Parsing out the role of the mesopontine tegmentum in alcohol-related behaviors

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Dedication

Is it cheesy to dedicate your thesis to your family? I would like to thank my family for the support during my time in graduate school. Asking for and receiving help is never easy, and they were able to support me through COVID and 2022 in ways I will be able to repay. Thank you to Heather Markun-Heard for being my best friend and to my brother Graham Schneider for allowing me the safest space to be, live and lament as a graduate student. I would also like to dedicate this thesis to Barret Schultz, who showed me how to always find the beauty and silliness in music and life.

Abstract

N/A. This thesis is an F31-style Specific Aims and Research Strategy that was completed to satisfy requirements for a master's degree in Neuroscience from the University of Minnesota—Twin Cities Department of Neuroscience.

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SPECIFIC AIMS.

The estimated cost of excessive drinking is $223.5 billion and is the third-leading preventable cause of death in the US. Alcohol exerts its effects on multiple neurotransmitter systems, including nicotinic acetylcholine receptors (nAChRs). NAChRs are ligand-gated cation receptors primarily located on the presynaptic terminals and soma of neurons. NAChRs in the ventral tegmental area (VTA), a midbrain region that plays an important role in drugs of abuse, have been shown to mediate aspects of drug-related behavior, including the effects of alcohol. As alcohol is not an agonist of nAChRs, this suggests that acetylcholine (ACh), the endogenous ligand of nAChRs, is a mediator of the effects of alcohol in the VTA. ACh input into the VTA originates from a singular location, the mesopontine tegmental nucleus (MPT). The MPT is made up of the pedunculopontine nucleus (PPN) and laterodorsal tegmental nucleus (LDT). The PPN and LDT are closely related structures that project to the VTA and other forebrain regions. The MPT contains cholinergic, glutamatergic and GABAergic neurons. However, the exact roles of the neuronal cell types in the MPT and the nAChRs they contain in the processing of alcohol-related phenotypes are unclear. Previous research has shown that injections of the GABAa agonist muscimol into the PPN decreased ethanol drinking, suggesting a role for the PPN in the processing of ethanol. Recent work shows that ethanol potentiates LDT ($\alpha3\beta4$) nAChR inward currents, which is blocked by the selective $\alpha3\beta4$ antagonist SR16584. This suggests that LDT nAChRs modulate responses to ethanol. Overall, both regions of the MPT have been implicated in preclinical alcohol-related phenotypes. However, a causal relationship has not been established between ethanol exposure and nAChR expression in the MPT.

As the MPT has been associated with the regulation of ethanol phenotypes and contains nAChRs, the goal of this fellowship is to examine if a causal relationship exists between ethanol exposure and nAChR subunit expression in the MPT. In addition, we will examine how modulation of specific nAChR subunits influence alcohol-related phenotypes. The hypotheses of this proposal are that 1) chronic ethanol exposure influences the expression of nAChR subunits in cholinergic neurons of the MPT and 2) modulation of these subunits influences future ethanol self-administration and behavioral response(s) to ethanol. We hypothesize that isolating and modulating relevant nAChR subunits will influence alcohol behaviors that will better inform future pharmacotherapies targeting nAChRs for AUD. This will be investigated in two specific aims.

Aim 1. Examine if there is a causal relationship between chronic ethanol exposure and the expression of nAChR subunits on cholinergic neurons.

We will examine the relationship between ethanol exposure and nAChR expression using a combination of a chronic ethanol exposure paradigm and *in situ* hybridization. Alcohol-naive, unmanipulated male and female C57BL/6J mice will be given four weeks of voluntary chronic intermittent 2-bottle choice to establish a consistent and high level of drinking or given water only (either ethanol/water or water/water). Then, we will determine how chronic ethanol exposure affects specific cellular transcript expression and nAChR subunit expression using HiPlex RNAScope in the MPT. Previous research has shown that voluntary ethanol consumption is positively correlated with nAChR expression, and so our working hypothesis is that chronic ethanol exposure will increase nAChR subunit expression. Cellular and nAChR subunit mRNA expression will be compared between alcohol-exposed and water control animals. These data will better inform how ethanol exposure modulates neurobiology of the MPT. This will allow us to identify specific nAChR subunits relevant in chronic ethanol drinking, which has the potential to inform the development of pharmacotherapy for AUD.

Aim 2. Determine the role of MPT nAChRs in ethanol drinking as a potential target for AUD pharmacotherapies.

We will investigate the role of MPT nAChRs on alcohol consumption and reward. As nAChRs in the MPT have been previously positively correlated with ethanol consumption levels, nAChRs will be modulated via nAChR antagonists microinjected into the MPT. These animals will be compared to control (sham) injected animals and the effect of nAChR antagonism will be evaluated in a chronic intermittent two-bottle choice ethanol consumption paradigm. This will determine if nAChRs targeted via antagonists can modulate and reduce alcohol drinking by measuring the preference and amount consumed of alcohol between experimental and control animals.

