

BRAIN REGION ACTIVATION ACROSS ACUTE AND PROTRACTED ALCOHOL
WITHDRAWAL IN MALE MICE

A THESIS
SUBMITTED TO THE FACULTY OF
THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

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May 2025

Acknowledgments

First and foremost, I'd like to thank my advisor, Dr. Anna Lee, for the mentorship and encouragement she has given me throughout this whole journey. Her style of mentorship and my style of learning fit perfectly together. She allowed me to be independent most of the time, but despite her many obligations, DGS and professor and mom duties, she always was available for guidance when I needed it. She is an excellent mentor at more than just research, an amazing human in general, and I feel privileged to have worked alongside her.

I would like to thank the members of my lab, Sophia Scott, Nichole Payne, Ruth Dobbelmann, and Matt Scalf for helping me along the way and making my time here fun. I enjoyed the social times where we got to goof off and do something like play hockey against each other, even though we had never played before. I was always looking forward to going to lab when I knew they would be there. Sophia and I swear we still got work done even between meeting for our two-person book club or when we would enable each other's chaos in lab.

I would also like to thank all the people who supported me with my ability to pursue my sport while getting my degree. My academic advisor, Meleah, and our program coordinator, Marie Lindloff, for helping me navigate the NCAA not understanding that graduate thesis research is, in fact, a full-time job. My coach, for believing in me and supporting me in making the best decision for my life when my original plans did not come to fruition. My athletic trainer, Alex Fruetel, and my sports psych, Kirby Kidd, for helping me navigate a difficult set of injuries. Lastly, I want to thank all my teammates for giving me an amazing experience, even if I didn't get to compete alongside them very often.

Finally, I want to thank my family for all the love and support they have given me throughout my entire life to get to where I am now. My mom, for picking up my daily calls and listening to me rant about things she wasn't really interested in. My sister for

actually understanding my research and giving me guidance on the college experience. My dad for all the support and encouragement he has given me in my time here. And my grandma for making sure I always had food. I am so privileged and eternally grateful to have such a close and loving family.

Abstract

Alcohol use disorder (AUD) remains a significant public health issue, with high relapse rates complicated by its poorly understood neurochemical mechanisms. Both acute and protracted withdrawal symptoms contribute to relapse vulnerability, yet the brain regions involved in the development of these symptoms have not been extensively studied in this context. This study aims to bridge this gap of knowledge by investigating changes in the Ventral Tegmental Area (VTA), Habenula (Hb), and Mesopontine Tegmentum (MPT), areas that literature suggests may have plausible involvement, during acute and protracted alcohol withdrawal in male mice. Mice underwent 9 days of passive alcohol administration (2.5 g/kg, i.p. with 4-Methylpyrazole) and brain tissue was collected at either 24 hours (acute), or 7 days (protracted) after cessation. Brain region activity was assessed using immunohistological labeling of cFos as a proxy for neuron activation. During acute withdrawal, we observed a significant decrease in the activity of cholinergic neurons in the Pedunclopontine Tegmental Nucleus (PTg) but found no substantial changes in the other regions listed above. Activity in the Lateral Habenula (LHb), and specifically, cholinergic activity in the MPT, increased during protracted withdrawal. No changes were observed in the VTA or Medial Habenula at 7-day withdrawal. These findings suggest that the LHb and MPT may play a role in modulating neurochemical changes resulting from alcohol withdrawal. Further investigation into these regions may provide insight on the neural basis of relapse vulnerability and lead to more efficacious treatments for AUD.

Table of Contents

| | |
|---|-------------------|
| Acknowledgments | <i>i</i> |
| Abstract | <i>iii</i> |
| Table of Contents | <i>iv</i> |
| Chapter 1 INTRODUCTION | 1 |
| 1.1 Prevalence of Alcohol Use Disorder and Impact on Individuals and Society | 1 |
| 1.2 Shortcomings of Current Therapies | 2 |
| 1.3 Alcohol Withdrawal as a Risk Factor for Relapse | 4 |
| 1.4 Brain Regions Involved in Alcohol Withdrawal | 5 |
| 1.4.1 Ventral Tegmental Area | 5 |
| 1.4.2 Habenula | 6 |
| 1.4.3 Mesopontine Tegmentum..... | 9 |
| 1.5 Animal Model | 11 |
| 1.5.1 Active vs Passive Administration..... | 11 |
| 1.5.2 Length of administration | 11 |
| 1.5.3 Classification of Acute and Protracted Withdrawal in Mice..... | 12 |
| Chapter 2 METHODS | 13 |
| 2.1 Chronic Alcohol Model | 13 |
| 2.2 Tissue Preparation | 13 |
| 2.3 Immunohistochemistry (IHC) | 13 |
| 2.4 Data Analysis | 14 |
| Chapter 3 RESULTS | 16 |
| 3.1 Brain Region Activation Patterns during Acute Withdrawal | 16 |
| 3.1.1 Hb cFos expression levels remain unchanged during acute withdrawal | 16 |
| 3.1.2 PTg cholinergic activation decreases during acute withdrawal, LDT activation does not change.. | 17 |
| 3.2 Brain Region Activation Patterns during Protracted Withdrawal | 19 |
| 3.2.1 VTA dopaminergic activation unchanged during protracted withdrawal | 20 |
| 3.2.2 LHb cFos expression increased during protracted withdrawal, MHb remains unchanged | 21 |
| 3.2.3 PTg and LDT percent activated cholinergic neurons increases during protracted withdrawal..... | 22 |
| Chapter 4 DISCUSSION | 25 |
| 4.1 LHb cFos expression increases during protracted, but not acute, withdrawal. MHb expression remains unchanged | 25 |

| | |
|---|-----------|
| 4.2 PTg exhibits changes in activity during both states of withdrawal, LDT only affected during protracted withdrawal..... | 27 |
| 4.3 Total Chat+ neurons in the LDT and PTg | 28 |
| 4.4 No changes in VTA activity observed, passive administration may be the cause | 30 |
| 4.5 Other limitations and Broader Implications..... | 31 |
| <i>References</i> | 34 |

List of Figures

| | |
|---|----|
| Figure 1: <i>Visualization of the VTA</i> | 5 |
| Figure 2: <i>Visualization of the Medial and Lateral Habenula</i> | 7 |
| Figure 3: <i>Visualization of MPT subregions: PTg and LDT</i> | 9 |
| Figure 4: <i>Basic connectivity of VTA, Hb, MPT in mouse brain from sagittal view</i> -- | 10 |
| Figure 5: <i>Examples of each IHC counting phenotype</i> | 14 |
| Figure 6: <i>cFos expression in Hb during acute withdrawal</i> | 16 |
| Figure 7: <i>cFos and Chat expression in PTg during acute withdrawal</i> | 18 |
| Figure 8: <i>cFos and Chat expression in the LDT during acute withdrawal</i> | 19 |
| Figure 9: <i>cFos expression in the Hb during protracted withdrawal</i> | 21 |
| Figure 10: <i>cFos and Th expression in the VTA during protracted withdrawal</i> | 22 |
| Figure 11: <i>cFos and Chat expression in the PTg during protracted withdrawal</i> | 23 |
| Figure 12: <i>cFos and Chat expression in the LDT during protracted withdrawal</i> | 24 |

List of Abbreviations

4-MP – 4-Methylpyrazole

AUD – Alcohol Use Disorder

BAC – Blood Alcohol Content

Chat – Choline acetyltransferase

DSM-V – Diagnostic and Statistical Manual of Mental Disorders, 5th edition

DA – Dopamine

IHC – Immunohistochemistry

IPN – Interpeduncular Nucleus

LDT – Laterodorsal Tegmental Nucleus

LHb – Lateral Habenula

MHb – Medial Habenula

MPT – Mesopontine Tegmentum

OCT – Optimal Cutting Temperature compound

PAWS – Post-Acute Withdrawal Syndrome

PFA – Paraformaldehyde

PTg - Pedunculo-pontine Tegmental Nucleus

RMTg – Rostromedial Tegmental Nucleus

ROI – Region of Interest

SEM – Standard Error of the Mean

Th – Tyrosine Hydroxylase

VTA – Ventral Tegmental Area

Chapter 1 INTRODUCTION

1.1 Prevalence of Alcohol Use Disorder and Impact on Individuals and Society

Excessive alcohol use is a major public health concern. Excessive drinking encompasses binge drinking (four or more drinks in a day for women; five or more for men), heavy drinking (eight or more drinks in a week for women; fifteen or more drinks in a week for men), as well as alcohol consumption during pregnancy or under the age of 21 (CDC 2025).

Excessive drinking can be detrimental to an individual's health, causing complications such as liver cirrhosis, heart problems, and increased risk of cancer, among many others (CDC 2025). All these negative health outcomes can collectively lower the life expectancy of excessive drinkers by one to five years (Wood 2018). Beyond its impact on individual health, alcohol misuse also imposes a significant burden on society. It is estimated that excessive drinking cost the United States around 250 million dollars in the form of lost labor productivity, property damage, criminal justice needs, and health care costs in 2010 (Sacks 2015). Excessive drinking was also responsible for an estimated 95,000 deaths in the US per year from 2011-2015, shortening the lifespan of those affected by 29 years (Esser 2020).

While not all individuals who engage in excessive drinking develop an Alcohol Use Disorder (AUD), excessive drinking is a major risk factor for it (NIAAA 2025). AUD is a chronic disease defined in the Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-V) as “a problematic pattern of alcohol use leading to clinically significant impairment or distress”. This encompasses things such as loss of control over alcohol consumption, compulsive drinking behaviors, and negative emotional and physical consequences upon cessation (DSM-V 2013). In the United States, around 10 percent of the population aged 12 or older are affected by AUDs every year

(SAMHSA 2024). Given the prevalence of AUDs and its impact on individual and societal well-being, effective treatment options are essential for improving outcomes and minimizing broader health and societal consequences.

