed on a clean temperature regulating pad and secured in place using ear bars. The scalp was opened and an approximately 3 mm hole was drilled in the skull above the ventral tegmental area. Recording electrodes were pulled using a vertical pipette puller (puller: Narishige Cat. No. PE-2, capillary tubes: A-M Systems Cat. No. 603000) and the tips broken under microscopic control to 1-2 μm internal diameter and an impedance of 1-3 MOhm tested at 1000 Hz and filled with 1.0 M KCl (Macron Fine Chemicals Cat. No. 6858-04). Using stereotaxic manipulators, the recording electrode was navigated to the VTA [from bregma: 2,900 μm anteroposterior (AP); 300 μm mediolateral (ML)]. The electrode was maneuvered from 3,700 μm to 5,200 μm dorsoventral (DV), and the number of GABA neurons encountered and baseline firing rate for each was recorded for the tract. This was then repeated for multiple tracts per animal prior to recording transient GABA activity. Following the tract recordings, a stable GABA neuron was

located with a firing rate of between 10-40 Hz and a recording of the discriminated spikes began. Following stabilization of firing rate, an IP saline injection was administered. The behavior of the neuron was recorded for 30 min and then an IP injection of EtOH (0.75 g/kg) was administered. The neuron was then monitored for another 30 min prior to terminating the experiment. At the conclusion of the experiment, the animals were humanely euthanized by isoflurane overdose and cervical dislocation to confirm death.

Results

Knockout Characterization

The first imperative was to confirm that the knockout mouse was a valid model for a CD5 deficient organism. We accomplished this using flow cytometry. We observed that the CD5 population that was present in the C57 control group was notably absent in the CD5 KO group (Fig. 1).

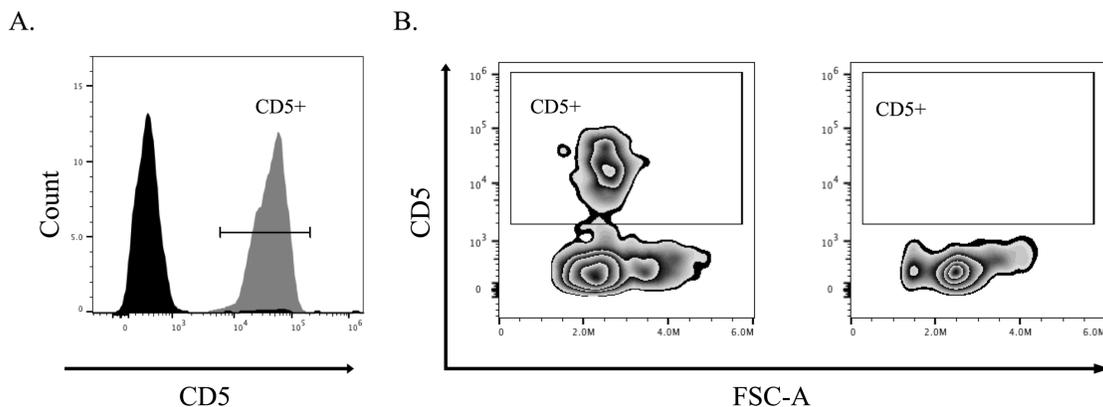


Figure 4-1: CD5 Knockout (KO) mice exhibit a lack of CD5 expression. A) Histogram illustrating the number of particles counted that are CD5+ between the KO (left) and the wildtype (right). B) Flow cytometry scatter diagrams illustrating the CD5+ population in wild type (left) and CD5 KO (right) mice.

Alcohol-Induced Sedation

Under typical circumstances, EtOH at low doses reduces locomotor activity, and at high doses it induces ataxia and unconsciousness, both of which are manifestations of the sedative properties of EtOH. To assess the sedative effects of EtOH in a CD5 deficient mouse population, we measured locomotor activity and loss of righting reflex (LORR) in both C57 and CD5 KO mice.

Locomotor activity following treatment with saline or EtOH (0.5 – 2.0 g/kg, IP) was assessed using a mixed model ANOVA with genotype as a between-subjects factor and dose as a within-subjects factor. Subjects were 15 mice (8 WT and 7 CD5 KO) who underwent all treatment conditions. Locomotor activity was normalized to the saline condition in each mouse.

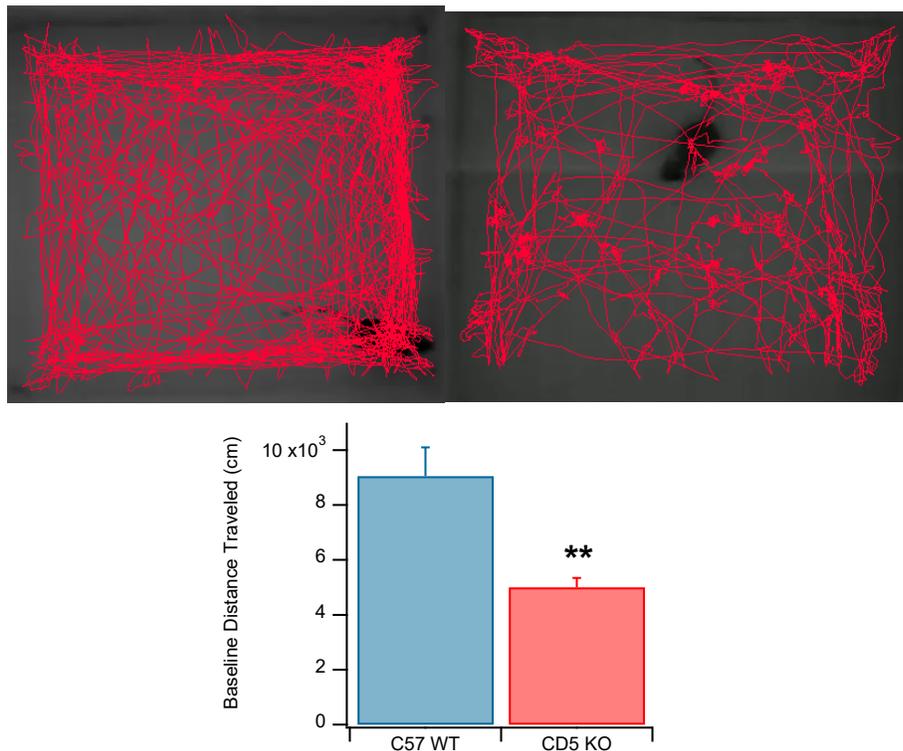


Figure 4-2: CD5 KO mice have lower baseline locomotor activity. (Top left) Representative trace from a C57 saline run, (Top right) Representative trace from a CD5 KO saline run, (Bottom) The baseline distance traveled compared between C57 and CD5 KO mice.

The Greenhouse-Geisser correction for sphericity was applied to all within-subjects factors in this analysis.

Prior to analysis all data were assessed for normality and outliers. The data were found to be roughly normally distributed. One outlier was identified and fenced to the relevant limit. The treated data was analyzed alongside the original untreated data. As there were no differences in which terms were found to be significant between the two data sets and the effect size estimates were nearly identical, we report only the results from the analysis of the untreated data. The results of the analysis of the treated data are available upon request.

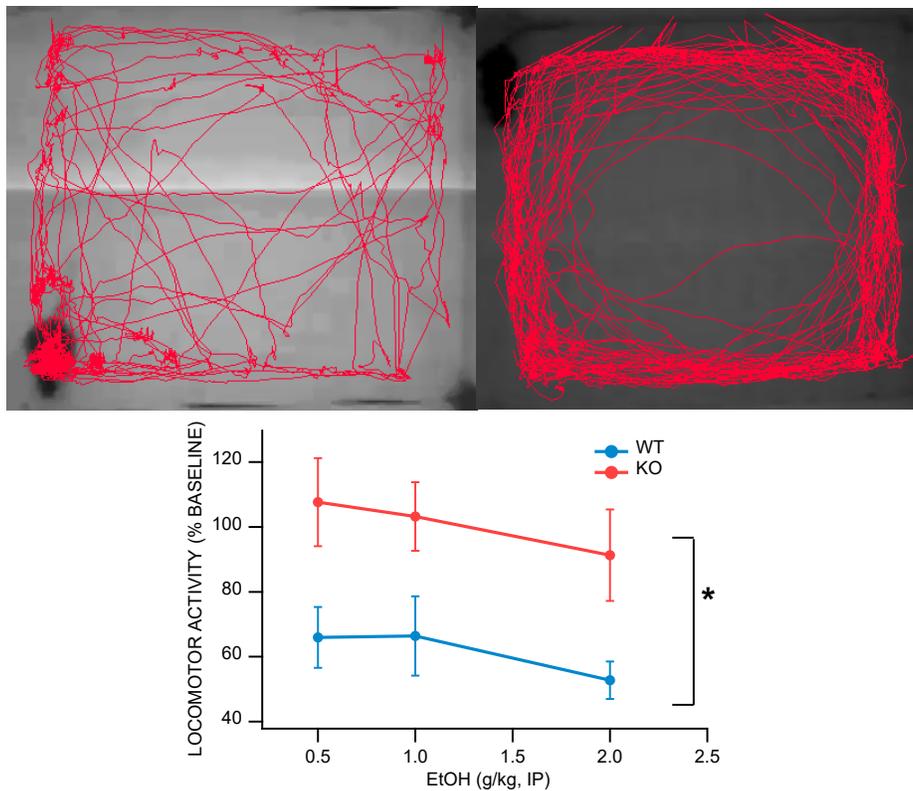


Figure 4-3: CD5 KO mice have blunted EtOH reduction to locomotor activity. (Top left) Representative tracing of a C57 2.0g/kg run, (Top right) Representative tracing of a CD5 KO 2.0g/kg run, (Bottom) Dose response curve showing the impact of EtOH on locomotor activity in C57 and CD5 KO mice.