Successful completion of this project will provide a better understanding of the role of brainstem cholinergic signaling in AUD and better inform the development of future treatments for AUD that target neurobiology. Based on this previous research and literature, the knowledge gained over the course of this project will better inform future formation of therapeutics for AUD.

RESEARCH STRATEGY: Background and Significance

Alcohol Use Disorder and its public health implications. 40.3 million Americans had a substance use disorder in the past year and 28.3 million of those had alcohol use disorder (AUD)[1]. Excessive drinking results in approximately $223.5 billion in costs per year[2], however only 1% of those with AUD in 2020 received medication-assisted treatment[1]. Three medications are FDA approved to treat AUD, but come with a variety of side effects and require the user to be abstinent from alcohol use[3]. Thus, there is a critical need to completely understand the neurobiology of alcohol use to better inform the development of additional future medication treatments.

The role of the cholinergic brainstem in VTA activity. Drugs of abuse modulate the mesocorticolimbic system by stimulating dopamine (DA) neurons in the ventral tegmental area (VTA), increasing DA release in the nucleus accumbens[4]. The only source of endogenous acetylcholine (ACh) to the VTA is from the cholinergic mesopontine tegmentum (MPT), consisting of the pedunculopontine nucleus (PPN) and laterodorsal tegmental nucleus (LDT)[5,6]. These structures modulate DAergic activity. Optogenetic stimulation of the PPN and LDT excite VTA DA neurons[5]. Stimulation of the PPN induces single-spike firing to burst firing in the VTA, whereas the LDT provides permissive glutamate and afferent (Glu/ACh)-induced burst firing[7]. Taken together these data provide strong evidence of MPT influence on VTA cellular activity. Cholinergic systems act through ionotropic nicotinic receptors (nAChRs)[8], and the MPT contains nAChRs on cells that project to the VTA[6].

The exact role of cholinergic midbrain nuclei in alcohol-related behaviors is unknown. The PPN and LDT have been shown to regulate cocaine[9,10], nicotine[9,11,12], opioid[9,13], ethanol[13–16] self-administration, reward and/or induced cellular activation. The exact cellular mechanism(s) and circuitry of the MPT's contribution to alcohol-related phenotypes is unclear. PPN lesions have been shown to block ethanol conditioned place preference[13]. Cholinergic modulators microinjected into the PPN regulate alcohol drinking behavior[14]. Focal acamprosate (FDA-approved drug to reduce ethanol intake) application to the PPN reduces ethanol intake and preference[15] and high-dose muscimol ($GABA_A$ agonist) to the PPN reduces ethanol-self administration[16]. However, due to non-specific cellular targeting in these studies, *the specific contributions of PPN and LDT neurons to alcohol-related phenotypes are still unknown.*

Midbrain cholinergic nuclei projections to the VTA regulate alcohol-related phenotypes. The PPN and LDT are closely associated structures that contain cholinergic, glutamatergic and GABAergic cells and project to the VTA[6,17–20]. Voluntary ethanol intake increases ACh in the VTA[21], and antagonism of nAChRs by mecamylamine (reducing ACh) reduces both voluntary ethanol intake and the DA-activating effects of ethanol[22,23]. These data suggest that cholinergic signaling from the VTA is important for the rewarding effects of ethanol. As the PPN and LDT are the only source of ACh to the VTA, they are likely involved in mediating ethanol's effects. The non-specific cellular targeting of previous studies regarding the MPT in alcohol phenotypes also suggests a potential role of non-cholinergic neurons in regulating ethanol behavior. MPT nAChR currents are potentiated by ethanol and there is a correlation between MPT nAChR currents and ethanol self-administration, however, chronic exposure to ethanol and functional implications of MPT nAChRs have not been indexed[24].

The exact role of PPN and LDT cholinergic and non-cholinergic cells and the nAChRs they contain have not been directly linked to alcohol related phenotypes. Parsing out the role of these regions and their cellular contributions to ethanol phenotypes will allow for a greater understanding of how the brainstem regulates drug processing and allow for more efficacious therapeutic treatments for AUD.

Our objective is to determine if there is a causal connection between ethanol exposure and expression of nAChRs. We hypothesize that chronic alcohol consumption induces nAChR expression on cholinergic MPT neurons, and targeted disruption of nAChRs in the MPT will reduce ethanol consumption. We will identify how MPT nAChR expression is affected by a voluntary chronic ethanol exposure paradigm (Aim 1) and how antagonizing these receptors affect ethanol consumption (Aim 2). The goal of this proposal is to establish if nAChRs located in the MPT are an effective AUD target to better inform future therapies to reduce ethanol drinking and use.

