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NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS:

MOLECULAR TARGETS FOR ALCOHOLISM AND

ETHANOL REWARD

A Dissertation Presented By

LINZY MARIN HENDRICKSON

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JANUARY 28, 2011

PROGRAM IN NEUROSCIENCE

NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS: MOLECULAR
TARGETS FOR ALCOHOLISM AND ETHANOL REWARD

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LINZY MARIN HENDRICKSON

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Looking back, I can see exactly how I've grown through my years of PhD research, and it is all in direct relation to how Andrew has been as a mentor. In my first year, he treated me as a first year student, discussing projects and teaching me first hand. My second through fourth years, I was a graduate student and he informed me that graduate students are supposed to, "work…that's it, no life…just work" and I did, day and night, too! Then, in the middle of my fourth year, Andrew surprised me the most by telling me that my graduate school career was coming to an end, that I should start looking for postdocs, that I was done. I was almost certain that he was kidding, but here I am, graduating in the middle of my fifth year, with three first author papers and two more on the way, and I owe it all to him. Thanks, Andrew.

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phone. At this point, I am almost sick of hearing you say how proud you are of me. Thanks, Mom. But seriously, my success is in direct relation to the love and guidance you have both given me throughout my life. You've always known what to say, whether I want to hear it or not. I also want to thank my brother, Brandon. I knew that once you received your PharmD, that I had to get my PhD. You have always set the bar high and I have always loved trying to raise it. Thanks, B.

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ABSTRACT

While it is clear that most drugs of abuse act to increase extracellular dopamine levels in the nucleus accumbens (NAc), the molecular mechanisms mediating this process vary depending on the molecular target each drug acts on. The rewarding properties of most drugs of abuse including cocaine, amphetamine, and heroin have been well established for some time; however, the molecular mechanisms by which ethanol acts to mediate reward have not been fully elucidated. In this thesis, I have examined the role of nicotinic acetylcholine receptors (nAChRs), known molecular targets for nicotine addiction, in mediating the initial rewarding properties of alcohol. Using a mouse model of voluntary ethanol consumption called Drinking in the Dark (DID), in combination with nAChR pharmacology and mouse genetics, we have provided further evidence for the role of nAChRs in mediating the initial rewarding properties of ethanol. Because of the vast number of possible functional nAChR subtypes present in the brain, I sought to investigate which subtype of nAChR may be responsible for ethanol reinforcement. To accomplish this, I used two complementary nAChR mouse models. The first is a knock-out line that does not express the α4 subunit (α4 KO) and the second is a knock-in line that expresses α4* nAChRs that are hypersensitive to agonist (Leu9′Ala). We have also shown, for the first time, that a specific nAChR subtype, those that contain the α4 subunit (α4*), mediate voluntary ethanol consumption and reward. Next, I examined the role of α4* nAChRs in modulating voluntary ethanol consumption after systemic

administration of the FDA approved smoking cessation drug varenicline, a partial agonist of α4* nAChRs. We showed that varenicline and nicotine both reduced acute ethanol consumption in an α4* nAChR dependent mechanism. Taken together, our data indicate that activation of α4* nAChRs is necessary and sufficient for reduction of ethanol consumption and further supports the hypothesis that α4* nAChRs are molecular targets for alcohol cessation therapies.

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List of Abbreviations

5-HT	5-hydroxytryptamine (serotonin)
5-HT$_3$	5-HT receptor (serotonin-gated ion channel receptor)
α4*	alpha 4 nAChR subunit containing
α4 KO	alpha 4 nAChR knock-out
ACh	acetylcholine
AChR	acetylcholine receptor
aCSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
AP	action potential
β2 KO	beta 2 knock-out (nAChR knock-out)
β4 KO	beta 4 knock-out (BK knock-out)
BEC	blood ethanol concentration
BK	large-conductance calcium- and voltage-gated potassium channel
BNST	bed nucleus of the stria terminalis
BSR	brain stimulation reward
CASA	National Center on Addiction and Substance Abuse at Columbia University
CBP	CREB-binding protein
CNS	central nervous system
CPP	conditioned place preference
DA	dopamine
DAergic	dopaminergic
DHβE	dihydro-β-erythroidine
DID	drinking in the dark
DALYs	Disability-Adjusted Life Years

DSM-IV	Diagnostic and Statistical Manual of Mental Disorders (4th Edition)
EtOH	ethanol
GABA	γ-amino butyric acid
GIRKS	G-protein-activated inwardly rectifying K$^+$ channels
i.p.	intraperitoneal
IPN	interpeduncular nucleus
KI	knock-in
KO	knock-out
LCM	laser capture microdissection
LHb	lateral habenula
Leu9'Ala	leucine 9' alanine
LDT	laterodorsal tegmental nucleus
MEC	mecamylamine
MFB	medial forebrain bundle
MHb	medial habenula
MLA	methyllycaconitine
NAc	nucleus accumbens
nAChR	nicotinic acetylcholine receptor
NIC	nicotine
NMDAr	*N*-methyl *D*-aspartate receptor
PBS	phosphate-buffered saline
PFC	prefrontal cortex
PKA	cAMP dependent protein kinase
PNS	peripheral nervous system
PPTg	pedunculopontine tegmental nucleus
qRT-PCR	quantitative real time polymerase chain reaction
SAL	saline
SEM	standard error of the mean

TH	tyrosine hydroxylase
TM	transmembrane domain
VEH	vehicle
VTA	ventral tegmental area
WHO	World Health Organization
WT	wild-type

Copyright Page

Some of the chapters of this dissertation have appeared in separate publications:

Chapter 2:

Hendrickson LM, Zhao-Shea R, Tapper AR. Modulation of ethanol drinking-in-the-dark by mecamylamine and nicotinic acetylcholine receptor agonists in C57BL/6J mice. *Psychopharmacology (Berl).* 2009 Jul;204(4):563-72.

Chapter 4:

Hendrickson LM, Zhao-Shea R, Pang X, Gardner PD, Tapper AR. Activation of α4* nAChRs is necessary and sufficient for varenicline-induced reduction of alcohol consumption. *J. Neurosci.* 2010 30: 10169-10176.

Chapter 5:

Hendrickson LM, Gardner PD, Tapper AR. Nicotinic acetylcholine receptors containing the α4 subunit are critical for nicotine-induced reduction of acute voluntary ethanol consumption. *Channels.* 5: 2, 1-4; March/April 2011.

Appendix 1:

Martin GE, Hendrickson LM, Penta KL, Friesen RM, Pietrzykowski AZ, Tapper AR*, Treistman SN*. Identification of a BK channel auxiliary protein controlling molecular and behavioral tolerance to alcohol. *Proc Natl Acad Sci.* U S A. 2008 Nov 11;105(45):17543-8. Epub 2008 Nov 3. * Authors contributed equally

CHAPTER I.

BRAIN MECHANISMS THAT MODULATE DRUG AND ALCOHOL ADDICTION

I.A. Introduction

Addiction is a chronic brain disorder, just as is dementia, epilepsy, and multiple sclerosis. It affects people of all cultures, ages, socio-economic status, and gender. According to the National Institute On Drug Abuse website, addiction is said to be, "An equal opportunity destroyer," and that, "No population group is immune to substance abuse and its damage." However, unlike other diseases, the social stigma of drug addiction is very negative. People suffering from addictions are often labeled as addicts and drug users, and this stigma often prevents them from seeking and receiving treatment.

In reality, addiction is a chronic disease that involves several factors. Similar to other chronic diseases, addiction can run in families, be influenced by environmental factors and behaviors, respond to treatment, and include life-long lifestyle changes (McLellan et al., 2000). While the most commonly thought of addiction disorder would be an addiction to a substance such as drugs or alcohol, addictions can occur to anything that produces a high or alters ones senses. For example, acts that produce feelings of pleasure, such as overeating, pathological gambling, and internet use can result in behavioral addictions (Grant et al., 2010; Holden, 2010).

Currently, there are several terms used to describe 'addiction' and they are often used interchangeably. While manifestation of addictions can change

(i.e., alcohol, cocaine, nicotine, gambling, etc.), addictions can be classified as either 'substance abuse' or 'substance dependence'. According to the current Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), substance abuse occurs, "When an individual persists in use of alcohol or other drugs despite problems related to use of the substance." Accordingly, the symptoms that must be present to be diagnosed with a substance abuse disorder, also defined by the DSM-IV are, "(1) recurrent use resulting in a failure to fulfill major obligations at work, school or home; (2) recurrent use in situations which are physically hazardous (e.g., driving while intoxicated); (3) legal problems resulting from recurrent use; or (4) continued use despite significant social or interpersonal problems caused by the substance use." However, with prolonged and persistence substance abuse, the diagnosis changes from 'abuse' to 'dependence'. This occurs when a person (1) has a diagnosed substance abuse disorder, (2) continues use despite related problems, (3) has an increase in tolerance, and (4) has withdrawal symptoms. Together, substance abuse and substance dependence are considered to be 'substance use disorders'.

To further complicate the matter, the term 'addiction' is often interchanged with 'substance dependence' and is essentially the same. Drug addiction is defined as a chronically relapsing disorder characterized by the compulsion to seek and take the drug, loss of control in limiting intake and the emergence of a negative emotional state during drug withdrawal (Koob and Le Moal, 1997). Recently, drug addiction has been described as a cycle comprising both impulse

control and compulsive disorders. The addiction cycle begins with impulsivity, which is associated with the positive reinforcement that drives a motivated behavior, and then develops into compulsivity in later stages, which is driven by negative reinforcement (Koob, 2004). Additionally, this cycle can be broken into three stages of addiction: binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation (Koob et al., 2009). To fully understand a complex disease such as addiction, it is necessary to elucidate the molecular, cellular, and circuit level mechanisms of each stage to determine what causes a person to develop from a drug user, to abuser, to addict. Fortunately, there exist several animal models of addiction that mimic each stage of the addiction cycle and allow researchers to elucidate the molecular mechanisms at each stage.

THESIS OVERVIEW

This thesis will examine one type of addiction, alcohol dependence or more commonly referred to as alcoholism (as referred to in the rest of this thesis), during the binge/intoxication stage, from the molecular to behavioral level. To understand and study addiction disorders, it is first necessary to become familiar with the brain reward pathways and how drugs of abuse can hijack the endogenous machinery resulting in feelings of reward. This first chapter will introduce current theories on the molecular mechanisms of drug addiction. I will give a brief background on alcoholism and the currently available therapeutic options followed by a discussion of the dopamine reward pathway. Next, I will discuss what is known about how alcohol (also referred to as ethanol)

interacts with the dopamine reward pathway, the known primary targets of ethanol and discuss mouse models of alcoholism. Finally, I will give an introduction to neuronal nicotinic acetylcholine receptors (nAChRs) and what is currently known about the role of nAChRs in ethanol reward, which will be the focus of the rest of my thesis.

The second chapter will explore the role of wild type (WT) nAChRs in an assay designed to measure acute voluntary ethanol consumption, termed Drinking in the Dark (DID). Because the role of nAChRs in voluntary ethanol intake was unknown, we sought to measure ethanol intake in WT mice after acute injections of several different nAChR agonists and antagonists. We also evaluated the effect of nAChR drugs on the activation of dopaminergic (DAergic) neurons of the ventral tegmental area (VTA). Our results indicate that the nAChR antagonist mecamylamine, as well as two nAChR agonists, nicotine and cytisine, can decrease acute ethanol intake. Additionally, we showed that while nicotine and ethanol can both activate DAergic neurons of the VTA, mecamylamine can block the ethanol induced activation of dopamine (DA) neurons. This result suggests that ethanol activates DAergic neurons of the VTA via neuronal nAChRs and that nAChRs modulate acute ethanol voluntary intake (Hendrickson et al., 2009).

Because of the vast number of possible functional nAChR subtypes present in the brain (Grady et al., 2007), the next area we sought to investigate was which subtype of nAChR may be responsible for ethanol reinforcement.

Several reports have shown that the two most commonly co-abused drugs are nicotine, the primary psychoactive ingredient found in tobacco products, and alcohol (Falk et al., 2006; Funk et al., 2006). The molecular mechanisms of nicotine addiction are well established and studies have shown that nAChRs containing the alpha 4 subunit (termed α4* nAChRs) are necessary and sufficient for nicotine addiction (Tapper et al., 2004). Thus, due to the common co-abuse of nicotine and alcohol, it is thought that these two drugs may have similar or overlapping molecular targets in the brain. Therefore, we sought to determine if this subtype of nAChR might also play a role in alcohol addiction. To accomplish this, we used two complementary nAChR mouse models. The first is a knock-out line that does not express the α4 subunit (α4 KO) and the second is a knock-in line that expresses α4* nAChRs that are hypersensitive to agonist (Leu9′Ala). The third chapter of this thesis will examine the contribution of α4* nAChRs to ethanol reward using several alcohol assays including voluntary consumption, ethanol conditioned place preference and ethanol induced activation of the VTA in both the α4 KO and Leu9′Ala nAChR mouse models. Combining these two mouse lines, our results show both the necessity and sufficiency of α4* nAChRs in ethanol reward.