We found that under baseline conditions CD5 KO mice exhibit significantly less locomotor activity than their wild type counterparts (**Fig. 2**). Additionally, EtOH significantly reduced locomotor activity in a dose dependent manner (**Fig. 3**; $F_{(3,39)} = 3.90, p = 0.018$, partial $\eta^2 = 0.23$ [0.01, 0.39]). The reduction in locomotor activity occasioned by ethanol administration was smaller in CD5 KO mice as compared with WT mice (**Fig. 3**; $F_{(1, 13)} = 10.18, p = 0.007$, partial $\eta^2 = 0.44$ [0.04, 0.66]). The degree to which the EtOH mediated decrease in locomotor activity was attenuated in CD5 KO mice was not dose dependent (**Fig. 3**; $F_{(3, 39)} = 2.86, p = 0.052$).

When injected with a high dose of EtOH (4.0 g/kg), CD5 KO mice showed a considerable trend toward a lower duration of LORR than the C57 controls, indicating a reduced response to the sedating effects of EtOH in the CD5 KO population (**Fig. 4**). However, this effect was not statistically significant, and as such, conclusions cannot yet be drawn based on these data. Additional experiments are underway to validate this trend with a more appropriately powered sample.

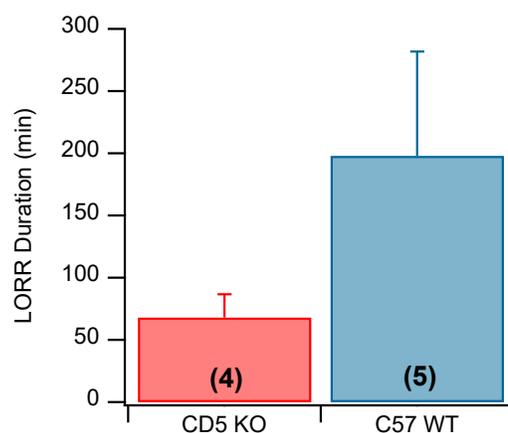


Figure 4-4: CD5 KO mice show reduced EtOH sedation (Preliminary). Preliminary data suggesting that CD5 mice exhibit a reduced loss of righting reflex with exposure to alcohol. Effect is currently underpowered and therefore not statistically significant.

Ethanol Consumption

Perhaps of greatest importance was to understand the impact of CD5 on EtOH seeking behavior. To measure this, we utilized a 24-hr access two-bottle choice drinking paradigm. Ethanol consumption in this paradigm was assessed using a mixed model ANOVA with genotype as a between-subjects factor and week as a within-subjects factor. Subjects were 12 mice (6 CD5 KO and 6 WT). Prior to analysis all data were assessed for normality and outliers as described above. These data were found to be roughly normally distributed. One outlier was identified and fenced to the relevant limit. The treated data were analyzed alongside the original untreated data. There were no differences in which terms were found to be significant between the two sets of data however it appeared that the effect size estimates were inflated by the outlier. As such we are reporting the results from analysis of the treated data. The results from the analysis of the untreated data will be made available upon request.

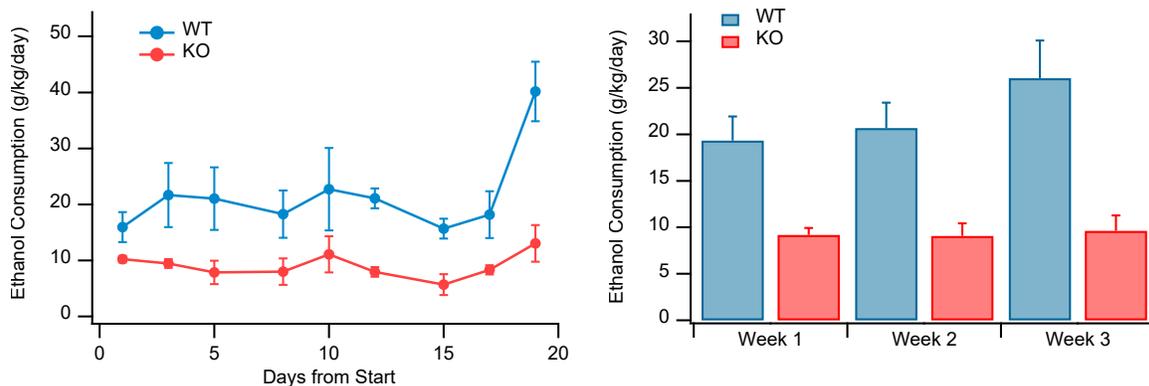


Figure 4-5: CD5 KO mice drink less ethanol than wildtype controls. (Left) CD5 KO mice consistently drank less ethanol than WT mice over three weeks. (Right) Weekly averages for WT and CD5 KO mouse ethanol consumption.

Ethanol consumption was lower in CD5 KO mice than in WT mice (**Fig. 5**; $F_{(1, 10)} = 9.64$, $p = 0.011$, partial $\eta^2 = 0.49$ [0.03, 0.71]). Additionally, EtOH consumption increased in both

groups over time (Figure 5. $F_{(2, 17)} = 4.99$, $p = 0.028$, partial $\eta^2 = 0.37$ [0.01, 0.58]). The increase in EtOH consumption over time was not genotype dependent (**Fig. 5**; $F_{(2, 17)} = 0.64$, $p = 0.514$).

Spontaneous Dopamine Release

We wanted to understand if these effects were mediated by DA release mechanisms, and so we compared the spontaneous DA release between the knockout mice and the controls. Spontaneous dopamine release events were measured in the slice preparation using FSCV. Utilizing a simple Student's T test, there was no difference observed between slices obtained from CD5 KO mice and slices obtained from C57 mice (**Fig. 6**; $p > 0.05$).

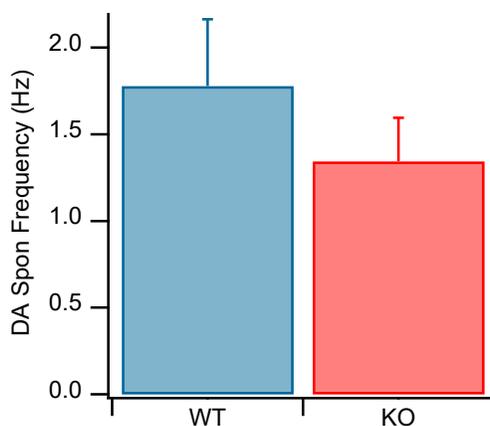


Figure 4-6: CD5 KO mice do not differ from WT controls on spontaneous DA release. CD5 KO mice do not exhibit altered frequency of spontaneous DA release in the NAc under baseline conditions.

Ethanol Effects on VTA GABA Neurons in CD5 KO Mice

Baseline firing rate data from 312 neurons recorded from 39 mice (see Table 1 for complete breakdown) were analyzed using a two-way analysis of variance (ANOVA) with sex and genotype as between subject factors.

Genotype	Sex	# Mice	# Tracts	# Neurons
C57 WT				
	Female	9	27	72
	Male	14	42	88
CD5 KO				
	Female	7	21	79
	Male	9	27	73

Table 1: Breakdown of the number of animals, tracts, and neurons recorded by group.

Post-hoc tests with a Bonferroni correction were performed following detection of a significant interaction term. Prior to analysis, data were assessed for outliers and normality using the interquartile range (IQR) rule and the Shapiro-Wilk test, respectively. The data were found to be non-normally distributed. Tukey’s ladder indicated that applying a log transformation to the data would result in a normal distribution. As such a set of transformed data was created where a log transform was applied and the outliers (3% of all data) were fenced to the relevant limit (median \pm 1.5 IQR). These data were analyzed alongside the original untransformed data. As there were no differences in which terms were found to be significant between the two data sets and the effect sizes were comparable (slightly smaller in the untransformed data set) we report

only the results from the analysis of the untransformed data. The results of the analysis of the transformed data are available upon request.

The baseline firing rate of VTA GABA neurons was found to be higher in female mice than in male mice (**Fig. 7**; $F_{(1, 308)} = 23.98, p < 0.001$, partial $\eta^2 = 0.07$ [0.03, 0.13]). There was not a significant effect of knockout of CD5 on baseline VTA GABA neuron firing rate (**Fig. 7**; $F_{(1, 308)} = 2.51, p = 0.114$). There was, however, a significant interaction between sex and genotype (**Fig. 7**. $F_{(1, 308)} = 5.53, p = 0.019$, partial $\eta^2 = 0.02$ [0.00, 0.06]). This significant interaction was likely due to the reduction in the baseline firing rate of VTA GABA neurons in CD5 KO females ($t = 2.74, p = 0.012$) but not CD5 KO males ($t = 0.55, p = 1.000$), relative to their sex-matched WT controls.