RESEARCH STRATEGY: Approach

Ethanol consumption. The Lee lab is proficient in multiple ethanol exposure paradigms, including the two-bottle choice chronic intermittent ethanol exposure[25,26]. Single-housed experimental group (ethanol exposed) mice are given the choice of either 20% ethanol solution or water in two separate bottles for 24 hours on Monday, Wednesday and Friday. The animals are presented with two water bottles at all other times on Tuesday, Thursday, Saturday and Sunday. Weekly, animals body weights are collected to assess general body health. We will calculate the amount of ethanol consumed (g/kg/session) and the percent preference for ethanol compared with total fluid consumption. The placement of ethanol or water bottles is counterbalanced to reduce risk of associating a side of the home cage with drug availability. After four weeks of chronic intermittent ethanol (CIE) exposure, animals are sacrificed 24 hours after their last exposure to ethanol. This captures a window of time in which animals are experiencing acute withdrawal from ethanol. Brains are removed and flash-frozen in isopentane to prepare for slicing and histology experiments. The two-bottle choice exposure model allows for an escalation of drinking where animals achieve high levels of ethanol drinking by the end of the exposure paradigm as well as clinically relevant blood ethanol concentrations following the intermittent ethanol exposure.

Fluorescent *in situ* hybridization: HiPlex RNAScope. HiPlex RNAScope[27] allows mRNA present in tissue to be quantified in a high-throughput manner. HiPlex allows for nine genes of interest to be identified in a single slice of tissue. Probes specific to the gene(s) of interest are applied to tissue and mRNA present (corresponding to probes/genes of interest) in tissue fluoresces under light microscopy. Our lab has written custom MATLAB code along with a CellProfiler[34-37] pipeline to quantify fluorescent puncta of mRNA in tissue based on cell nuclei. Readings of percent positivity (percentage of cells that are positive for the target mRNA) and percent occupancy (percent of mRNA present in cell area) are identified.

Cannula injection of nAChR antagonists into the MPT. To target specific nicotinic receptor subunits in the MPT, we will utilize a pharmacological approach. In this approach, nAChR antagonists DHBE (antagonist of β2 containing nAChRs) and mecamylamine (global nAChR antagonist that is FDA-approved) will be injected into the MPT. This will

functionally antagonize nAChR activity in the MPT. The advantages of using this technique is that no transgenic mice are required, the nAChR system is not genetically manipulated and it will allow us to isolate nAChRs located in the MPT specifically. Using two drugs, one specific to the β2 subunit (DHBE) in conjunction with a global nAChR antagonist (mecamylamine) will allow us to differentiate the effects on ethanol drinking behaviors on a subunit-specific level.

Feasibility. We have successfully conducted RNAScope HiPlex in our lab, resulting in a submitted publication in revision (Gao*, Schneider* et al., 2022, in revision). Microinjection of nAChR pharmacological agents into the mouse brain has been performed successfully in our lab[28].

Sex as a biological variable and mice. All experiments will use purchased C57BL/6J mice from The Jackson Laboratory. Based on the existing literature and our experiences, male and female mice on a JAX C57BL/6 background show differences in ethanol consumption and preference levels. Based on this, we will conduct all analyses in both male and female mice separately. Using appropriate numbers of both sexes of animals will be performed to determine true statistical differences between groups. In our experience with ethanol exposure experiments, RNAScope and power analyses, this will be n=5 per sex for each experimental group for Aim 1, and n=8 per sex for each group for Aim 2. Our RNAScope data will be analyzed using a nested design that will allow for additional power in detecting significant differences across experimental groups. No previous data suggests that estrous cycle affects phenotypes being assayed in this proposal. In addition, female mice are intact and freely cycling, which will be more relevant in assaying the human drinking experience.

Scientific rigor and reproducibility. Group sizes will be determined using a power of .8 (80% power to detect a 30% difference). For all experiments, a control group (water, Aim 1) or a control, non-nAChR modulator (Aim 2) will be included. Comparisons between all groups will not be completed until tests of normality are conducted to ensure proper distributions are considered in analyses. If data are normal, nested T-tests and nested one- or two-way ANOVAS (nesting only for Aim 1 HiPlex data) will be used with appropriate post hoc testing. If abnormal (determined using the normality tests in Graphpad Prism 9), data will be analyzed using nonparametric methods and appropriate

post-hoc testing (such as the Mann-Whitney test to substitute for the Student's T-test *or* Friedman's test or Kruskal-Wallis test to substitute for ANOVAs). Cannula implantation site will be verified postmortem using appropriate histological and fluorescent microscopy assays. Behavioral experiments will be conducted in multiple cohorts at different times to minimize environmental and/or experimenter differences.

