The Thesis Committee for Tira Anne Meyers

Certifies that this is the approved version of the following thesis:

Ethanol Priming and Its Effects on Consumption and Accumbal Plasticity

APPROVED BY

SUPERVISING COMMITTEE

Richard Morrisett, Supervisor

Christine Duvauchelle

Ethanol Priming and Its Effects on Consumption and Accumbal Plasticity

By

Tira Anne Meyers BSCh, PharmD

Thesis

Presented to the Faculty of the Graduate School

of the University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Master of Science in Pharmaceutical Sciences

The University of Texas at Austin

December 2015

In dedication to my parents,

Without whom none of my success would be possible.

Ethanol Priming and Its Effects on Consumption and Accumbal Plasticity

By

Tira Anne Meyers, MSPS

The University of Texas at Austin, 2015

SUPERVISOR: Richard Morrisett

Alcohol abuse and dependence are major concerns in the United States because of their chronic, detrimental health effects and societal costs. The mesocorticolimbic pathway and the nucleus accumbens (NAc), in particular, play critical roles in the formation of drug dependence and expression of drug related behaviors. The NAc contains two subregions, the core and shell, which encode different aspects of drug responding and have distinct dopamine responses. The accumbens can be further divided into two subpopulations of D1-dopamine receptor (D1) or D2-dopamine receptor (D2) expressing medium spiny neurons (MSNs) that precipitate different intracellular cascades and actions upon the reward circuitry. Alterations in the expression N-methyl-D-aspartate receptor (NMDAR)-dependent long-term depression (LTD) in the D1 MSNs of the NAc shell are seen following chronic intermittent ethanol (CIE) exposure and sustained operant responding. To our knowledge, however, LTD induction has not been extensively studied in volitional self-administration. Prior to operant training, pre-exposure of ethanol, or ethanol priming, is necessary to develop the association of the positive effects of ethanol, but it has not been shown if similar changes in LTD induction occur during this brief ethanol exposure. The purpose of this thesis is to review the current research studying alcohol and its effects on accumbal plasticity, a preview of my work depicting an ethanol-only operant self-administration protocol, and the future directions of alcohol research.

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Chapter One

Ethanol priming and its effect on accumbal plasticity

Alcohol abuse and dependence

Alcohol use disorders (AUDs) are conditions characterized by a problematic pattern of ethanol consumption that can lead to alcohol abuse and alcoholism. Typically, AUDs initially begin with moderate alcohol drinking that progressively increases with the development of tolerance and can lead into an uncontrollable, compulsive drinking behavior. It is hypothesized that the development of addiction is modeled by an allostatic process where the reward set point inevitably deviates due to excessive alcohol intake, thus requiring increasing amounts of ethanol to be consumed for the same effect (1,2). This then can develop into a repeated cycle of intoxication, withdrawal, and relapse (3–5), which can persist despite adverse health, economic, and societal consequences (6–10). To this end, alcohol abuse and alcoholism are major and prevalent concerns within the United States. More than 85,000 deaths annually are attributed to alcohol-related causes (11,12), and costs the U.S. more than $200 billion annually due to medical expenditures and indirect costs such as loss of productivity (13–15).

Neurobiological and environmental factors influence ethanol-drinking behaviors (16), but specific individual risk factors are still undetermined. The focus of pre-clinical research then has been on the brain's reward circuit, the

mesocorticolimbic pathway, and developing models to understand ethanol self-administration and dependence.

Mesocorticolimbic pathway and ethanol

The mesocorticolimbic pathway plays a significant role in the development of reinforcement processing, motivation, and goal-directed behavior (17,18). This reward circuit, as depicted in **Figure 1.1**, originates from the ventral tegmental area (VTA) where dopamine (DA) neurons project to various forebrain regions that include the hippocampus, amygdala, prefrontal cortex (PFC), and nucleus accumbens (NAc) (19–21). Additionally, the NAc also receives glutamatergic projections from the PFC and other mesolimbic structures (21). The principal γ-aminobutyric acid (GABA)-ergic neurons of the NAc, the medium spiny neurons (MSNs), makes up approximately 90% of the neurons found in this brain region (21–23), and provide inhibitory projections back to the VTA and ventral pallidum (19,21,24,25).

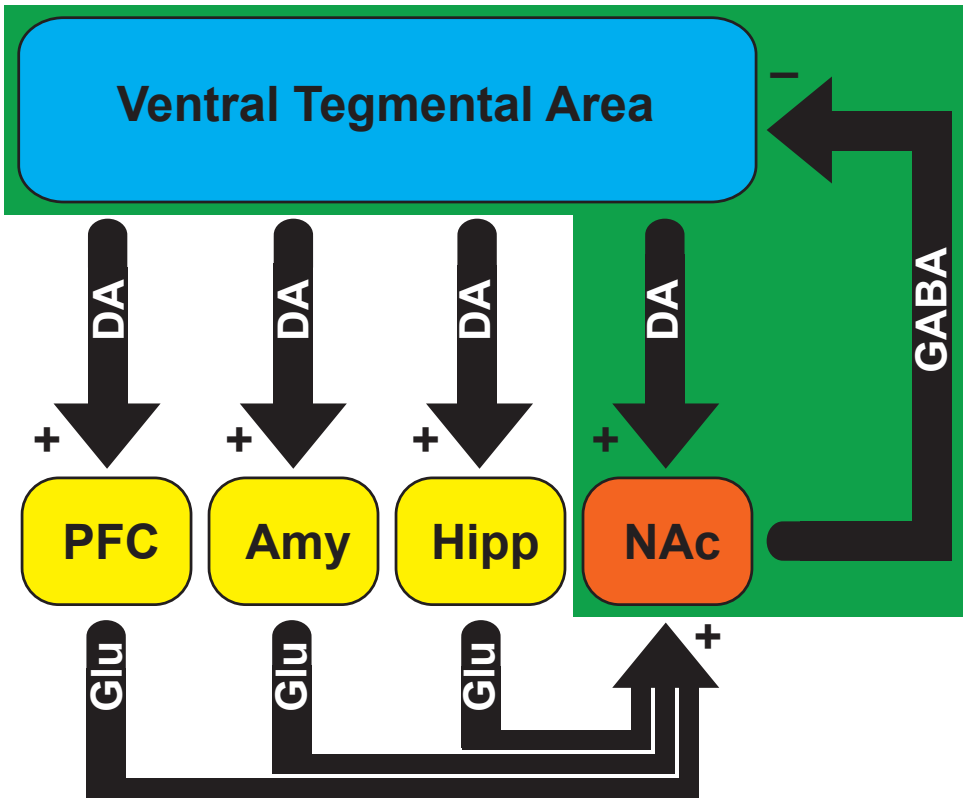

Figure 1.1. Schematic of mesocorticolimbic pathway. From the ventral tegmental area (VTA), dopamine (DA) neurons project to pre-frontal cortex (PFC), amygdala (Amy), hippocampus (Hipp), and nucleus accumbens (NAc). Similarly, glutamate (Glu) neurons provide excitatory projections also to the NAc. The γ-aminobutyric acid (GABA) neurons of the NAc provide inhibitory projections back on to VTA, thus completing the circuit. The focus of this thesis is on the VTA-NAc circuit as contained in the green box.

Under normal circumstances, the mesocorticolimbic pathway governs an individual's response to a stimulus, such as a natural reward (i.e. food, water, or sex), which subsequently leads to a conditioned-approach behavior. However, during exposure to ethanol or other drugs of abuse, this reward circuit is taken over to influence the learning and development of addictive behaviors that then lead to compulsive drug use and negative consequences (26–31). Early work has suggested that psychostimulant sensitization requires the VTA and

modifications within the NAc (32). For this reason, the focus of this thesis will be on the VTA-NAc circuit.

It is hypothesized that adaptations within the mesocorticolimbic pathway underlies the development and expression of dependence with drugs of abuse and ethanol (19,33,34). Substantial evidence has shown that addictive drugs increase DA concentrations within the VTA and its forebrain projections (18,35–43). Work has shown within the VTA that ethanol exposure increased dopaminergic neuron firing (44,45), and withdrawal following chronic ethanol exposure reduced this excitability (46). The VTA is believed to have a greater impact on learned behavior versus ethanol consumption (18,21), while its projections to the NAc and increased DA concentrations may lead to neurological modifications and increased ethanol intake (32,47,48).

Ample work has implicated the NAc in the neural organization involved in reward processing and the development of addiction (25,49–54). It is postulated that the increase of DA levels within the NAc could be due to increased release of DA at VTA DA neuron terminals (55), or indirectly through increased inhibition upon VTA GABA interneurons thereby disinhibiting VTA DA neuron action (56,57). Microdialysis studies have shown that cocaine (58), amphetamine (59), nicotine (60), and ethanol (61), stimulated DA transmission to the NAc preferentially over the dorsal caudate putamen. Similarly, systemic ethanol exposure caused a dose-dependent release of DA into the NAc (36,37,43,48,61–64), while withdrawal from ethanol decreased DA release to the accumbens

(45,62). Furthermore, the expectation of receiving an ethanol reinforcement via self-administration stimulated the release of DA in the NAc (62), but this same action is not seen following intra-peritoneal (IP) injections of alcohol (65). This increase in DA concentration in the NAc following ethanol exposure also has an effect on consumption behavior. Intra-accumbal administration of a DA agonist resulted in an increase of ethanol consumption and preference, whereas DA antagonists attenuated this action (66–69). Six-hydroxydopamine (6-OHDA) lesions of the accumbens disrupted the acquisition of ethanol consumption, but not the maintenance of drinking once the associated behavior was acquired (66,70). From these studies, it is evident that the NAc plays a significant role in the development of addiction, but because of its complexity, a further understanding of the accumbal subregions and its neuronal activity is essential to discerning neurobiological changes occurring with ethanol exposure.