One of the main goals in alcohol addiction research, aside from elucidating the molecular mechanisms contributing to alcohol abuse, is identifying possible molecular targets for alcohol therapeutics. Currently, there are alcohol cessation drugs available; however, there exists a large variability in

response such that only 20 % - 30 % of patients respond to treatment (Spanagel, 2009). Chapter four of this thesis will examine α4* nAChRs as molecular targets for alcohol cessation therapies. First, we show that ethanol induces c-Fos expression in a selective region of the brain, the posterior VTA. Additionally, the DAergic neurons that were activated by ethanol have higher gene expression of specific nAChR subunit genes, namely the α4, α6 and β3 subunits. Next, using the two aforementioned nAChR mouse models, we examined the role of α4* nAChRs in modulating voluntary ethanol consumption after systemic administration of the FDA approved smoking cessation drug varenicline (Coe et al., 2005; Jorenby et al., 2006), a partial agonist of α4* nAChRs. We showed that varenicline reduced acute ethanol consumption in an α4* nAChR dependent mechanism. Finally, we show that an infusion of varenicline directly to the posterior VTA, but not the anterior VTA, decreases voluntary ethanol intake (Hendrickson et al., 2010).

The fifth chapter of this thesis expands on chapter four by showing that an additional nAChR agonist, nicotine, also decreases ethanol intake in an α4* nAChR dependent manner (Hendrickson et al., 2011). Taken together, our data indicate that activation of α4* nAChRs is necessary and sufficient for reduction of ethanol consumption and further supports the hypothesis that α4* nAChRs are molecular targets for alcohol cessation therapies.

The sixth and final chapter of this thesis will be a discussion of the research presented including the contribution to the field as well as future directions and concluding remarks.

I.B. Alcoholism: Background and current therapies

Approximately 4 % of US adults, over 12.5 million people, are dependent on alcohol (Hasin et al., 2007) and alcoholism is the third leading cause of preventable mortality in the US (Mokdad et al., 2004). The estimated economic cost of alcoholism in the US, due to health care costs as well as productivity impacts such as lost wages, was $220 billion in 2005, which was significantly higher than cancer ($196 billion) or obesity ($133 billion) (CASA, 2005). Worldwide, about 2 billion people consume alcohol, with 76.3 million who have diagnosable alcohol use disorders. Additionally, when analyzing the global burden of this disease, alcohol causes 1.8 million deaths (3.2 % of the worldwide total) and accounts for 4 % of the total Disability-Adjusted Life Years (DALYs) (World Health Organization. Dept. of Mental Health and Substance Abuse., 2004).

However, while alcohol use is common in most populations, not every person that uses alcohol will become addicted to it. Why do some people lose control over their alcohol use or drink irresponsibly at times while others are able to drink responsibly? What causes one person's alcohol consumption to escalate to the point of a physical dependence on alcohol, such that they

experience withdrawal symptoms when they discontinue alcohol use? These are just a few of the questions that the neuroscience field is currently working on.

One area of alcoholism research seeks to understand the genetic, circuit, cellular and molecular mechanisms that underlie the transition from drug use to drug addiction. This would most closely align with the first addiction cycle, as discussed previously, which is associated with positive reinforcement and motivated behaviors, i.e., the binge/intoxication stage. Research on this stage of addiction focuses on the acute response of the drug in specific brain regions that are known to modulate reward, the dopaminergic mesolimbic pathway, which is the focus of this thesis and described in more detail in the next section. Other areas of alcohol research that study chronic alcohol consumption, alcohol withdrawal, and alcohol craving and relapse are analogous to the second and third stages of addiction and focus this research on brain regions including the central and extended amygdala, bed nucleus of the stria terminalis (BNST) and the nucleus accumbens.

While several areas of alcoholism research exist, the end goal of the majority of research is to identify new and improved treatment options for those suffering from alcoholism. Currently, there are three FDA approved medications for treating alcoholism. The first, disulfriam, was approved in 1954, and is classified as an anti-relapse medication (Christensen et al., 1991). It is an acetaldehyde dehydrogenase inhibitor, which after drinking alcohol allows the buildup of acetaldehyde in the blood, causing symptoms including headache,

nausea, vomiting, weakness, mental confusion, or anxiety (Christensen et al., 1991). However, in recent years, many doctors have stopped prescribing this drug because of the severe symptoms it causes and the fact that if a patient wished to drink again, they could simply not take their medication. Naltrexone, available since 1994, is a competitive opioid receptor antagonist that works by decreasing the euphoric effects produced by alcohol. It is considered to be an anti-relapsing drug because it decreases heavy drinking in patients with alcoholism and prevents relapse to heaving drinking (O'Malley et al., 1992; Volpicelli et al., 1992). The third drug, acamprosate, is a partial agonist of NMDA glutamate receptors and an antagonist of metabotropic glutamate receptors and is thought to act as an anti-craving medication by inhibiting glutamate signaling (Mason, 2003; Mason et al., 2006). While European studies have reported modest benefits with acamprosate, these studies have not been reproducible in the US (Pettinati et al., 2006).

Unfortunately, while these medications have been effective for some, only 20-30 % of treated patients respond to the anti-craving and anti-relapsing compounds (Spanagel, 2009). Interestingly, new studies have shown that people with different genetic profiles may drink for different reasons, and also that they may respond better to one type of medication versus another. For example, populations with a specific type of mu opioid receptor respond to naltrexone better than others, and this group has been described as 'feel good drinkers' (Anton et al., 2008; Oslin et al., 2006). Another population of alcoholics

report that they drink to relieve feelings of stress and anxiety (Kuehn, 2009) for which new medications are currently being tested (George et al., 2008). This large variability in patient response is a driving force in identifying new molecular targets for improved pharmacotheraputic drugs. Consequently, the main focus of alcoholism treatments has been to restore the balance to the different biochemical pathways in the brain that are disrupted during alcohol dependence.

I.C. Brain reward mechanisms

Species that learned to respond to natural rewards, like when and where they could obtain food and opportunities for mating, ensured their survival and achieving these goals function as rewards (Hyman et al., 2006). Consequently, many neural substrates that modulate reward systems are conserved across species from *Drosophila*, mice, and rats, to humans and include dopamine (DA), G-proteins, protein kinases, amine transporters, and transcription factors including the cAMP response element-binding protein (CREB) (Kelley and Berridge, 2002). Later, humans found that this endogenous system can be exogenously altered with various drugs. We now know that responses to natural rewards and addictive drugs have many similarities and shared pathways. In fact, one study has shown a cross-sensitization between the natural reward sugar, and the drug amphetamine (Avena and Hoebel, 2003).

One common effect of natural rewards and most drugs of abuse is an enhancement of activity in the mesolimbic dopamine system (discussed in more

detail below), leading to an increase of dopamine release in the nucleus accumbens (Di Chiara and Imperato, 1986; Koob and Bloom, 1988; Wise, 1998). While it is widely accepted that the epicenter of reward in the brain, whether natural or drug, is the mesolimbic dopamine system, much controversy exists regarding the role of dopamine in modulating goal-directed behavior. Mesolimbic dopamine has been proposed to play a role in functions such as movement, motivation, reward, learning, arousal, attention and emotion (Gonzales et al., 2004). This makes sense because each of these individual behavioral components is necessary for the outward, measurable behavior of reward (i.e., an organism must locate a reward, pay attention and learn where to find it, like it and have a desire to return to it).

MESOCORTICOLIMBIC DOPAMINE PATHWAY

It widely accepted that the mesocorticolimbic dopamine system plays a central role in modulating the rewarding effects of drugs of abuse (Koob, 1992; Wise and Bozarth, 1987). Olds and Milner first identified this pathway in 1954. Using brain stimulation reward (BSR) they discovered that rats returned to the same region of a testing apparatus where they had received electrical stimulation to the septal area of the brain (Olds and Milner, 1954). Upon further examination using mapping and lesion studies, it was determined that the most sensitive sites in the brain (i.e., lowest stimulation threshold) were along the medial forebrain bundle (MFB) which connects the ventral tegmental area (VTA) to the basal forebrain (Corbett and Wise, 1980; Olds and Milner, 1954; Wise, 1981). Next,

using pharmacology, studies showed that dopaminergic receptor blockade attenuated brain stimulation reward (Liebman, 1983; Wise, 1978), suggesting that specific neurotransmitter systems were involved in reward mechanisms (Wise, 1987).

Flash-forward almost 60 years and what was once commonly referred to as the 'reward circuit' is now known as the mesolimbic dopamine pathway. This pathway consists of dopaminergic neurons whose cell bodies originate in the ventral tegmental area (VTA), a region of the midbrain, and project to regions of the limbic system including the nucleus accumbens (NAc), amygdala and hippocampus. An additional dopaminergic pathway, the mesocortical pathway, also originates in the VTA and project to regions of the prefrontal cortex. These pathways are shown in a simplified diagramed in Fig. I-1.
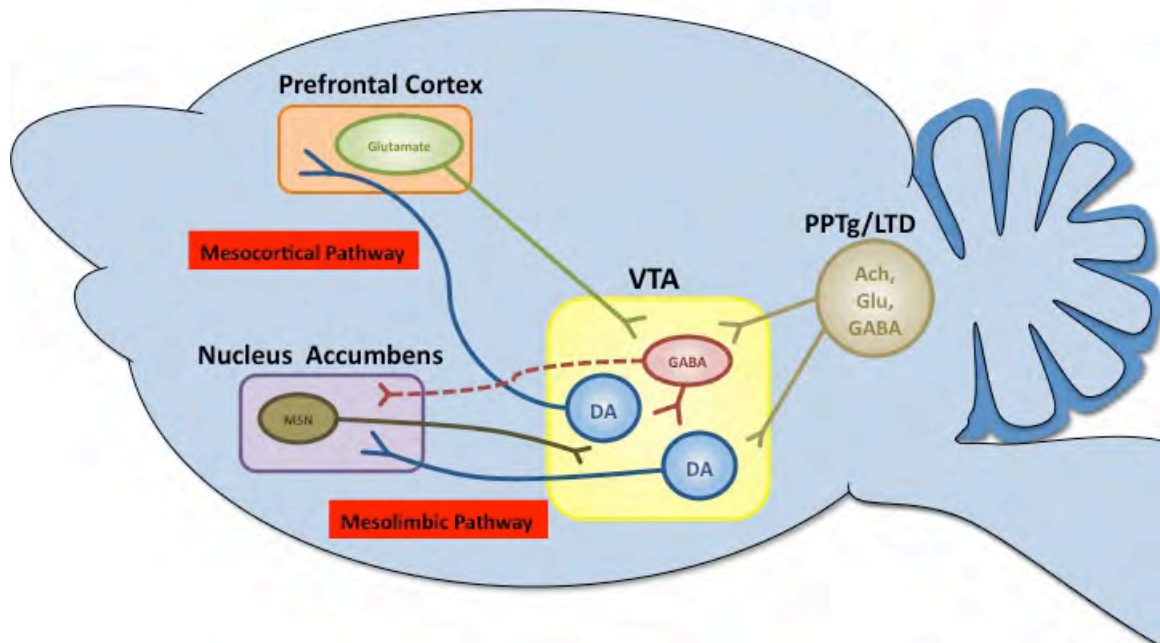
Figure I-1. The Mesocorticolimbic Dopamine Pathway in a rodent brain. DAergic neurons originate in the VTA and project to the NAc making the mesolimbic pathway and to the prefrontal cortex, making the mesocortical pathway. The majority of GABAergic neurons in the VTA are interneurons while a small subset also project from the VTA to the NAc as well as other forebrain regions. The VTA receives ACh, glutamate and GABAergic inputs from the PPTg and LTD and glutamate inputs from the prefrontal cortex.

THE VENTRAL TEGMENTAL AREA

The VTA is known to at least partially mediate the rewarding effects of nicotine, opiates, ethanol and cannabinoids (Ikemoto, 2007). For example, rats and mice will self-administer opiates (Bozarth and Wise, 1981), cannabinoids (Zangen et al., 2006), cocaine (Rodd et al., 2005), nicotine (David et al., 2006) or ethanol (Gatto et al., 1994; Rodd-Henricks et al., 2000) directly into the VTA. Additionally, intravenous nicotine self-administration is attenuated by either selective lesions of VTA dopaminergic neurons in rats (Corrigall et al., 1992) or by a local VTA infusion of a nicotinic receptor antagonist (Corrigall et al., 1994).

The ventral tegmental area is located in the midbrain, medial to the substantia nigra and ventral to the red nucleus (Paxinos, 2000). It is referred to as an 'area' and not considered to be a 'nucleus' because the cryoarchitecture of the region is not well defined such that the boundaries of the VTA are determined by its neighboring structures (Fields et al., 2007; Ikemoto, 2007). Within the VTA are two main cell populations, the A10 DAergic projection neurons, which comprise ~60% of cells in this region (Swanson, 1982), as well as local GABAergic interneurons (Carr and Sesack, 2000; Margolis et al., 2006b). The VTA receives inputs from regions throughout the CNS (Geisler and Zahm, 2005) including glutamatergic projections from the prefrontal cortex (Sesack and Pickel, 1992), as well as glutamatergic, cholinergic and GABAergic projections from two groups of mesopontine tegmental area neurons, the pedunculopontine tegmental nucleus (PPTg) and the laterodorsal tegmental nucleus (LDT) (Cornwall et al.,

1990; Oakman et al., 1995; Semba and Fibiger, 1992). Other regions projects to the VTA include the NAc, amygdala, ventral pallidum, superior colliculus and lateral hypothalamus (Fields et al., 2007). Additionally, the lateral habenula, a small nucleus that is a part of the epithalamus, has been shown to project to midbrain areas that modulate the release of dopamine including the VTA and substantia nigra pars compacta (Herkenham and Nauta, 1979; Ji and Shepard, 2007; Matsumoto and Hikosaka, 2007).