The peak reduction in VTA GABA neuron firing rate following EtOH administration was analyzed across 31 mice (n by group: female/WT = 6; female/KO = 6; male/WT = 11; male/KO = 8) using a two-way ANOVA with sex and genotype as between subject factors. Data were assessed for outliers and normality as stated previously. Data were found to be relatively normal

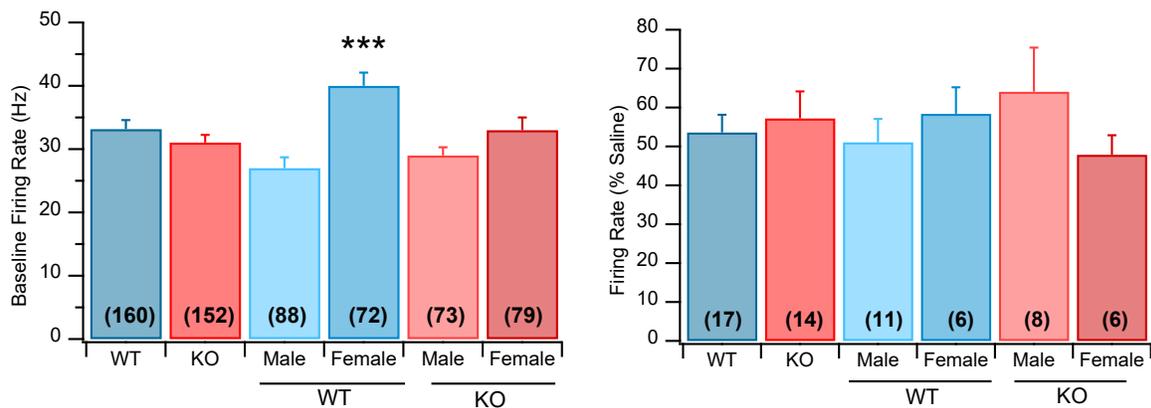


Figure 4-7: CD5 KO mice do not differ from WT controls in basal firing rate or EtOH dependent firing rate reduction. (Left) Baseline firing rate for GABA neurons. (Right) The peak reduction of firing rate induced by a 0.75g/kg intraperitoneal injection of EtOH.

and no outliers were detected. The firing rate of VTA GABA neurons following EtOH administration was not significantly affected by sex (**Fig. 7**; $F_{(1,27)} = 0.29, p = 0.595$), genotype (**Fig. 7**; $F_{(1,27)} = 0.03, p = 0.875$), or sex by genotype interaction (**Fig.7**; $F_{(1,27)} = 2.04, p = 0.165$).

Discussion

Alcohol-Induced Sedation

Alcohol has various effects on motor function and consciousness. In humans and in mice it is well known to cause ataxia, sedation, and unconsciousness at high doses. However, at present there is still much to be learned about the exact mechanism by which these and other symptoms occur. We observed that a genetic knockout of the T cell marker CD5 blunted some of EtOH's sedative effects, namely the reduction in locomotor activity and the loss of the righting reflex (preliminary). We also observed that the wild type mice exhibited greater locomotor behavior prior to any EtOH exposure. It is possible that the effect we observe in the blunting of the effect of EtOH-induced sedation is related to the differences in baseline activity. After exposure to EtOH, the raw distance traveled scores between the two genotypes were very similar (data not shown). It seems possible that we are observing somewhat of a floor effect with the CD5 KO mice where they began with low locomotor activity and did not get any lower with EtOH exposure while the WT controls began with very high locomotor activity that decreased with exposure to EtOH.

In addition to a mitigated effect of EtOH sedation, we also observed a shift in number of visits to the center region. The wild type controls again had a high number of visits to the center region on average, which reduced with exposure to EtOH while the CD5 KO mice began with a low number of visits and stayed low. The subjective observation that this trend mirrors the trend

observed in overall distance traveled hints faintly that there could be a causal relationship between these parameters. This becomes more clear when we look at the time spent in the center region. There was no difference between strains in the time spent in the center region, and both strains saw a decrease in time spent in the center region when they were exposed to EtOH. This means for the C57s that had a high number of visits to center that their visits must have been shorter in order to have more visits in the same amount of time. It is worth noting at this point that some of the CD5 KO mice exhibited the qualitative trait of stopping periodically in the center of the chamber. While the cause of this stopping behavior is unknown, it could be the cause of CD5 KO mice having fewer visits to the center but spending the same amount of time there.

While the loss of righting reflex did not reveal a statistically significant difference between genotypes, it seems evident that there is a clear trend present that could be verified with a properly powered sample. Therefore, additional experiments are underway to supplement this preliminary finding and evaluate whether this effect is real or happenstance. If we assume that the trend is supported by the results from the current experiments, this would provide parallel evidence that CD5 KO mice exhibit resistance to the sedative effects of EtOH.

Ethanol Consumption

There are very few treatments currently approved to treat AUD, and the treatments that do exist have mixed efficacy. It is therefore essential to continue to elucidate factors that impact EtOH seeking behavior. We decided to evaluate the differences between the CD5 KO and WT mice in regards to EtOH drinking behavior and found that CD5 KO mice drink less EtOH in a 24hr access two-bottle choice paradigm. This pattern was persistent throughout the entire 21-day testing period.

We have investigated the role of IL-10 in DA dynamics and found that while IL-10 was not intrinsically rewarding in a conditioned place preference paradigm, it does directly increase the firing rate of DA neurons in the VTA and increase DA release in the NAc (data not published). It has been shown that B cells that are CD5⁺ express more IL-10 than cells that are CD5⁻ (Gary-Gouy et al., 2002). It may be that IL-10 secretions from CD5⁺ B cells occur in response to EtOH and mediate some of the reinforcing properties of EtOH. This could explain why CD5 KO mice exhibit reduced EtOH consumption behavior as compared to their WT counterparts. Future experiments will need to be conducted to validate this model.

Ethanol Effects on GABA Neuron Inhibition

We have repeatedly reported that EtOH acutely inhibits GABA neuron firing rate at physiologically-relevant levels (Gallegos et al., 1999; Ludlow et al., 2009; Steffensen et al., 2009; Stobbs et al., 2004). We anticipated that CD5 KO mice would exhibit reduced inhibition to EtOH in this same measure. However, we observed that there were no baseline differences in GABA neuron firing rate between CD5 KO mice vs WT mice and likewise that both genotypes are equally affected by a 0.75 g/kg IP injection of EtOH. This was initially surprising given the differences in EtOH consumption and sedation that we observed. It is possible that the effects we observe elsewhere are mediated by direct activity onto DA neurons rather than indirect activity on GABA neurons. In future work we will measure DA neuron activity to evaluate this hypothesis.

We also observed a sex difference between the average baseline firing rate of GABA neurons in C57 WT mice, which was not seen in the CD5 KO mouse. Because the WT mice were not the focus of this study, we did not follow this up with additional experiments, but it could be worth exploring in a future study design.

Taken together, these results suggest that CD5 plays a role in the sedative effects of EtOH as well as EtOH consumption. Considering the tremendous need for new and improved treatments for alcohol use disorder, the latter will be the focus of future work and could result in the discovery of a novel treatment target for AUD.

CHAPTER 5: Interleukin-10 effects on VTA neurons

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Abstract

Dopamine (DA) transmission is a key player for the reinforcing properties of various drugs of abuse. The current dogma is that DA transmission is increased via the inhibition of ventral tegmental area (VTA) GABA neurons and that excitation of VTA GABA neurons results in decreased DA transmission. Microglia, the major neuroimmune effector in the brain, may be a key mediator in this process by releasing cytokines following activation. We used standard cell-attached and whole cell mode electrophysiological techniques to evaluate the effects of select cytokines on VTA neuron firing rate *in vitro*. We found no change in firing rate in VTA DA or GABA neurons in response to IL-6, but an increase in firing rate in VTA DA neurons response to IL-10. Consistent with the changes in firing rate, optically evoked IPSCs were found to be decreased in response to IL-10, and mIPSCs were found to be decreased in amplitude. *Ex vivo* voltammetry and *in vivo* microdialysis were performed to determine whether IL-10 can directly result in an increase in DA release. Although *ex vivo* voltammetry showed no change in DA release, IL-10 increased DA release *in vivo*. Intra- cisterna magna IL-10 administration produced conditioned place aversion. These findings suggest that cytokines such as IL-10 may play a role in the reinforcing properties of drugs of abuse.

Introduction

The mesolimbic dopamine (DA) system originates in the midbrain ventral tegmental area (VTA) and projects to the nucleus accumbens (NAc) area of the striatum. This system is involved in motivation and the reinforcement of behaviors (Wise, 2008). Current dogma maintains that addiction is based on the dysregulation of the mesolimbic DA system. Ventral tegmental area DA neurons are regulated by inhibitory γ -aminobutyric acid (GABA) neurons found locally in the VTA and in other brain areas. Drugs of abuse such as alcohol and opioids alter GABAergic transmission onto DA neurons, providing evidence that they play a critical role in the rewarding properties of drugs (Bonci & Williams, 1997; Gallegos et al., 1999; Theile et al., 2011).