The nucleus accumbens and ethanol

The NAc is located anterior to the posterior border of the anterior commissure and is subdivided into two subregions, the core and shell, which encode different aspects of drug responding, have distinct DA responses, and have different projection targets (71–78). The NAc shell is presumed to be related with initial, unconditioned drug reward which may have a key role in the induction of drug seeking behavior, whereas the core is involved in the processing of motivation and may be more significant in the acquisition of cue

conditioned behavior (52,78–83). Psychostimulant self-administration showed that animals preferentially infused drugs into the shell subregion versus the core (84,85), and this action has been replicated in alcohol studies (86). Lesions in the core prevented acquisition of first and second order schedules of reinforcement of heroin, but established responding was not abolished (87). Additionally, work showed blockade of the core attenuated conditioned responding, but inactivation of the shell reduced the renewal of responding suggested to mimic relapse (83). Taken together, this evidence indicates that the relationship of the two subregions of the NAc both effects the development of drug seeking and consumption and that their relationship is multifaceted and still not completely understood.

The complexity of the NAc can be taken one step further with the homogenous distribution of two populations of MSNs: D1-dopamine receptor (D1) or D2-dopamine receptor (D2) expressing neurons. These MSNs not only differ on DA receptor expression, but also upon their expression of releasable peptides and projection targets (88–91). D1-like receptors, which consist of D1 and D5 receptors, are related to the control of movement and functions (92), co-localized with the signaling of peptide dynorphin and substance P, are coupled to Gs/olf protein signaling which stimulates adenylyl cyclase activity and results in cyclic adenosine monophosphate (cAMP), and stimulates phospholipase kinase c (PKC) (93–95). D2-like receptors, which includes D2, D3, and D4 receptors, show high affinity for phenothiazines and thioxanthines (96), are co-localized with

encephalin, and are coupled to Gi/o protein signaling which leads to the inhibition of adenylyl cyclase (97–100). Application of D1-antagonists such as SCH23390 resulted in a reduction operant ethanol responding (66,101–106), and animals lacking D1 receptors have reduced ethanol self-administration (107–109). Conversely, D2 agonist exposure lead to a deficit in reward learning (110), and reduced drug sensitization (111), but D2 antagonist application showed a reduction in drinking (112,113). In summary, these findings suggest that the contrasting activity D1 and D2 MSNs within the NAc both influence the development and expression of ethanol dependence, but it has not been elucidated how the loss of neuronal control of one subtype may affect the other. Furthermore, it is then essential to study behavioral adaptations that occur due to ethanol exposure.

Modeling ethanol drinking and the significance of ethanol priming

It is well-known that both genetic and environmental factors influence ethanol self-administration (114,115), thus the development of alcohol drinking models to mimic alcohol abuse and dependence is crucial and is done by a variety of techniques. Much like their human counterpart, the development of specific strains of rodents that preferably consume ethanol has allowed for researchers to analyze alcoholic tendencies (116–119), however, this does not provide true face validity to the human population since it focuses on a small subset of the society.

Animal drinking models employ two different methods to establish ethanol consumptions as either passive or volitional administration. Commonly, passive administration models ethanol dependence by maintaining high blood ethanol concentrations (BECs) in animals for varying lengths of time followed by periods of abstinence to replicate withdrawal. Examples of passive administration include ethanol vapor exposure via inhalation chambers (120–126), intravenous (IV) injections (127), intra-gastric (IG) infusion (128–132), or IP injections (133,134). Using ethanol vapor or injections provides construct validity in terms of developing ethanol dependence and models an aspect of withdrawal and relapse (53,135). Specifically, inhaled ethanol vapor increased ethanol consumption (136), but did not increase water or other palatable fluid intake (137,138). IV/IG/IP injections have been shown to increase DA levels (134,139–141), and helped to avoid the aversive taste and smell of alcohol (142). Though passive administration allows for the study of ethanol dependence, these techniques lack face validity since humans generally consume ethanol orally.

In order to control for face validity and better mimic human alcohol consumption, the development of volitional administration is used. Ethanol self-administration is either achieved by allowing animals to have free access to an ethanol solution or by conditional training for alcohol access via operant self-administration. Free-choice drinking can model consummatory aspects of ethanol intake and is often done as two-bottle choice (TBC). Briefly, animals are provided bottles of either an ethanol solution or water within their home cage for

a specified amount of time. This model can either be intermittent access where animals are given 24 hours on/off access to the bottles or they can be given limited access for two to four hours on consecutive days at the same start/stop times. Studies have shown that significant levels of ethanol consumption and preference can be produced with either intermittent (143–147), or limited access (148–151), and that TBC can be correlated with measureable BECs (120,122,124), however, the model is limited because it does not allow in-depth ethanol-seeking behavior analysis.

Operant self-administration allows for modeling of both consummatory and appetitive behavior, and for the assessment of motivation by the amount of work (i.e. lever presses or nose pokes) an animal is willing to perform for alcohol reinforcement. Ethanol self-administration using standard operant conditioning procedures is firmly established (152) and enables a more refined analysis of ethanol consumption patterns and drug seeking (132). Furthermore, operant self-administration allows for the study of drug dosing and schedule of reinforcement to analyze a substance's reinforcing efficacy. Evidence has shown that ethanol reinforcement via operant self-administration involved the activation of the VTA-accumbal system and released DA into the NAc (43,48,64,153,154). Application of DA agonists in the NAc increased ethanol intake, whereas DA antagonists attenuated consumption (101,113,155–158). It can be then concluded that volitional of administration allows for another layer of

understanding of ethanol addiction, but it still has limitations in the extent an animal will consume.

While using either model of passive or volitional administration to model ethanol drinking behavior, it is first essential to have ethanol pre-exposure to acquire the association of positive effects of alcohol (2,47,159–163). This pre-exposure, or ethanol priming, must occur early on in ethanol exposure (164), and be completed first prior to the development of dependence (2,162). If these positive effects are not established, the animals may then reject consuming alcohol (120,138). Common approaches used for ethanol priming include food and water deprivation (144,165), ethanol-containing liquid diets (121,166–168), sucrose fading (152,169–172), or a TBC paradigm (2,146,147). Initially, ethanol consumption begins at low levels, but escalates following TBC (146,173), and increases conditioned place preference (CPP) (140,174). As seen with chronic ethanol administration, increased DA patterns during ethanol priming can occur within the first week of alcohol exposure (175,176), illustrating that this time period may be essential in addiction. However, even with the benefit of increasing alcohol consumption and developing drinking behavior, ethanol priming can have limitations. Excessive food or water deprivation or ethanol-containing diets could force animals to consume ethanol for its caloric value (177,178), while causing unnecessary stress that may influence drug self-administration (179,180). Work has shown that ethanol is an effective reinforcer without the need of food or water restrictions (181), so this technique can be

avoided. Though the sucrose fading technique provides face validity within the aspect that humans generally initiate with mixed drinks before progressing to more potent alcohols, this method also provides a potential confound because of sweetener's own rewarding and reinforcing effects (182,183).

Undoubtedly, the development of ethanol drinking models has provided another tool in understanding alcohol abuse and dependence, however, a more in depth study would require also the considering the neuronal activity that may be creating this phenotypic change.

Accumbal plasticity and ethanol

It is well established that both natural rewards and drugs of abuse are intricately tied with synaptic and cellular mechanisms that are shared with learning and memory processes (184), and that neuroadaptions in the mesocorticolimbic pathway contribute to the development and expression of addiction (16,19,28,33,34,185,186). Synaptic plasticity allows for the reorganization of neural circuits to either strengthen or weaken transmissions between neurons with various forms being found in the NAc (187–189). Of these, the best-characterized forms are long-term depression (LTD) and long-term potentiation (LTP) (190). It was first suggested that excitatory glutamatergic transmissions played a significant role in behavioral adaptations following drug exposure (32,191), in particular because of interactions with glutamate receptors,

such as the N-methyl-D-aspartate receptor (NMDAR), within mesocorticolimbic structures contribute to various forms of ethanol neuroadaptions (156,192).

In vivo MSNs have two membrane potential states: a hyperpolarized downstate at approximately -80mV and a more depolarized upstate where action potential discharge occur at about -50mV (193,194). For ethanol or drug naïve MSNs, alterations in glutamate excitation by prolonged low frequency stimulation (LFS) (1 Hz) paired with postsynaptic depolarization (-50mV) that mimics the depolarized upstate resulted in LTD expression of excitatory postsynaptic currents (EPSCs) (188,195), which is believed to model excitatory transmission from the PFC and the amygdala (196). However, the dysregulation of these two states is proposed as a major neuroadaption underlying addiction (197), and has been shown with the exposure to drugs of abuse. Drug sensitization studies using cocaine (188,198,199), amphetamine (200), or heroin (201), have shown a disruption in NMDAR-dependent LTD. Ethanol-induced disruption of NMDAR-dependent LTD has also been observed in mice that experienced alcohol sensitization (202–207).

Although there is work that showed the application of NMDA antagonists attenuated ethanol responding (208,209), and reduced consumption (141,210,211), it is unclear how alcohol is facilitating this activity on the glutamatergic receptor. Evidence has suggested that NR1/NR2B-containing NMDARs may be inhibited by ethanol (212), while others believed the pairs of residues in the third and fourth membrane spanning domains of NR1/NR2A

subunit interfaces are significant in controlling ethanol sensitivity (213). Additionally, others have considered the NR2C or NR2D subunits to be more preferentially effected by alcohol than NR2A or NR2B (214). Work has shown NR1B-containing NMDAR action is reduced in the presence of a D1 receptor activation (215,216), which is mediated by the phosphorylation of DA and cAMP-regulated phosphoprotein-32 kD (DARPP-32), an intracellular signaling molecule used by DA (109,215). Furthermore, a single bout of CIE exposure has been shown to induce LTP due to NR2B-containing NMDAR, where the response is dose-dependent and the activation of D1 receptors occludes ethanol inhibited LTD (125). Taken together, it is evident that NMDAR-dependent LTD is affected by the presence of ethanol and D1-expressing MSNs are critical in this neuroadaption.