Projections from the VTA are primarily to the ventromedial striatum including the nucleus accumbens shell and core as well as smaller projections to the prefrontal cortex (PFC), hippocampus, entorhinal cortex and lateral septal areas (Fields et al., 2007). Furthermore, studies using retrograde tracers have shown that distinct groups of neurons originating in the VTA project to specific forebrain regions (Fallon et al., 1984; Margolis et al., 2006a). Projections to the NAc contain the largest proportion of DA neurons, with 65-85% being DAergic, while the PFC projections are only 30-40% DAergic (Fallon et al., 1984; Swanson, 1982). The remaining component of VTA afferents to the NAc and PFC arise from GABAergic neurons (Carr and Sesack, 2000). Recently, evidence has shown that the VTA is not a homogeneous region and can be divided into three subregions, the anterior VTA, posterior VTA and the tail VTA. Additionally, evidence indicates that each region may project to distinct regions of the striatum and may also respond differently to drugs of abuse (Ikemoto, 2007; Shabat-Simon et al., 2008).

THE NUCLEUS ACCUMBENS

For decades, the nucleus accumbens has been a main focus of mesolimbic dopamine in studies of natural and drug reward (Gonzales et al., 2004). It is located in the ventromedial striatum and is primarily composed of GABAergic medium spiny neurons (~95 %) and to a lesser extent, cholinergic interneurons (1-2 %). Two distinct regions of the nucleus accumbens have been described, the core and shell, based on differences in functions and anatomical connectivity (Heimer et al., 1991; Zahm, 1999). Additionally, studies have shown that the response to extracellular dopamine release of these two regions differs. For example, it has been shown that the dopamine release induced by a food reward is rapidly habituated in the shell, but not the core (Bassareo et al., 2002). Another study showed differential NAc shell and core Fos immunolabeling of cholinergic interneurons after cocaine self-administration (Berlanga et al., 2003). These and other data suggest the possibility that the shell may act to modulate the initiation of drug seeking behavior by mediating the hedonic states associated with reward (Pecina and Berridge, 2000; Rodd-Henricks et al., 2002) while the core may modulate acquisition and maintenance of drug seeking (Ito et al., 2004).

The extracellular dopamine concentration in the NAc is regulated by two main factors; (1) the rate of release of dopamine from DAergic neurons that originate in the VTA and (2) by dopamine uptake through dopamine transporters located in perisynaptic areas (Nirenberg et al., 1997). DAergic neurons of the

VTA are known to be the main input source of extracellular DA in the NAc. Under normal conditions, the action potential (AP) firing rate of DAergic neurons is tonic with spike activity at 1-5 Hz (Grace and Bunney, 1984). However, when an unexpected presentation of a primary reward or a reward-predicting stimulus occurs, the firing rate increases to 2-10 APs at 10-30 Hz (Pan et al., 2005; Schultz, 1998).

DRUGS OF ABUSE

Drugs of abuse are varied in chemical structure and act on several different molecular targets to hijack the endogenous brain reward circuitry. While drugs can have different physiological effects (i.e., sedative or stimulant) it is clear that they all act, at least acutely, on the mesolimbic dopamine pathway to increased dopamine in the NAc (Wise and Rompre, 1989). The primary molecular targets for most drugs of abuse including nicotine, cocaine, amphetamines and opioids has been extensively studied and well defined (Gonzales et al., 2004). Some drugs act on the primary, dopamine containing neurons, while others act on secondary neurons within the circuit. For example, nicotine binds to and activates nicotinic acetylcholine receptors (nAChRs), ligand gated ion channels that modulate presynaptic dopamine release, while cocaine binds to and blocks the presynaptic dopamine transporter (Koob et al., 1998). Other drugs such as morphine and heroin increase dopamine release by acting at the circuit level. These drugs bind G-protein coupled opioid receptors located on GABAergic interneurons and cause them to become hyperpolarized. This

reduces the GABA input to the DAergic neurons, leading to a disinhibition of DA neurons, which results in an increase of DA release. Thus, the effect of most drugs of abuse in the brain is the result of the molecular interactions between the drug and the primary molecular targets found on neurons that result in an increase of DA release in the NAc. However, the molecular mechanisms of how ethanol alters the activity of mesolimbic dopamine system are not entirely clear.

I.D. Alcohol's action in the mesolimbic pathway

There are likely to be similarities and overlapping mechanisms in how the brain is affected by alcoholism compared to other drugs of abuse. Although it is clear that most addictive drugs produce an increase in mesolimbic DA release, the precise mechanisms that lead to an increase in extracellular DA vary according to the molecular targets upon which each specific drug acts. So understanding how alcohol produces the disease alcoholism requires knowledge of how ethanol affects particular molecular, cellular and system level functions. ACUTE EFFECTS OF ETHANOL ON DOPAMINERGIC NEURONS OF THE VTA

Several papers published in the late 1980s and early 1990s further implicated the role of the mesolimbic DA system in mediating the reinforcing properties of ethanol. In 1985, ethanol was first shown to cause a dose-dependent increase in the spontaneous firing rate of DAergic neurons *in vivo* (Gessa et al., 1985). This paper also showed that DAergic neurons of the VTA

were activated by lower doses of ethanol compared to the neighboring DAergic neurons in the substantia nigra. This suggested that ethanol preferentially activated the DAergic neurons of VTA. Soon after, it was shown that systemic administration of several drugs of abuse, including ethanol, stimulated DA transmission, resulting in increased dopamine levels in the NAc (Di Chiara and Imperato, 1988; Imperato and Di Chiara, 1986). Later, additional studies found that rats will self-administer ethanol directly into the VTA (Gatto et al., 1994), and more specifically, the posterior VTA (Rodd et al., 2004). Interestingly, studies also found that ethanol induced a dose-dependent increase in spontaneous firing of VTA DAergic neurons that were placed in media that blocked synaptic transmission or even in acutely dissociated DAergic neurons (Brodie et al., 1999; Brodie et al., 1990) suggesting that ethanol acts directly on these DAergic neurons. Thus, the search for primary targets of ethanol has been a main focus of the alcohol research field.

THE PROTEIN THEORY: ETHANOL'S PRIMARY TARGETS

Until the 1980s, it was believed that alcohol affected the central nervous system by acting on and perturbing membrane lipids of CNS neurons (Spanagel, 2009). Several lipid theories were proposed and these suggested that alcohol affected the membrane fluidity, disordering, volume occupation or expansion (Peoples et al., 1996). This theory however, has fallen out of favor in recent years due to several restrictions of the hypothesis. For example, to induce membrane disorder, extremely high concentrations of alcohol (>500 mg/dl blood

alcohol level (BAL)) that are close to the LD_{50} of ethanol in humans is necessary. Even at high physiological concentrations of ethanol (~300 mg/dl) that induce loss of consciousness, only 1 alcohol molecule would be present per ~200 lipid molecules (Peoples et al., 1996; Spanagel, 2009). These and other observations led to an ideological switch in the 1970s to a theory of alcohol that suggested that alcohols bound directly to proteins, producing conformational changes that diminished or abolished their activity (Eyring et al., 1973).

Three general mechanisms of action were proposed as to how alcohols could interact with proteins such as ligand-gated ion channels (Peoples et al., 1996). Alcohols could interact with the ligand-binding site and act as agonists or competitive antagonists, they could act on modulatory sites of the receptor to change agonist binding or cause the opening of the channel to occur more or less frequently, or alcohols could act as a physical channel blocker by binding a site within the ion channel lumen (Peoples et al., 1996). Therefore, determining which proteins are sensitive to ethanol and elucidating how ethanol affects the structure and function of those proteins to alter cellular physiology are of particular importance. Additionally, the protein theory is favorable from a therapeutic standpoint because developing pharmacotheraputic drugs targeted for proteins tend to be more specific than drugs targeted to lipids.

Indeed, this theory was validated by the findings that physiological concentrations of ethanol, ranging from 30 to 100 mM, directly affect the function of several ligand-gated ion channels including glutamate, $GABA_A$, glycine,

nAChR and 5-hydroxytryptamine 3 (serotonin) (5-HT_3) receptors (Lovinger, 1997). Several studies have identified a role for alcohol action on glycine and $GABA_A$ chloride-sensitive ion channels, with the most common effect being an enhancement of receptor function (Mihic et al., 1997; Suzdak et al., 1986; Wick et al., 1998). Similarly, alcohol has been shown to potentiate 5-HT_3 receptors (Lovinger, 1999) and neuronal nAChR function (Narahashi et al., 1999), which will be discussed in more detail in the next section. Interestingly, this potentiating effect of ethanol is similar in these receptor channels that are all part of the cys-loop family. This family shares similar features including a pentameric structure, four transmembrane (TM) domains per subunit, a membrane-spanning pore forming region in TM2, and a short loop defined by a disulfide bond in the amino terminal region (Lovinger, 1997). Unlike the previously mentioned receptors, alcohol was shown to inhibit the ionotropic glutamate receptors including the AMPA, NMDA and kainate, but with varied mechanisms and effects (Dopico and Lovinger, 2009).

Several non-ligand-gated ion channels have also been implicated as primary targets of alcohol. For example, alcohol inhibits L-type Ca^{2+} channels (Wang et al., 1994), opens G-protein-activated inwardly rectifying K^+ channels (GIRKS) (Kobayashi et al., 1999), and modulates the steady-state activity of large-conductance calcium- and voltage-gated potassium (BK) channels (Dopico et al., 1996).

While these receptors and ion channels are the currently known primary targets of ethanol, the action of ethanol depends on several variables including the concentration of ethanol and subunit composition of the channel or receptor (Spanagel, 2009). Additionally, the overall effect of how alcohol's modulation of these proteins at the molecular level contributes to altered neuronal cell physiology, and eventually to changes in circuit level processing to mediate the rewarding properties of alcohol remains an open question.

MOUSE MODELS OF ALCOHOLISM

The disease alcoholism is a very complex disorder that cannot easily be reproduced in its entirety. Fortunately, several animal models exists that focus on specific aspects of human addictions including self-administration, reward, withdrawal and locomotor activation (Schlaepfer et al., 2008b). Furthermore, the animal models used to represent the human aspects have strong face validity (Koob et al., 2009). This means that the animal model seems to be a valid representation of what is supposed to be measured (i.e., the animal phenotype resembles a human trait). It is useful to develop animal models defined by the symptoms that are specific for each stage of the addiction cycle including the binge-intoxication stage, the withdrawal-negative affect stage and the preoccupation-anticipation stage. For the purpose of this thesis, I will focus on animal models of the binge-intoxication stage.

To create an accurate representation of alcohol addiction in an animal model, certain criteria have been proposed: 1) ethanol should be orally self-

administered; 2) the amount of ethanol consumed should elevate blood ethanol concentration (BEC) to pharmacologically significant levels; 3) ethanol should be consumed for its pharmacological effects, rather than calories, taste or smell; 4) ethanol should be positively reinforcing; 5) chronic ethanol consumption should produce metabolic and functional tolerance; and 6) chronic ethanol consumption should produce signs of physical dependence (Cicero, 1980; Rhodes et al., 2005). In reality, it would be quite difficult to include all of these criteria into one model, but partial models do exist.

Preference drinking is one of the most widely used partial mouse models of alcoholism (Belknap et al., 1993; McClearn and Rodgers, 1959; Metten et al., 1998). In this assay, animals receive a bottle of plain water and a bottle of an ethanol solution in their home cage and consumption of each solution is measured over a 24hr period for several days to weeks to determine the animal's preference for ethanol or water. This assay led to the discovery of certain mouse lines that have genetically based preferences for ethanol.

For instance, the C57BL/6 strain of inbred mouse has an inherently high preference for ethanol, CBA/J mice have a moderate preference for alcohol and the DBA/2 strain will almost completely avoid ethanol (McClearn and Rodgers, 1959; Rhodes et al., 2007). Indeed, several studies have confirmed that C57BL genes are a strong predictor for high alcohol drinking by showing that other strains in the C57 lineage including C57/LJ, C57BR/cdJ, C58/J and C57BL/10J also show high ethanol preferences (Belknap et al., 1993; Rhodes et al., 2007;

Rodgers, 1966). One proposed hypothesis for the difference in alcohol preference in C57BL/6J mice is that this strain contains fewer dopamine D2 receptors in the hypothalamus and hippocampus compared to strains that avoid alcohol such as DBA/2J mice (Ng et al., 1994). This same result has also been found in rats that are selectively bred for high alcohol preference compared to rats with low alcohol preference (McBride et al., 1997; Stefanini et al., 1992). Additionally, C57BL/6J mice treated with dopamine receptor agonists have decreased ethanol consumption (Ng and George, 1994), suggesting that reduced dopamine function may result in the high ethanol preference seen in C57BL/6J mice (Kamdar et al., 2007).