1.2 Shortcomings of Current Therapies

Current therapies for AUD consist of pharmacological approaches and behavioral interventions, often used in conjunction for greater effectiveness. Behavioral interventions are typically the first line of treatment for AUD and can be used independently. The most common behavioral interventions are cognitive behavioral therapy, motivational interviewing, and 12 step approaches like Alcoholics Anonymous (Breuninger et al. 2020). These methods aim to equip individuals with coping strategies and support systems to reduce drinking, helping to alleviate the distress or impairment associated with AUDs.

Pharmacological treatments, when used in conjunction with behavioral therapies, may enhance treatment outcomes. There are currently three FDA approved treatments for AUD: naltrexone, disulfiram, and acamprosate (NIH 2021). Disulfiram is an acetaldehyde dehydrogenase inhibitor that induces unpleasant physiological reactions when alcohol is consumed, thereby promoting abstinence (NIH 2021). Although it has demonstrated some efficacy in treating AUD, its clinical utility is limited by poor patient compliance (Lanz et al. 2023) and it does not address any of the underlying neurochemical disruptions that result from AUD.

In contrast, naltrexone and acamprosate target neurochemical mechanisms implicated in AUD (opioid signaling and glutamatergic balance, respectively) (NIH 2021), and have shown short-term efficacy in reducing alcohol consumption and increasing abstinent days (Srisurapanont & Jarusuraisin 2005; Rosner et al. 2010). However, their long-term effectiveness remains limited.

In a pooled clinical investigation, acamprosate was found to improve the primary outcome of continuous abstinence, with 36.1% of treated patients

remaining abstinent at 6 months compared to 24.3% of those taking the placebo (Mann et al. 2004). Though it is significant, the overall effect is modest. Despite treatment, this data shows 63.9% of individuals recovering from AUDs will still relapse within 6 months. Thus, while acamprosate may support recovery in some individuals, it fails to achieve the primary outcome of 6 months of continuous abstinence for the majority.

Naltrexone shows a slightly different pattern of efficacy. It is particularly effective at reducing the amount of heavy drinking days (Garbutt et al. 2005) and is associated with improvements in short term abstinence (Jonas et al. 2014). However, it fails to demonstrate lasting efficacy, as studies show no significant difference in the continuous abstinence rates between the treatment and placebo groups at the 6-month mark (Garbutt et al. 2005). These findings highlight important limitations in current treatment options for AUD. Although available pharmacological therapies can provide partial benefits, achieving sustained, continuous abstinence (the gold standard outcome in clinical trials) remains a significant challenge in most patients.

Even intensive treatment options, such as inpatient rehabilitation programs, struggle with this challenge. Rehabilitation clinics provide structured environments that integrate behavioral and pharmacological interventions, offering the most comprehensive approach to AUD management. This intensive setting makes clinics especially effective at preventing relapse in the short term because patients are monitored closely, and treatment compliance is high. Despite this, the long-term outcomes are poor. Studies show that 60-70% of individuals relapse within 6-12 months of discharge (Sinha 2011; Nguyen et al. 2020). These high relapse rates following discharge suggest that immediate relapse risks can be effectively managed, but there is a prolonged period of relapse vulnerability following treatment that is not being adequately addressed.

This highlights the shortcomings of current treatment options and the need for more effective, long-term solutions to support sustained recovery from AUD.

1.3 Alcohol Withdrawal as a Risk Factor for Relapse

A key factor contributing to relapse in the short and long term is the development of withdrawal symptoms, which can intensify cravings and make continuous abstinence difficult. Withdrawal symptoms can be distinguished temporally, with different manifestations emerging during the early and later phases of cessation. Acute alcohol withdrawal encompasses an array of physiological (i.e. tremor, nausea, seizures) and psychological (i.e. anxiety) symptoms that develop within **hours to days** after cessation from chronic alcohol consumption (DSM-V 2013). Intervention during this phase is more feasible, as individuals are typically under structured care in rehabilitation facilities and the symptoms of acute withdrawal are both well documented and widely studied.

However, emerging research supports the idea of post-acute withdrawal syndrome (PAWS), a less understood, but relevant phase of withdrawal. Unlike acute withdrawal, which is dominated by physical symptoms, PAWS is characterized by persistent psychological changes that include increased irritability, anxiety and anhedonia (Bahji et al. 2022), which is collectively described as a state of negative affect. Anhedonia, the inability to feel pleasure, has been linked to increased risk of relapse during recovery (Nyguen et al. 2020). Because this state of negative affect can persist up **to 4-6 months** after cessation of drinking (Bahji et al. 2022), PAWS poses a prolonged vulnerability period that significantly heightens the risk of relapse, making it a significant, yet often overlooked, aspect of alcohol withdrawal. The prolonged presence of these affective disturbances likely contributes to the high relapse rates observed following discharge from rehabilitation clinics, and the limited long-term effectiveness of current pharmacotherapies.

Despite its clear relevance to alcohol relapse, there remains a significant gap in knowledge concerning PAWS. Most research, both clinically and molecularly, is focused on acute withdrawal. This leaves key questions unanswered, such as when acute withdrawal progresses into PAWS and the underlying neurochemical mechanisms behind this change. Our study aims to bridge this gap by investigating shifts in brain region activity during both phases of withdrawal. Insights gained from this work could ultimately guide the development of more effective, long-lasting treatments for AUD.

1.4 Brain Regions Involved in Alcohol Withdrawal

1.4.1 Ventral Tegmental Area

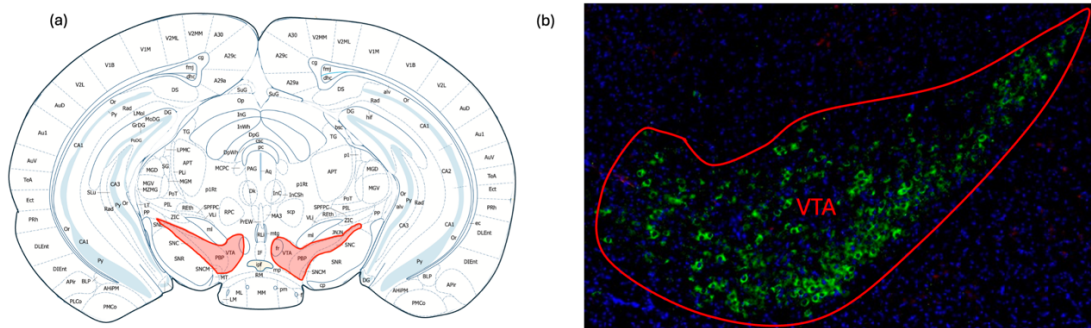


Figure 1 *Visualization of the VTA.* (a) Image of the VTA outlined in red (adapted from Paxinos & Franklin brain atlas). (b) Representative fluorescent image of immunohistochemically stained VTA tissue. Dopaminergic neurons (Th+) are shown in green, and neuron nuclei (DAPI+) are shown in blue. The VTA boundary is outlined in red. (Image collected by M. Lanz).

The Ventral Tegmental Area (VTA) is a central area of study in alcohol addiction, specifically the role of its dopaminergic neurons in modulating reward behavior. Acute doses of alcohol result in increases in the intrinsic firing rate of

dopaminergic neurons both *in vitro* and *in vivo* (Morikawa & Morrisett 2010; Juarez et al. 2017), leading to increased dopamine (DA) release to downstream areas of the brain such as the nucleus accumbens (NAc) (Lyness & Smith 1992). This increased DA release in the NAc is responsible for modulating feelings of reward (Hamid et al. 2016). Though this pathway has been extensively studied with acute doses of alcohol, newly emerging literature is examining its role in withdrawal. Current evidence indicates that dopaminergic neuron activity in the VTA is reduced during acute withdrawal (Shen 2003; Diana et al. 1996; Bailey et al. 2001). Interestingly, this reduction appears to reverse during protracted withdrawal, with studies reporting increased VTA dopaminergic activity at 7 and 21 days of abstinence (Hopf et al. 2007; Hirth et al. 2015). Our study aims to replicate these changes in VTA activity during both acute and protracted withdrawal using our own experimental approach.

1.4.2 Habenula

The Habenula is composed of two subregions of different neuronal populations: the Medial (MHb) and Lateral (LHb) Habenula.

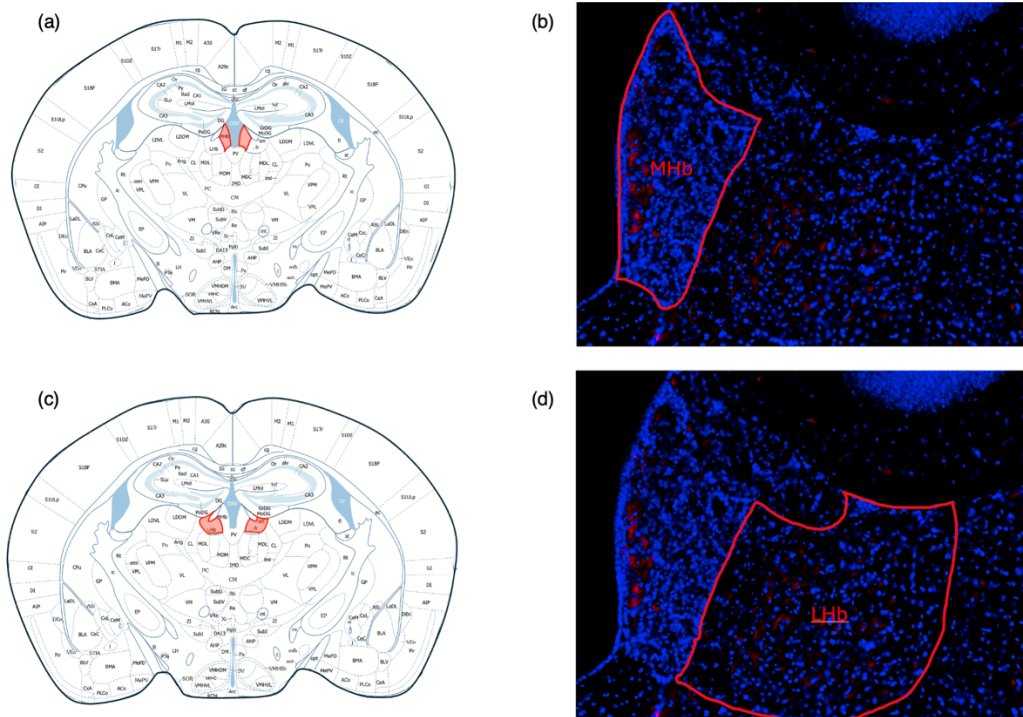


Figure 2 Visualization of the Medial and Lateral Habenula. (a) Image of the Medial Habenula outlined in red (adapted from Paxinos & Franklin brain atlas) (b) Representative fluorescent image of immunohistochemically stained MHb tissue. Active neurons (cFos+) are shown in red, and neuron nuclei (DAPI+) are shown in blue. The MHb boundary is outlined in red (c) Image of the Lateral Habenula outlined in red (adapted from Paxinos and Franklin brain atlas) (d) Representative fluorescent image of immunohistochemically stained LHb tissue. Active neurons (cFos+) are shown in red, and neuron nuclei (DAPI+) are shown in blue. The LHb boundary is outlined in red. (Image collected by M. Lanz).