Further studies into the mechanism of NMDAR-dependent LTD have found alterations in plasticity mediated by the α-amino-3-hydroxy-5-methylsoxazole-4-proprionate (AMPA) receptor (217,218). Activation of the NMDA receptor increases calcium concentrations in the post-synaptic terminal. This increase allows for the activation of calcineurin and protein phosphatase (PPI). The dephosphorylation AMPA receptors at the C-terminal tail, which contains the consensus sequence, leads for their removal via a clathrin- and dynamin-dependent endocytotic process (219–224). Previous study (225), showed NAc induced LTD was blocked by active dynamin-derived peptide (GluR23Y), while the inactive peptide (GluR23A) maintained LTD. Additionally,

in vivo exposure to drugs of abuse modulated AMPA receptor signaling in the NAc (188,195,220,226–229), and the suppression of AMPA:NMDA ratio (188,225). Similarly, alcohol studies have shown the occlusion of AMPA-mediated LTD by acute ethanol application, and chronic intermittent ethanol (CIE) exposure via alcohol vapor and the same stimulation protocol disrupts LTD within the NAc shell (125,126). Further evidence has shown that ethanol-induced disruption of NMDAR-dependent LTD is dependent on GluA2-containing AMPA receptors following extended withdrawal (126,230,231), indicating that exposure to drugs of abuse have long-lasting effects on neuronal plasticity.

These electrophysiological studies have modeled ethanol consumption to investigate possible neuroadaptions from repeated exposures via passive administration (74,120,232). However, little has been done to test if oral ethanol consumption via operant self-administration would produce similar effects on accumbal plasticity. In my preliminary work, I created a protocol (as described in detail Chapter Two), which trained animals to press for an ethanol-only solution via operant chambers. With the assistance of Dr. Mangieri of the lab, EPSC recordings from D1 MSNs in both the core and shell subregions were completed following 24 hours after the final operant session. Our work, as depicted in **Figure 1.2**, showed that NMDAR-dependent LTD is reduced in the shell, but not core subregion and that the reduction of magnitude of LTD is inversely related to the amount of ethanol consumed during the final session (data not shown).

These results show that even moderate amounts of ethanol consumed can affect neuroadaptions within the NAc once the conditioned behavior is sustained.

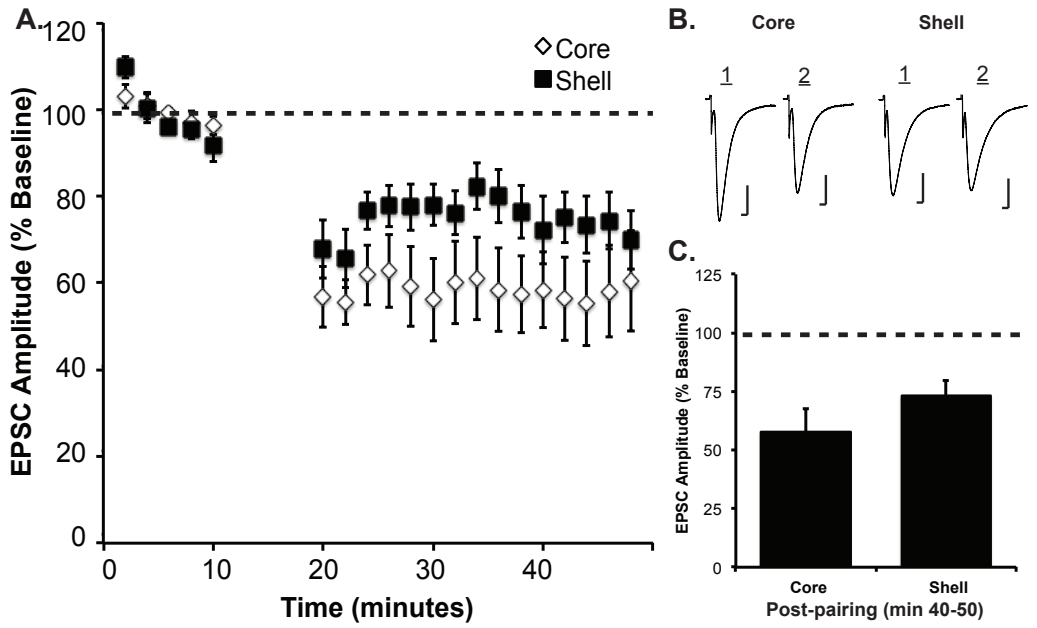

Figure 1.2. NAc shell, but not core, has a reduction in LTD following operant self-administration of ethanol. A) Pairing LFS stimulation with post synaptic depolarization resulted in LTD of AMPA-mediated EPSCs of the core (open diamond, 62.0 ± 11.9% of baseline, n = 8, $p < 0.05$) and a reduction of LTD is seen within the shell (closed squares, 75.1 ± 4.8% of baseline, n = 6, $p < 0.05$). B) Sample traces of averaged baseline and post-pairing EPSCs. Scaled bars represent 5 ms (horizontal) and 50pA (vertical). C) Bar graph representing the percentage of baseline ± standard error of the mean for averaged EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50). Two of 14 recordings (1 core and 1 shell), were completed by Dr. Mangieri. Post-pairing was significantly different between core and shell ($p < 0.01$).

While the focus of work has been on excitatory projections to the NAc, it is necessary to also consider the inhibitory action of MSNs on the VTA. Both glutamatergic and GABAergic play a role in ethanol's acute and long-term effects (233–236). More than 30% of the neurons found within the VTA are GABAergic

and include both interneurons and those that project to other brain regions (237). With repeated cocaine exposure, D1 MSNs exerted preferential projections to the GABA inter-neurons of the VTA and occluded inhibitory long-term potentiation (iLTP) (57). When studying the effects of alcohol, evidence has shown that ethanol dose-dependently reduced GABAergic spontaneous inhibitory postsynaptic currents (sIPSCs) and also had an inverse correlation with VTA DA neuron firing (238). Work also has shown that ethanol inhibited the VTA GABAergic inter-neurons (239–241), and this action is attenuated with the application of a $GABA_A$ receptor antagonist (241,242). It is likely that the increased VTA DA neuronal activity is attributed to the disinhibition produced by the inhibition of GABA inter-neurons of the VTA (243). Given these points, both the actions upon the NAc and by MSNs are significant in regulating the development of ethanol addiction.

Conclusions

From these findings, it is evident that the interactions within the VTA-NAc circuit play a crucial role in the development of addiction. The effects of ethanol on learning-related plasticity involves multiple neurotransmitters and a host of receptors cellular signaling (244). However, the underlying mechanism of ethanol priming is still unknown. At this moment, the next essential step is to model ethanol self-administration while controlling for potential confounds.

Chapter Two

Sucrose-free ethanol operant self-administration in C57BL/6J mice

Tira A. Meyers, PharmD, Esther Maier, PhD, Richard Morrisett, PhD

College of Pharmacy, The University of Texas, Austin, Tx 78712-1074

Division of Pharmacology and Toxicology (TAM, EM, RAM),

Waggoner Center for Alcohol and Addiction Research (RAM),

Institute for Molecular and Cellular Biology (RAM), and

Institute for Neuroscience (RAM)

Correspondence:

Richard Morrisett PhD

The University of Texas at Austin

PHAR-Pharmacology

1 University Avenue, A1915

Austin, Tx 78712-0125

Phone #: (512) 471-1911

Fax #: (512) 475-6068

Email: ramorris@austin.utexas.edu

Abstract

Rodent models of operant self-administration have frequently used a sucrose-fading technique to enhance lever responding acquisition and to increase ethanol consumption. The use of a sweetener, however, constitutes a potential confound likely stemming from its own reinforcing effects. In the current study, we sought to determine if C57BL/6J (B6) mice could be conditioned to self-administer an ethanol-only solution in a time frame similar to traditional sucrose-fading procedures. Three different treatments were tested on male B6 mice that employed 1) a sucrose-fading technique, 2) a pre-exposure to ethanol using a limited access two-bottle choice (TBC) paradigm before operant training, and 3) prolonged habituation sessions in the chamber with limited ethanol access prior to operant training. The cumulative number of sessions to acquire fixed ratio schedule (FR) 4 of reinforcement were compared in addition to behavioral data during sustained FR4 sessions following acquisition to an ethanol-only solution. Finally, mice were tested for ethanol preference and blood ethanol concentrations (BECs) measured following a truncated FR4 session. Results concluded that animals given only ethanol during operant training required significantly fewer sessions to achieve FR4 acquisition. Mice receiving prior TBC had similar ethanol intake as sucrose-faded mice. Following operant training, no difference in ethanol preference was seen between treatment groups and BECs were positively correlated with ethanol consumption via operant conditioning. Taken together, these results demonstrate that B6 mice can be easily trained to

self-administer sustaining levels of ethanol alone while controlling for potential confounds.