However, even when using high alcohol preferring mouse strains in preference drinking assays, mice will rarely reach BEC levels that produce measurable effects on behavior and physiology and the measured BECs also tend to fluctuate with drinking throughout the 24 hour period (Dole and Gentry, 1984). As a result, this would not be an appropriate assay to use to test the efficacy of pharmacotherapeutic drugs for reducing ethanol intake (Le et al., 1994; Rhodes et al., 2005).

When the period of alcohol intake is limited in the two-bottle assay, from 24 hours to a short period each day, several studies found that the rate of ethanol consumption and BEC is increased relative to continuous access (Linseman, 1987; Macdonall and Marcucella, 1979); however, the BECs achieved in the limited access assay remained low (~0.50 mg/ml). Interestingly,

in a study of the temporal pattern of ethanol intake, it was shown that rats consumed ethanol in discrete bouts mostly during the dark phase of the light-dark cycle (Gill et al., 1986).

Many labs have developed models of alcohol self-administration in which mice do reach pharmacologically significant levels of BEC. One paradigm requires a 35-day ethanol training period, food restriction to 80 % of baseline weight, and periodic water deprivation for 22 hours followed by placement of food in the cage 1h prior to placement of the ethanol bottle to induce "high thirst motivation" (Middaugh et al., 1999). While this assay results in mice reaching high ethanol intake (~8 g/kg) and BECs (~3.5 mg/ml), it does not have high face validity (Rhodes et al., 2005) because of the altered motivational state of the mice. Variations on this assay include using a sucrose fading design where mice are initially exposed to high sucrose and low ethanol concentrations and over the course of several days the sucrose concentration decreases while the ethanol concentration increases (Samson, 1986; Tolliver et al., 1988). In this assay, mice are trained to consume up to 3 g/kg ethanol and reach BECs averaging 2.5 mg/ml (Ryabinin et al., 2003). Similarly, water deprivation fading prior to ethanol placement results in behavioral intoxication in motor coordination tests (Cronise et al., 2005; Finn et al., 2005).

An additional model of alcoholism that has greater face validity is operant conditioning. In this assay the mice learn to perform a behavior such as a nose poke or lever press in order to receive ethanol. While this assay does produce

pharmacologically significant drinking in rodents, it requires a lengthy training period and much attention to individual animals (Rhodes et al., 2005)

In 2005, a new rodent model of alcoholism was introduced that does not require food or water restriction and produces pharmacologically significant drinking. This model, now referred to as "Drinking-In-The-Dark" (DID), is a relatively simple, limited access procedure that allows for high-throughput screening of drugs that may act to alter ethanol consumption (Rhodes et al., 2005). The procedure works by taking advantage of the normal diurnal rhythm of consumatory behavior of mice (Freund, 1970; Goldstein and Kakihana, 1977). Mice eat and drink according to a sinusoidal curve and the peak time period of increased eating and drinking occurs within the first few hours into the dark cycle (Kurokawa et al., 2000). Thus, the assay begins 2 hours after the lights are off when the water bottle is removed from the home cage and replaced with a single ethanol bottle that is left in place for 2 hours. Ethanol intake is recorded and converted to grams ethanol per kilogram body weight of the mouse.

The DID assay has many benefits over the previously described methods. For instance, from the first day of presenting the ethanol bottle, mice will consume high concentrations of ethanol. Interestingly, the concentration of ethanol (10 %, 20 %, or 30%) does not affect the quantity of ethanol consumed in g/kg, but does affect the volume consumed over the 2-hour period (Rhodes et al., 2005). This is important for screening drugs that may act to alter acute ethanol intake. By eliminating the acclimation process of slowly increasing ethanol

concentrations over periods of weeks, any changes that would be induced by chronic ethanol consumption are also eliminated. Then, when the assay is repeated for 2 or more days, mice will regularly consume ethanol to physiologically relevant concentrations of > 1.0 mg/ml BEC. One pitfall of this assay is that it only works well with the high alcohol preferring strain of mice, C57BL/6J; however, this happens to be the genetic background strain used in our lab. Thus, the DID procedure was adopted and modified for use in our lab.

I.E. nAChRs: Structure, function and role in addiction

nAChRs are fast ionotropic receptors that are activated by ACh and nicotine (Albuquerque et al., 2009). They belong to the superfamily of Cys-loop ligand-gated ion channels that include receptors for γ-amino butyric acid (GABA$_A$ and GABA$_C$), glycine and 5-hydroxytryptamine (5-HT$_3$) (Changeux and Edelstein, 1998; Le Novere and Changeux, 1995). These ligand-gated ion channels have similar structural and functional features. All subunits in this family contain a pair of disulfide-bonded cysteines separated by 13 residues (Cys-loop) in their extracellular amino terminus (Karlin, 2002) (Fig. I-2A). Nicotinic receptors are formed by an arrangement of 5 subunits around a central pore with an extracellular endogenous ligand-binding domain that is distinct from the channel pore (Albuquerque et al., 2009).

In vertebrate species, 17 nAChR subunits have been identified (α1-α10, β1-β4, γ, δ, and ϵ) all of which can be found in humans and other mammalian

species, except for α8 which has only been identified in avian species (Millar and Gotti, 2009). Subunits are classified as either α-, by the presence of a Cys-Cys pair near the start of TM1, or non-α when the Cys pair is missing (Changeux and Edelstein, 1998; Le Novere and Changeux, 1995) (Fig. I-2A). Within the family of nAChRs, one more classification must be made between the muscle-type nAChRs and neuronal-type nAChRs. The distinction between these types of receptors was first discovered by performing binding assays using radiolabled nicotinic agonists. Muscle-type nAChRs bind the snake venom αBungarotoxin (αBgtx) at nM affinity, while neuronal-type nAChRs bind other ^{3}H-agonists with nM affinity but do not bind αBgtx (with the exception of α7 containing nAChRs) (Lukas and Bencherif, 1992). Later, it was discovered that the subunit composition as well as the major sites of expression differs between muscle and neuronal nAChRs (Millar and Gotti, 2009). The muscle nAChRs consist of the α1, β1, γ, δ, and ϵ subunits and can form functional receptors with only two subunit compositions, α1, β1, γ, δ in adult tissues or α1, β1, γ, ϵ in embryonic tissues (Mishina et al., 1986; Takai et al., 1985). Muscle nAChRs mediate the transmission of nerve signals to skeletal muscle, which is critical for the operation of voluntary and involuntary muscle response (Lindstrom, 1997). Thus, dysfunction of these receptors results in neuromuscular disorders that affect muscle control such as Myasthenia Gravis (Lindstrom, 1997).

NEURONAL nAChRs: STRUCTURE

Neuronal nAChRs regulate processes including neurotransmitter release, and cell excitability (Gotti and Clementi, 2004; Role and Berg, 1996). These receptors, as a part of the cholinergic system, have been implicated in modulating many physiological functions including anxiety, pain, arousal, and food intake (Changeux and Edelstein, 2001; Gotti and Clementi, 2004; Gotti et al., 1997). Further, disruption of neuronal nAChR function and cholinergic transmission has been implicated in several CNS diseases including Alzheimer's disease, Parkinson's disease, drug addiction, and schizophrenia among others (Hogg et al., 2003; Lindstrom, 1997).

Neuronal nAChRs, like all members of the cys-loop family of ligand-gated channels are formed by the arrangement of five subunits to create a central pore (Albuquerque et al., 2009). The structure of neuronal nAChRs is homologous to muscle nAChRs (Karlin, 2002), for which the atomic structure has been determined from electron microscopy studies from the fish electric organ (*Torpedo* nAChRs) (Miyazawa et al., 2003; Unwin, 2005). Each nAChR gene encodes a protein subunit consisting of a large amino-terminal extracellular domain composed of β-strands, four transmembrane α-helices segments (M1-M4), a variable intracellular loop between M3 and M4, and an extracellular carboxy-terminus (Corringer et al., 2000) (Fig I-2A). The extracellular N-terminus contains the ACh binding domain that forms a hydrophobic pocket located between adjacent subunits in an assembled receptor (Sine, 2002). The M2

segment of all five subunits forms the conducting pore of the channel, and regions in the M2 intracellular loop contribute to cation selectivity and channel conductivity (Corringer et al., 2000).
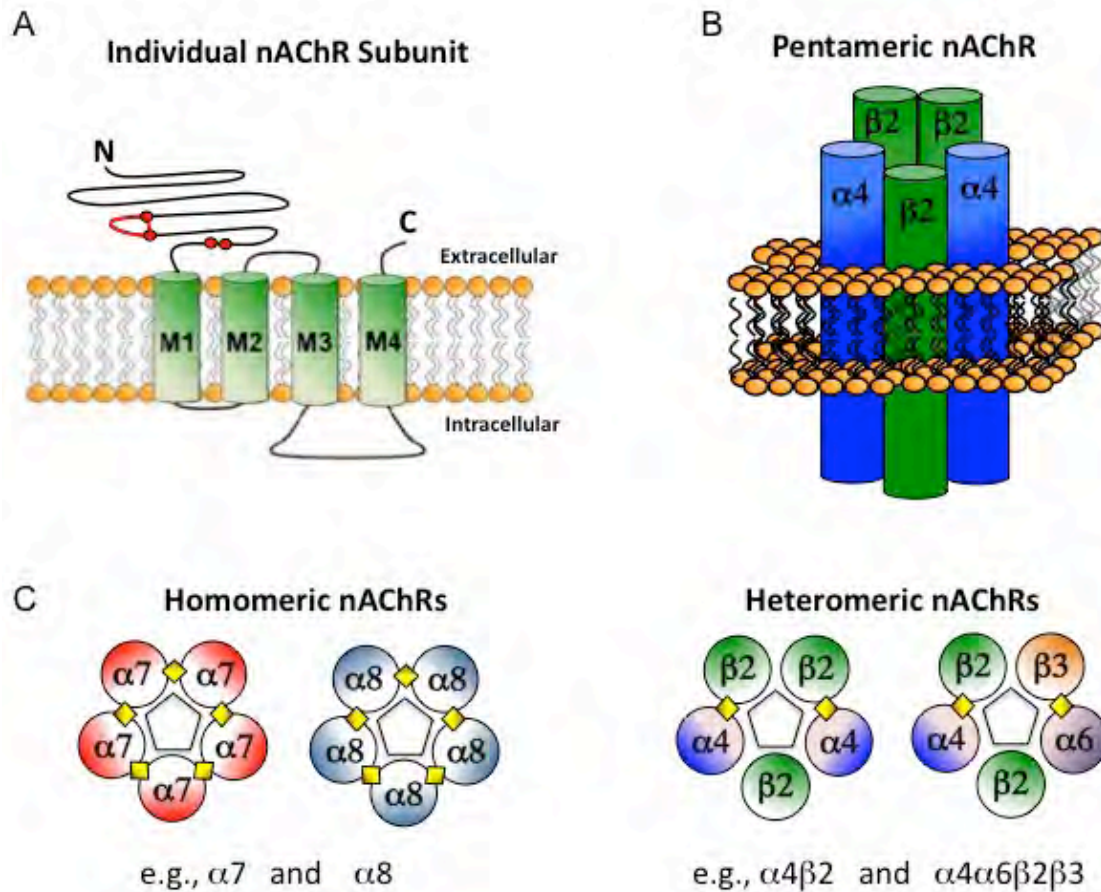
Figure I-2. Structure of nicotinic acetylcholine receptors. A) The membrane topology of an individual nAChR subunit. The N-terminal extracellular domain contains two cysteine pairs (red circles) near the beginning of TM1 which would designate this as an α subunit. Also shown in red is the Cys-Cys loop, characteristic of all Cys-loop ligand-gated ion channel subunits. B) Diagram of the pentameric subunit arrangement of an assembled heteromeric nAChR. C) Subunit composition of two examples of homomeric nAChRs (left) and two heteromeric nAChRs (right). The yellow diamonds indicate the proposed location of the ACh binding site(s). Figure modified with permission from (Improgo et al., 2010).

Of the 17 total nAChR subunits, twelve have been identified as neuronal subunits and consist of nine α subunits (α2- α10) and three β subunits (β2-β4) (Le Novere and Changeux, 1995). As previously mentioned, α subunits contain two adjacent cysteines at positions that are homologous to those present at the α1 muscle-type nAChR while the β subunits lack the cysteine pair (Changeux and Edelstein, 1998). Five subunits combine to form two classes of receptors: homomeric receptors containing only α subunits (α7- α9) or heteromeric receptors that contain α and β subunits (α2- α6 and β2- β4) (Dani and Bertrand, 2007) (Fig. I-2B, 2C). The most commonly found subtypes in the brain are the low affinity α7 homomeric and high affinity α4β2* heteromeric nAChRs. An asterisk in nAChR nomenclature (i.e., α4*, α4β2*) indicates that other nAChR subunits are also present and can be read as "α4 containing nAChRs". This subunit diversity contributes to nAChRs with distinct pharmacological and biophysical properties (Gotti et al., 2007; McGehee and Role, 1995).