Aim 1 - Examine if there is a causal relationship between chronic ethanol exposure and the expression of nAChR subunits on cholinergic MPT neurons.

The MPT has been previously associated with mediating the rewarding properties of drugs of abuse such as morphine and amphetamine[29], where PPN lesions block conditioned place preference. The LDT is also associated with regulation of the mesocorticolimbic dopaminergic system as the LDT is required for burst firing of DA cells in the VTA[30]. While previous research suggests a role for the MPT in mediating the effects of drugs of abuse, there has been little investigation of nAChRs of the MPT and their regulation of drug phenotypes. Past work showed that injection of the muscarinic ACh antagonist scopolamine into the LDT reduces dopamine release in the nucleus accumbens[31]. More recently, ethanol consumption levels have been shown to positively correlate with LDT $\alpha3\beta4$ nACh receptor inward currents[24]. However, this study was correlational and only exposed rats to an acute (5-day two bottle choice) ethanol consumption paradigm. No long-term drug exposure paradigm was utilized and causal effects of ethanol exposure were not investigated. The goal of this aim is to determine if chronic ethanol exposure induces nAChR transcript expression in neurons and astrocytes of the MPT. **I hypothesize** that chronic voluntary ethanol exposure will induce nAChR subunit expression in cells of the MPT. In Aim 1, I will test this hypothesis by exposing male and female C57BL/6J mice to voluntary chronic ethanol exposure followed by *in situ* hybridization to assess nAChR expression levels in cells of the MPT.

Experimental Design: We will use a two-bottle choice chronic intermittent ethanol procedure as described above. After four weeks of chronic intermittent ethanol (CIE) exposure, animals are sacrificed 24 hours after their last exposure to ethanol. 24 hours following their last ethanol exposure captures a window of time where animals are

experiencing acute withdrawal. Brains will be extracted, flash-frozen and kept at -80°C to preserve mRNA in tissue. RNAScope HiPlex will be utilized to assess cellular and nAChR

mRNA levels in tissue. Brains will be sliced coronally at 16μm at the level of MPT where the mid-to-anterior LDT is present as well as the posterior PPN (pPPN, approximately -4.96 to -4.84 bregma[38-42]). Cholinergic MPT cells from this region project to the VTA[5]. Probes specific to 9 genes of interest will be applied to tissue (for nAChR subunits: *Mm-Chrna3* for α3, *Mm-Chrna4* for α4, *Mm-Chrna5* for α5, *Mm-Chrna6* for α6, *Mm-Chrnb2* for β2, *Mm-Chrnb3* for β3, *Mm-Chrnb4* for β4; for cellular markers: *Mm-Slc17a6* for VGLUT2 [glutamatergic transporter, marker for glutamatergic cells], *GFAP* for astrocytes, *Mm-Slc32a1* for VGAT [GABAergic transporter, marker for GABAergic cells], *Mm-ChAT* for cholinergic cells). Following imaging of the pPPN and LDT at 40x magnification, mRNA fluorescence will be quantified. Fluorescent puncta surrounding cell nuclei (via DAPI) will be quantified. Readings of percent positivity (percentage that a cell is positive for the mRNA of interest) and percent occupancy (percent of mRNA present in cell area) are identified for each region of interest. The average % target transcript positivity for each cell type will be calculated. Custom MATLAB code sets and a CellProfiler[34-37] pipeline allows for a reduction of experimenter error as computer automation generates quantification of fluorescent puncta. This code has been used previously by the Lee lab in a publication in revision, verifying its significance and usability. Following quantification of mRNA in tissue, expression levels of cells and nAChRs will be correlated with consumption levels. Expression levels and consumption levels will be compared across experimental groups by way of nested ANOVA. Patterns of expression across groups will be identified as significant or otherwise.

Predicted outcomes and interpretations: As ethanol has been shown to potentiate MPT currents[24], it is predicted that ethanol will cause an increase in nAChRs in the MPT compared with the water control animals. As the hypothesis is that levels of drinking correlate with nAChR expression, we will expect to see the most significant increase in nAChR expression among animals with the highest levels of drinking.