Neuroimmune mechanisms may underlie some of the intoxicating, rewarding, and neuroadaptive effects of drugs of abuse. Mice administered lipopolysaccharide (LPS), a bacterial signal that triggers immune system activation, show decreased firing in VTA DA neurons (Blednov et al., 2011) and increased DA release in the NAc in vivo (Borowski et al., 1998). These findings suggest that neuroimmune activation alters the mesolimbic DA system and could contribute to the pathology of addiction.

To date, only a few studies have evaluated the effects of cytokines on VTA neurons, and none has addressed cytokine effects on VTA GABA neurons. Although no studies have tested the effects of cytokines on VTA neurons, the anti-inflammatory cytokine interleukin (IL)-10 has been shown to reduce GABA inhibition in the hippocampus (Suryanarayanan et al., 2016). Thus, IL-10 is a likely mediator of the effects of drugs of abuse on GABA synaptic transmission in VTA neurons and subsequent DA release. In the current study, we investigated the effects of IL-10 on VTA neuron firing rate and GABA transmission on VTA DA neurons. We also evaluated

IL-10 effects on DA release in the NAc and in the conditioned place preference (CPP) procedure. We hypothesized that IL-10 would inhibit GABA synaptic transmission on VTA DA neurons and increase DA release in the NAc, and exhibit rewarding properties.

Methods

Animal Subjects

Male C57BL6 (black) mice, VGAT-ChR2-EYFP mice, and glutamate-decarboxylase-67 (GAD-67)-green fluorescent protein (GFP) knock-in (GAD67-GFP) on a CD-1 (white albino) mouse (Tamamaki et al., 2003) were bred and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For each methodology to be employed, animals were treated in strict accordance with the Brigham Young University Animal Research Committee (IACUC) guidelines, which incorporate and exceed current NIH guidelines. Once weaned at PND 21, all mice were housed in maximum groups of five and given *ad libitum* access to water and solid food and placed on a reverse light/dark cycle with lights ON from 10 PM to 10 AM.

Preparation of Brain Slices

All brain slice preparations were performed in P28-60 day old C57BL/6-ChR2-VGAT and GAD67-GFP knock-in mice. Brains were extracted following brief isoflurane anesthesia (5%). Upon extraction, the brain was secured onto a cutting stage using a cyanoacrylate adhesive. The brain was then sectioned in artificial cerebral spinal fluid (ACSF; in mM: 126 NaCl, 11.1 Glucose, 2.5 KCl, 1.2 NaH₂PO₄, 21.4 NaHCO₃, 1.2 MgCl₂, 2.4 CaCl₂) bubbled with carbogen, comprised of 95% O₂ and 5% CO₂. Targeting the VTA, horizontal slices (220 μM thick) were then placed in an incubation chamber containing ACSF supplied with carbogen for at least 30 min. After 30 min, brain slices were then placed in a recording tissue chamber with

ACSF continuously flowing at physiological temperature (35 °C). Cytokines were reconstituted and frozen in aliquots until ready to use: IL-10 (20 ng/ml, Peprotech), IL-6 (300 ng/ml, BioLegend).

Electrophysiology Recordings in Brain Slices

Electrophysiology studies used electrodes pulled from borosilicate glass capillaries (2.5-6 M Ω) and then filled with one of four solutions: an ACSF solution previously described, a potassium-gluconate pipette solution [in mM: 123 K-gluconate, 0.2 EGTA, 10 HEPES, 8 NaCl, 2 Mg-ATP, 2 Na₃-GTP] for studies investigating optogenetically-evoked inhibitory post-synaptic currents (oIPSC) or miniature IPSCs (mIPSC), or a K-gluconate solution spiked with a phosphoinositide 3-kinase (PI3K) inhibitor (LY294002, Cayman Chemical, Item #: 70920) or its inactive structural homolog (LY303511, Cayman Chemical, Item #: 15514). Pipettes having tip resistances of 2.5 – 5 M Ω , and series resistances typically ranging from 7 to 15 M Ω were used. Positive pressure was applied to the electrode when approaching the neuron. For cell-attached recordings, a seal (10 M Ω – 1 G Ω) was created between the cell membrane and the recording pipette by applying suction to the electrode. Spontaneous spike activity was then recorded in voltage-clamp mode with an Axon Instruments Multiclamp 700B amplifier and sampled at 10 kHz using an Axon 1440A digitizer and collected and analyzed using pClamp10 software. A stable baseline recording of firing activity was obtained for 5-10 min before adding any substances. Whole cell voltage clamp recordings were filtered at 2 kHz with an Axon Instruments Multiclamp 700B amplifier and digitized at 5 to 20 kHz respectively using an Axon 1440A digitizer. Axon Instruments pClamp ver10, Mini Analysis (Synaptosoft: Decatur, GA), and Igor Pro (Wavemetrics: Oswego, OR) software packages were utilized for data collection and analysis. A 470 nm blue light was used to evoke oIPSCs in VGAT-ChR2-EYFP mice. Using a paired-pulse stimulus, the stimulation was adjusted to a half-max level in order to allow the

measurement of changes that increase or decrease oIPSC levels. For experiments where boiled IL-10 was used, the solution containing the IL-10 was placed in a microcentrifuge tube and submerged in boiling water for at least 1 min. Recordings of mIPSCs and oIPSCs were done in the presence of 3 mM kynurenic acid (Sigma, Cat. No. K3375) and 500 μ M lidocaine (Sigma, Cat. No. L5647) to block glutamatergic excitatory synaptic input.

Ex Vivo Voltammetry

Slices were prepared as previously described, transferred to the recording chamber, and perfused with ACSF (34 °C) at a rate of \sim 1.8 ml/min. Fast scan cyclic voltammetry recordings were performed and analyzed using Demon Voltammetry and Analysis software (Yorgason et al., 2011) (RRID:SCR_014468). The carbon fiber electrodes used in voltammetry experiments were made in-house. To make these electrodes, the carbon fiber (7 μ m diameter, Thornel T-650, Cytec) was aspirated into a borosilicate glass capillary tube (TW150, World Precision Instruments). Electrodes were then pulled on a P-87 Horizontal pipette puller (Sutter Instruments) and cut so that 100–150 μ m of carbon fiber protruded from the tip of the glass. The electrode potential was linearly scanned as a triangular waveform from -0.4 to 1.2 V and back to -0.4 V (Ag vs AgCl) with a scan rate of 400 V/s (Yorgason et al., 2011). Before inserting the carbon fiber electrode into the brain slice, the voltage ramp was applied every 16 msec (60 Hz) for a 5–10 min period. After this electrode conditioning period, the scanning interval was changed to 100 msec (10 Hz). If electrodes had low electrical noise (median SD < 0.07 nA averaged across six 1 s bins), then they were used for experiments. Carbon fibers were advanced completely into the tissue at a 20° angle with the tip positioned \sim 85 μ m below the slice surface. Dopamine release was evoked through electrical stimulation (1 pulse/min) via a glass

micropipette (30 μ A, monophasic⁺, 0.5 ms). Paired pulse stimulations were performed with interstimulus intervals of 0.5, 1, 2, 4, 8, 12, and 16 Hz.

Microdialysis and High-Performance Liquid Chromatography

These experiments were conducted in 8 mice per treatment condition. A microdialysis probe (CMA 7, Harvard Apparatus, Holliston, MA, USA) was stereotaxically inserted into the nucleus accumbens (NAc) at the following coordinates (relative to bregma): +1.5 anteroposterior (AP), \pm 0.6 mediolateral (ML), -5.0 dorsoventral (DV). Artificial cerebrospinal fluid was perfused through the probe at a rate of 2.0 μ l/min. Samples were collected every 20 min for a baseline period of at least 2 hr. Once a stable baseline was established, an intracerebroventricular microinjection of 60 ng of IL-10 in 1 μ l of ACSF or 1 μ l of ACSF alone was administered. The microinjection was carried out using a 10 μ L Hamilton syringe (Reno, NV, USA) with a 25 gauge needle in conjunction with a microsyringe pump injector (UMP3, World Precision Instruments [WPI], Sarasota, FL, USA) attached to a microdrive controller (Micro4, WPI). The coordinates for the microinjection were as follows: -0.5 AP, \pm 1.1 ML, -2.4 DV. Microdialysis sampling continued for an additional 2 hr following the microinjection. All mice were anesthetized for the duration of the procedure using isoflurane (1.2% - 1.8%). Determination of the dopamine (DA) concentration in the microdialysis samples was performed using a high-performance liquid chromatography (HPLC) system (Ultimate 3000, Thermo Fisher Scientific, Waltham, MA, USA) coupled to an electrochemical detector (Coulchem III, ESA). The electrochemical detector included a guard cell (5020, ESA) set at +275 mV, a screen electrode (5014B, ESA) set at -100 mV, and a detection electrode (5014B, ESA) set at +220 mV. Dopamine was separated using a C18 reverse phase column (HR-80, Thermo Fisher Scientific). Mobile phase containing 75 mM H₂NaO₄P, 1.7 mM sodium octane sulfonate, 25 μ M ethylenediaminetetraacetic acid, 0.714 mM trimethylamine, and 10% acetonitrile was pumped

through the system at a flow rate of 0.5 ml/min. Dopamine levels following the microinjection were expressed as percentage of baseline, with the baseline being determined by the average DA concentrations of 3 consecutive stable collections occurring prior to the microinjection. The first hour of microdialysis data on DA release in the NAc was analyzed using a mixed model analysis of variance (ANOVA) with time as a within-subjects factor, treatment condition as a between-subjects factor, and a time by treatment condition interaction term. A Greenhouse-Geisser correction for sphericity was applied to the results of the ANOVA. Prior to analysis, the data were checked for outliers (classified as a datum falling ± 2 interquartile ranges [IQR] beyond the median). Two data points were identified as outliers using this method. These outliers were subsequently fenced to the upper limit of the outlier test (median + 2 IQR) as we did not find any evidence of data input error or variation from experimental protocol that would have justified the removal of these data points. The data were also found to be relatively normal using the Shapiro-Wilk test.