Using heterologous expression systems, the subunit composition and resulting pharmacological and biophysical properties of functional nAChRs has begun to be elucidated. When each nAChR subunit was expressed in *Xenopus* oocytes alone, the only functional homomeric receptors preferentially formed were those composed of α7, α8, or α9 nAChR subunits (Couturier et al., 1990; Gerzanich et al., 1994; Gotti et al., 1994). Together, these homomeric nAChRs have high Ca^{2+} permeability, a rapid desensitization rate and are blocked by nanomolar concentrations of αBgtx and are therefore termed, αBgtx-nAChRs

(Gotti and Clementi, 2004). Notably, α8 nAChRs have only been identified in the chick nervous system (Gotti et al., 1994) and while the α9 nAChR subunit can form homomeric nAChRs (Elgoyhen et al., 1994), studies show that it forms functional nAChRs much more efficiently when co-expressed with the α10 subunit (Elgoyhen et al., 2001; Sgard et al., 2002).

α7-containing nAChRs are one of the most abundantly expressed subtypes in the brain and account for the majority of high affinity αBgtx binding (Gotti and Clementi, 2004). In the hippocampus, α7 nAChRs presynaptically modulate glutamate and GABA release (MacDermott et al., 1999). Additionally, in other perisynaptic areas α7 nAChRs regulate other inputs to neurons as well as activate downstream cell signaling pathways (Berg and Conroy, 2002; Shoop et al., 1999). Recently, evidence from heterologous expression systems has indicated that α7 can also form functional heteromeric receptors when co-assembled with β2 (Khiroug et al., 2002) and this finding was later confirmed by the discovery of similar α7-containing heteromeric nAChRs in rat brain (Liu et al., 2009). However, these α7-containing heteromeric nAChRs have distinct ion permeability and pharmacological properties compared to α7 homomeric nAChRs (Khiroug et al., 2002). The remaining neuronal nAChR subunits (α2-α6 and β2-β4) do not form functional nAChRs when expressed alone in *Xenopus* oocyte systems (Boulter et al., 1987; Luetje and Patrick, 1991) and are found to be contained within the cell and not expressed on the cell surface (Cooper et al., 1999; Millar and Gotti, 2009).

In addition to α7 homomeric receptors, nAChRs containing the α4 and β2 subunits (i.e., α4β2* nAChRs) are the most abundant and widely distributed heteromeric receptors in the brain and contribute to the high affinity nicotine binding sites (Champtiaux and Changeux, 2002). In heterologous expression systems, additional pair-wise combinations of functional nAChRs include α2β4, α3β2, α3β4, and α4β4 (Duvoisin et al., 1989; Papke et al., 1989). The α5 and α6 nAChR subunits are unable to form functional nAChRs unless co-expressed with additional α and β subunits to form triplet nAChRs. For example, α5* nAChRs include α3α5β2, α3α5β4 and α4α5β2 and α6* nAChRs include α6β3β4, α3α6β4 and α3α6β2 (Millar and Gotti, 2009).

Through the use of nAChR knock-out and knock-in mice, *in situ* hybridization and subtype-specific ligands and antibodies, much work has been done to elucidate the native nAChR subtypes present in nervous system. As discussed previously, α7 homomeric and α4β2 heteromeric nAChRs are the most abundant subtypes in mammalian brain. However, studies now indicate that approximately 20 % of all native α4β2 nAChRs also contain the α5 subunit (Brown et al., 2007). And, in specific brain regions, additional nAChR subtypes have been found (Millar and Gotti, 2009). For example, the α3β4 subtype is the most abundant nAChR in the autonomic ganglia and subsets of the medial habenula and interpeduncular nucleus (Millar and Gotti, 2009). In the rodent mesostriatal pathways, 40 - 60 % of all α6* nAChRs are present as α4α6β2β3 with the remaining subtype as α6β2β3 (Zoli et al., 2002) and each of these

subtypes have different affinities for the antagonist α-conotoxin MII (Salminen et al., 2007; Zoli et al., 2002).

This vast diversity of nAChR subunit expression allows for specialization of nAChR functions including receptor open times and single channel conductance as well as pharmacological properties such as agonist and antagonist binding affinities (McGehee and Role, 1995). Additionally, the selective expression of nAChR subunits also allows for specific brain regions to be more or less affected by exposure to nicotine (Albuquerque et al., 2009). For instance, DA neurons in the VTA express the α6 and β3 subunits in addition to α4 and β2 (Hendrickson et al., 2010) and this subtype (α4α6β2β3*) has been shown to have one of the highest affinities for nicotine (Salminen et al., 2007).

Unlike muscle-type nAChRs, neuronal nAChRs can have various receptor stoichiometries, which can affect agonist specificity and the function and regulation of the receptor (Nelson et al., 2003; Zwart and Vijverberg, 1998). For example, α4β2 nAChRs can be formed by either two α and three β subunits ($(\alpha4)_2(\beta2)_3$) or three α and two β subunits ($(\alpha4)_3(\beta2)_2$) (Moroni et al., 2006; Nelson et al., 2003; Zwart and Vijverberg, 1998). Interestingly, while these α4β2 nAChRs have similar high affinities for nicotine, the $(\alpha4)_2(\beta2)_3$ subtype has been found to be most sensitive to upregulation by nicotine (Albuquerque et al., 2009), thus, adding another layer to the complexity of nAChR structure and function.

NEURONAL nAChRs: FUNCTION

Upon ligand binding, a conformational change of the receptor occurs, opening the channel within microseconds, allowing the passage of small monovalent (K^+ and Na^+) and divalent (Ca^{2+}) cations through the pore, down their electrochemical gradient (Dani and Bertrand, 2007; Laviolette and van der Kooy, 2004). The ligand-binding site is a hydrophobic pocket at the interface between adjacent α subunits for homomeric nAChRs or between the α subunit and the 'back' face of the neighboring β subunit for heteromeric nAChRs (Gotti and Clementi, 2004). For all nAChRs, the 'front' side of the ligand-binding site is formed by the α subunit, where the Cys-Cys pair is required (Albuquerque et al., 2009).

Neuronal nAChRs can exist in three conformational states and are regulated by exposure to agonist: closed at rest, when the receptor has low affinity for agonist and the channel is closed; the active state, when agonist occupies the ligand binding site and the channel is open allowing cations to flow down their electrochemical gradient; and the desensitized state, when the channel is closed and the receptor is not responsive to ligand (Albuquerque et al., 2009; Dani and Bertrand, 2007). The wide distribution of nAChRs on cellular membranes results in several physiological outcomes, depending on the location of the receptor.

Interestingly, while nAChRs mediate fast, direct synaptic transmission at neuromuscular junctions and autonomic ganglia, there are very few examples of

fast nicotinic transmission in the mammalian brain (Dani and Bertrand, 2007). Instead, studies show that a significant proportion of nAChRs are located presynaptically (Role and Berg, 1996) where they facilitate Ca^{2+} dependent release of neurotransmitters (Wonnacott, 1997). This may occur indirectly as a result of Na^+ influx causing membrane depolarization and activation of voltage-gated Ca^{2+} channels or directly through Ca^{2+} influx through the channel itself (α7 nAChRs, see below) (Albuquerque et al., 2009). Additionally, nAChRs are found on the somas of both GABAergic and DAergic neurons of the VTA (Wooltorton et al., 2003). Activation of these somatodendritic nAChRs by nicotine mediates neuronal excitability (Pidoplichko et al., 1997) as well as modulates gene expression through the local increases of Ca^{2+} in the cytoplasm (Dajas-Bailador and Wonnacott, 2004). For example, α7 homomeric nAChRs have a higher $Ca^{2+}:Na^+$ permeability ratio compared to other nAChRs (Albuquerque et al., 2009). Thus, activation of an α7 nAChR can affect several Ca^{2+} dependent second messenger systems (Suzuki et al., 2006).

nAChRs MEDIATE NICOTINE ADDICTION

Several studies have shown that the mesolimbic DA pathway mediates nicotine addiction because DA antagonists or lesions of DA neurons of this pathway reduce nicotine self-administration (Corrigall and Coen, 1989; Corrigall et al., 1994; Corrigall et al., 1992; Di Chiara, 2000). Nicotine from tobacco smoke rapidly crosses the blood brain barrier, binds and activates its molecular targets, nAChRs. The activation of nAChRs located on DAergic neurons of the

VTA causes an increase in burst firing as well as overall firing rates (Dani and Bertrand, 2007) resulting in DA release in the NAc (Nisell et al., 1994). This nicotine-induced increase of DA release in the NAc, and the resulting rewarding properties, are mediated by nAChRs because infusion of a nAChR antagonist into the VTA blocks the development of nicotine reward behaviors (Corrigall et al., 1994; Corrigall et al., 1992).

The subtype of nAChR found on the DAergic and GABAergic neurons of the VTA are predominantly the high affinity α4β2* nAChRs and after nicotine activates these receptors, they transition to a desensitized state (Dani and Bertrand, 2007; Pidoplichko et al., 1997). At the same time, nicotine activates presynaptic α7 nAChRs located at the terminals of glutamate neurons, thus increasing glutamatergic transmission onto the DA neurons (Mansvelder and McGehee, 2000; Schilstrom et al., 2000). The α7 nAChRs rapidly desensitize at high agonist concentrations; however, at the nicotine concentrations achieved through smoking, the receptors do not significantly desensitize (Wooltorton et al., 2003). Thus, the net effect of decreased GABAergic transmission and increased glutamatergic transmission results in excitation of the mesolimbic DA reward system (Mansvelder et al., 2002).
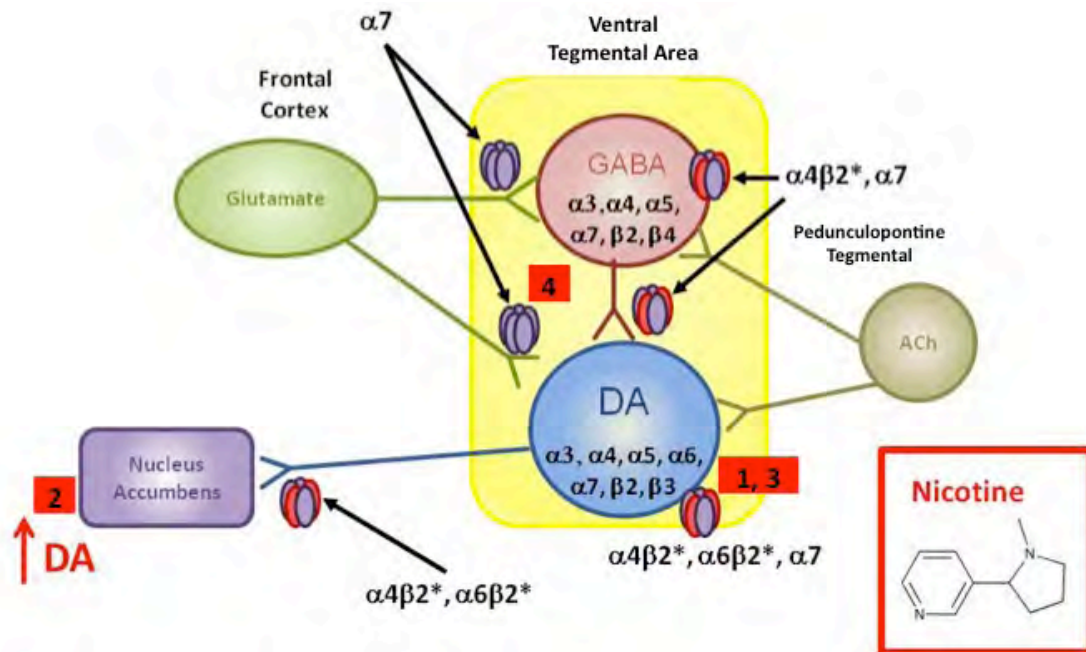
FIGURE I-3. nAChR subtypes within the VTA & implications for nicotine addiction. Schematic diagram of the mesolimbic DA pathway, originating in the ventral tegmental area (yellow box) and projecting to the NAc (purple box). DAergic projection neurons (blue) express the α3, α4, α5, α6, α7, β2 and β3 nAChR subunit genes (depicted inside the soma) and the known functional receptor subtypes found on this neuron include the α4β2*, α6β2*, and α7 subtypes (depicted as a receptor on the cell membrane and pre-synaptic terminal). GABAergic interneurons (pink) express the α3, α4, α5, α7, β2, and β4 nAChR subunit genes and have known functional nAChRs of α4β2* and α7 subtypes. Cholinergic afferents from the PPTg/LDT synapse onto both cell types in the VTA. Glutamatergic inputs from the prefrontal cortex also synapse in the VTA, and have functional α7 nAChRs on the pre-synaptic terminals. Nicotine addiction, beginning on the right: (1) nicotine binds and activates the high affinity α4β2* nAChRs located on the DAergic neurons of the VTA, (2) causing DA release in the NAc. (3) The high affinity nAChRs rapidly desensitize and stay in the desensitized state for a long period of time. (4) Low affinity α7 nAChRs are activated and desensitize; however, the α7 nAChRs are able to recover from desensitization very quickly, such that they are able to be activated again, increasing glutamate release in the VTA.

GENETICALLY MODIFIED nAChR MOUSE MODELS

Because of the large number of possible combinations of functional nAChRs present in the brain, traditional pharmacological approaches are difficult. Thus, genetically modified nAChR mouse models have been invaluable to determine the specific nAChR subtypes that contribute to nicotine addiction (Mineur and Picciotto, 2008). Genetically modified nAChR mice, in combination with nAChR pharmacology and behavioral assays of nicotine addiction, are even more powerful tools.