The LHb is comprised mainly of glutamatergic neurons that project to and regulate many downstream targets including VTA dopaminergic neurons. (Olmelchanko et. al 2020). The LHb has been consistently linked to aversive behaviors, with studies reporting increased activation in response to negative stimuli such as foot shock or looming threats in mice (Lecca et. al 2017; Wang et

al. 2017). This is proposed to happen through its glutamatergic projections to a region known as the rostromedial tegmental nucleus (RMTg), which inhibits the VTA. Given its well-established role in aversive behaviors and regulatory influence on the VTA, the glutamatergic neurons in the LHb may be important in modulating negative affect during withdrawal.

The Medial Habenula (MHb), on the other hand, consists predominantly of cholinergic neurons that project to the interpeduncular nucleus (IPN), another region associated with aversive processing (Lee et al. 2019). Some studies have shown this region to be responsible for modulating somatic signs of acute withdrawal (McLaughlin 2017; Perez et al. 2015), though a clear consensus has not been reached. Furthermore, the involvement of if the MHb in modulating protracted withdrawal signs has not been investigated, despite its neurochemical connectivity to the IPN suggesting plausible involvement.

Together the LHb and MHb are compelling targets for further investigation, as both regions are functionally capable of influencing affective and somatic aspects of alcohol withdrawal across acute and protracted phases.

1.4.3 Mesopontine Tegmentum

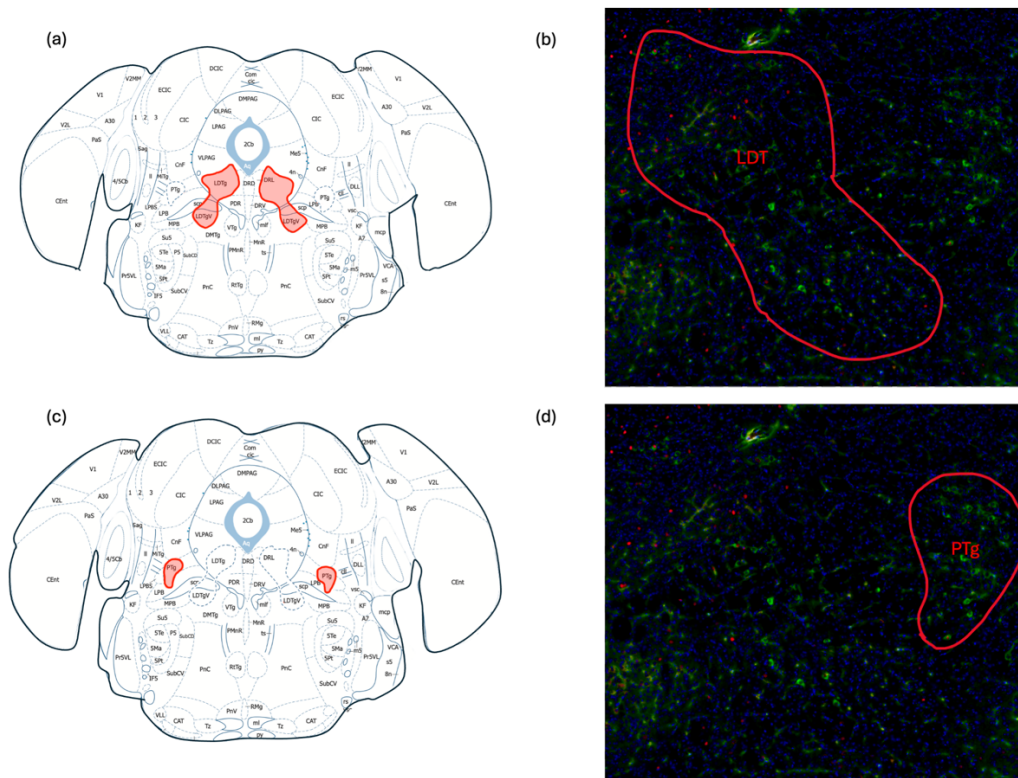


Figure 3 Visualization of MPT subregions: PTg and LDT. (a) Image of the LDT outlined in red (adapted from Paxinos & Franklin brain atlas) (b) Representative fluorescent image of immunohistochemically stained LDT tissue. Active neurons (cFos+) are shown in red, cholinergic (Chat+) neurons in green, and neuron nuclei (DAPI+) in blue. The LDT boundary is outlined in red (c) Image of the PTg outlined in red (adapted from Paxinos and Franklin brain atlas) (d) Representative fluorescent image of immunohistochemically stained PTg tissue. Active neurons (cFos+) are shown in red, cholinergic (Chat+) neurons in green, and neuron nuclei (DAPI+) in blue. The PTg boundary is outlined in red. (Image collected by M. Lanz).

The mesopontine tegmentum (MPT), a hindbrain structure that modulates VTA activity, has not been widely studied in the context of alcohol withdrawal.

However, given its cholinergic modulation of the dopaminergic reward pathways in the VTA, it is a compelling candidate for further investigation. The MPT is comprised of two subregions, the pedunculopontine tegmental nucleus (PTg) and the laterodorsal tegmental nucleus (LDT). Both regions contain cholinergic, glutamatergic, and GABAergic neurons (Wang 2009). The cholinergic neurons can be considered master modulators of cholinergic signaling in the brain. They contain many varicosities along their axons allowing for volume transmission of acetylcholine to a variety of downstream targets, including the VTA (Xiao et. al 2016). Previous studies from our lab have shown increases in activity of the PTg, but not the LDT after chronic alcohol administration in mice (Mulloy et al. 2024). Though many have speculated on its role in AUD (Maskos 2008; Steidal et al. 2017), little research has investigated the effects of acute or protracted alcohol withdrawal in the MPT. In this study, we aim to evaluate the role that cholinergic activity of both the LDT and PTg play in alcohol withdrawal and how this relates to changes in Hb and VTA.

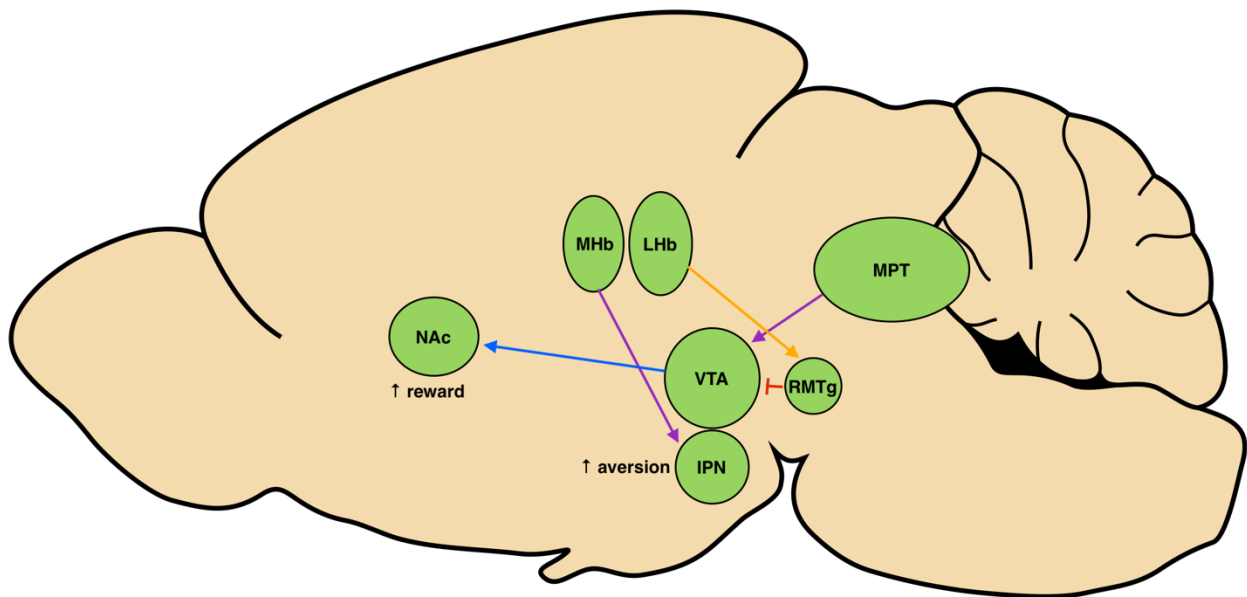


Figure 4 *Basic connectivity of VTA, Hb, MPT in mouse brain from sagittal view.*

Cholinergic signaling is pictured with purple, glutamatergic with orange, GABAergic with red, and dopaminergic with blue

1.5 Animal Model

1.5.1 Active vs Passive Administration

Most models of chronic alcohol consumption in mice generally fall into two categories: active and passive administration. Active administration models, such as the two bottle choice paradigm and operant self-administration, are designed to investigate the reinforcing and motivational properties of alcohol and require voluntary consumption of alcohol (Becker & Ron 2014). These models are well suited for assessing reward directed behavior in mice. In contrast, models of passive administration like vapor inhalation, gastric intubation, and i.p. injections (Heilig et al. 2010), allow for precise control of dosage and timing of alcohol administration. This is particularly advantageous when assessing specific stages of withdrawal, as it ensures consistent blood alcohol concentrations across subjects and enables researchers to precisely define the onset and duration of abstinence periods.