Alternative outcomes, approaches and interpretations: If animals do not show a difference in nAChR expression levels between the ethanol and water consuming groups, we would then examine if there is a positive correlation between nAChR expression levels

and the amount of ethanol consumed within the ethanol exposed group, as we would then hypothesize that the baseline expression of nAChR directly influences the amount of ethanol consumed. In addition, if nAChR expression levels are not affected, it could be possible that channel conductance of existing nAChRs increases following ethanol exposure. This could be confirmed using electrophysiology. If we find that nAChRs are not involved in regulating ethanol phenotypes, this could mean that 1) non-ionotropic ACh receptors play a role. Perhaps muscarinic receptors, which are GPCRs that are activated by ACh, regulate responses to ethanol. 2) Cellular activity that is not related to nAChR expression, such as induction of protein(s) or cell activity marker(s), may be mediating the response to ethanol. For example, one way to assess neuronal activity in the MPT would be to measure DeltaFosB levels, a long-lasting marker of neuronal activity, following ethanol exposure. 3) Receptor systems other than cholinergic receptors may be responsible for modulating ethanol response. Cholinergic cells, glutamate cells and/or GABAergic cells likely contain a variety of other receptor types that may regulate ethanol phenotypes. In addition to this, more complex cell-to-cell interactions could be mediating responses to ethanol. For example, cross-talk between cholinergic cells to GABAergic cells within the MPT with efferents to the VTA could be mediating the response to ethanol. RNAScope would not be highly effective in assessing cellular and circuit interactions. 4) The chosen strain of mice may not show robust differences in consumption and/or nAChR expression following ethanol exposure. Genetically bred alcohol-preferring animals may show greater molecular differences in nAChR expression, so investigating a different mouse strain may reveal differences in receptor expression following ethanol exposure.

Consumption is only one behavioral phenotype being assayed in Aim 1. There are other phenotypes, such as ethanol induced loss-of-righting-reflex (LORR) duration, a measure of ethanol sedation, and ethanol-induced anxiety-like behavioral measurements (*e.g.* time spent on open arms in exploratory plus or zero maze tasks), which could be correlated with nAChR expression in the MPT. A neurobiological change (nAChR expression changing from drug exposure) may be subtle, whereas overt behaviors (LORR and open arm time) might be a more effective way to index differences caused by drug exposure. Global knockouts of the β2 nAChR subunit have been shown to have lower LORR duration and increase time spent on open arms in the plus maze task. However, β2 KOs have not

been shown to alter ethanol *preference,* which could be related to consummatory behaviors[32], explaining why a change in consumption may not be present following ethanol exposure, but other behavioral changes may be.

Aim 2 - Determine the role of MPT nAChRs in ethanol drinking as a potential target for AUD pharmacotherapies.

We postulate that levels of ethanol drinking are influenced by MPT nAChR expression. In this aim, we will use pharmacological nAChR antagonists to assess the contribution of nAChR expression to ethanol consumption. Although pharmacological nAChR antagonists are not as selective as targeted genetic manipulations, they are advantageous in that they do not involve genetic ablation of nAChRs, which may result in compensatory changes in the knockouts that can influence the effects of drugs of abuse. NAChRs have been shown to influence drug dependence, intake, negative affect, reinforcement and withdrawal[33]. Knockouts of various subunits may have altered responses thus, the use of genetic ablations of nAChRs require caution in the interpretation. Modifying nAChR activity via pharmacological antagonism and assessing the effects of pharmacology is a reversible, biologically relevant way to understand the nAChR system's influence on drug processing. Microinjection of scopolamine (muscarinic ACh antagonist) into the PPN has been shown to decrease drinking in rodents[14]. MPT nAChR expression has been correlated with ethanol intake where ethanol drinking resulted in increased nAChR current activity[24]. These studies suggest a role of MPT cholinergic receptors in ethanol behaviors, however, the exact role of nAChRs have not been assessed. The goal of this aim is to determine the role of MPT nAChR on ethanol consumption using a pharmacological approach.

Experimental Design: Male and female C57BL/6J mice will be anesthetized with isofluorane and implanted with bilateral guide cannula targeting the MPT (approximately -4.96 to -4.84 bregma[38-42]) and fixed with dental adhesive as described previously by our laboratory[28]. Following recovery, all mice will be exposed to the same two-bottle choice paradigm described in Aim 1. Twenty minutes prior to their *last* CIE session on week 4, there will be one microinfusion of either 1 mM dihydro-beta-erythroidine (DHBE; a4β2 nAChR antagonist), 1 mM mecamylamine (non-specific nAChR subtype antagonist) or a phosphate buffered saline (PBS) vehicle control. Following the session, bottles will be

removed and ink will be bilaterally injected into cannulae to visualize infusion sites. Animals with injections outside of the target MPT region will be excluded from analysis.

Scientific rigor and statistical analysis: Based on estimates of experimental variance and from previous experience[28], we estimate an n=8 for each of the 3 drug treatment groups (DHBE, mecamylamine or PBS) per sex, for a total of n=24 mice per sex, n=48 total. Male and female animals will be analyzed separately. Analyses will be calculated using Prism 9.0. Data will be tested for normality and variance using the Grubbs test. Welch's corrections will be used if variances are unequal. Comparison of data across drug exposure (DHBE analyses conducted independently of mecamylamine analyses) will be conducted using two-way ANOVA followed by Tukey's multiple comparisons tests.