The first genetically modified nAChR mouse was made by deleting a region of DNA that encodes the β2 subunit gene, *Chrnb2*, termed "β2 knock-out" and abbreviated as β2 KO (Picciotto et al., 1995). Importantly, these mice develop and breed normally and have normal behaviors compared to WT mice (Picciotto et al., 1995). However, the β2 KO appear to lack high affinity binding of α4β2 nAChR agonists, as well as have decreased nicotine-induced DA release in the striatum and an absence of nicotine self-administration (Grady et al., 2001; Mineur and Picciotto, 2008; Picciotto et al., 1998). Thus, using the β2 KO mouse, it was determined that β2* nAChRs were involved in the reinforcing properties of nicotine addiction (Picciotto et al., 1997). The sufficiency of high-affinity, α4β2* nAChRs in mediating the rewarding properties of nicotine was further validated with the Leu9′Ala α4 knock-in nAChR mouse model (Tapper et al., 2004) as well as targeted re-expression of the β2 subunit in β2 KO mice (Maskos et al., 2005).

In addition to assessing the normal, drug-naive state of the mouse (i.e., development, breeding, behavior) and the ability to develop (or not to develop) nicotine-induced reward, it is also important to measure other reward behaviors to verify that the effects of the nAChR subunit knock-out are specific for nicotine addiction. For example, β2 KO mice have an increased threshold for food reinforcement, an increased threshold for cocaine conditioned place preference, but maintained morphine self-administration to the VTA, among other tests (Mineur and Picciotto, 2008). Since 1995, this knock-out approach has been repeated with the α7, α4, α3, β4, α5, α6, and β3 nAChR subunits (Champtiaux et al., 2002; Cui et al., 2003; Marubio et al., 1999; Orr-Urtreger et al., 1997; Ross et al., 2000; Salas et al., 2003; Wang et al., 2002; Xu et al., 1999a; Xu et al., 1999b).

For the purposes of this thesis, only two additional nAChR mouse models will be described in detail. The first mouse model is the α4 knock out mouse (α4 KO) which does not express the gene encoding the α4 nAChR subunit, *Chrna4*, and therefore does not express functional α4* nAChRs (Ross et al., 2000). The homozygous α4 KO mouse was created by removing a 750 bp fragment from the fifth exon of the *Chrna4* gene (Ross et al., 2000). This fragment contains DNA encoding the first hydrophobic transmembrane domain through the second intracytoplasmic loop. Homozygous knockout mice are born in normally expected ratios, are capable of reproduction and have no physical abnormalities. Similar to the β2 KO, α4 KO mice do not show nicotine-induced DA release in the

striatum and are less sensitive to the locomotor depressant effects of nicotine (Marubio et al., 2003); however, α4 KO mice have higher baseline locomotor activity compared to controls (Ross et al., 2000). Importantly, there is no significant difference in the expression of other nAChR subunits such as α3, α5, or α6, indicating that deletion of the α4 gene does not induce compensatory changes in other nAChR subunit mRNAs or proteins (Marubio et al., 1999). However, it has been suggested that increased function of α6β2* nAChRs may be responsible for the differences seen in baseline locomotor activity of the α4 KO mice (Mineur and Picciotto, 2008).

The second mouse model discussed here is the Leu9′Ala knock-in mouse (Leu9′Ala). This mouse line contains a single Leucine to Alanine point mutation at the 9′ position of the transmembrane 2 pore-forming domain causing these receptors to be 50-fold more sensitive to nicotine and endogenous acetylcholine (Tapper et al., 2004). This hypersensitive mutation allows for selective activation of α4* nAChRs with low doses of nicotine that have no effect in WT mice. For example, mice with this mutation developed nicotine-induced locomotor sensitization and nicotine conditioned place preferences at very low doses of nicotine that have no effect in WT mice (Tapper et al., 2004). Both lines (α4 KO and Leu9′Ala) have been back-crossed at least ten times to a C57BL/6J background to minimize variability between strains due to genetic background.

I.F. Ethanol-nicotine interactions

ALCOHOL'S ACTION ON nAChRs

Research on alcohol modulation of neuronal nAChRs did not occur until the mid 1980s, when the development of patch clamp techniques allowed the study of ion channels and receptors (Narahashi et al., 1999). Neuronal nAChRs were of particular interest because of their structural similarity to $GABA_A$ receptors, one of the known primary targets of ethanol, as well as their modulation of pre-synaptic neurotransmitter release (Schlaepfer et al., 2008b). Several model systems including PC12 cells (Nagata et al., 1996), recombinant nAChRs expressed in *Xenopus* oocytes (Cardoso et al., 1999; Forman and Zhou, 1999, 2000), cultured neurons (Aistrup et al., 1999; Marszalec et al., 1999; Zuo et al., 2001), and transfected cell lines (Zuo et al., 2002) have been used to study alcohol modulation of nAChRs. However, the results of these studies are varied and depend on the receptor subunit composition, agonist concentration, ethanol concentration and experimental conditions (Dopico and Lovinger, 2009).

Although ethanol is known to target several ligand gated ion channels including GABA, glutamate and 5-HT, the concentration of ethanol required to modulate these channels (30 – 200 mM) is much higher than the concentration of alcohol in the blood that results in behavioral intoxication (10 – 50 mM) (Narahashi et al., 1999; Spanagel, 2009). Interestingly, studies using PC12 cells showed that ethanol potently modulates nAChRs at low concentrations of ethanol

(100 μM – 10 mM), identifying nAChRs as potential primary targets of ethanol (Nagata et al., 1996).

In cultured rat cortical neurons, ACh-evoked currents insensitive to αBgtx (i.e., heteromeric nAChRs) were significantly enhanced by physiologically relevant concentrations of ethanol while nAChRs sensitive to αBgtx (i.e., α7 homomeric nAChRs) were inhibited (Aistrup et al., 1999). More specifically, in *Xenopus* oocytes, acute alcohol (75 mM) potentiates ACh-induced current of α2β4, α4β4, α2β2 and α4β2 nAChRs while lower concentrations of ethanol (20-50 mM) inhibited nicotine-induced current of α7 nAChRs and all concentrations of ethanol tested had no effect on α3β2 or α3β4 nAChRs (Cardoso et al., 1999). Not surprisingly, concentrations of ethanol higher than 100 mM are less selective for specific nAChR subtypes and potentiate most nAChR receptors (Spanagel, 2009).

Similar to other ligand gated ion channels, ethanol potentiation of nAChRs is hypothesized to be a result of the ethanol-induced stabilization of the open channel state of the receptor (Forman and Zhou, 1999; Wu et al., 1994; Zuo et al., 2004). Additionally, it is possible that the ethanol-induced inhibitory effect seen with α7 nAChRs is due to the inherently fast desensitization rate of these receptors, implying that ethanol potentiation results in enhanced desensitization (Dopico and Lovinger, 2009). Thus, these and other *in vitro* and *in vivo* studies, suggest that alcohol modulation of nAChRs, either by enhancing or inhibiting function, may contribute to the common co-abuse of nicotine and alcohol.

CO-ABUSE OF NICOTINE AND ALCOHOL

Alcohol and nicotine are two of the most widely used drugs, and are often abused together. Several reports from the 1980s and 1990s have estimated that 80 % of alcohol-dependent people are also smokers (Bobo, 1992; Miller and Gold, 1998) and that smokers have an increased risk of developing alcohol use disorders (DiFranza and Guerrera, 1990; Grant et al., 2004). Interestingly, while the smoking rates in the general population have dramatically decreased over the past two decades, smoking has remained high in alcoholic individuals (Meyerhoff et al., 2006), with current estimates still between 70-75 % (Bobo and Husten, 2000). These high rates of co-abuse of nicotine and alcohol have led some researchers to define this population as 'alcoholic smokers' as compared to 'smokers' (Littleton et al., 2007). In fact, this population has increased risk factors for cardiovascular and lung diseases (Benowitz, 2003) and for some forms of cancer (Sasco et al., 2004). The risks of cancer of the mouth, throat, or esophagus for the smoking drinker are more than the sum of the risks posed by these drugs individually (Room, 2004).

GENETIC LINKS BETWEEN ALCOHOLISM AND nAChRs

Many hypotheses have been proposed as to the basis of the high rates of nicotine and alcohol co-abuse. For example, it is possible that alcohol use leads to nicotine use or vice versa (Tyndale, 2003) or that because alcohol and nicotine are legal and readily available, the likelihood of their co-use is increased (Funk et al., 2006). However, there is much evidence that suggests that common genes

may influence the development of alcohol and nicotine abuse behaviors individually as well as contribute to both disorders in humans (Bierut et al., 2000; Madden and Heath, 2002; True et al., 1999). Using twin studies, it was determined that identical twins are two times as likely to be dependent on alcohol and/or nicotine if the other twin is dependent, compared to fraternal twins (Heath et al., 1997). Additionally, experimental studies using rodents have indicated a genetic linkage between the effects of alcohol and nicotine by selectively breeding mice and rats for different responses to alcohol. Animals that were more sensitive to alcohol's sedative effects (long sleep mice (LS), and high alcohol sensitivity rats (HAS)) were more sensitive to the locomotor effects of nicotine when compared to animals with low sensitivity to alcohol's effects (short sleep mice (SS) and low alcohol sensitivity rats (LAS)) (De Fiebre et al., 1987; de Fiebre et al., 2002).

Recent genetic association studies have identified several nAChR subunit genes as common genetic targets associated with both nicotine and alcohol addiction. The CHRNA5/A3/B4 nAChR gene cluster was identified as a genetic locus that influences the development and level of alcohol use and nicotine addiction in human populations (Joslyn et al., 2008; Schlaepfer et al., 2008a). Variation in CHRNA5, the gene encoding the α5 nAChR subunit, has been associated with a genetic risk for alcoholism (Wang et al., 2009) and the β2 nAChR subunit, encoded by CHRNB2, has also been associated with the subjective responses to alcohol and nicotine (Ehringer et al., 2007). Using

nAChR mouse models, a polymorphism in the α4 nAChR subunit gene has been shown to influence the initial sensitivity of the α4β2* nAChR to ethanol (Butt et al., 2003) as well as partially mediate the difference between the LS and SS mice, discussed above (Stitzel et al., 2001). Similarly, CHRNA3, the gene encoding the α3 nAChR subunit, was expressed at a higher rate in the brains of mice that were less sensitive to ethanol-induced stimulation, thus identifying CHRNA3 as a candidate for modulating the acute locomotor stimulant response to ethanol (Kamens et al., 2009).

I.G. Evidence for nAChR modulation of ethanol reward

One of the goals of alcohol research is to identify molecules that may play a significant role in ethanol's euphoric effects that promote voluntary drinking and acute intoxication (Hendrickson et al., 2009). Because of the strong evidence of the co-abuse of nicotine and ethanol, and the fact that both drugs act on the mesolimbic pathway, nAChRs have emerged as target molecules in at least partially mediating the reinforcing properties of alcohol (Soderpalm et al., 2000). Indeed, there is ample of evidence that ethanol can modulate nAChR function *in vitro*, through re-expression in *Xenopus* oocytes (Cardoso et al., 1999), in cultured PC12 cells (Nagata et al., 1996), and dissociated rat cortical neurons (Aistrup et al., 1999). However, the effects of ethanol on nAChRs of these systems produces varied results and greatly depends on the receptor subunit composition, concentration of ethanol and other agonists used, presence or lack

of synaptic terminals from afferent regions and experimental conditions (Dopico and Lovinger, 2009). Thus, an important question is: what is the effect of ethanol modulation of nAChRs *in vivo*?