1.5.2 Length of administration

In addition to method of administration, the length of alcohol administration is also an important variable to consider in mouse models of AUD. Historically, the accepted standard for inducing physiological dependence was a 9-week ethanol liquid diet paradigm in rats (Uzbay & Kayaalp 1995). While effective at mimicking chronic alcohol consumption in humans by maintaining elevated blood alcohol content (BAC) over time and producing symptoms of alcohol withdrawal upon cessation, this approach was time consuming and resource intensive.

Recent literature has focused on developing a more efficient chronic administration model. Studies have shown that 9 days of chronic ethanol

injections paired with an alcohol dehydrogenase inhibitor 4-methylpyrazole (4-MP), produces similar levels of alcohol dependence to that of the liquid diet model. Importantly, both models induced similar intensities of somatic (i.e. shakes, chewing, grooming) and affective (i.e. anxiety-like and compulsive-like behaviors) signs of withdrawal in mice (Perez & Biasi 2015).

The use of 4-MP is particularly important given that mice metabolize alcohol 5x faster than humans (Chancharoenthana *et al.* 2025). By inhibiting alcohol dehydrogenase, 4-MP prolongs elevated blood alcohol levels in mice after exposure, better approximating the pharmacokinetics of alcohol in humans. Data from our lab confirm that our injection model, described below and in the methods, successfully achieves BAC within the range of human intoxication (above 0.08%). Specifically, average blood alcohol levels of mice in this paradigm reach $0.1\% \pm .015\%$ at 45 minutes post-injection and remain elevated at $0.1\% \pm .013\%$, at 90 minutes post injection (unpublished data).

1.5.3 Classification of Acute and Protracted Withdrawal in Mice

Regarding the classification of withdrawal stages in a mouse model, literature indicates acute withdrawal symptoms typically emerge within 2-48 hours after cessation of alcohol exposure, peaking around 24 hours and resolving within a week (Heilig et al. 2010). As such, withdrawal related phenomena observed one week or later are typically classified as protracted withdrawal.

In this study, we utilized a passive administration model (i.p. injections) of alcohol (2.5 g/kg) paired with 4-MP (9 g/kg) over nine days, followed by immunohistochemistry (IHC), to assess the changes in activity in the VTA, Hb, and MPT during both acute (24 hour) and protracted (7 day) withdrawal in male mice.

Chapter 2 METHODS

2.1 Chronic Alcohol Model

Adult male C57BL/6J mice (Jackson Laboratory) 8-12 weeks of age were given daily injections of alcohol (2.5 g/kg, i.p.) or saline, both paired with the alcohol dehydrogenase inhibitor 4-MP (9 mg/kg, ip) for nine days. Mice were sacrificed and perfused with 4% paraformaldehyde (PFA) at 24 hours (n=4) or 7 days (n=5) after last alcohol injection.

2.2 Tissue Preparation

Following fixation in PFA for 2-4 hours, brain tissue was transferred to a 30% solution of sucrose in PBS for cryoprotection. Tissue was immersed in 30% sucrose for 2-5 days. Tissue was then frozen on dry ice and stored at -80°C until it was ready to be sectioned. Perfused mouse brains were allowed to acclimate to -20°C for one hour before being mounting on the cryostat using an optimal cutting temperature compound (OCT). 30 µm coronal sections were taken of the Hb, VTA, and MPT and were stored free-floating, in PBS, at 4°C, until ready for further processing.

2.3 Immunohistochemistry (IHC)

Preparation: Free-floating tissue was processed using double-labeling IHC. Tissue was washed 3x for 5 minutes with PBS to remove excess OCT. Sections were then blocked for 1 hour at room temperature with 5% normal donkey and alpaca serum to prevent non-specific binding. Tissue was washed again 3x for 5 minutes to remove blocking solution before being incubated overnight at 4°C with primary antibodies.

Primary: Primary antibody for the Hb an anti-cFos rabbit monoclonal antibody only (1:1000, Abcam ab190289). The MPT was treated with the same anti-cFos antibody and an anti-Chat (choline acetyltransferase) goat monoclonal antibody (1:500, Invitrogen PIPA518518). The Chat enzyme catalyzes the rate

limiting step in the production of acetylcholine, making it a good marker for visualizing cholinergic neuronal populations. Similarly, tyrosine hydroxylase (Th) catalyzes the rate limiting step in the production of dopamine, making it a good probe target for visualizing dopaminergic populations in the VTA. Therefore, VTA was treated with anti-Th monoclonal antibody (1:500, Synaptic Systems 213004) and the same anti-cFos antibody as the other two.

Secondary: Following primary incubation, tissue was washed again 3x for 5 minutes with PBS and then treated for 2-3 hours at room temperature with secondary antibody solutions. The Hb was treated with anti-rabbit alpaca (1:400 Alexa Fluor 594), a secondary antibody targeting the rabbit (anti-cFos) primary antibody. The MPT and VTA were treated with the same anti-rabbit alpaca antibody and an anti-goat donkey antibody (1:400, Alexa Fluor 488), targeting the goat (anti-Th/Chat) primary antibody. Sections were then mounted onto slides with Prolong Gold antifade mountant with DAPI.

2.4 Data Analysis

Slides were imaged using the Keyence BZ-X800 microscope. Each region of interest (ROI), defined as one side of a single tissue section, was imaged separately. Cell counting was performed by a blinded reviewer using ImageJ to identify probe-positive cells based on signal intensity and morphology.

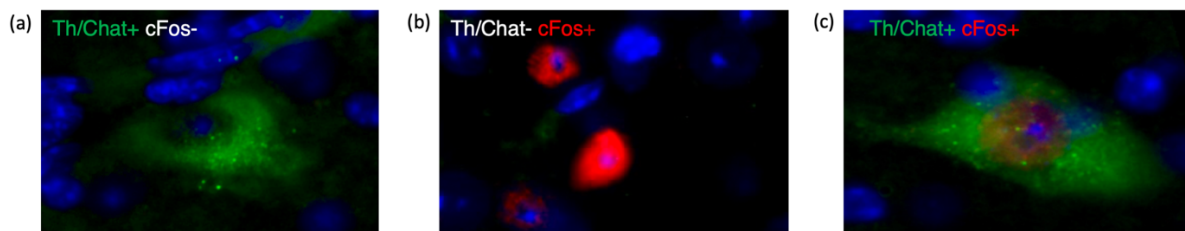


Figure 5 Examples of each IHC counting phenotype. (a) Th⁺/Chat⁺ cFos⁻ neurons are green only under fluorescent microscope. This marks inactive dopaminergic (VTA) or cholinergic (MPT) cells, depending on the primary antibody used (anti-Th, anti-Chat,

respectively) (b) Th-/Chat- cFos+ neurons are red only under a fluorescent microscope. This marks unknown cell types that are active. (c) Th+/Chat+ cFos+ neurons are both green and red under a fluorescent microscope. This marks active, dopaminergic or cholinergic neurons.

GraphPad Prism (V.10) was used for statistical analysis. All statistics were calculated using a nested t-test analysis, with ROI from the same animal being grouped together. Outliers were determined and excluded using the ROUT method. Data for all regions are reported as mean +/- standard error of the mean (SEM). **In the MPT:** ROI containing less than 10 total Chat+ cells were excluded; The percentage of activated cholinergic neurons (cFos+Chat+/all chat+) was calculated for each ROI and analyzed using a nested t-test. In addition, cFos+Chat- and total Chat+ neurons were also analyzed. **For the VTA:** The percentage of activated dopaminergic neurons (cFos+Th+/all Th+) was calculated for each ROI and analyzed using a nested t-test. In addition, cFos+Th- and total Th+ neurons were also analyzed. The results for the acute withdrawal timepoint for the VTA were not obtained. **For the Hb:** The amount of cFos+ cells for each region was counted and analyzed in a nested t-test.

Chapter 3 RESULTS

3.1 Brain Region Activation Patterns during Acute Withdrawal

We assessed neuronal activity across multiple brain regions (Medial and Lateral Habenula, PTg, and LDT) at 24 hours following the final alcohol injection, a time point corresponding to acute withdrawal.

3.1.1 Hb cFos expression levels remain unchanged during acute withdrawal

Due to the absence of a reliable marker for glutamatergic neurons in IHC approaches, we evaluated overall neuronal activity in the Medial and Lateral Habenula using total cFos expression (cFos+ cell counts) within each region. We did not find any significant change of cFos expression between the alcohol and saline mice in the Medial ($p=0.41$, $t=0.89$) or the Lateral ($p=0.37$, $t=0.75$) Habenula during acute withdrawal (Figure 6).