Predicted outcomes and interpretations: In the previous aim, it was hypothesized that ethanol increases the expression of nAChRs located in the MPT. We predict that antagonizing nAChR activity with pharmacological interventions will decrease the amount of ethanol consumed compared with animals that receive the PBS control. Decreased ethanol consumption in animals receiving nAChR antagonists would implicate the role of MPT β2*-containing and/or global nAChR influence on ethanol behaviors.

Alternative outcomes, approaches and interpretations: One limitation is that all mice will receive ethanol. Follow-up control experiments can determine the effect of MPT nAChR antagonism on water consumption to determine if MPT nAChRs influence fluid intake, or on sucrose consumption to determine their effect on a non-drug reward. We would hypothesize that water and sucrose consumption will not be altered by DHBE or mecamylamine injection into the MPT.

Ethanol consumption is the only behavior being examined in this aim. Using this pharmacological approach, other ethanol-related behaviors could be investigated. For example, to determine the effects of nAChR antagonists on ethanol reward, conditioned-place preference could be utilized. If MPT nAChRs mediate ethanol reward, we hypothesize that antagonism of MPT nAChRs would attenuate the expression of ethanol conditioned place preference compared with mice that receive PBS control micro-infusions. Other behavioral indicators, such as ethanol aversion and withdrawal could be utilized to determine the contribution that MPT nAChRs have on these ethanol-related behaviors.

If there is no effect of MPT nAChR antagonism on ethanol consumption, it is possible that MPT nAChRs could have an influence on non-consummatory behaviors that are not measured by the proposed experiments. Other behavioral experiments such as LORR could be used to indicate if MPT nAChRs have an influence on ethanol sedation. Another limitation is that one dose of DHBE and mecamylamine will be used. It is possible that a higher pharmacological dose is required to modulate ethanol consumption and testing a dose-response effect could be a future experiment.

A proposed alternative approach to this aim includes the creation of a viral-mediated knockdown of nAChRs that are specific to one nAChR subunit. The knockdown of a particular nAChR subunit in the MPT would allow us to determine the effects of a singular subunit on ethanol behaviors. In addition to a viral-mediated intervention of nAChRs; different pharmacological interventions could be utilized. For example, sazetidine-A has been shown to reduce alcohol consumption when injected into the VTA of male mice, and is thought to act on high-affinity nAChR subtypes, so other nAChR agonists or antagonists may affect other related ethanol behaviors. In addition to examining the MPT, the contribution of nAChRs in other addiction-related brain regions could be investigated. For example, the nAChR containing locus coeruleus (brainstem region adjacent to the MPT) may be involved in the regulation of ethanol phenotypes. Last, another receptor subtype or family could be responsible for regulating ethanol consumption. GABAa receptors in the MPT could regulate ethanol consumption; a viral approach such as DREADDs to create non-functional GABAa receptors could be utilized to index the role of GABA receptors in the brainstem in ethanol consumption.

Impact/Summary: Focusing on a specific subunit (β2*-containing nAChRs) of a receptor family that may affect a singular drug behavior (ethanol consumption) could be a narrow, targeted approach for future drug discovery for medications that treat AUD. If targeting a particular nAChR subunit is an effective way to reduce ethanol consumption, a medication that works via reducing nAChR expression in targeted brain regions (or cellular types) would be an effective medication for individuals with AUD. Future research on nAChRs in all brain regions implicated in addiction will further inform the alcohol field in potential ways to reduce risky ethanol consumption.

The goal of this proposal is to determine how ethanol affects nAChRs in the MPT, and to investigate how antagonizing nAChRs of the MPT affects ethanol consumption. More

research is needed to understand how nAChRs in the brain are involved in regulating responses to drugs of abuse. Learning more about this receptor family and the regions they are located in will inform future pharmacotherapies that aim to reduce unsafe drug use behaviors.