Introduction

Operant self-administration is a proven, reliable tool for studying drug-seeking behaviors and is extremely useful in understanding both, appetitive and consummatory components of addiction (152,245). Ethanol studies have reliably shown that alcohol can serve as an effective reinforcer, however, training rodents to lever respond for an alcohol solution has proven to be difficult, most likely due to its aversive taste (142,246), and delayed pharmacological effects (163). A variety of different approaches have been used to overcome these adversities in operant responding for ethanol in rodents which includes food and water deprivation (144,147,162,165,247), having extended operant training periods (248), intermittent intra-peritoneal ethanol injections (133), chronic intermittent ethanol vapor exposure (120), a two bottle choice (TBC) paradigm (146,147,249), or by the most widely used manipulation, a sucrose-fading procedures (152,169,250–252). Briefly, animals are initially trained to press for a sucrose solution during operant sessions and once a desired level of lever acquisition is attained, ethanol is gradually introduced and the sucrose content is subsequently reduced or faded out. This manipulation has been shown to improve ethanol preference and to facilitate learning in rats and mice (250,251,253), however, using a sweetener to achieve ethanol self-

administration introduces a potential confound. Because sucrose possesses caloric value (178,254), and innate rewarding properties to support operant self-administration alone (182,183), the influence of ethanol on seeking and consummatory behaviors may be masked when both substances are co-administered.

In comparison to other mouse strains such as BALB/CJ, DBA/2J, and C58/J, the C57Bl/6J (B6) strain has commonly been used for ethanol studies. B6 mice readily consume ethanol at pharmacologically significant levels (250,255,256), and can be trained to self-administer ethanol using a TBC paradigm (117,120,150,246,257), and by operant models (114,256,258,259). Such operant self-administration results, however, have to date only been achieved using either sucrose-fading techniques (114,133,165,181), or with extensive food and water deprivation (144,147,162,165,247).

Therefore, the purpose of this study was to determine if B6 mice can be trained to respond for ethanol without the initial use of sucrose and extensive water or food deprivation. Three treatments were compared: 1) a modified sucrose-fading technique (181), 2) pre-exposure to ethanol using a limited access TBC paradigm (120), with habituation sessions prior to operant conditioning, and 3) habituation sessions alone prior to operant training. Here we present data showing that B6 mice trained only to lever respond for ethanol not only required significantly fewer sessions to achieve the fixed ratio (FR) 4 schedule of requirement, but also animals with TBC pre-exposure prior to

operant training consumed a similar amount of ethanol as the sucrose-faded animals.

Materials and Methods

Animals

Forty-three male, C57BL/6J mice, were obtained from Jackson Laboratory (Bar Harbor, ME) at three to four weeks of age. Animals were individually housed in a temperature-controlled environment under a reversed 12-hour light/dark cycle (lights off at 1200h) for at least one week prior to experiments. All animals were given *ad libitum* access to food and water unless otherwise stated. Operant sessions were conducted five days a week (Monday-Friday, weekends off) between 1100h and 1500h. Age-matched mice (five to six weeks) were randomly assigned into the three treatment groups with water controls as diagramed in **Figure 2.1**: sucrose fade (SF), pre-exposure TBC (Pre-TBC), and prolonged habituation (Pro-Hab) treatments. The University of Texas at Austin Institutional Animal Care and Use Committee (IACUC) approved all procedures used.

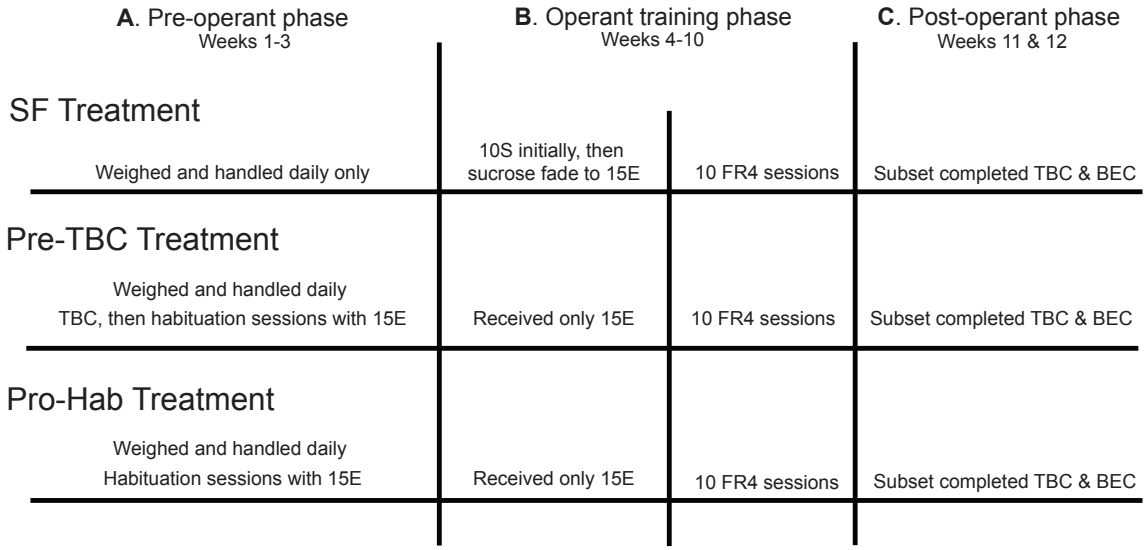

Figure 2.1. Schematic of experimental design. Animals were divided into one of three treatment groups with water controls. A) Pre-operant phase: all animals were weighed and handled daily. Pre-exposure two-bottle choice (Pre-TBC) mice received limited access 2-hour TBC within their home cage for 9-10 sessions, and then habituation sessions for five sessions. Prolonged habituation (Pro-Hab) animals received only habituation sessions for 10-15 sessions. Sucrose-fade (SF) mice only received two weeks of weighing and handling. B) Operant training phase: All mice received similar lever response training. SF-trained animals were initially trained on a 10% sucrose (10S) solution, while Pre-TBC and Pro-Hab-trained animals only received 15% ethanol (15E) throughout all operant sessions. Once fixed ratio (FR) 4 acquisition was achieved (~10 sessions), SF mice had ethanol introduced into the 10S solution, and over 6 sessions, the alcohol concentration was incrementally increased to 15E, and then the sucrose concentration was subsequently faded out. All animals continued a minimum of 10 FR4 sessions for data analysis once FR4 acquisition on an ethanol-only solution was achieved. C) Post-operant phase: A subset of animals from each treatment group received TBC for five days to test for ethanol preference. Following preference testing, another subset of animals receiving post-operant TBC completed three to five FR4 operant sessions for blood ethanol concentration (BEC) analysis.

Apparatus

Modular operant chambers (15.9 x 14.0 x 12.7 cm; Med Associates, St. Albans, VT) were housed inside a sound-attenuating cabinet outfitted with an exhaust fan. Each chamber was equipped with stainless steel grid floors, a custom-made, extending/retracting 10 mL graduated sipper, two retractable

response levers positioned on either side of the sipper with stimulus lights above each lever, and a house light placed on the wall opposite to the sipper. A lickometer circuit was connected to each sipper tube and grid floor to monitor lick patterns. All modules were controlled and monitored by MED Associates interfaces and software (Med Associates Inc., St. Albans, VT).

Drinking solutions

Animals received either solutions of sucrose and/or ethanol, or tap water during all experimental manipulations. Solutions were made weekly with 95% ethanol and/or sucrose mixed in tap water. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Lever response training

Prior to operant training, active lever assignment was alternated between all animals within each treatment group to control for side preference and animals were water restricted for 16 hours prior to the first operant training session. The goal of the initial operant session was to train the animals to associate the lever with solution availability. This process was comprised of three stages that proceeded as follows: During Stage One (five minutes) the sipper was extended into the chamber to allow for free access to the drinking solution and no levers were presented. Immediately following, Stage Two (15 minutes) initially retracted the sipper, and after four minutes, both levers were

extended into the chamber. After one minute, a sipper presentation program was initiated. During the presentation program, the assigned active lever would retract and be accompanied by the blinking of the stimulus light above the retracted lever port. The sipper would then be extended into the chamber for 20 seconds of access. After this presentation, the sipper would retract and the active lever was re-extended into the chamber. The first operant training session consisted of five sipper presentations with 100-second intervals of no sipper access in between each presentation. Once the final scheduled sipper presentation was completed, animals received two minutes of lever access where lever presses were recorded and no sipper access could be elicited. Finally, during Stage Three (160 minutes) the animals were given a FR1 schedule of contingency for the active lever, where the inactive lever had no consequence.

Upon completion of the first operant training session, the following day animals received a two-hour, modified training sessions with Stage One consisting of only 15 seconds of free sipper access, and Stage Two containing five sipper presentations, as previously described, during the first three minutes of the session. The remainder of the modified training session was on a FR1 contingency schedule. All subsequent FR1 sessions had no free sipper access and were only a FR1 schedule contingency programs for two hours.

In order to proceed to higher lever response requirements with the maximum being FR4 (i.e., FR2, FR3, FR4, respectively), animals were required

to complete two consecutive sessions where the active lever responses were greater than 50% of total lever presses (active + inactive) for the operant session. The first FR2 session was two hours in length, and all following FR2 sessions, as well as FR3 and FR4 sessions, were one hour in duration. Once acquisition to FR4 on ethanol-only solution was achieved, a minimum of 10 additional sessions were completed and used for data analysis. For more details, refer to the timeline in **Figure 2.2**.