INITIAL STUDIES: A DOGMA IS SET

In the past two decades, much of the work characterizing the role of nAChRs in mediating the behavioral and neurochemical effects of ethanol in rodents came from the Soderpalm and Engel research group in Sweden, among others. This group was the first to show that ethanol-induced activation of the mesolimbic DA system, as measured by extracellular DA release in the NAc, could be blocked with a systemic injection of the nAChR antagonist mecamylamine (Blomqvist et al., 1993). This antagonizing effect was then localized to the VTA by a local perfusion of mecamylamine (Blomqvist et al., 1997). Next, using rats that were trained to voluntarily consume ethanol, it was shown that both systemic (Blomqvist et al., 1996) and local VTA infusion (Ericson et al., 1998) of mecamylamine decreased ethanol intake and preference in the high-alcohol preferring rats. As a result, these early studies were key in developing a hypothesis that has persisted in the field; that at least a part of the rewarding effects of ethanol are mediated by nAChRs located in the VTA (Blomqvist et al., 1992; Chatterjee and Bartlett, 2010).
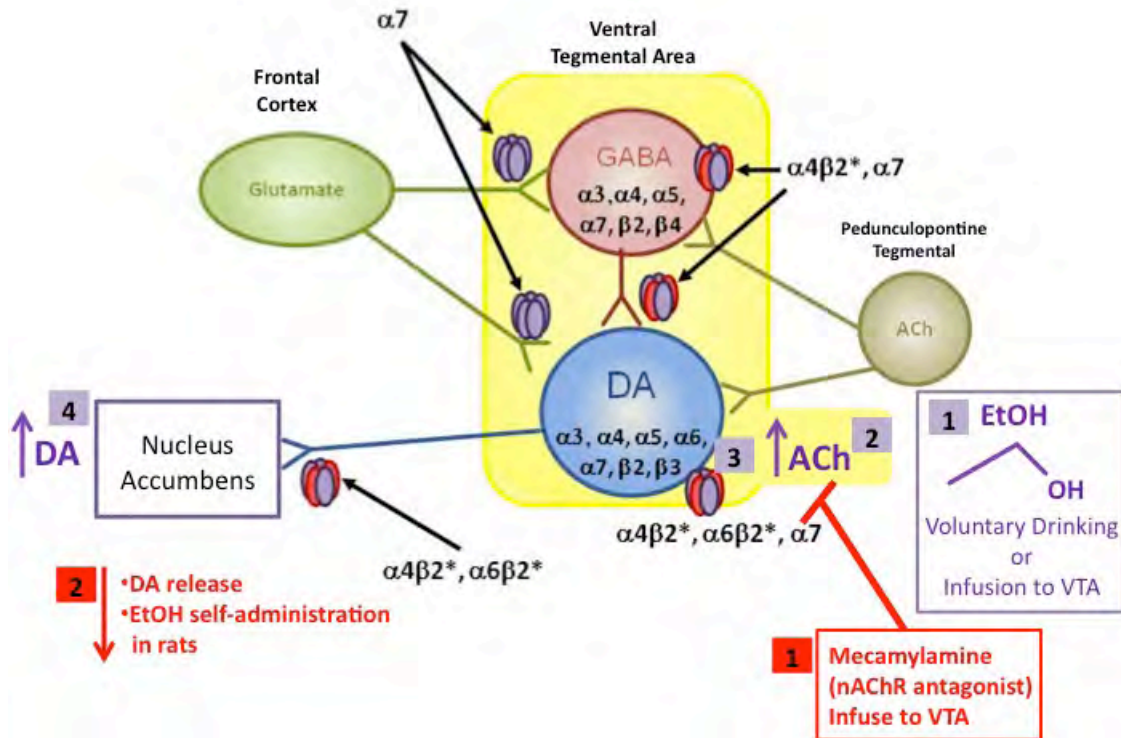
FIGURE I-4. Alcohol's action in the mesolimbic DA pathway, a role for nAChRs. Beginning on the right, then moving leftward, in purple: (1) Ethanol administration, via voluntary drinking or a direct infusion into the VTA, activates DAergic cell bodies by either (2) increasing the release of ACh into the VTA or (3) directly activating / modulating nAChRs, with the end result being (4) increased DA release in the NAc. Evidence for nAChRs, beginning on the right, in red: (1) pre-application of the nAChR antagonist mecamylamine, either by systemic injection or direct infusion to the VTA, (2) blocks the ethanol-induced DA release in the NAc as well as decreases ethanol self-administration in rats.

NICOTINE'S EFFECT ON ETHANOL SELF-ADMINISTRATION

Several labs have investigated the effect of nicotine treatment on alcohol self-administration, with varying results. In one study, researchers gave sub-chronic nicotine (0.35 mg/kg, daily) to a group of medium alcohol preferring rats and saw a marked increase in ethanol intake and preference (Blomqvist et al., 1996). This is similar to a previous study that found that alcohol consumption was increased in rats with nicotine pellets implanted subcutaneously (Potthoff et al., 1983). Interestingly, two additional studies found that acute nicotine administration has the opposite effect and actually decreases ethanol drinking (Gauvin et al., 1993; Le et al., 2000). The differences in the effects of acute and chronic nicotine treatment on ethanol self-administration may be attributed to the development of locomotor sensitization to chronic nicotine (Benwell and Balfour, 1992), or, that rats develop tolerance to the initial depressant effect of nicotine (Stolerman et al., 1995).

ETHANOL'S ACTION ON nAChRs: DIRECT OR INDIRECT?

While there is considerable evidence that ethanol directly acts on several ligand-gated ion channels (Grant, 1994), most studies of ethanol's action on nAChRs has demonstrated that it is more likely a co-agonist, acting to potentiate the effects of acetylcholine (Larsson et al., 2002; Marszalec et al., 1999). However, it has remained unclear whether ethanol acts directly on nAChRs or indirectly through an enhancement of cholinergic transmission. To elucidate this mechanism, one study measured extracellular concentrations of ACh in the VTA

of rats that voluntarily consumed ethanol and found that ACh levels were increased after ethanol consumption and shortly thereafter, DA concentrations were elevated in the NA as well (Larsson et al., 2005). The cholinergic afferents to the VTA are predominantly from the pedunculopontine and the laterodorsal tegmental area (Oakman et al., 1995), brain regions that have also been implicated in mediating natural as well as drug-reward behavior (Yeomans et al., 1993).

LOCALIZING ETHANOL'S EFFECT: ANTERIOR VS. POSTERIOR VTA

Recent studies have indicated that the VTA is not a homogeneous region and can be divided into at least two distinct brain regions, the anterior and posterior VTA (aVTA and pVTA) (Ikemoto, 2007; Shabat-Simon et al., 2008). While both regions consist of predominantly two types of neurons, DAergic projection neurons and GABAergic interneurons, studies have found that neurons within the anterior and posterior VTA project to different regions of the striatum and respond differently to drugs of abuse (Ikemoto, 2007; Shabat-Simon et al., 2008; Zangen et al., 2006). Furthermore, differential expression of nAChR subunits has been identified in both types of neurons (Champtiaux et al., 2003; Klink et al., 2001; Wooltorton et al., 2003) and regions within the VTA (Zhao-Shea et al., 2011). While there is a general consensus in the field that the VTA is critical for at least partially mediating the initial rewarding properties of drugs of abuse, there are conflicting reports of which region is more important. For example, one study found that blocking nAChRs of the anterior, but not posterior

VTA, blocked NAc DA release when ethanol was directly infused to the NAc, implicating a role for the anterior VTA in a neuronal NAc-aVTA-NAc feedback loop (Ericson et al., 2008).

Despite this study, the evidence suggesting that the pVTA plays a critical role in modulating ethanol reward is much more compelling than for the aVTA. For instance, one study showed that alcohol-preferring rats will self-administer ethanol directly into the pVTA (Gatto et al., 1994). Similarly, this result has been repeated, but instead showed that male and female non-alcohol preferring rats will also self-administer ethanol directly to the posterior, but not anterior VTA (Rodd et al., 2004; Rodd-Henricks et al., 2000). Furthermore, this effect is attenuated with co-application of the DA D2 receptor agonist, quinpirole, indicating that the activation of pVTA DA neurons is involved in the process (Rodd et al., 2004). Another more recent study showed that infusion of ethanol to pVTA is sufficient for increased DA release in the NAc (Ding et al., 2009).

nAChR SUBTYPE SPECIFICITY: CURRENT APPROACHES

Recently, the scope of nAChR/ethanol reward research has changed from an examination of the role of nAChRs in ethanol reward to a more focused study of specific nAChR subtypes that mediate ethanol reward and therefore may be possible targets for the development of alcohol cessation therapies. The most common approach used to determine the nAChR subtype associated with the behavioral effects of ethanol are to use a variety of nAChR antagonists that are

selective for one subtype of nAChR versus another (Chatterjee and Bartlett, 2010).

The non-competitive nAChR antagonist mecamylamine has been shown to decrease ethanol intake in several mouse and rat models of ethanol intake and/or preference (Blomqvist et al., 1996; Dyr et al., 1999; Ericson et al., 1998; Farook et al., 2009; Kuzmin et al., 2009; Le et al., 2000; Nadal et al., 1998). However, because mecamylamine is non-selective for a particular subtype of nAChR, it does not further elucidate which nAChR is critical for the effect.

Antagonists that are nAChR selective include DHβE, selective for α4β2 and MLA, selective for α7 nAChRs; however prior studies using these compounds have found no effect on ethanol consumption (Kuzmin et al., 2009; Le et al., 2000). An additional compound, α-conotoxin MII, is an antagonist selective for α3β2*, β3* and/or α6* nAChRs. This peptide, while potent and selective, does not cross the blood brain barrier, and must be locally administered via cannula or intracranial injection. Interestingly, α-conotoxin MII, when infused into the VTA, was shown to significantly reduce ethanol-induced DA release in the NAc, reduce the locomotor stimulatory effect as well as reduce ethanol intake in both rats and mice (Jerlhag et al., 2006; Larsson et al., 2004)

Several studies have had success using the newly discovered compound varenicline, a high affinity α4β2* nAChR partial agonist that is FDA approved as a smoking cessation medication (Coe et al., 2005). Varenicline selectively binds and partially activates the nAChRs that modulate nicotine intake (i.e., α4β2*

nAChRs) thus occupying or desensitizing the relevant receptors, precluding any further activation by nicotine . Additionally, the selective activation of α4* nAChRs themselves may act to increase DA release in the NAc such that nicotine has no further enhancing effect. Recently, studies have found that varenicline significantly reduced ethanol seeking and consumption in rats (Steensland et al., 2007), significantly reduced alcohol consumption in a group of heavy drinking smokers (McKee et al., 2009), and attenuated alcohol stimulated DA release in the NAc of rats (Ericson et al., 2009). Although varenicline was designed as a potent high affinity partial agonist of α4β2* nAChRs (Coe et al., 2005; Jorenby et al., 2006), it also acts as a low affinity, partial agonist of α3β2* and α6* nAChRs and a low affinity, full agonist of α7 and α3β4* nAChRs (Mihalak et al., 2006). Thus, the nAChR subtypes that mediate varenicline's effect on alcohol consumption and stimulated DA release in the NAc remain unknown.

Surprisingly, even though nAChRs have long been molecular targets of interest in mediating the rewarding properties of ethanol, and the α4 KO and β2 KO mice have been available for over a decade, very few studies have used these mice to examine the role of nAChRs in ethanol consumption or other behavioral responses to ethanol. To date, only one published study has reported baseline ethanol consumption in the β2 KO and α7 KO mice, and this was just last year. This study found that female, but not male, α7 KO mice consumed less ethanol than WT mice and there was no difference in ethanol intake in β2 KO mice (Kamens et al., 2010). Additionally, both β2 KO and α7 KO mice had

decreased ethanol intake after varenicline treatment, suggesting that varenicline does not act solely through α7 or β2* nAChRs (Kamens et al., 2010). Incredibly, no published study, to our knowledge, has examined baseline ethanol consumption or the acute rewarding effects of ethanol in the α4 KO mouse line. This is even more baffling since two polymorphic forms of the α4 nAChR subunit gene were shown to be responsible for the differences in the behavioral sensitivity to ethanol and nicotine in mice almost 10 years ago (Butt et al., 2003; Dobelis et al., 2002; Stitzel et al., 2001).

Preface to Chapter II

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Author contributions:

Hendrickson LM, Tapper AR designed experiments.

Hendrickson LM performed experiments.

Zhao-Shea R performed imaging of c-Fos immunostaining.

Hendrickson LM, Tapper AR wrote the paper.

CHAPTER II

Modulation of ethanol drinking-in-the-dark by mecamylamine and nicotinic

acetylcholine receptor agonists in C57BL/6J mice

II.A. INTRODUCTION

Alcoholism is the third preventable cause of mortality in the world and few therapeutic treatments are available highlighting the importance of understanding the underlying molecular mechanisms of ethanol's reinforcing properties (CDC, 2004). Animal models of voluntary alcohol drinking provide a unique tool to study potential pharmacological means to reduce ethanol intake, but few of these models yield intoxicating blood alcohol levels. Recently a straight forward voluntary drinking paradigm has been established whereby high alcohol preferring C57BL/6J mice are exposed to 20 % ethanol for two or four hours during the dark cycle. Termed "Drinking in the Dark" (DID), this novel assay reliably produces pharmacologically relevant blood ethanol concentrations even upon first exposure and has been utilized as a mouse model of "binge drinking" (Rhodes et al., 2005; Rhodes et al., 2007).

A major goal of alcohol addiction research is to identify molecules that may play a significant role in ethanol's euphoric effects that could promote persistent voluntary drinking and acute intoxication. Achieving this goal has proven problematic due to ethanol's properties to interact with a myriad of proteins expressed in the CNS (Harris, 1999). Neuronal nicotinic acetylcholine

receptors (nAChRs) have emerged as candidate molecules in at least partially mediating the reinforcing properties of alcohol (Soderpalm et al., 2000). Neuronal nAChRs are ligand-gated cation channels that are activated by the endogenous neurotransmitter, acetylcholine, as well as the addictive component of tobacco smoke, nicotine. Currently, 12 mammalian neuronal nicotinic acetylcholine receptor subunits have been identified (α2-10 and β2-4). The majority of high affinity nAChRs are heteromeric pentamers consisting of α and β subunits. Thus, multiple receptor subtypes with varying subunit compositions and electrophysiological properties exist (Jones et al., 1999; Laviolette and van der Kooy, 2004; Lindstrom et al., 1996). Indeed, many neuronal nAChR subtypes are expressed throughout the mesocorticolimbic reward pathways especially in the VTA in both DAergic neurons projecting to nucleus accumbens and in local GABAergic interneurons (Klink et al., 2001; Wooltorton et al., 2003). How does ethanol interact with these receptors? Interestingly, systemic ethanol has been shown to increase acetylcholine concentrations in the VTA, presumably, activating nAChRs in this area (Ericson et al., 2003). In addition, ethanol can directly modulate nAChR activity depending on the subtype of nicotinic receptor expressed (Forman and Zhou, 2000; Zhou et al., 2000; Zuo et al., 2002). Because a variety of subtypes exist in these nuclei, identification of the specific nicotinic receptor subtype(s) that may underlie ethanol reward is paramount.