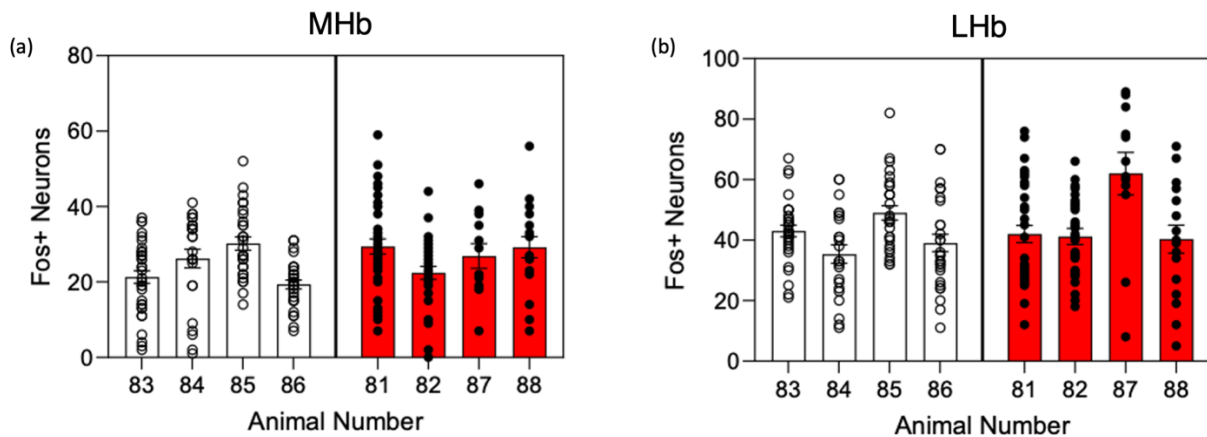


Figure 6 *cFos* expression in Hb during acute withdrawal. Bar graphs comparing number of cFos expressing neurons between saline-treated (white, open dot) and alcohol-exposed (red, closed dot) mice at 24 hours of withdrawal in both the Medial and Lateral Habenula. Error bars represent the SEM. (a) No significant difference in number of cFos expressing neurons in the MHb ($p=0.41$, $t=0.89$). $n=12-38$ ROI per animal, $n=4$ animals per group (b) No significant difference in the number of cFos expressing neurons in the LHb ($p=0.48$, $t=0.75$). $n=12-35$ ROI per animal, $n=4$ animals per group.

3.1.2 PTg cholinergic activation decreases during acute withdrawal, LDT activation does not change

We also examined the changes in activity of cholinergic neurons in the PTg and LDT. To assess cholinergic activity in the MPT, we quantified the percentage of activated cholinergic neurons (cFos+Chat+ neurons/all Chat+). We found a significant ($p=0.037$, $t=2.68$) decrease in the percent of activated cholinergic neurons in the PTg at 24 hours of withdrawal (Figure 7). Here, the alcohol-treated group showed an $11 \pm 4\%$ decrease in the percent activated cholinergic neurons compared with the control group. This change was not observed in the LDT ($p=0.87$, $t=1.16$) (Figure 8). We did not find a significant difference in number of non-cholinergic active neurons (Fos+Chat-) of the PTg ($p=0.18$, $t=1.53$) (Figure 7) or LDT ($p=0.87$, $t=1.58$) (Figure 8). There was a trend ($p=.051$, $t=2.44$) for an increase in total number of cholinergic neurons (all Chat+) in the LDT during acute withdrawal (Figure 8) that was not reflected in the PTg ($p=0.62$, $t=0.52$) (Figure 7).

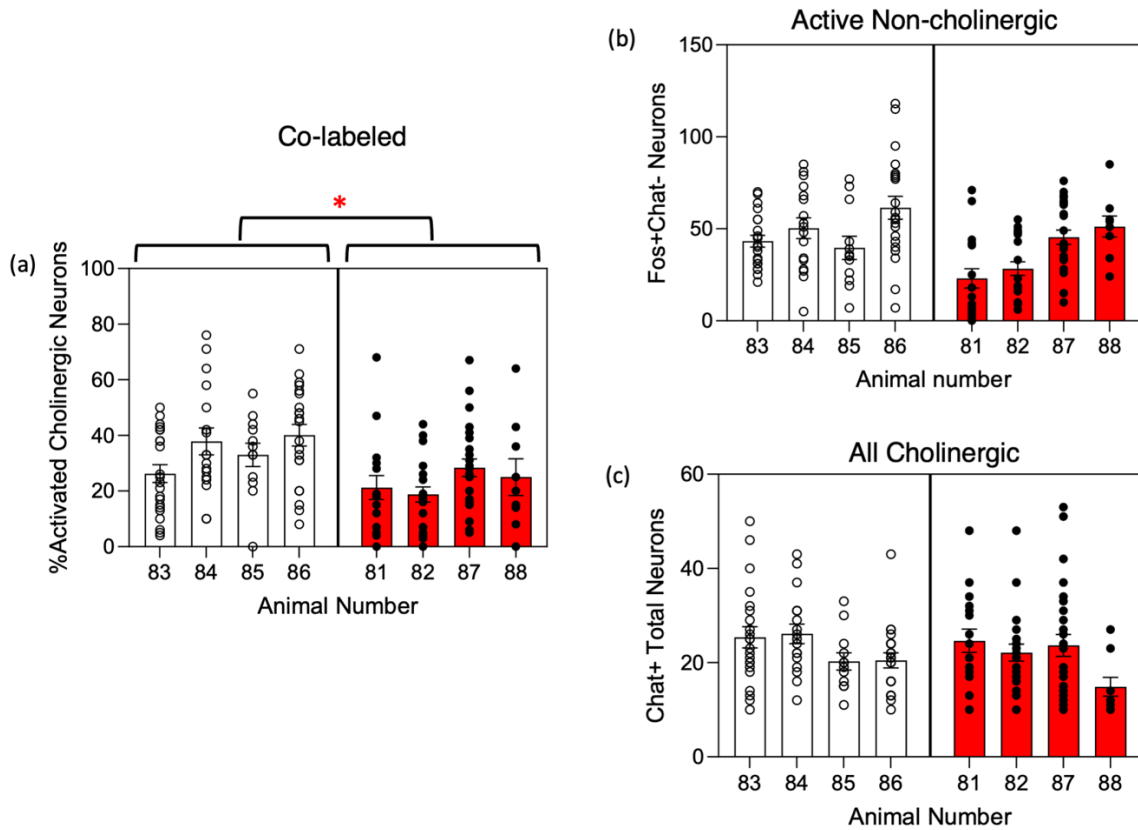


Figure 7 *cFos* and *Chat* expression in PTg during acute withdrawal. Nested graphs comparing saline-treated (white, open dot) and alcohol-exposed (red, closed dot) mice at 24 hours of withdrawal in the PTg. Data includes 9-22 ROI per animal, n=4 animals per group. Error bars represent the SEM. (a) There was a significant difference ($p = 0.037$, $t=2.68$) in the percentage of activated cholinergic neurons between saline-treated and alcohol-exposed mice with alcohol exposed mice showing an average decrease of $11 \pm 4\%$ (SEM). (b) No difference in the amount Fos+Chat- neurons between the groups ($p=0.18$, $t=1.53$). (c) No difference in total number of Chat+ neurons between the groups ($p=0.62$, $t=0.52$)

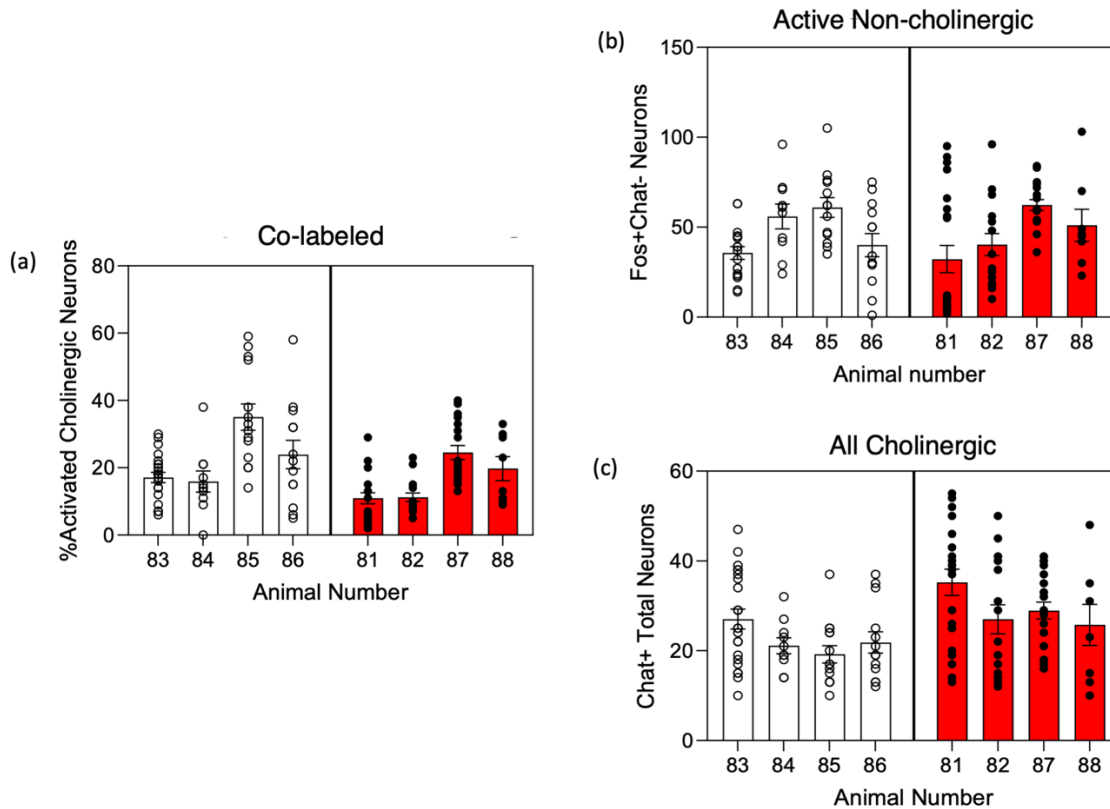


Figure 8 *cFos* and *Chat* expression in the LDT during acute withdrawal. Nested graphs comparing saline-treated (white, open dot) and alcohol-exposed (red, closed dot) mice at 24 hours of withdrawal in the LDT. Data include 8-21 ROI per animal, $n=4$ animals per group. Error bars represent the SEM. (a) No difference ($p = 0.29$, $t=1.16$) in the percentage of activated cholinergic neurons between saline treated and alcohol exposed mice. (b) No difference in the amount Fos+Chat- neurons between the groups ($p=0.87$, $t=1.58$) (c) A trend towards an increase in the total number of cholinergic neurons in the alcohol exposed mice ($p=0.051$, $t=2.44$) compared to control.