Conditioned Place Preference Procedure

Conditioned place preference (CPP) procedure experiments were carried out in 10 mice per treatment condition. Place conditioning occurred in a 16" x 16" x 16" apparatus constructed out of Plexiglass. The floor of the apparatus was divided into an 8" x 16" rough compartment and an 8" x 16" smooth compartment on testing days by the insertion of a 1/8" thick piece of textured acrylic. During conditioning days, the insertion or removal of a 16" x 16" piece of textured acrylic allowed pairing of the different treatment conditions with the different contexts. The CPP procedure consisted of 3 phases: pretest, conditioning, and posttest. The pretest and posttest phases occurred over one day each with each mouse placed into the apparatus and allowed to choose between the two contexts for 30 minutes. The pre-test and post-test sessions occurred prior to beginning and after completion of the conditioning trials, respectively. The

conditioning trials occurred over the course of three days. Each day the mice were lightly anesthetized with isoflurane (3%) and given an intra-cisterna magna (ICM) injection of ACSF (1 μ l) in the morning and an ICM injection of IL-10 (60 ng in 1 μ l ACSF) or vehicle (1 μ l ACSF) in the afternoon. Immediately following the injections, the mice were transported to the CPP apparatus and placed in the smooth compartment (after an ACSF injection) or the rough compartment (after an IL-10 injection) after waking up from the anesthesia. The mice were left in the compartment for 10 min for each conditioning trial. The apparatus has been found to be biased, with the mice showing an initial preference for the smooth compartment. The time spent in the IL-10 or vehicle paired chamber was analyzed using a mixed models ANOVA with treatment condition as a between-subjects factor, test (pre or post) as a within-subjects factor, and treatment condition by test as an interaction term. A Greenhouse-Geisser correction for sphericity was applied to the results of the ANOVA. Data were cleaned as described in the microdialysis and high-performance liquid chromatography section. One datum was identified as an outlier and bounded to the median + 2 IQR limit for outlier detection. The data were found to be relatively normal using the Shapiro-Wilk test.

Statistical Analyses

All results are presented as raw mean values and percent control \pm SEM. Results before and after drug exposure were compared using a two-tailed paired *t*-test. Experiments relying on variance in time or current were analyzed using an ANOVA with post hoc *t*-test at individual points. Statistical significance required \geq 95% level of confidence ($P \leq 0.05$). Analysis software included Microsoft Excel, STATA, and Igor Pro (Wavemetrics, Oswego, OR). Significance levels are indicated on graphs with asterisks *, **, *** and correspond to significance levels $P < 0.05$, 0.01 and 0.001, respectively. Figures were constructed with Igor Pro software.

Results

IL-10 Modulates the Firing Rate of VTA Neurons

We tested the effects of select cytokines (IL-6 and IL-10) on VTA DA and GABA neuron firing rates in cell-attached mode, which is a stable recording technique that does not alter the intracellular milieu. We found that the anti-inflammatory cytokine IL-10 increased the firing rate of VTA DA neurons by $70.63 \pm 20.87\%$ (**Fig. 1**; $n=13$, $p=0.0204$, by paired t-test), but had no effect on the firing rate of VTA GABA neurons $0.63 \pm 5.32\%$ (**Fig. 1**; $n=13$; $p=0.4476$). Interleukin-6, a pro-inflammatory cytokine, did not have any effect on firing rate of VTA GABA or DA neurons (**Fig. 2**; DA: $113.98 \pm 9.48\%$; $n=4$, $p=0.3349$; **Fig. 2**; GABA: $1.17 \pm 11.24\%$;

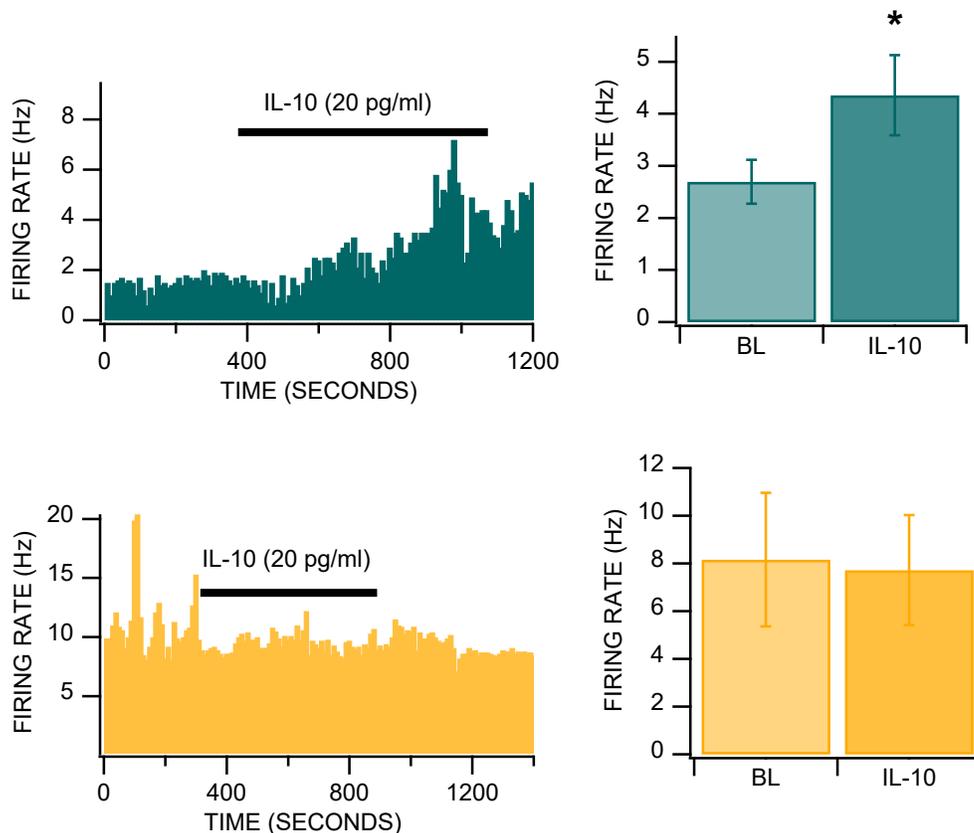


Figure 5-1: Effects of IL-10 on VTA Neuron Firing Rate. (Top left) Representative trace of the effects of IL-10 on the firing rate of a DA neuron. (Top right) IL-10 increased VTA DA neuron firing rate. (Bottom left) Representative trace of the effects of IL-10 on the firing rate of a GABA neuron. (Bottom right) IL-10 had no effect on VTA GABA neuron firing rate.

n=5; p=0.9061). These results suggest that IL-10 is modulating the firing rate of VTA DA neurons by an alternative mechanism than by changing the firing rate of VTA GABA neurons. As IL-6 did not significantly alter firing rate, it suggests that the effect of increasing firing rate is specific to IL-10, or perhaps it is an effect specific to anti-inflammatory cytokines.