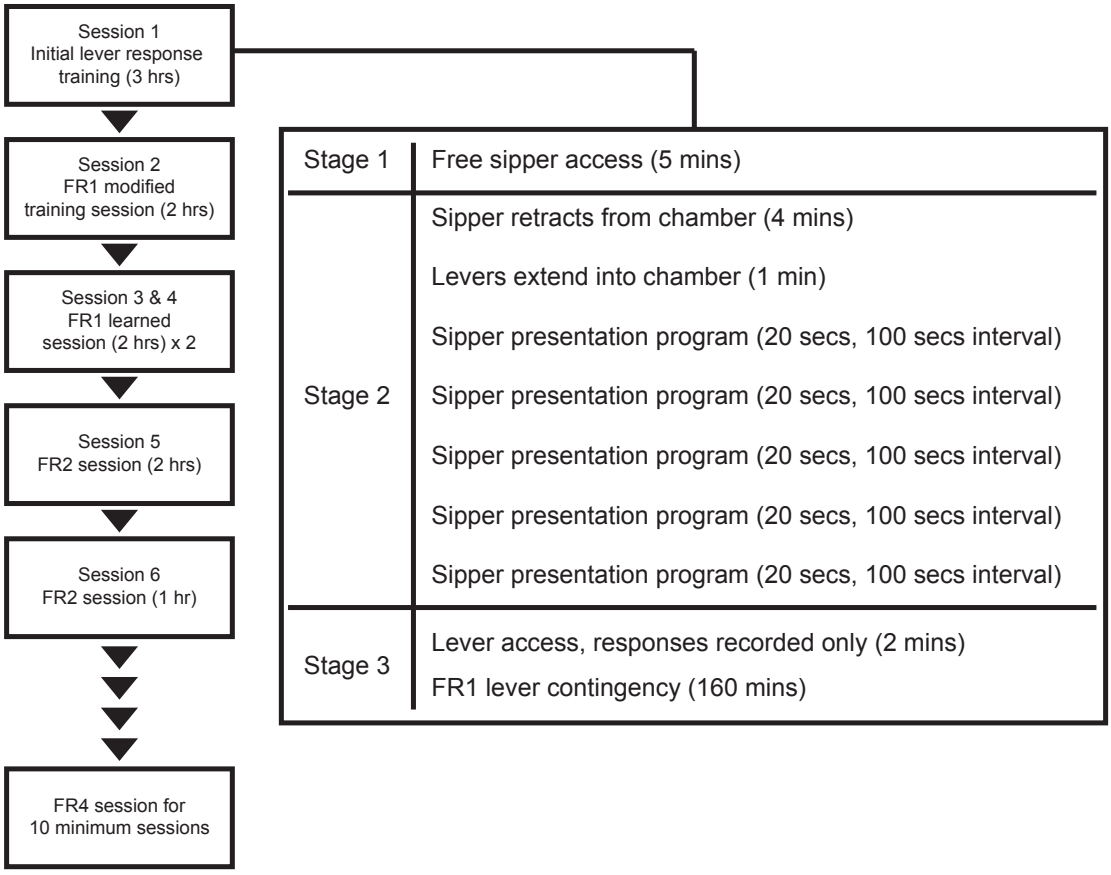

Figure 2.2. Timeline of lever response training. All treatment groups received the initial lever response training. Session 1 (3 hours) is described in more detail within the inset. Following initial training (Session 2), mice received a modified fixed ratio (FR) 1 session (2 hours) with Stages One and Two completed in the first three minutes of the session. Sessions 3 and 4 consisted of a FR1 contingency program with no free access to ethanol and all remaining operant sessions had a lever response contingency program to receive sipper access. To proceed to higher lever response requirements with the maximum being FR4 (i.e., FR2, FR3, FR4, respectively), animals were required to complete two consecutive sessions where the active lever responses were greater than 50% of total lever presses (active + inactive) for the operant session. Once acquisition to FR4 on ethanol-only solution was achieved, a minimum of 10 additional sessions were completed and used for data analysis.

If lever acquisition did not occur after the first training session, up to two additional days of water restriction and up to five additional modified FR1 training

sessions were used. If mice still did not achieve lever acquisition after this time, then the animals were excluded from further experimentation.

Sucrose fade (SF) treatment

SF-trained mice and their water controls were handled and weighed during weekdays (Monday through Friday) for two weeks prior to operant training for age matching to the other treatment groups. Animals initially received 10% w/v sucrose (10S) solution for lever response training. Upon achieving FR4 acquisition and completing two consecutive successful sessions, SF mice drinking solution had ethanol incrementally introduced at 10S/7.5% ethanol v/v solution (10S/7.5E). For every two successful sessions, the concentration of ethanol increased to 15E and then the sucrose concentration was gradually removed until the mice only received ethanol (10S/15E, 5S/15E, and 15E, respectively).

Pre-exposure two-bottle choice (Pre-TBC) treatment

Following assignment, Pre-TBC mice received nine to 10 consecutive days of TBC (15E and tap water) in their home cages between 1130h and 1330h. All custom-built bottles were weighed before and after the drinking sessions and the positions of the bottles were alternated daily. Animals were weighed daily to calculate ethanol intake. Following TBC, mice received five habituation sessions in the operant chamber prior to lever response training. These two hour-long

habituation sessions consisted of free sipper access to 15E with no levers available for the entire session. Following habituation sessions, mice proceeded into the lever response training to achieve acquisition to the FR4 for 15E drinking solution.

Prolonged habituation (Pro-Hab) treatment

Animals in this group initially completed two to three weeks of habituation sessions as previously described prior to the lever response training. Animals in this group received only 15E solution throughout all operant sessions.

Post-operant ethanol preference and blood ethanol concentration analysis

Upon completion of the additional 10 FR4 sessions, a subset of animals from each group received five days (Monday through Friday) of TBC as previously described. Following post-operant TBC and to validate lick data, another subset of mice that completed the post-operant TBC and consumed greater than a mean of 0.1 mL of 15E during the final 10 FR4 operant sessions completed three to five days of FR4 operant sessions, where the final session was shortened to 30 minutes in length to collect trunk blood samples for blood ethanol concentration (BEC) analysis. The start times for each mouse were staggered by five minutes to allow for blood collection with equal amounts of time between sample collections. Of each animal, duplicate samples of 10 µL of trunk blood were collected and added to 90 µL of a saturated saline solution (~5.5 M)

in 10 mL vials. All samples and external ethanol standards (freshly prepared; 12.5, 25, 50, 100, and 200 mM) were analyzed using a Bruker 430 gas chromatographer (GC) fitted with a flame ionization detector and CombiPAL autosampler (Bruker Corporation, Fremont, CA). Air control vials were run between the external standards and blood samples.

Data and statistical analysis

Ethanol intake (grams of ethanol per kilogram of animal body weight; g/kg) was calculated from the difference in the initial and final volumes of ethanol solution following TBC and operant session and pre-session body weights. Ethanol preference was determined by the percentage of ethanol solution in relation to the total fluid intake (tap water and 15E intake). Cumulative records of lever and sipper responding were recorded by MED-PC IV software (MED Associates, Inc., St. Albans, VT). Percent active presses were determined by number of active lever presses over total lever (active + inactive) responses from an operant session. For GC data, a standard curve was derived from the external standards (peak heights) and was used to calculate individual BECs using CompassCDS Workstation software (Bruker Corporation, Fremont, CA).

All statistical analyses were performed using StatPlus:mac version 2009 (AnalystSoft Inc., Alexandria, VA). Statistical analyses tested for significance at $p < 0.05$. The number of sessions required during lever response training was used to determine acquisition (sessions) to achieve FR4 on an ethanol-only

solution and was analyzed using the student's t-test. Lever presses, total licks and ethanol intake during operant the final 10 FR4 sessions were analyzed via mixed two-way ANOVA (Treatment x Day). Post hoc t-tests were used for individual comparisons using the Bonferroni correction. Missing values due to software and hardware malfunctions (i.e. cracked and/or leaking sipper tubes, improper contact of leads with metal sipper) were replaced with the average of the ten sessions, an adjustment in the degrees of freedom was made, and p values were recalculated (<1%).

Results

We excluded seven of the 43 mice due to the animals not achieving FR4 acquisition. Of the remaining animals, 33 mice received post-operant ethanol preference test and 14 animals of this subset were used for BEC analysis.

Acquisition to FR4

The mean number of operant sessions required for FR4 acquisition on an ethanol-only solution for each treatment group and water control can be seen in **Figure 2.3**. Analysis comparing treatments with SF water-controlled- and Pre-TBC-trained mice required significantly fewer sessions to achieve FR4 acquisition than their respective counterparts ($t(11) = 3.07$, $p < 0.05$; $t(13) = 2.63$, $p < 0.05$, respectively). Pro-Hab-trained mice and their water controls showed no difference ($t(12) = 1.18$, $p = 0.26$). Further analysis between treatments found

that while SF-trained animals reached acquisition to FR4 first, animals then achieved acquisition on an ethanol-only solution last because of the sessions required to fade out sucrose. Both Pre-TBC- and Pro-Hab-trained mice required fewer sessions to achieve an ethanol-only FR4 acquisition than SF-trained animals ($t(14) = 7.05$, $p < 0.001$; $t(15) = 4.05$, $p < 0.01$, respectively), and there was no difference between Pre-TBC and Pro-Hab mice ($t(16) = 1.64$, $p = 0.12$). Acquisition data is further summarized in **Table 2.1**.