3.2 Brain Region Activation Patterns during Protracted Withdrawal

We also assessed the brain region activity for the VTA, Medial and Lateral Hb, PTg and LDT at 7 days after last alcohol injection (protracted withdrawal).

We used the same parameters to assess each brain region. In this study we chose to do only within-cohort analyses. For example, we compared saline and alcohol treated groups within the protracted cohort, but did not perform cross-cohort comparisons (e.g. acute alcohol vs. protracted alcohol).

3.2.1 VTA dopaminergic activation unchanged during protracted withdrawal

To assess dopaminergic activity in the VTA, we quantified the percentage of activated dopaminergic neurons (cFos+Th+ neurons/all Th+). We found no changes in the percentage of activated dopaminergic neurons in the VTA during protracted withdrawal ($p=0.78$, $t=0.28$) (Figure 9). We also did not find any significant differences in the number of non-dopaminergic cFos expressing (Th-cFos+) neurons ($p=0.93$, $t=0.92$) between groups (Figure 9). No difference in the amount of dopaminergic (all Th+) ($p=0.34$, $t=0.09$) neurons between groups was observed (Figure 9).

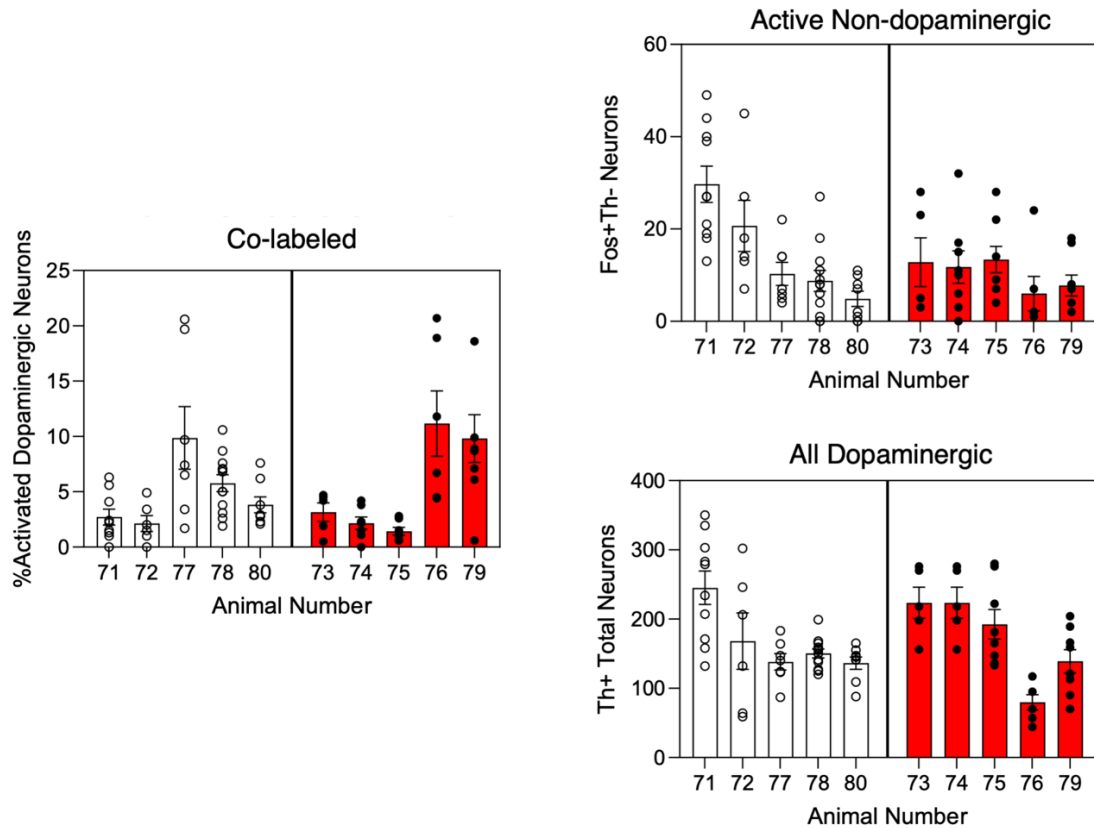


Figure 9 *cFos* and *Th* expression in the VTA during protracted withdrawal. Nested graphs comparing saline-treated (white, open dot) and alcohol-exposed (red, closed dot) mice at 7 days of withdrawal in the VTA. Data include 5-13 ROI per animal, n=5 animals per group. Error bars represent the SEM. (a) No difference ($p = 0.78$, $t=0.28$) in the percentage of activated dopaminergic neurons between saline treated and alcohol exposed mice. (b) No difference in *cFos*+*Th*- neurons between the groups ($p=0.93$, $t=0.92$) (c) No difference in total number of *Th*+ neurons between the groups ($p=0.34$, $t=0.09$).

3.2.2 LHb *cFos* expression increased during protracted withdrawal, MHb remains unchanged

We observed a significant increase ($p<.05$, $t=2.31$) in the amount of *cFos*+ neurons in the LHb during protracted withdrawal (Figure 10). Alcohol exposure

resulted in an increase of 10.5 ± 4.5 cFos+ neurons, corresponding to $67.5 \pm 29.2\%$ increase compared to the saline treated mice in the LHb. This trend was not reflected in the MHb ($p=0.12$, $t=1.75$) (Figure 10).

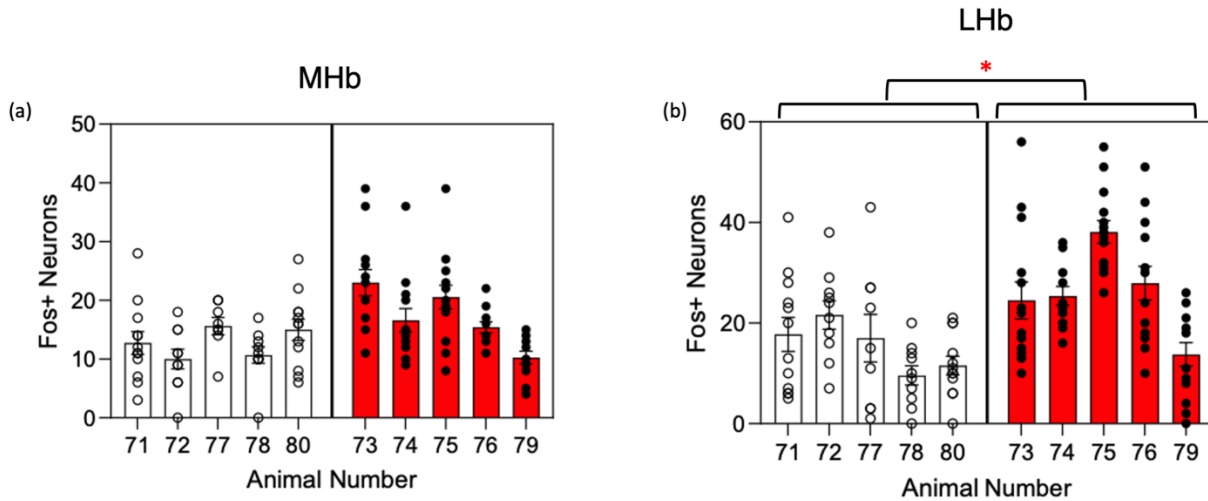


Figure 10 *cFos* expression in the Hb activity during protracted withdrawal. Nested graphs comparing number of cFos expressing neurons between saline-treated (white; open dot) and alcohol-exposed (red; closed dot) mice at 7 days of withdrawal in both the Medial and Lateral Habenula. Error bars represent the SEM. (a) The number cFos+ neurons in the MHb is unchanged ($p=0.12$, $t=1.75$) between groups. Data include 8-14 ROI per animal, $n=5$ animals per group (b) Chronic alcohol exposure resulted in a $67.5 \pm 29.2\%$ (SEM) (10 ± 4.5 (SEM) cFos+ neurons) increase ($p<.05$, $t=2.31$) in number of cFos+ cells compared to the control in the LHb. Data include 9-14 ROI per animal, $n=5$ animals per treatment group.

3.2.3 PTg and LDT percent activated cholinergic neurons increases during protracted withdrawal

Both the PTg and LDT saw a significant ($p=0.023$, 0.015 , respectively) ($t=2.82$, 3.09 , respectively) increase in activity of cholinergic neurons during

protracted withdrawal (Figure 11 & 12). Alcohol exposure resulted in a $15 \pm 5\%$ increase in percent activated cholinergic neurons in the PTg and an $18 \pm 6\%$ increase in LDT compared to saline controls. There was no significant difference in the number of active non-cholinergic (cFos+Chat-) neurons in the PTg ($p=0.72$, $t=0.36$) (Figure 11) or LDT ($p=0.14$, $t=1.20$) (Figure 12). There was a trend ($p=0.09$, $t=1.96$) for a decrease in the total number of cholinergic neurons in alcohol exposed mice the PTg compared to the saline control (Figure 11). No differences ($p=0.26$, $t=1.20$) in cholinergic neuron number were detected in the LDT (Figure 12).