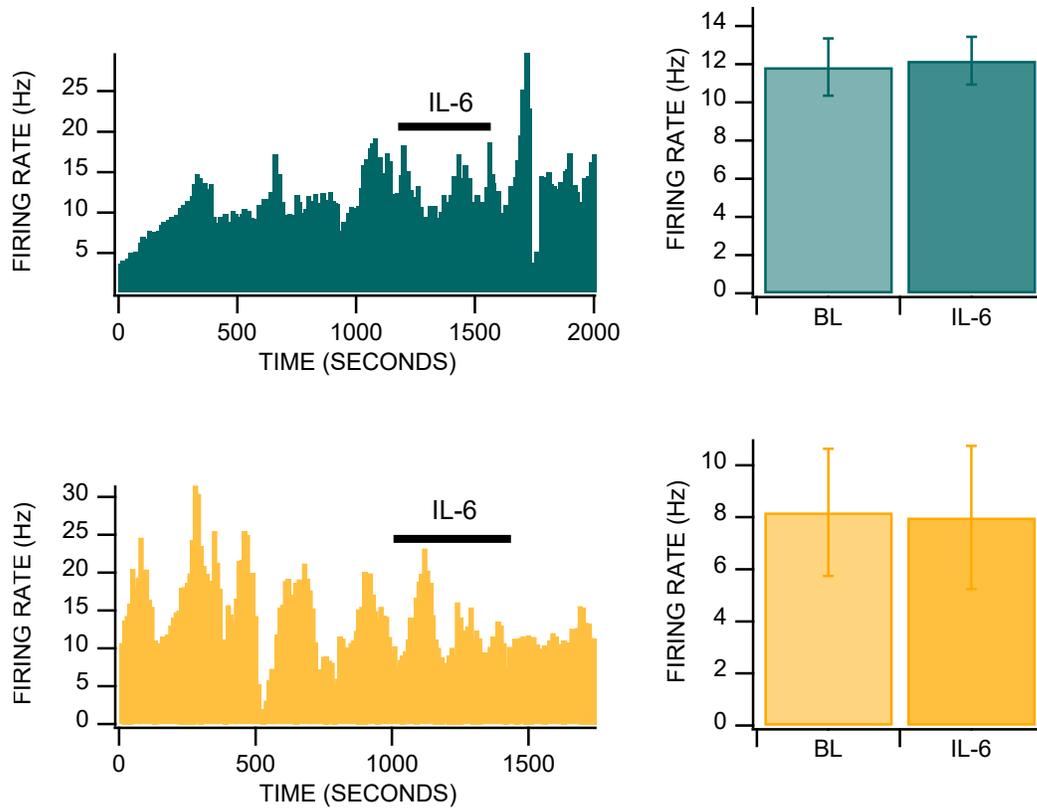


Figure 5-2: Effects of IL-6 on VTA Neuron Firing Rate. (Top left) Representative trace of the effects of IL-6 on the firing rate of a DA neuron. (Top right) IL-6 had no effect on VTA DA neuron firing rate. (Bottom left) Representative trace of the effects of IL-6 on the firing rate of a GABA neuron. (Bottom right) IL-6 had no effect on VTA GABA neuron firing rate.

Effects of IL-10 on Optogenetically-Evoked IPSCs on VTA DA Neurons

Based on the findings that IL-10 increased DA neuron firing rate, we tested the effects of IL-10 on oIPSCs in DA neurons. Previous studies have shown that IL-10 inhibits GABAergic synaptic inhibition in the hippocampus (Suryanarayanan et al., 2016), suggesting that IL-10 may be acting through a similar mechanism to increase VTA DA neuron firing rate. We used

channelrhodopsin-2 (ChR2) vesicular GABA transporter (VGAT) mice to activate GABA neurons and then recorded from DA neurons to study the effects of IL-10 on GABA transmission on DA neurons. We found that IL-10 decreased the amplitude of the oIPSC on DA neurons by $21.69 \pm 6.31 \%$ (**Fig. 3**; $n=10$; $p=0.0397$). This suggests that IL-10 may contribute to the disinhibition of DA neurons by attenuating GABAergic synaptic transmission on VTA DA neurons.

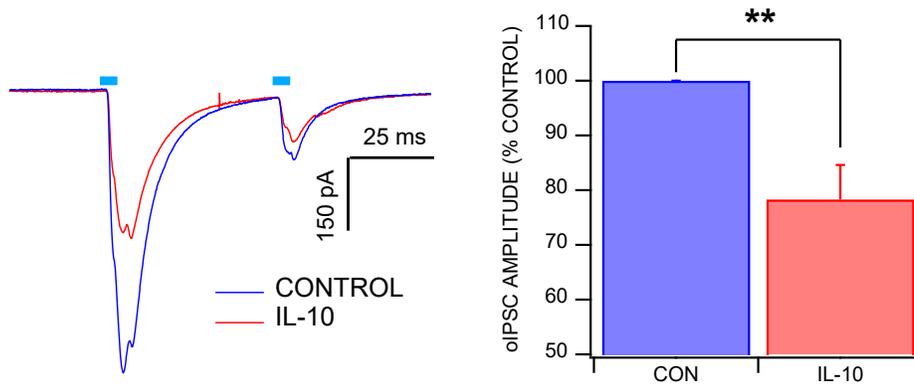


Figure 5-3: Effects of IL-10 on Optogenetic IPSCs on VTA DA Neurons. (Left) Representative trace of the effects of IL-10 on the optogenetically-evoked IPSC in a VTA DA neuron. This neuron had an IPSC that was decreased from 340pA to 190pA during IL-10 perfusion. (Right) IL-10 decreased the amplitude of oIPSCs in 9 out of 10 neurons.

Effects of IL-10 on mIPSCs on VTA DA Neurons

In order to understand how IL-10 was affecting GABAergic synaptic release, we tested the effects of IL-10 (10 pg/ml, 20 pg/ml and 40 pg/ml) on mIPSCs on VTA DA neurons, which were recorded in the presence of ? to block action-potential mediated IPSCs. We found that IL-10 did not affect the frequency of mIPSCs (**Fig. 4**; $n=7$) although there was a decreasing trend. However, IL-10 significantly decreased the amplitude of mIPSCs in a dose-dependent manner (**Fig. 4**; $n=7$). This effect was blocked by boiling the IL-10, confirming that it is the intact cytokine that is causing the effect (**Fig. 4**; $n=3$). These results suggest that IL-10 has post-synaptic effects on GABAergic synaptic transmission on VTA DA neurons.

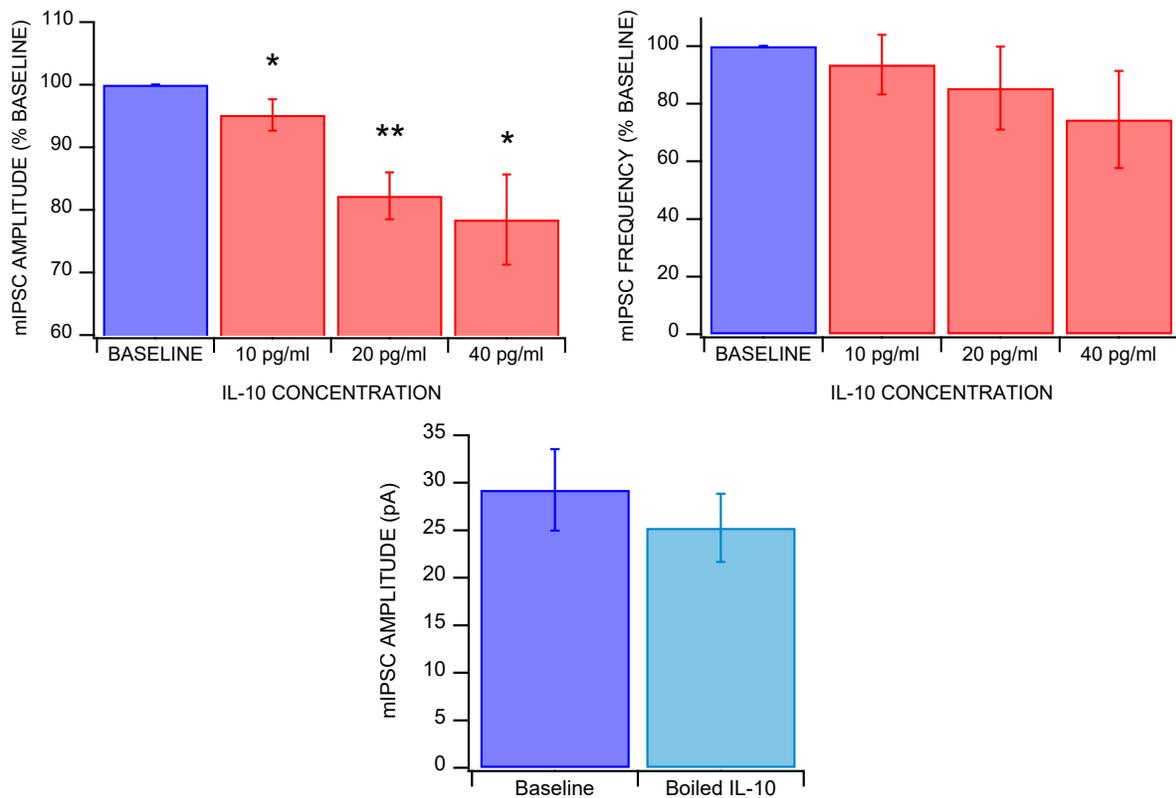


Figure 5-4: Effect of IL-10 on mIPSCs in VTA DA Neurons. (Top left) IL-10 decreased the amplitude of mIPSCs on VTA DA Neurons. (Top right) IL-10 had no significant effect on the frequency of mIPSCs on VTA DA neurons. (Bottom) Boiling IL-10 eliminates its reduction in mIPSCs in VTA DA neurons.

PI3K Inhibitor Blocks IL-10 Effects

To evaluate the mechanism by which IL-10 elicits these effects, we measured the amplitude of mIPSCs in the presence of the PI3K inhibitor LY294002 (10 μ M). Preliminary data indicate that having LY294002 in the pipette during the measurement of mIPSCs may block and possibly even reverse the inhibitory effects of IL-10 on mIPSC amplitude (data not shown). Additional experiments will confirm this.