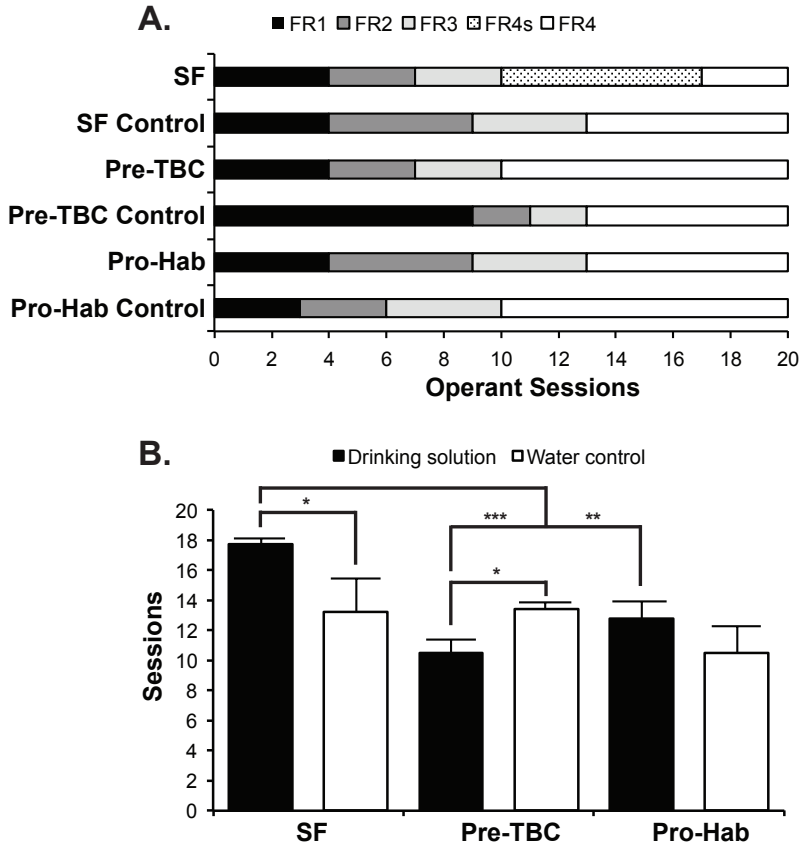

Figure 2.3. Fixed ratio (FR) 4 acquisition of treatment groups. A) Depicts mean number of sessions per FR schedule for animal to achieve ethanol-only self-administration of FR4. FR1, FR2, FR3, and FR4 indicate increased fixed ratio requirement. FR4s indicates sessions when sucrose fading occurred. B) Bar graph depicts mean number of sessions ± standard error of the mean (SEM) to FR4 acquisition on an ethanol-only solution. Even though SF-trained mice reached FR4 first, these animals required additional operant sessions to achieve FR4 acquisition for an ethanol-only solution. Both SF water-controlled and Pre-TBC animals required fewer sessions than their counterparts to achieve FR4 acquisition. Both Pre-TBC and Pro-Hab mice required fewer sessions to achieve FR4 acquisition than SF mice. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

	SF (n=7)	SF Control (n=4)	Pre-TBC (n=8)	Pre-TBC Control (n=5)	Pro-Hab (n=8)	Pro-Hab Control (n=4)
FR4 Acquisition (sessions)	17.7 ± 0.4	13.3 ± 2.2	10.5 ± 0.9	13.4 ± 0.5	12.8 ± 1.2	10.5 ± 1.8

Table 2.1. Fixed ratio (FR) 4 acquisition. Table shows mean number of sessions ± standard error of the mean (SEM) of operant sessions required by treatment groups to achieve acquisition of FR4 on an ethanol-only solution.

Operant behavior analysis

Data depicted in **Figure 2.4** shows operant behavior observed during the final 10 FR4 sessions on 15E. No significant difference was seen in percent active presses ($p = 0.99$, data not shown), however, analysis of the number of active lever responses showed a significant main effect by Treatments ($F(5, 30) = 2.83$, $p < 0.05$), but no effect by Day ($F(9, 45) = 0.78$, $p = 0.63$) or Treatment X Day interaction ($F(45, 270) = 0.83$, $p = 0.77$). Post-hoc analysis revealed that both Pre-TBC and Pro-Hab mice pressed the active lever significantly greater than their water controls ($p < 0.001$ and $p < 0.01$, respectively), and that Pre-TBC mice pressed the active lever significantly greater than both SF and Pro-Hab mice ($p < 0.001$, for both). For inactive lever responses, no difference was seen by Treatments ($F(5,30) = 1.05$, $p = 0.41$), by Day ($F(9, 45) = 0.60$, $p = 0.80$), or Treatment X Day interaction ($F(45,270) = 0.66$, $p = 0.95$). Total licks during the mean operant sessions showed a significant main effect by Treatments ($F(5, 30) = 5.16$, $p < 0.01$), but no effect by Day ($F(9, 45) = 0.49$, $p = 0.88$) or Treatment X Day interaction ($F(45, 270) = 1.30$, $p = 0.11$). Post-hoc analysis revealed that all treatment groups licked significantly greater than their water controls ($p < 0.001$, for all), and Pre-TBC mice had significantly greater licks than both SF and Pro-Hab mice ($p < 0.01$ and $p < 0.001$, respectively). No difference was seen between SF- and Pro-Hab-trained animals ($p = 1$).

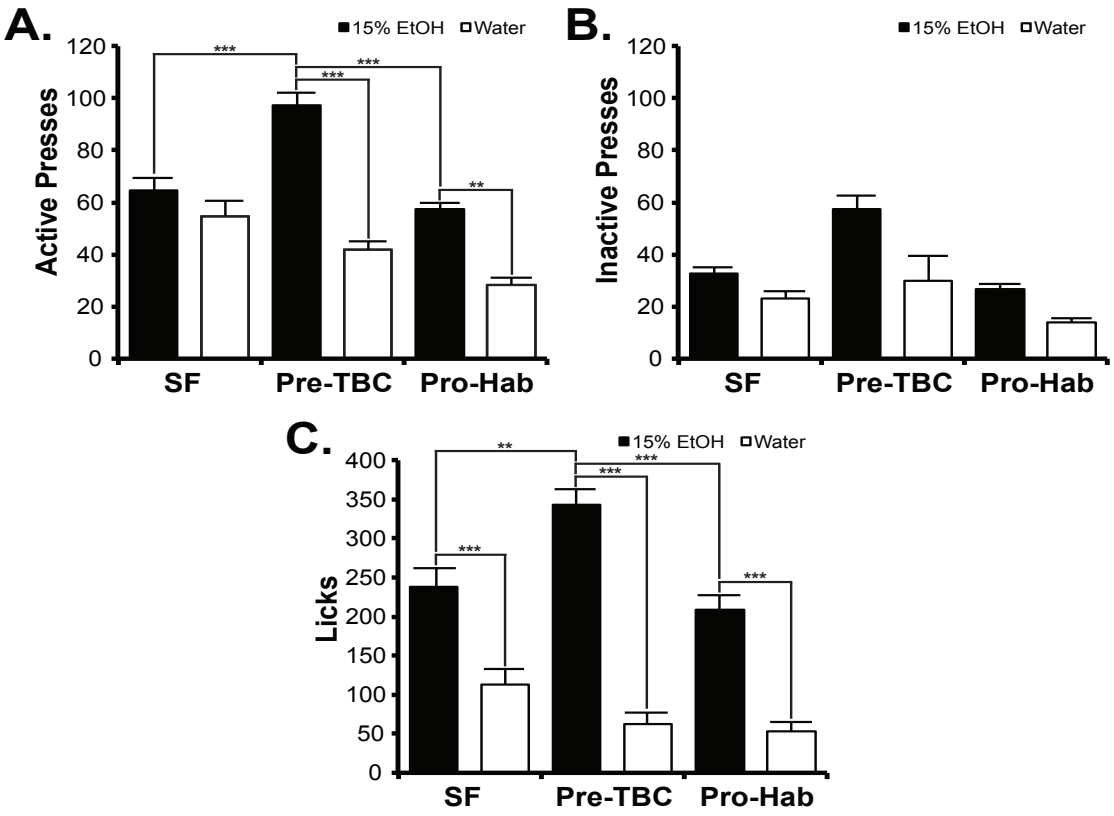

Figure 2.4 Behavior data for final 10 fixed ratio (FR) 4 sessions. A) Bar graph depicts mean active presses ± standard error of the mean (SEM) of the final 10 FR4 sessions. There was a significant main effect by Treatments ($F(5, 30) = 2.83$, $p < 0.05$), but no effect by Day ($F(9, 45) = 0.78$, $p = 0.63$) or Treatment X Day interaction ($F(45, 270) = 0.83$, $p = 0.77$). Post-hoc analysis revealed that Pre-TBC and Pro-Hab mice pressed significantly more than their water controls ($p < 0.001$, $p < 0.01$, respectively). When comparing treatment groups, Pre-TBC-trained animals pressed the active lever significantly more than SF- and Pro-Hab-trained mice ($p < 0.001$, for each). B) Bar graph of mean inactive presses ± SEM showed no significant differences between water controls or treatment groups. C) Bar graph depicts mean total licks ± SEM and showed a significant main effect by Treatments ($F(5, 30) = 5.16$, $p < 0.01$), but no effect by Day ($F(9, 45) = 0.49$, $p = 0.88$) or Treatment X Day interaction ($F(45, 270) = 1.30$, $p = 0.11$). Post-hoc analysis revealed all treatment groups licked significantly more than their water controls ($p < 0.001$, for all). Pre-TBC mice also had significantly greater total licks than SF and Pro-Hab mice ($p < 0.01$, $p < 0.001$, respectively). ** = $p < 0.01$, *** = $p < 0.001$.

For ethanol intake, analysis showed there was a significant main effect by Treatment (**Figure 2.5**; $F(2, 20) = 7.24$, $p < 0.01$), but there was no difference by Day ($F(9, 18) = 0.87$, $p = 0.56$), or by Treatment X Day interaction ($F(18,180)$

=0.49, p = 0.96). Post-hoc comparison found that Pro-Hab-trained mice consumed significantly less ethanol than both SF- and Pre-TBC-trained animals ($p < 0.001$, for both). There was no difference between the SF and Pre-TBC treatment groups for mean ethanol intake ($p = 1$). For better comparison and control data, results are summarized in **Table 2.2**.