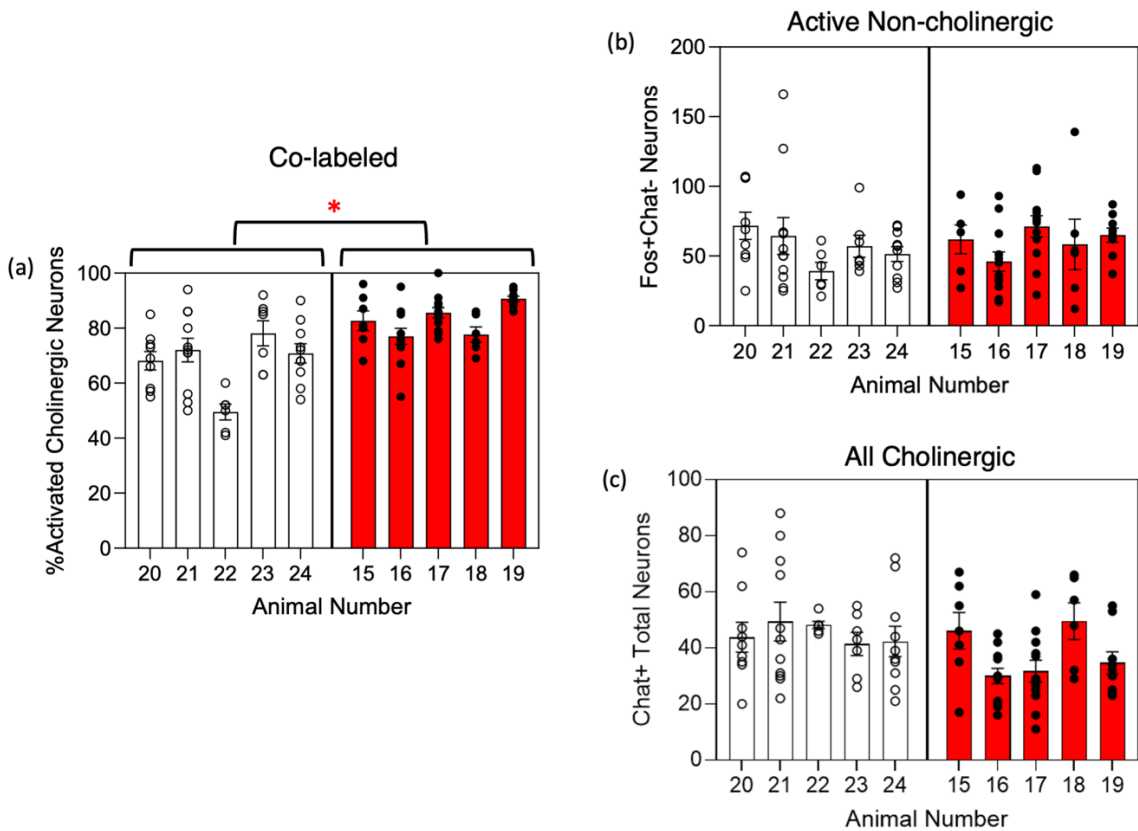


Figure 11 *cFos* and *Chat* expression in the PTg during protracted withdrawal. Nested graphs comparing saline-treated (white, open dot) and alcohol-exposed (red, closed

dot) mice at 7 days of withdrawal in the PTg. Data includes 6-12 ROI per animal. Error bars represent the SEM. (a) There was a significant difference ($p = 0.023$, $t=2.82$) in the percentage of activated cholinergic neurons between saline-treated and alcohol-exposed mice, with alcohol-exposed mice showing an average increase of $15 \pm 5\%$ (SEM). (b) No difference ($p=0.72$, $t=0.36$) in the amount cFos+Chat- neurons between the groups. (c) Total number Chat+ neurons in the PTg show a trend for a decrease ($p=0.09$, $t=1.96$) in the alcohol group compared to the control.

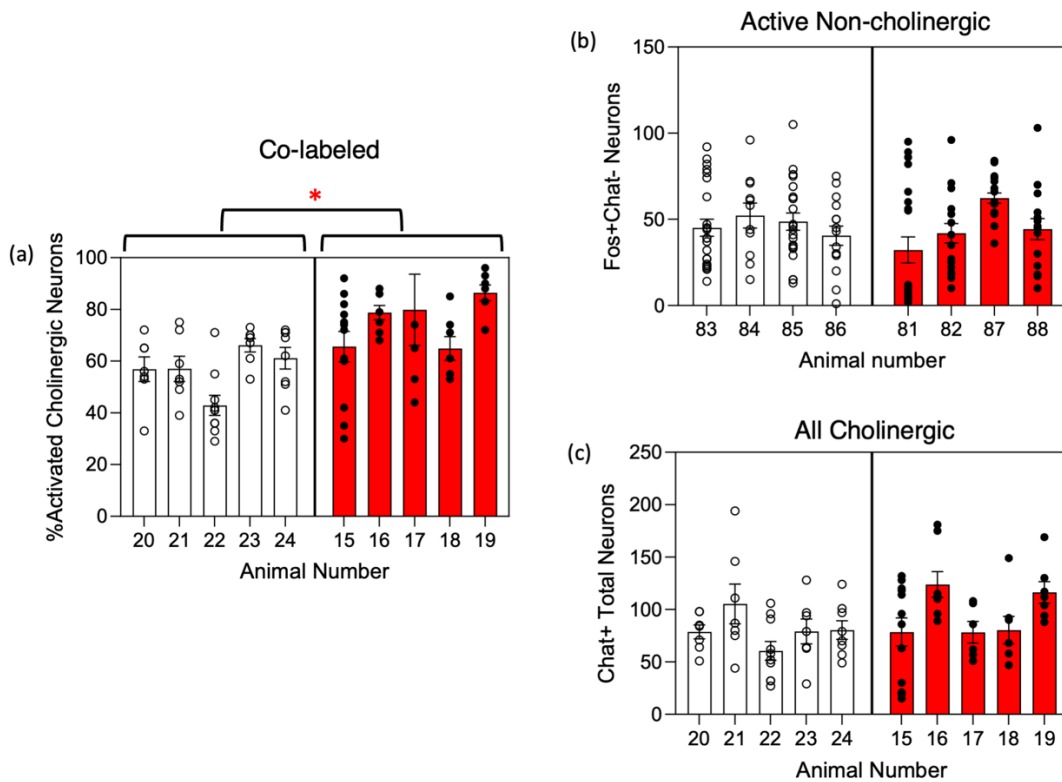


Figure 12 *cFos* and *Chat* expression in the LDT during protracted withdrawal. Nested graphs comparing saline-treated (white, open dot) and alcohol-exposed (red, closed dot) mice at 7 days of withdrawal in the LDT. Data includes 6-12 ROI per animal. Error bars represent the SEM. (a) There was a significant difference ($p = 0.02$, $t=3.09$) in the

percentage of activated cholinergic neurons between saline-treated and alcohol-exposed mice, with alcohol-exposed mice showing an average increase of $18 \pm 6\%$ (SEM). (b) No difference in the amount cFos+Chat- neurons between the groups ($p=0.14$, $t=1.20$) (c) No difference in total number of Chat+ neurons between the groups ($p=0.26$, $t=1.20$).

Chapter 4 DISCUSSION

The objective of this study was to investigate region-specific changes in neuronal activity during acute and protracted withdrawal in mice. Using a passive chronic alcohol administration model followed by immunohistochemistry to detect changes in cFos expression, we aimed to characterize the activity patterns of dopaminergic neurons in the VTA, cholinergic neurons in the MPT, and basic activity patterns in the Hb. By comparing alcohol exposed and saline treated mice at 24 hours and 7 days after last alcohol exposure, we aimed to identify molecular changes in alcohol withdrawal that may provide insight into the mechanisms underlying relapse susceptibility across the entire alcohol withdrawal period.

4.1 LHb cFos expression increases during protracted, but not acute, withdrawal. MHb expression remains unchanged

We assessed activity in the Habenula by quantifying the total number of cFos+ neurons. During protracted withdrawal, we observed a significant increase in cFos expression within the LHb, supporting its involvement in mediating aversive states. Unlike the acute phase of withdrawal, where somatic symptoms dominate, protracted withdrawal is characterized by persistent negative affect, including increased anxiety, irritability and anhedonia (Heilig et al. 2010). These symptoms often drive relapse, not in pursuit of reward, but through the desire to alleviate the distress caused by them. The selective increase in LHb activity during protracted, but not acute withdrawal, aligns with its established role in

aversive processing and suggests that LHb hyperactivity may contribute to development of withdrawal symptoms during protracted withdrawal.

As discussed earlier, glutamatergic signaling within the LHb is known to mediate these aversive behaviors via projections to RMTg, which subsequently inhibits VTA activity. Therefore, we hypothesize that this hyperactivity of the LHb during protracted withdrawal is driven by activation of glutamatergic neurons. Because of the limitations of the IHC assay, namely the absence of an antibody capable of distinguishing glutamatergic neurons, we were unable to confirm the LHb hyperactivity we observed is driven by glutamatergic neurons. This is because proteins that most reliably distinguish glutamatergic neurons from other populations, vesicular glutamate transporters (Vgluts), are expressed in both the cell bodies and synapses of glutamatergic neurons. The resulting staining of both the cell bodies and processes makes it difficult to distinguish glutamatergic cells under the microscope. Future experiments could employ RNAscope, a technique that probes for mRNA rather than proteins, which are more localized in the nucleus of the neurons and more easily visualized. This would allow for clear resolution of glutamatergic neurons using Vgluts and activity level quantification with cFos.

We did not observe changes in the MHb during acute or protracted withdrawal. This may also be a result of the limitations of using an IHC assay. Because the MHb interfaces with a central ventricle, there is an increased chance of edge artifacts, resulting in the reduction of the precision of the assay. This makes it more difficult to detect significant changes in cFos expression. Although evidence is still emerging, the current literature suggesting potential involvement of the MHb in mediating somatic signs of acute withdrawal (McLaughlin 2017; Perez et al. 2015) indicates that further investigation is warranted before drawing definitive conclusions. Future studies could employ

other methods, such as electrophysiology or RNAscope, to get a more accurate representation of activity of this region.