REFERENCES / BIBLIOGRAPHY

1. Key Substance Use and Mental Health Indicators in the United States: Results from the 2020 National Survey on Drug Use and Health. 156 (2020).
2. Bouchery, E. E., Harwood, H. J., Sacks, J. J., Simon, C. J. & Brewer, R. D. Economic Costs of Excessive Alcohol Consumption in the U.S., 2006. *Am. J. Prev. Med.* **41**, 516–524 (2011).
3. Substance Abuse and Mental Health Services Administration & National Institute on Alcohol Abuse and Alcoholism. *Medication for the Treatment of Alcohol Use Disorder: A Brief Guide*. vol. 15 (Substance Abuse and Mental Health Services Administration, 2015).
4. Koob, G. F. & Volkow, N. D. Neurobiology of addiction: a neurocircuitry analysis. *Lancet Psychiatry* **3**, 760–773 (2016).
5. Dautan, D. *et al.* Segregated cholinergic transmission modulates dopamine neurons integrated in distinct functional circuits. *Nat. Neurosci.* **19**, 1025–1033 (2016).
6. Maskos, U. The cholinergic mesopontine tegmentum is a relatively neglected nicotinic master modulator of the dopaminergic system: relevance to drugs of abuse and pathology: Nicotinic master modulation. *Br. J. Pharmacol.* **153**, S438–S445 (2008).
7. Chen, L. & Lodge, D. J. The lateral mesopontine tegmentum regulates both tonic and phasic activity of VTA dopamine neurons. *J. Neurophysiol.* **110**, 2287–2294 (2013).
8. Jean-Pierre Changeux & Edelstein, S., J. *Nicotinic Acetylcholine Receptors: From Molecular Biology to Cognition*. (Odile Jacob, 2005).
9. Corrigall, W. A., Coen, K. M., Adamson, K. L. & Chow, B. L. C. Manipulations of mu-opioid and nicotinic cholinergic receptors in the pontine tegmental region alter cocaine self-administration in rats. *Psychopharmacology (Berl.)* **145**, 412–417 (1999).
10. Schmidt, H. D., Famous, K. R. & Pierce, R. C. The limbic circuitry underlying cocaine seeking encompasses the PPTg/LDT. 26 (2010).
11. José Lança, A., Sanelli, T. R. & Corrigall, W. A. Nicotine-induced Fos expression in the pedunculopontine mesencephalic tegmentum in the rat. *Neuropharmacology* **39**, 2808–2817 (2000).
12. Alderson, H., Latimer, M. & Winn, P. Intravenous self-administration of nicotine is altered by lesions of the posterior, but not anterior, pedunculopontine tegmental nucleus. *Eur. J. Neurosci.* **23**, 2169–2175 (2006).
13. Ting-A-Kee, R., Dockstader, C., Heinmiller, A., Grieder, T. & Van Der Kooy, D. GABAA receptors mediate the opposing roles of dopamine and the tegmental pedunculopontine nucleus in the motivational effects of ethanol. *Eur. J. Neurosci.* **29**, 1235–1244 (2009).
14. Katner, S. Alcohol Intake of P Rats Is Regulated by Muscarinic Receptors in the Pedunculopontine Nucleus and VTA. *Pharmacol. Biochem. Behav.* **58**, 497–504 (1997).
15. Brager, A., Prosser, R. A. & Glass, J. D. Acamprosate-responsive brain sites for suppression of ethanol intake and preference. *Am. J. Physiol.-Regul. Integr. Comp. Physiol.* **301**, R1032–R1043 (2011).
16. Samson, H. H. & Chappell, A. Injected muscimol in pedunculopontine tegmental nucleus alters ethanol self-administration. *Alcohol* **23**, 41–48 (2001).
17. Wang, H.-L. & Morales, M. Pedunculopontine and laterodorsal tegmental nuclei contain distinct populations of cholinergic, glutamatergic and GABAergic neurons in