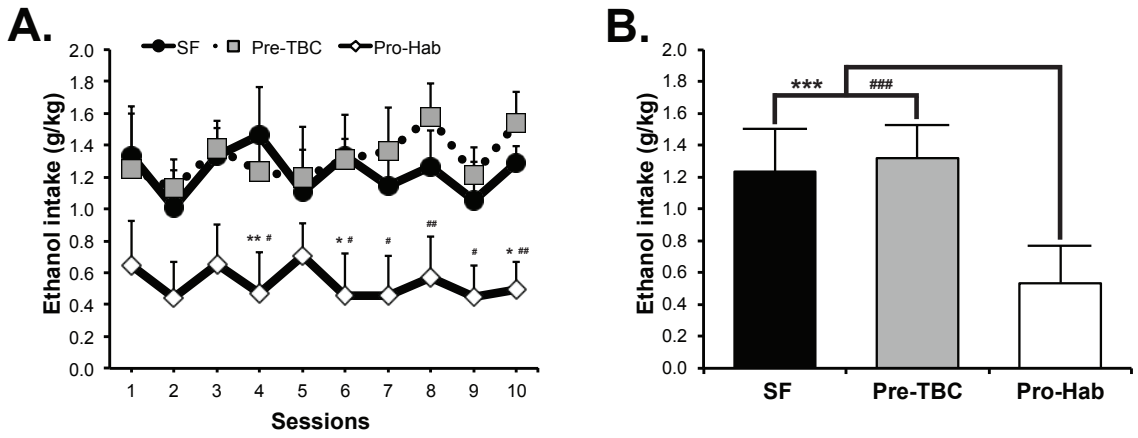

Figure 2.5. Mean ethanol consumption for the final 10 fixed ratio (FR) 4 sessions. A) The line graph depicts mean consumption ± standard error of the mean (SEM) of the treatment groups for the final 10 FR4 sessions. Analysis showed there was a significant main effect by Treatment ($F(2, 20) = 7.24$, $p < 0.01$), but there was no difference by Day ($F(9, 18) = 0.87$, $p = 0.56$), or by Treatment X Day interaction ($F(18,180) = 0.49$, $p = 0.96$). Post-hoc analysis showed that Pre-TBC and SF animals consumed significantly more ethanol than Pro-Hab animals on certain days. * = $p < 0.05$, ** = $p < 0.01$ for SF mice. # = $p < 0.05$, ## = $p < 0.01$ for Pre-TBC mice. B) Bar graph depicts mean consumption ± SEM of the final 10 sessions. Post-hoc analysis showed that Pro-Hab-trained mice consumed significantly less ethanol than both SF- and Pre-TBC-trained animals ($p < 0.001$, for both). No difference was seen in SF and Pre-TBC mice.

	SF (n=7)	SF Control (n=4)	Pre-TBC (n=8)	Pre-TBC Control (n=5)	Pro-Hab (n=8)	Pro-Hab Control (n=4)
Active Presses (#)	64.8 ± 9.9	54.8 ± 15.6	97.3 ± 26.4	42.0 ± 16.7	57.2 ± 11.6	28.3 ± 8.0
Inactive Presses (#)	32.9 ± 2.4	23.3 ± 2.7	57.4 ± 5.4	30.1 ± 9.4	26.7 ± 2.1	14.0 ± 1.6
Licks (#)	238.2 ± 24.0	112.7 ± 20.2	343.4 ± 19.7	62.0 ± 15.5	209.1 ± 18.1	52.8 ± 11.9
Ethanol Intake (g/kg)	1.23 ± 0.27		1.32 ± 0.21		0.53 ± 0.24	

Table 2.2. Summarized operant data. The table includes mean behavior data ± standard error of the mean (SEM) of the treatment groups for the final 10 FR4 sessions.

Ethanol preference and BECs analysis

There was no difference between treatment groups for ethanol preference following operant training ($p = 1$) and no difference in the mean ethanol intake during post-operant TBC (**Figure 2.6**; $p = 0.12$). Following an abbreviated final session, BECs were significantly correlated to the estimated ethanol consumption (**Figure 2.7;** $R(14) = 0.54$; $p < 0.05$).

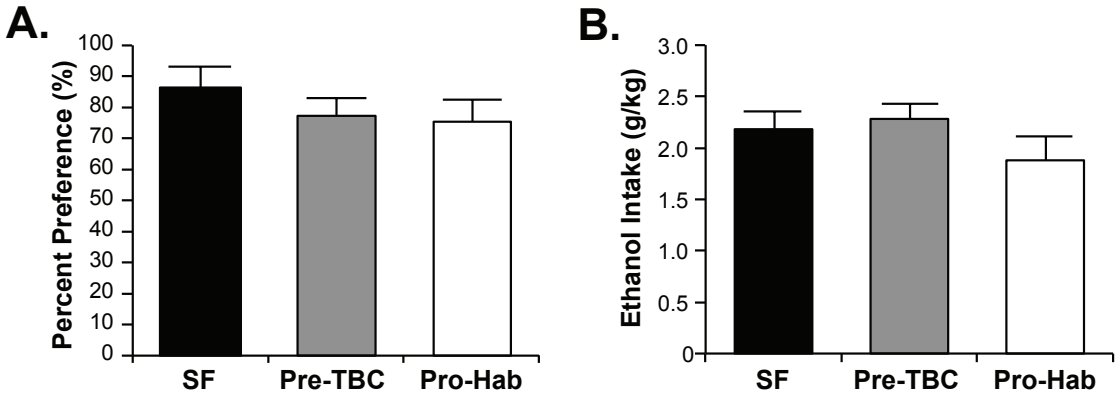

Figure 2.6. Ethanol preference testing following operant. A) Bar graph depicts mean percent ethanol preference ± standard error of the mean (SEM) of the treatment groups for five post-operant two-bottle choice (TBC) sessions. Percent ethanol preference, calculates as the combined intake of five 15% ethanol (15E) in relation to the total fluid intake (tap water and 15E intake). No difference was seen between treatment groups ($F(3,28) =$, $p = 1$). B) Bar graph depicts mean ethanol intake ± SEM of the five TBC sessions. Analysis showed there was no difference by treatment groups ($F(2, 21) = 2.40$, $p = 0.12$).

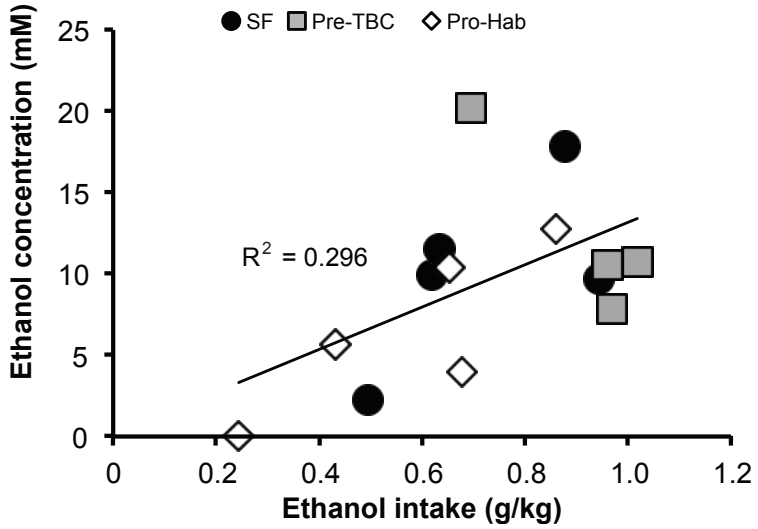

Figure 2.7. Blood ethanol concentrations (BECs) and ethanol intake of final FR4 operant session. Scatterplot depicts a subset of treatment mice and the relationship of measured BECs versus calculated ethanol intake of final truncated FR4 session. BECs were significantly correlated to ethanol consumption ($p < 0.05$).

Discussion

In previous research, operant training of mice for ethanol-only solutions has proven to be challenging and to require the use of additional manipulations, in particular with the use of a sweetener such as sucrose. Previous studies have commonly employed the sucrose-fading technique (152,169,250–252), and has shown to improve ethanol preference and to facilitate learning (250,251,253). Sucrose, however, is a powerful reinforcer (182,183), and can play a role in ethanol drinking behavior (178,260). By comparing sucrose fading with two ethanol-only treatments, the goal of this study was to develop a method to devoid of such confound. Our data show that B6 mice can be easily trained to self-administer ethanol without the use of sucrose, and in significantly less time than previously achieved. A number of prior reports initially utilizing a sweetener indicate that acquisition of ethanol self-administration can easily require more than four weeks (169,181,261,262), and this delay is predominantly due to the fading out of the sweetener after lever-pressing acquisition is obtained. The present study also illustrates this finding; SF-trained mice achieved acquisition to FR4 first, but then required additional sessions to reach lever responding for an ethanol-only solution.

Even though the seeking of a drug reinforcer can be seen as one aspect in understanding addiction, this behavior alone cannot be directly correlated to increased ethanol consumption. Indeed, all animals showed lever responding preference for the active lever (treatment groups and water controls), but

experimental groups showed differences in solution consumption. Finn and colleagues (2008) reported that during extinction of a sucrose solution, B6 mice preferentially continue to show preference for the active lever, but overall lever responding extinguishes over time. This behavior supports the concern that sucrose, as a natural reinforcer, may activate the mesolimbic dopamine pathway (182,183), and may constitute a confound in training animals to self-administer ethanol by showing an appetitive behavior towards the faded sucrose.