4.2 PTg exhibits changes in activity during both states of withdrawal, LDT only affected during protracted withdrawal

Findings from both this study and previous work from our lab suggest PTg activation patterns vary significantly over the course of withdrawal. In this study we found a significant decrease during acute withdrawal, and a significant increase during protracted withdrawal of the percent of activated cholinergic neurons in the PTg of alcohol exposed mice. These changes in activity were specific to cholinergic neurons, as there no change in cFos+ neurons in non-cholinergic populations during either withdrawal period. Previous studies from our lab investigated the cholinergic activation two hours after last alcohol injection using a similar chronic administration paradigm (2g/kg of alcohol for 15 days) (Mulloy et al. 2024). In that study, an increase cholinergic activation was observed in ethanol exposed mice 2 hours after the last alcohol injection. Taken together, these results suggest that cholinergic activity in the PTg initially increases shortly after alcohol cessation, decreases by the 24-hour withdrawal time point, and subsequently returns to elevated levels by 7 days. This suggests that chronic alcohol consumption causes dysregulation of the PTg across acute and protracted withdrawal.

We also observed a significant increase in the percent of activated cholinergic neurons in LDT during protracted withdrawal only. Unlike the PTg, the LDT has not been implicated in any of the early withdrawal time points (i.e. 2 hours and 24 hours after cessation of alcohol) (Mulloy et al. 2024). This may be due to different underlying neural circuitry of the two subregions. In addition to the sharing a projection pathway to the VTA with the PTg, the LDT is also reciprocally linked to the LHb through its own distinct circuit (Bueno et al. 2019). Given our findings of elevated LHb activity during protracted withdrawal, there

could be interplay between excitatory glutamatergic neurons in the LHb and cholinergic neurons in the LDT. These distinct neural pathways, which are not present in the PTg, could explain the differences in temporal activation of the two subregions and suggest that the activation of these two subregions in withdrawal does not occur through the same mechanism.

Considering the limited efficacy of current pharmacotherapies in preventing long-term relapse, and the persistence of withdrawal many months into recovery, identification of novel pharmacological targets could improve AUD outcomes. Our findings, showing significant changes in cholinergic activity in the MPT during withdrawal, could lend itself to the discovery of one such target. Current approved therapies for AUD target primarily opioid and glutamatergic signaling, and there is a notable absence of pharmacological treatments that modulate cholinergic signaling. However, emerging data supports the use of varenicline, a partial agonist of the nicotinic receptor, to treat AUD. Clinical trials show that varenicline significantly increased the percentage of abstinence days in those taking the drug versus controls (Phimarn et al 2023). Though the effect was modest (an average increase of 4.2 abstinent days in those taking the drug), the efficacy of the drug in the context of AUD reinforces the idea that cholinergic signaling is an important driving factor of relapse. Further research into how these cholinergic systems modulate withdrawal behaviors and could lend itself to more specific, and therefore, more efficacious treatments for AUD. For example, interventions targeting specific cholinergic receptor subtypes could enable selective modulation of cholinergic signaling, potentially minimizing side effects, alleviating withdrawal symptoms, and ultimately decreasing relapse vulnerability.

4.3 Total Chat+ neurons in the LDT and PTg

The MPT had some unusual trends for changes in the total number of cholinergic neurons during withdrawal. Specifically, the PTg showed a trend towards a decrease in the number of cholinergic neurons during protracted

withdrawal. One interpretation is that this chronic alcohol exposure can cause neurodegeneration of cholinergic neurons in the PTg. Some evidence supports this possibility, such as findings that chronic alcohol exposure during adolescence reduces the number of cholinergic neurons in the basal forebrain in mice (Verterno & Crews 2018). However, studies in adult mice, assessing cholinergic number numbers in the MPT, did not find any changes as a result of chronic alcohol exposure (Pereira et al. 2020). Another interpretation of the data is that the neurons themselves are not degenerating but undergoing a phenotypic shift. Previous research from our lab showed that around 27% of the cholinergic neurons in the PTg are also glutamatergic (Mulloy et al. 2024). It is possible that alcohol alters the expression balance of these dual phenotype neurons, leading to a reduction of detectable Chat expression and thus fewer cells being classified as cholinergic in our analysis.

In contrast, the LDT showed a trend for an increase in the number of cholinergic neurons during acute withdrawal. Given the lack of evidence to suggest that chronic ethanol exposure could cause neurogenesis of cholinergic neurons, it is unlikely the trend is reflecting this. A more plausible explanation is the shift in phenotype proposed in the PTg may occur in the opposite direction during acute withdrawal in the LDT. That is, glutamatergic and cholinergic co-expressing neurons may upregulate Chat during acute withdrawal, leading to their increased classification as cholinergic. Because these interpretations have little support in broader literature, a more plausible explanation is that these trends are an artifact of variability in our sectioning regime. In coronal sections, both the PTg and LDT have a small cross-sectional area that spans many planes. Because a set number of sections were taken per animal, slight differences in the anterior-posterior positioning could have led to variability in the number of cholinergic neurons counted. Given the lack of supporting literature of phenotypic shifts and potential for anatomical variability in sectioning, these

patterns most likely represent an artifact of our methods, rather than a true biologic effect.

In addition, there were some inconsistencies when comparing the number of Chat+ neurons to previous experiments in our lab. The average number of Chat+ neurons in the acute withdrawal cohort of this experiment was around 30 for each ROI in the PTg. Previously studies from our lab found the average number of Chat+ neurons to be around 60 per ROI in the PTg (Mulloy et al. 2024), which is around the number reported in our protracted withdrawal cohort. The difference in the number of cholinergic neurons between our acute withdrawal cohort and previous experiments in our lab raises concerns regarding tissue quality or staining. However, the percent activated cholinergic neurons for the saline group was similar between experiments (around 20% cholinergic activation), which lends some consistency to the data. Nonetheless, the difference in the number of Chat+ neurons in the acute withdrawal cohort highlights the need for replication of this data.

4.4 No changes in VTA activity observed, passive administration may be the cause

We did not observe any significant differences in the percentage of activated dopaminergic neurons during protracted withdrawal. Studies have reported a decrease during acute withdrawal (Shen 2003, Diana et al. 1996, Bailey et al 2001) and an increase during protracted withdrawal (Hopf et al. 2007; Hirth et al. 2015) in activity of VTA dopaminergic neurons. Our findings, that VTA activity remained unchanged during protracted withdrawal may not reflect these trends in literature due to study limitations such as passive administration and use of cFos as a proxy for activation. Literature has criticized passive administration models for being unable to elicit reward responses from the VTA (Becker and Ron 2014), however, other studies demonstrating VTA hypofunction as a result of acute withdrawal have not been hindered by this limitation (Diana et

al. 1996; Bailey et al. 2001). These studies, however, measure firing rates of neurons as a proxy of VTA activity rather than immunohistological characterization of cFos protein expression. Studies that use cFos quantification to measure activity levels also failed to find any significant changes in the VTA during acute or protracted withdrawal (Smith et. al 2020). This suggests that cFos-based IHC may not be an accurate measure of VTA activity in the context of alcohol withdrawal.

If the limitations in our methodology prevented accurate assessment of VTA activity, and the trends reported in literature are valid, then the observed temporal shifts in VTA function during withdrawal support our hypothesis that the MPT plays a modulatory role in VTA activity. Specifically, the decrease in PTg cholinergic activity we observed during acute withdrawal aligns with the reported VTA hypofunction during this stage. Similarly, increased PTg activity during protracted withdrawal corresponds with the hyperactivity of the VTA during protracted withdrawal. Together, these findings suggest a temporal link between the PTg cholinergic and VTA dopaminergic activity across different stages of alcohol withdrawal. This temporal alignment supports the hypothesis that the MPT lays a modulatory role in VTA activity during both acute and protracted withdrawal.

4.5 Other limitations and Broader Implications

Although we observed several region-specific changes in cFos expression, we cannot directly link these neural changes to withdrawal behaviors. However, our lab has shown that drugs that modulate cholinergic activity, like mecamylamine and varenicline, can attenuate alcohol withdrawal behaviors in male mice (unpublished data). Future experiments employing techniques such as optogenetic or pharmacological manipulations could target specific neural populations within the PTg, LDT, or LHb and directly assess their

effect on modulating withdrawal behaviors. This will be essential to establish a direct connection from the neurochemical changes to the withdrawal behaviors.

It is also important to note that this study was conducted exclusively in male mice. Research from our lab suggests that male and female mice share few similarities in molecular and behavioral changes associated with alcohol withdrawal. For example, previous research from our lab demonstrated that female mice did not exhibit the same increases in PTg activity following chronic alcohol administration (Mulloy et al. 2024). Furthermore, we have observed that female mice manifest certain signs of withdrawal differently compared to the males. Specifically, females showed no significant changes in measures of anxiety and compulsive-like behaviors during acute withdrawal, despite displaying comparable levels of alcohol metabolism and intoxication to male mice (unpublished data). Broader literature also supports the idea of significant sex differences in withdrawal symptoms. One study found the male, but not female mice, experience anhedonia (measured via sucrose preference) during acute withdrawal (Metten et al. 2018). This pattern seems to extend to humans as well, where a review of clinical studies investigating sex differences found that men were more likely to develop alcohol withdrawal symptoms than females (Unlu et al. 2023). Taken together, all these findings support the idea of fundamentally different neural mechanisms underlying alcohol withdrawal in males and females, highlighting the need to optimize experimental designs to investigate each sex separately.

The findings of this study contribute to a growing body of literature aimed at disentangling the neural circuits involved in alcohol withdrawal, particularly during protracted abstinence when the risk of relapse remains high. Identifying differences in brain region activity, such as increased activation of LHb and MPT supports the hypothesis that these regions may serve as modulators of affective symptoms during protracted withdrawal. Since many of the current therapies for

AUD do not effectively address these symptoms, these data highlight potential novel targets for the development of pharmacological interventions. Ultimately, integrating these findings with behavioral approaches and pharmacological developments could eventually help improve long-term outcomes for those struggling with AUDs.

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