Effects of IL-10 on DA Release in the NAc

Based on previous evidence of increased DA neuron firing and decreased GABAergic synaptic inhibition, the next question was whether DA release in the NAc is influenced by IL-10. Therefore, we tested the effects of IL-10 on *ex vivo* evoked DA release in the core of the NAc. A paired-pulse protocol of evoked DA release was used to determine if the interstimulus interval combined with IL-10 had an effect on DA release (**Fig. 5**). From this data, a subtraction was

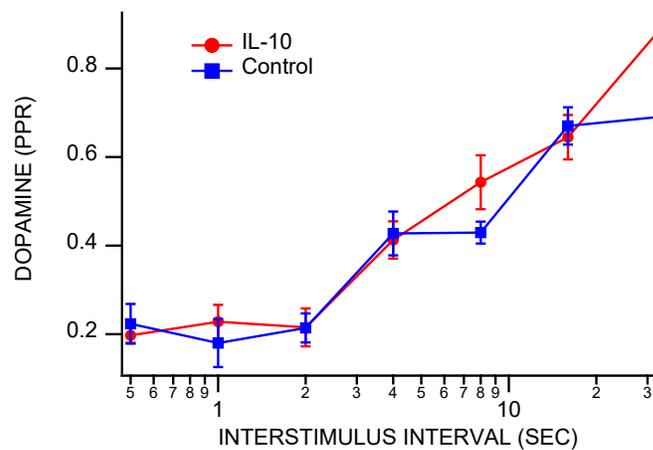
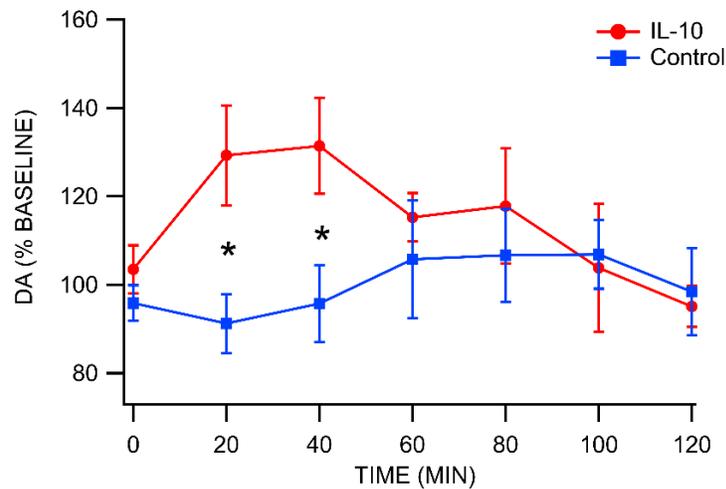


Figure 5-5: Effects of IL-10 on Evoked DA Release in the NAc Ex Vivo Using Fast Scan Cyclic Voltammetry. A paired-pulse ratio protocol was tested and then repeated with the superfusion of IL-10. There was no significant difference between the control and IL-10 at any of the stimulation intervals.

performed (data not shown) to determine whether IL-10 had an effect on evoked DA release. In slices containing NAc terminals, without connections to the cell bodies in the VTA, IL-10 had no effect on evoked DA release. Additionally, there was no interstimulus interval where a significant difference was seen between control and IL-10. This suggests that IL-10 has no effect on DA neuron terminals in the NAc. Dopamine release was also measured *in vivo* by microdialysis following an intracerebroventricular (ICV) injection of IL-10 (60 ng/mouse). Intracerebroventricular microinjections of IL-10 were found to modestly enhance DA release in the NAc during the first hour following the microinjection (**Fig. 6**; $F_{(1,14)} = 10.97$, $p = 0.005$; η^2_p

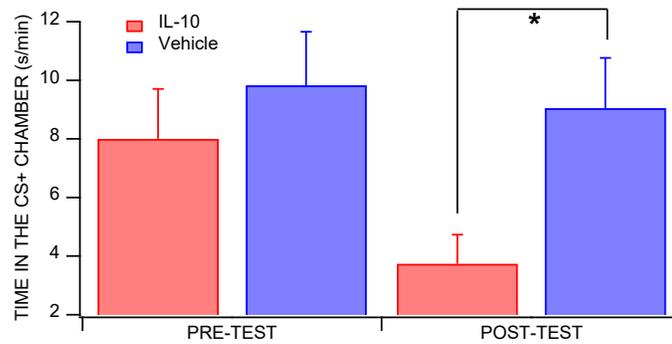
= 0.439). Comparisons of individual timepoints during the first hr using t-tests revealed that DA release in the NAc was enhanced specifically at 20 ($t_{(14)} = -2.903$, $p = 0.005$) and 40 ($t_{(13)} = -2.589$, $p = 0.011$) minutes post-microinjection. Based on the ex vivo DA release data above, only the first hr of the microdialysis data was analyzed. There was not a significant effect of time ($F_{(3,41)} = 1.12$, $p = 0.347$) or a time-by-treatment condition interaction on DA release ($F_{(3, 41)} = 2.08$, $p = 0.135$).



*Figure 5-6: Microinjections of IL-10 enhance DA release in the NAc. Microinjections of IL-10 (60 ng, ICV) moderately enhance DA release in the NAc at 20 and 40 minutes after the microinjection. Microinjections of vehicle (1 μ l aCSF, ICV) failed to enhance DA release in the NAc. * denotes significance level $p < 0.05$.*

Effects of IL-10 on Conditioned Place Preference

Intra-cisterna magna injections of IL-10 were found to produce a conditioned place aversion (**Fig. 7**; $F_{(1,18)} = 6.21$, $p = 0.023$, $\eta^2_p = 0.256$) as evidenced by decreased time spent in the IL-10/vehicle paired chamber by IL-10-treated mice as compared with vehicle-treated mice at the posttest ($t_{(17)} = 2.718$, $p = 0.015$) but not at the pretest ($t_{(18)} = 0.7334$, $p = 0.473$). This finding was inconsistent with our hypothesis that ICM injections of IL-10 would be reinforcing. Given that the apparatus has been found to be biased we may have encountered a floor effect on the aversive properties of the IL-10 injection.



*Figure 5-7: ICM administration of IL-10 produced conditioned place aversion. IL-10 administration (60 ng, ICV) produced a conditioned place aversion. Avoidance of a chamber paired with IL-10 administration was increased following the pairings as compared with a group that received only vehicle pairings (1 μ l aCSF, ICV) in the same chamber. * denotes significance level $p < 0.05$.*

Discussion

The purpose of this study was to investigate the effects of cytokines, particularly IL-10, on VTA neurons to identify its potential role in modulating VTA neuronal activity, DA release, and reward. Interleukin-10 modulates the firing of VTA DA neurons by decreasing GABAergic synaptic inhibition onto VTA DA neurons. However, IL-6 had no effect on GABA or DA neuron firing rate. We tested the effects of IL-10 on GABA synaptic transmission onto VTA DA neurons and found that IL-10 inhibits GABAergic synaptic transmission. Our data also suggest that IL-10 increases DA release in the NAc *in vivo*, although increased release is not seen in *ex vivo* slice preparations. Surprisingly, IL-10 administration was not shown to be rewarding in the CPP procedure,

Cytokine Effects on VTA Neuron Firing Rate

We observed that IL-10 increased the excitability of VTA DA, but not GABA neurons. The increase in firing rate is consistent with the IL-10-dependent decrease in oIPSC and mIPSC amplitude in VTA DA neurons, suggesting that decreased post-synaptic inhibition explains

some, if not all, of the increased excitability of VTA DA neurons. Little is known about the role of the anti-inflammatory cytokine IL-10 in the brain, particularly in regard to how it may be involved in the response to ethanol and other drugs of abuse. Recent evidence has shown that IL-10 inhibits mIPSCs in the hippocampus (Suryanarayanan et al., 2016). Interleukin-1 β (IL-1 β) inhibits LTP induction, and this effect is prevented by IL-10 (Kelly et al., 2001).

We tested the effects of IL-6 on firing rate in order to compare pro-inflammatory vs anti-inflammatory cytokines. No difference was observed on the firing rate of DA or GABA neurons, although these results are not entirely conclusive due to the low sampling of neurons and the abnormally high firing rate of the putative DA neurons. However, because no difference was seen, we did not perform any further experiments with IL-6. Additional evidence suggests that IL-6, a pro-inflammatory cytokine, may affect synaptic transmission. Interleukin-6 has been shown to enhance excitatory postsynaptic potentials in the hippocampus (Nelson et al., 2012). Additionally, overexpression of IL-6 alters the effects of acute ethanol on field excitatory postsynaptic potentials and LTP (Hernandez et al., 2016).

Effects of IL-10 on Synaptic Transmission

GABAergic synaptic transmission was inhibited by IL-10. However, we did not test whether glutamatergic (GLUergic) synaptic transmission is modified by IL-10. A recent study showed that IL-10 modulates GLUergic synaptic activity in pyramidal neurons suggesting that changes in GLUergic synaptic transmission may also be an important consideration in the VTA (Nenov et al., 2019).