In comparison to all methods used, SF- and Pre-TBC-trained mice showed no difference in ethanol consumption, but both consumed significantly greater amounts of alcohol than Pro-Hab-trained mice. This behavior implies that pre-exposure to ethanol in the home environment prior to the operant conditioning may promote persistent ethanol self-administration over prior habituation to the operant chamber and that use of a sweetener may be avoided. In the present study, the mean consumption of Pre-TBC mice reached 1.32 g/kg in a one-hour session, whereas other studies have shown that B6 mice can achieve greater ethanol intake (2-4 g/kg) during shorter operant sessions. This could be attributed to the time of day when the drinking studies were conducted, specifically where delaying volitional drinking to occur three or four hours into the animal's active dark phase can increase ethanol consumption (170,250,255,256). Despite the time of alcohol presentation, Pre-TBC mice consumed similar amounts of ethanol to SF mice in this study, but without the aid of the potential

confound. This finding further illustrates that operant conditioning of B6 mice can forego the use of a sweetener to study the reinforcing effects of ethanol.

To determine if the differences in alcohol consumption between the treatment groups were due to variability in ethanol preference, a subset of animals from each group received a TBC paradigm following sustained FR4 operant sessions. Our data suggests that there are no differences between treatment groups and that the SF training paradigm was neither more efficient nor more effective in increasing ethanol consumption. While numerous prior investigators have reported that the addition of a sweetener to alcohol solutions can help to improve ethanol preference and increase appetitive and consummatory behavior (152,251,263–266), our findings show that use of sucrose is unnecessary.

Furthermore, we wanted to verify that the calculated ethanol consumption resulted in pharmacologically relevant BECs by analyzing trunk blood for its ethanol content. The BECs obtained after a 30-minute operant session confirmed that ethanol consumption correlated with elevated BECs, however, all BECs detected were below that of alcohol intoxication and showed high variability among the animals. This finding could be due to several factors, such as ethanol consumption at different time points over the 30 minute session (255,267), effects of consumption attributed to circadian rhythm (170,250,268,269), or general differences in metabolism of the individual animal. Nevertheless, inter-animal variability in BEC is a well-known and little understood

phenomenon; for instance, our lab previously noted large differences in BEC following vapor exposure to cage-mates that were treated identically (unpublished data).

Interestingly, mice receiving only water during their operant training also achieved acquisition to FR4 and had similar percent active lever presses, but showed little to no water consumption during sessions. To our knowledge, only one study has shown that mice press for water when expecting an ethanol reward (i.e., during extinction) (165), but there are no studies reporting animals receiving only water as a control during operant training. It has been shown that B6 mice perform lever responses simply for a stimulus light, a phenomenon functionally defined as operant sensation seeking (OSS) (270). OSS is observed when increasing the novelty of the stimulus light results in lever pressing not associated to the conditioned reinforcer. To address this phenomenon further, a subset of mice was tested without the blinking of the stimulus light. We observed no difference in operant responding after the removal of the light cue (data not shown), however, the incentive of operant responding of the water controls can at this point only be speculated.

In summary, the current study demonstrates that the use of sweeteners for operant conditioning in B6 mice to self-administer ethanol is unnecessary. Furthermore in this study, the use of pre-exposure to ethanol in the form of the TBC paradigm significantly decreases the time to achieve lever response acquisition and attains similar ethanol consumption when compared to sucrose-

faded animals. Therefore, this method should prove to be useful for removing (i.e., sucrose) a powerful confound, and therefore aid in modeling aspects of ethanol self-administration.

Acknowledgements

The authors thank Joel Kent and David Hernandez for their assistance in collecting operant data and James Reno for providing assistance in writing our MatLab program for MedPC-IV analysis.

Chapter Three

Future directions of ethanol research

In summary, alcohol abuse and dependence are serious concerns in the U.S. due their effects on health and costs on society. Substantial evidence has shown the mesocorticolimbic pathway plays a significant role in motivation and reward processing and that neuroadaptions within this system contribute to the development of addiction to ethanol and other drugs of abuse (19,33,34). Dopaminergic projections from the VTA innervate forebrain regions, in particular, the NAc (21), and neuroadaptions within this brain region are implicated in the formation and expression of drug-related behaviors (184).

Undoubtedly, neuroadaptions to the NAc can occur after sustained ethanol and drug exposure. Cocaine studies showed disruption of NMDAR-dependent LTD following two to three weeks of daily injections (188,198), and a similar disruption in LTD was seen following 14 days of amphetamine administration (200). Furthermore, NMDAR-dependent LTD was also occluded after establishing two weeks of heroin administration and following an additional two weeks of abstinence (201), illustrating that this alteration in plasticity has long lasting effects. Passive ethanol administration via CIE showed occlusion of NMDAR-dependent, AMPA-mediated LTD within D1 MSNs of the NAc shell and these effects persist for up to two weeks following ethanol exposure (126). In my own preliminary work, I have shown that B6 mice not only self-administer an

ethanol-only solution via operant chambers without the use of the potential confounds such as sucrose or extensive food or water deprivation, but also a significant reduction in the magnitude of LTD is seen in D1 MSNs of the shell, but not core. Furthermore, the degree in magnitude of LTD is inversely correlated with the amount of ethanol consumed following the final operant sessions (unpublished data). Taken together, this evidence shows that sustained drug exposure induces metaplasicity, however, little is known of the underlying mechanisms that leads to the development of ethanol addiction.

Extensive evidence shows that to establish the alcohol's positive effects, ethanol priming must first occur before compulsive drinking behavior can be developed (2,161,163,271). Future work should then test if there are neuronal changes occurring with plasticity following ethanol priming and if modulating this critical period could have effects on ethanol seeking and conditioned learning. Work within the NAc has often used conventional methods to block accumbal activity by means of accumbal lesions or application of agonists or antagonists. Accumbal lesions disrupted the acquisition of ethanol drinking and renewal of conditioned responding (52,83,87), as well as the application of D1 agonists or antagonists have facilitated or attenuated alcohol consumption (102,103,155). However, these methods provide confounds to the study of the ethanol addiction due to lesioning of the accumbens is non-reversible and limits the understanding of the efferent and afferent inputs to the NAc, whereas D1 compounds lack complete specificity to D1 MSNs and also have off-target effects. Fortunately,

the development of new technology can provide additional support in studying alcohol addiction that overcomes these limitations.

Optogenetics employs both a light-sensitive microbial opsin-expressing virus and a light source to allow for precise manipulation of cells, both in slice and in behaving animals (272–275). Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) provide a chemogenetic approach to studying ethanol addiction where reversible modulation of modified G-coupled protein receptors are activated with an inert and extrinsic ligand, clozapine-n-oxide (CNO) (276–278). Both of these techniques allows for reversible, selective functional modulation without the occurrence of off-target effects and work utilizing these tools for the study of addiction has only just begun. For optogenetics, inhibition of the NAc core reduced aversion-resistant alcohol intake, which is used as a model of compulsive drug taking (279), while tonic activation of VTA DA neurons has been shown to decrease ethanol self-administration (280). Recent work in accumbal-related addiction using DREADDs investigated the opposing roles of D1 and D2 MSNs on amphetamine sensitization (281), and has shown an inhibition of the NAc core attenuated ethanol consumption (282). Taken together, these innovative techniques could then be applied during ethanol priming to see if the D1 MSNs play a critical for operant self-administration acquisition and ethanol consumption.

Though the focus of this thesis has been on the DA reward circuit, it is important to realize that this system is not the only mediator of addiction (283).

Astrocytes, a subset of glial cells, have historically been thought to “filler” around neural networks, but recent work as shown otherwise. Such tasks of astrocytes include preserving homeostasis of the extracellular space, providing lactate for the neuronal metabolic cycle and supplying glutamine for neurons (284). Work using astrocytes and DREADDs has shown with activation of the glia cells increased cytosolic calcium concentrations and facilitated brain stimulation responding while reduce ethanol reinstatement (285). Another issue to consider is that alcohol-dependent humans often are also smokers (286), and ethanol has been shown to modulate nicotinic acetylcholine receptors (nAChR) (287). Lesioning of the NAc reduced both ethanol and nicotine self-administration (70,265), and the nAChR antagonist, mecamylamine, reduce alcohol intake and responding (288,289). Furthermore, work has shown that elevated DA concentration in the NAc coincides with elevated acetylcholine (ACh) in the VTA after volitional ethanol consumption (290). Finally, work in non-dopaminergic brains regions such as the supramammilary nucleus (85,291,292), midbrain raphe nuclei (293), rostromedial tegmental nucleus (294–296), may also have effects on the mesocorticolimbic pathway that have otherwise be ignored. Taken together, all of these additional pathways and transmitters will need further study to discern if their role in ethanol addiction is significant.

Going forward, future research is needed to determine the underlying mechanism of ethanol priming and hopefully, if it is brought to light, then improved preventative initiatives and treatments could be created for society.

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VITA

Tira Anne Meyers was born in a military hospital at Fort Huachuca in Sierra Vista, Arizona. After completing her work at Haltom High School, Haltom City, Texas, in 2004, she entered into the University of Texas at Austin, Texas that fall. She received the degree of Bachelor of Science from UT in May of 2008 and continued her academic career by entering into the professional program of the UT College of Pharmacy in August of that year. She then received the degree of Doctor of Pharmacy from UT in May of 2012 and entered into the graduate program with the UT College of Pharmacy in the summer of that year. During her time in graduate school, she was also employed as a pharmacist at the Heart Hospital of Austin, Texas and remained highly active in her professional pharmacy fraternity, Phi Delta Chi, by serving in several appointed leadership positions regionally, facilitating national and regional meeting workshops, and volunteering as a preceptor and mentor to the local chapter.

Address: tirameyers@utexas.edu

This manuscript was typed by Tira A. Meyers.