the rat. *Eur. J. Neurosci.* **29**, 340–358 (2009).
18. Martinez-Gonzalez, C., Bolam, J. P. & Mena-Segovia, J. Topographical Organization of the Pedunculopontine Nucleus. *Front. Neuroanat.* **5**, (2011).
19. Mena-Segovia, J. Structural and functional considerations of the cholinergic brainstem. *J. Neural Transm.* **123**, 731–736 (2016).
20. Mena-Segovia, J. & Bolam, J. P. Rethinking the Pedunculopontine Nucleus: From Cellular Organization to Function. *Neuron* **94**, 7–18 (2017).
21. Larsson, A., Edström, L., Svensson, L., Söderpalm, B. & Engel, J. A. VOLUNTARY ETHANOL INTAKE INCREASES EXTRACELLULAR ACETYLCHOLINE LEVELS IN THE VENTRAL TEGMENTAL AREA IN THE RAT. *Alcohol Alcohol* **40**, 349–358 (2005).
22. Ericson, M., Blomqvist, O., Engel, J. A. & Söderpalm, B. Voluntary ethanol intake in the rat and the associated accumbal dopamine overflow are blocked by ventral tegmental mecamylamine. *Eur. J. Pharmacol.* **358**, 189–196 (1998).
23. Blomqvist, O., Engel, J. A., Nissbrandt, H. & Söderpalm, B. The mesolimbic dopamine-activating properties of ethanol are antagonized by mecamylamine. *Eur. J. Pharmacol.* **249**, 207–213 (1993).
24. Abburi, C. & McDaid, J. Ethanol interaction with α3β4 nicotinic acetylcholine receptors in neurons of the laterodorsal tegmentum. *Alcohol. Clin. Exp. Res.* **45**, 2495–2505 (2021).
25. Touchette, J. C., Maertens, J. J., Mason, M. M., O'Rourke, K. Y. & Lee, A. M. The nicotinic receptor drug sazetidine-A reduces alcohol consumption in mice without affecting concurrent nicotine consumption. *Neuropharmacology* **133**, 63–74 (2018).
26. O'Rourke, K. Y., Touchette, J. C., Hartell, E. C., Bade, E. J. & Lee, A. M. Voluntary co-consumption of alcohol and nicotine: Effects of abstinence, intermittency, and withdrawal in mice. *Neuropharmacology* **109**, 236–246 (2016).
27. Wang, F. *et al.* RNAscope: A Novel In Situ RNA Analysis Platform for Formalin-Fixed Paraffin-Embedded Tissues. *Mol Diagn.* **14**, 22–29 (2012).
28. Touchette, J. C., Moen, J. K., Robinson, J. M. & Lee, A. M. Enhancement of alcohol aversion by the nicotinic acetylcholine receptor drug sazetidine-A. *Addict. Biol.* **26**, (2021).
29. Bechara, A. & van der Kooy, D. The tegmental pedunculopontine nucleus: a brain-stem output of the limbic system critical for the conditioned place preferences produced by morphine and amphetamine. *J. Neurosci.* **9**, 3400–3409 (1989).
30. Lodge, D. J. & Grace, A. A. The laterodorsal tegmentum is essential for burst firing of ventral tegmental area dopamine neurons. *Proc. Natl. Acad. Sci.* **103**, 5167–5172 (2006).
31. Lester, D. B., Rogers, T. D. & Blaha, C. D. Acetylcholine-Dopamine Interactions in the Pathophysiology and Treatment of CNS Disorders: Dopamine-Acetylcholine Interactions in CNS Disorders. *CNS Neurosci. Ther.* **16**, 137–162 (2010).
32. Dawson, A., Miles, M. F. & Damaj, M. I. The β2 nicotinic acetylcholine receptor subunit differentially influences ethanol behavioral effects in the mouse. **25** (2014).
33. Zoli, M., Pucci, S., Vilella, A. & Gotti, C. Neuronal and Extraneuronal Nicotinic Acetylcholine Receptors. *Curr. Neuropharmacol.* **16**, 338–349 (2018).
34. Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O, Guertin DA, Chang JH, Lindquist RA, Moffat J, Golland P, Sabatini DM (2006) CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biol **7**:R100
35. Kamentsky L, Jones TR, Fraser A, Bray MA, Logan DJ, Madden KL, Ljosa V,

Rueden C, Eliceiri KW, Carpenter AE (2011) Improved structure, function and compatibility for CellProfiler: modular high-throughput image analysis software. Bioinformatics **27**:1179–1180

36. McQuin C, Goodman A, Chernyshev V, Kamentsky L, Cimini BA, Karhohs KW, Doan M, Ding L, Rafelski SM, Thirstrup D, Wiegraebe W, Singh S, Becker T, Caicedo JC, Carpenter AE (2018) CellProfiler 3.0: Next-generation image processing for biology. PLoS Biol **16**:e2005970
37. Stirling DR, Swain-Bowden MJ, Lucas AM, Carpenter AE, Cimini BA, Goodman A (2021) CellProfiler 4: improvements in speed, utility and usability. BMC Bioinformatics **22**:433
38. Allen Institute for Brain Science (2004). Allen Mouse Brain Atlas [dataset]. Available from mouse.brain-map.org.Allen Institute for Brain Science (2011).
39. Allen Reference Atlas – Mouse Brain [brain atlas]. Available from atlas.brain-map.org.
40. Daigle, T. L., et al. (2018). A Suite of Transgenic Driver and Reporter Mouse Lines with Enhanced Brain-Cell-Type Targeting and Functionality. Cell, **174**(2), 465–480.e22. https://doi.org/10.1016/j.cell.2018.06.035
41. Lein, E.S. et al. (2007). Genome-wide atlas of gene expression in the adult mouse brain, Nature **445**: 168-176. https://doi:10.1038/nature05453
42. Harris, J. A. et al. (2019). Hierarchical organization of cortical and thalamic connectivity. Nature **575**, 195-202. https://doi:10.1038/s41586-019-1716-z
43. Oh, S.W. et al. (2014). A mesoscale connectome of the mouse brain, Nature **508**: 207-214. https://doi:10.1038/nature13186