Activation of the IL-10 receptor (IL-10R) is mediated by the Jak/STAT pathway, and leads to decreased cytokine production and decreased inflammation (Murray, 2006). Currently the expression of IL-10 receptors in the brain is not well characterized. It has been shown that

IL-10Rs are expressed in the brain on neurons in the hippocampus, cortex, and spinal cord as well as microglia (Lim et al., 2013). It seems likely that IL-10Rs are expressed in the VTA as well, but whether they are expressed on GABA or DA neurons or both, remains unknown. The effects of IL-10 on GABA synaptic transmission could be due to activity on either presynaptic GABA terminals or DA neurons. Recent evidence has shown that IL-10 inhibits GABAergic synaptic transmission. For example, IL-10 inhibits both the frequency and amplitude of mini IPSCs in the hippocampus, at least partially due to the postsynaptic effects of PI3K on GABA(A)R expression (Suryanarayanan et al., 2016). It has also been shown that IL-10 modulates GLUergic synaptic plasticity. The induction of LTP is inhibited by IL-1 β , and this effect is prevented by IL-10 (Kelly et al., 2001). The lack of effect of IL-10 on VTA GABA neurons, but inhibition of oIPSC and mIPSC amplitudes, but not mIPSC frequency, suggests a post-synaptic effect on DA neurons.

IL-10 Increases DA Release

Increased VTA DA neuron firing logically results in increased DA release in the NAc. Many drugs of abuse act in the VTA to cause increases in DA release in the NAc, such as opioids, nicotine, and alcohol (Balfour, 2009; Deitrich et al., 1989; Fields & Margolis, 2015). Our findings suggest that IL-10 (and perhaps other cytokines) may be mediating some of the rewarding and reinforcing properties of drugs of abuse. Dopamine release is thought to correlate with the reinforcement of behavior and conditioned preferences (Salamone & Correa, 2002; Wise, 2008). In this study, we have shown that IL-10 increases DA release, which is suggestive of reward. However, place conditioning found that IL-10 was aversive rather than rewarding. Other studies have shown behavioral effects of anti-inflammatory cytokines on ethanol drinking behaviors. For example, IL-10 in the basolateral amygdala decreased ethanol consumption, but not sucrose consumption or performance in an open-field test (Marshall et al., 2017).

Interestingly, another study found that antagonizing the IL-1 receptor in the basolateral amygdala also decreased ethanol consumption (Marshall et al., 2016). These results suggest that anti-inflammatory cytokines play a role in ethanol-related behaviors.

Our results affirm the importance of the neuroimmune system in mediating reward, and particularly implicate IL-10 as a key mediator in these effects. Future studies are needed to investigate the effects of other cytokines, particularly anti-inflammatory cytokines, in the mesolimbic DA system in order to fully understand how cytokines may be involved in reward. Additionally, more pharmacological research is needed to identify whether cytokines affect GLUergic synaptic transmission in the VTA. Further research about IL-10's modulation of DA release will clarify the role of IL-10 in the neural circuitry of reward and could potentially lead to novel pharmacological and behavioral treatments.

CHAPTER 6: CONCLUSION

The field of alcohol research and treatment is in much need of advances, particularly advances that will result in novel treatments or in the improvement of existing treatments. The present work represents several discoveries that can help to advance alcohol research in support of the development of novel treatments.

There are two pathways investigated here that, with additional research and validation, could be utilized as novel drug targets for use in the treatment of alcohol use disorders with potential to be effective in other substance abuse disorders. The first is the BDNF/TrkB/KCC2 pathway and the second is the CD5/IL-10 pathway.

Manuscript One

In the first manuscript we demonstrated that chronic alcohol exposure leads to a decrease in sensitivity to the GABA_A agonist muscimol. Under baseline conditions, muscimol decreases the activity of VTA GABA neurons, but after chronic alcohol exposure this decrease is mitigated. This difference is not seen in VTA DA neurons, indicating that GABA neurons and DA neurons respond differentially to alcohol exposure as it pertains to the activity of GABA_A channels.

We also illustrated that DA neurons in the VTA are generally less sensitive to muscimol than are VTA GABA neurons. Since GABA_A channels are chloride dependent and GABA_B channels are potassium dependent, this could be due to the relative expression of GABA_A vs. GABA_B receptors on DA neurons. In future experiments we may measure the relative concentrations of GABA_A and GABA_B receptors on DA neurons to verify this hypothesis. We could also use the GABA_B blocker baclofen to produce a baclofen sensitivity curve and see if we observe the same behavior that we observe with muscimol in response to chronic alcohol

exposure. If we find a greater relative expression of GABA_B receptors in DA neurons than in GABA neurons, this could explain the differential response to muscimol we observed.

The reduction in sensitivity to muscimol's inhibition we call GABA switching. It may be that GABA switching is a result of a dysregulation of the chloride gradient, which we investigated in our second manuscript.

Manuscript Two

In our second manuscript, we demonstrated that chronic exposure to alcohol causes an increase in BDNF expression in the VTA and in the NAc with the more pronounced change in the VTA. Although BDNF typically has preservative functions, we conclude that in this case it is instigating a chain of events that in part leads to withdrawal symptoms and potentially dependence as well. We tested the firing rate of VTA GABA neurons in the presence of BDNF and found that there were no clear or obvious acute changes on the 5-30 minute time scale and concluded that any impact BDNF was having on these neurons must be on a longer time scale. To test longer time scales, we utilized the endogenous secretions of BDNF that occur during withdrawal from chronic alcohol exposure.

Prior to chronic EtOH exposure, activating TrkB using the selective agonist 7,8-DHF resulted in an increase in alcohol seeking and blocking TrkB resulted in a decrease in alcohol seeking. This clearly illustrates the impact that activity at TrkB receptors can have on alcohol drinking behavior. However, the clinical utility of this discovery is limited due to the results in chronic EtOH exposed animals. The effect observed in the air exposed control mice was not observed in the EtOH exposed animals, or, in other words, there was no difference in drinking when TrkB was activated or blocked in the dependent animals. This could be due to adaptation of TrkB receptors, which we may confirm in future experiments. Essentially, if TrkB is

downregulated due increased exposure to its endogenous agonist, BDNF, this could readily explain this reduced sensitivity to 7,8-DHF in the dependent animals.

Manuscript Three

In our third manuscript, we illustrated the importance of the T cell marker CD5 in the sedative effects of alcohol. In an open field measure of locomotor activity, a CD5 KO population of mice showed significantly less alcohol-induced sedation than wild type controls, suggesting that CD5 is somehow important to producing the sedative effects of alcohol. Additionally, in an LORR paradigm, a suggestive trend toward significance was observed. Again, the CD5 KO mice appeared to be resistant to the sedating effects of alcohol at a higher dose in that they recovered their righting reflex more quickly, although this trend was not statistically significant with the current sample. Prior to publication, additional replicates of this experiment will be run to confirm or deny the presence of a CD5-dependent effect on LORR.

We also demonstrated that the presence or absence of CD5 impacts alcohol seeking behavior. In a 24-hour access two bottle choice drinking paradigm, the CD5 KO population drank significantly less than the wild type controls. This is of critical importance, since a decrease in drug seeking behavior can be an early indicator of a potential treatment target for substance use disorders. However, considering the varied functions that CD5 has throughout the body and immune system, it may prove to be an elusive drug target as it would likely produce an array of unpleasant side effects. Additional work is needed elucidate the potential side effects of acting on CD5 or its downstream effectors in an effort to reduce drug seeking behavior.

It should be noted that as with any study conducted in a knockout mouse, there must be caution taken when interpreting results since knocking out certain genes from before birth can result in adaptations elsewhere in the brain and body that could confound the results of the study.

Correspondingly, future studies may include pharmacological agents that target CD5 and its downstream effectors to validate the results obtained in this study. Future work may also include additional studies investigating other differences in protein expression or behavioral traits between CD5 KO mice and C57 wild type mice to ascertain what types of adaptations may have occurred and whether they could be impacting the results obtained here.

Manuscript Four

In our fourth manuscript, we demonstrated that IL-10, which is secreted in greater quantities by CD5⁺ B cells than CD5⁻ B cells, has direct effects on DA neurotransmission. We showed that IL-10 directly increased the firing rate of VTA DA neurons but not VTA GABA neurons when perfused acutely. Concomitantly, we illustrated that IL-10 administered via ICV injection increased DA release in the NAc as measured by microdialysis. These two findings together would suggest that IL-10 administration would be intrinsically rewarding. However, contrary to our expectations, we found that IL-10 was aversive when administered via intracisternal magna injection in a conditioned place preference paradigm. We therefore conclude that IL-10 influences DA neurotransmission but not in ways that are rewarding. It is possible that this DA release could, however, still be reinforcing. For example, it has been shown that painful stimuli can also cause an increase in DA release (Taylor et al., 2016). Indeed it is crucial for the brain to be able to reinforce behaviors that lead to rewarding outcomes as well as avoid behaviors that lead to hurtful outcomes, and so it stands to reason that painful stimuli could also cause DA release which is critical for reinforcement mechanisms, both for the positive and for the negative.

In summary, we conclude that these findings increase our understanding of how alcohol elicits its effects on the brain and body, which understanding is crucial for the development of

novel treatments and improvement of existing treatments for alcohol use disorder. We have demonstrated two protein targets, TrkB and CD5, that appear to have influence on alcohol seeking behavior, which suggests therapeutic potential, albeit not without the possibility of significant side effects. Additionally, we have provided a framework for the discovery of a third treatment target, KCC2, which has the potential for a therapeutic benefit with very few side effects. Future work will focus on elucidating the therapeutic potential of KCC2 in substance use disorders.

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