The nonspecific antagonist, mecamylamine, when injected systemically or locally within the VTA, blocks ethanol self administration in high ethanol preferring rats that have acquired robust ethanol drinking through increasing concentration of ethanol exposure over a two week period (Blomqvist et al., 1996; Ericson et al., 1998). Using a similar paradigm in rats, studies have shown that DHβE and MLA, antagonists selective for α4β2 and homomeric α7 nAChRs, respectively, fail to block ethanol consumption (Le et al., 2000), and dopamine overflow in nucleus accumbens (Ericson et al., 2003; Larsson et al., 2002). On the other hand, it has been shown that the α3β2*, β3*, and α6* subunit specific antagonist, α-conotoxin MII, does inhibit ethanol consumption, activity, and dopamine release in nucleus accumbens (Jerlhag et al., 2006; Larsson et al., 2004). More recently, varenicline, an α4β2 partial agonist clinically approved as a smoking cessation therapeutic (Coe et al., 2005; Gonzales et al., 2006; Steensland et al., 2007; Tonstad et al., 2006), was found to reduce both ethanol intake and seeking in rats (Steensland et al., 2007). To our knowledge, the role of nAChRs in acute ethanol intake in mice has not been examined.

The goal of the current study was to test the hypothesis that nAChR signaling is involved in acute alcohol intake (i.e. "binge drinking") as measured using the DID assay in C57BL/6J mice. Toward this end, we exposed mice to a panel of nAChR antagonists and agonists prior to the presentation of ethanol and measured alcohol intake.

II.B. MATERIALS AND METHODS

Animals. Male C57BL/6J mice (Jackson Laboratory) used in the experiments were between 8-14 weeks of age and were housed 3-4 animals per cage up until the start of each experiment. During acclimation, animals were kept on a standard 12 hour light/dark cycle with lights on at 7:00 AM and off at 7:00 PM. The animals were given food and water *ad libitum*, except when ethanol was substituted for water for 2 hours at night as described below. All experiments were conducted in accordance with the guidelines for care and use of laboratory animals provided by the National Research Council (National Research Council, 1996), as well as with an approved animal protocol from the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Drugs and drinking solutions. Ethanol solutions were prepared from 190 proof absolute anhydrous ethanol (95 % ethanol, 5 % water; Pharmco-Aaper brand, Brookfield, CT) diluted to 20 % ethanol (v/v) using tap water. Sucrose (EMD) was dissolved in tap water to make a 10 % (w/v) concentration. Mecamylamine hydrochloride, hexamethonium hydrochloride, MLA, DHβE, nicotine hydrogen bitartrate, and cytisine (all purchased from Sigma-Aldrich, St. Louis, MO), were dissolved in 0.9 % saline and were administered via intraperitoneal (i.p.) injections at the indicated doses. Nicotine concentrations are reported as nicotine base.

Drinking in the dark (DID) procedure. Animals were placed in experimental chambers for 1 week prior to the beginning of the DID sessions. The mice received a 15 ml graduated cylinder water bottle fitted with a one holed rubber stopper with a stainless steel double-ball-bearing sipper tube which was sealed with parafilm to prevent leakage. Our drinking assay is a modified 2-day version of a limited access drinking procedure first described in Rhodes et al., 2005. On the first night, two hours after the lights were turned off, half of the mice were given an i.p. injection of saline and the other half were i.p. injected with drug. Immediately after the injections, the water bottle was removed and replaced with a single bottle of 20 % ethanol and left in place for two hours. On the second night, the injection groups were switched (i.e. mice that received saline on the first night received drug on the second; whereas mice that received drug on the first night received saline on the second) and again given a single 20 % ethanol bottle for two hours. The amount of ethanol consumed was recorded immediately after each two hour session and converted to g/kg per each animal's ethanol consumption and body weight. For control experiments, mice received 10 % sucrose for two hours instead of ethanol.

Experimental Design. Table 1 lists the experiment number, type, drug injected, and number of animals used. For DID experiments, each mouse received two DID sessions with a low and high dose of the same drug, except in experiments 1, 3, 4, 11 and 12 where the mice only received saline and one dose of drug.

Mice that received two doses of drug were given seven days of rest between two-day DID experiments, and tested again in the same two-day DID procedure. Lower doses were used in the initial two-day DID round followed by higher doses in the second DID round (see Table 1). In experiments 14 and 15, the DID procedure was exactly the same as described above except that ethanol measurements were taken in 15 minute intervals throughout the two hour drinking session.

Blood Ethanol Concentration. For experiment 5 (blood ethanol concentration (BEC) measurements), prior to ethanol drinking, one group of mice was injected i.p. with saline and a separate group was i.p. injected with 1 mg/kg mecamylamine. Trunk blood was obtained from the mice after completion of the two hour ethanol drinking assay. Blood was collected in heparinized capillary tubes, centrifuged at 1500 X g for 5 minutes and blood analyzed using an alcohol oxidase-based assay. Blood ethanol concentrations were measured on a GM7 Micro-Stat Analyzer (Analox Instruments Ltd.).

Immunohistochemistry. Mice were i.p. injected with saline for three days prior to the start of the experiment to habituate them to handling and to reduce c-Fos activation due to stress. Two groups of six mice were used. Mice from the first group received two injections: An i.p. injection of 3.0 mg/kg mecamylamine followed by a 2.0 g/kg ethanol injection, or a saline injection followed by a 2.0

g/kg ethanol injection, or a saline injection followed by a second saline injection. The time between the first and second injection was forty-five minutes and was estimated based on the delayed effect that mecamylamine had on drinking pattern (Fig. 4).   The second group of mice received an i.p. injection of 0.5 mg/kg nicotine followed by a saline injection, or a 0.5 mg/kg nicotine injection followed by a 2.0 g/kg ethanol injection, or a saline injection followed by a second saline injection.   The time between injections was fifteen minutes based on nicotine's more rapid effect on drinking pattern.

Ninety minutes after the second injection, all mice were deeply anesthetized with sodium pentobarbital (200 mg/kg, i.p.) and perfused transcardially with 10 ml of 0.1 M phosphate-buffered saline (PBS) followed by 10 ml of 4 % paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Brains were removed and post-fixed for 2 h with the same fixative and cryoprotected in sodium phosphate buffer containing 30 % sucrose until brains sank. VTA serial coronal sections (20μm) were cut on a microtome (Leica CM 3050S, Leica Microsystems Inc.) and collected into a 24-well tissue culture plate containing 1 X PBS. Slices containing VTA were collected between -2.92 mm and -4.04 mm from bregma. After rinsing sections in PBS twice for 5 min., they were treated with 0.4 % Triton X-100 PBS (PBST) twice for 2 min. followed by incubation in 2 % BSA/PBS for 30 min. Sections were washed with PBS once and then incubated in a cocktail of primary antibodies for Tyrosine Hydroxylase (TH, mouse monoclonal, 1:250, Santa Cruz Biotechnology, Santa Cruz, CA,

USA) and c-Fos (rabbit polyclonal, 1:400, Santa Cruz) in 2 % BSA/PBS overnight at 4° C. The sections were then washed with PBS three times for 5 min followed by incubation in secondary fluorescent labeled antibodies (goat anti-rabbit Alexa Fluor® 488 and goat anti-mouse Alexa Fluor® 594, 1:300, Molecular Probes, Inc., Eugene, USA) at room temperature in dark for 30 min. After washing with PBS five times for 5 min/wash, sections were mounted on slides by using VECTASHIELD® Mounting Medium (Vector laboratories, Inc., Burlingame, CA). The number of positive neurons was counted under a fluorescence microscope (Zeiss, Carl Zeiss MicroImaging Inc., NY) at a magnification of 400X. The intensity of fluorescence was quantified by using a computer-associated image analyzer (Axiovision Rel. 4.6). Neurons were counted as signal positive if intensities were at least two times higher than that of the average value of background (sections staining without primary antibodies).

Data Analysis. The effect of pre-injections of nicotinic agonists and antagonists on ethanol intake was compared to saline pre-injections using one of two statistical tests. In experiments where one group of mice received one dose of drug, One-Way ANOVAs followed by Tukey post-hoc tests were used. In experiments where one group of mice received two doses of drug, a Repeated measure ANOVA followed by Tukey post-hoc test was used. Data were analyzed using Graphpad software (Graphpad Software, Inc.). Student's t tests were used to analyze immunohistochemistry data. Results were considered

significant at $p<0.05$. All data are expressed as means ± standard errors of means (SEM).

II.C. RESULTS

Effects of mecamylamine on alcohol consumption in the DID assay.

To determine if mecamylamine can inhibit ethanol self-administration in the DID paradigm, mice were pre-injected, i.p. with 0.5, 1.0, or 3 mg/kg mecamylamine immediately prior to 20 % ethanol exposure. Mecamylamine dose dependently reduced the volume of ethanol drinking (Fig. II-1A). Mice receiving a pre-injection of 1.0 or 3.0 mg/kg mecamylamine consumed significantly less ethanol compared to saline injected mice (Fig. II-1B, 1.30 +/- 0.44 and 1.53 +/- 0.24 compared to 2.62 +/- 0.28 and 3.05 +/- 0.28 g/kg ethanol, respectively). Repeated measure ANOVA indicated an overall significant difference between saline and mecamylamine pre-injection on ethanol intake ($F_{3,18} = 9.33$, $p<0.001$). Tukey Post-hoc analysis indicated a significant effect of mecamylamine with a pre-injection dose of 1.0 and 3.0 mg/kg compared to respective saline values. The antagonist did not affect sucrose intake in mice at the tested doses of 1 or even as high as 6 mg/kg (Fig. II-1C, for 1 mg/kg $F_{1,12} = 0.54$, $p<0.05$, for 6 mg/kg $F_{1,10} = 3.23$, $p<0.05$). Pre-injection of the peripheral nAChR antagonist, hexamethonium, at a dose of either 1 or 3 mg/kg, also did not significantly reduce ethanol intake ($F_{3, 21} = 0.20$, $p>0.05$, data not shown).

To determine if the effect of mecamylamine ultimately resulted in a lower blood ethanol concentration, we acquired blood samples immediately following the two hour DID assay in mice that received either a saline or 1 mg/kg mecamylamine pre-injection (Fig. II-1D). Mice that received mecamylamine prior to their ethanol bottle exhibited significantly lower blood ethanol levels compared to mice that received a pre-injection of saline (Fig. II-1D, 13.5 +/- 3.9 mM compared to 25.8 +/- 2.8 mM ethanol, respectively $F_{1,9}$ = 6.2, $p<0.05$).

Effects of selective nAChR antagonists on ethanol consumption.

Pre-injection of a low (1 mg/kg) or high (3 mg/kg) dose of the nAChR competitive antagonist, DHβE, did not significantly affect ethanol intake in C57BL/6J mice compared to a pre-injection of saline. Repeated measure ANOVA yielded a non-significant effect of pretreatment: $F_{3,21}$ = 0.67, $p>0.05$ (data not shown). Similarly, pre-injection of a low (5 mg/kg) or high (10 mg/kg) dose of the α7 selective antagonist, MLA, did not significantly reduce ethanol intake ($F_{3,18}$ = 0.56, $p>0.05$, data not shown).

Effects of nAChR agonists on ethanol consumption in the DID assay.

To evaluate the effects of nAChR agonists on ethanol intake in the DID assay, we pre-injected C57BL/6J mice with nicotine immediately prior to presentation of the 20 % alcohol bottle. Compared to a saline injection, both 0.25 mg/kg and 0.5 mg/kg nicotine decreased the volume of ethanol drinking (Fig. II-2A). Repeated-measure ANOVA indicated an overall effect of pretreatment on intake ($F_{3,18}$ = 6.33, $p<0.01$, Fig. II-2B). Post-hoc comparisons

indicated that 0.5 mg/kg nicotine significantly reduced ethanol intake compared to saline (p<0.05, 2.42 +/- 0.32 compared to 3.76 +/- 0.36 g/kg ethanol). Pre-injection of either dose did not significantly reduce consumption of sucrose solution (Fig. II-2C, $F_{3,21}$ = 0.24, p>0.05). The β4* nAChR full agonist, and α4β2 selective partial agonist, cytisine also dose dependently reduced the volume of ethanol drinking compared to a saline injection (Fig. II-3A).   There was a significant effect of 3 mg/kg cytisine on ethanol intake ($F_{1,6}$ = 29.8, p<0.01, Fig. 3B, 1.37 +/- 0.39 compared to 4.01 +/- 0.39 g/kg ethanol) but not with 1 mg/kg ($F_{1,16}$ = 0.15, p>0.05). Repeated measure ANOVA on the effect of pre-injection on sucrose drinking revealed a significant interaction ($F_{3,21}$ = 9.63, p<0.01). However, post-hoc analysis revealed no significant difference between mice given 1 or 3 mg/kg cytisine compared to saline injected controls (Fig. II-3C, p > 0.05, NS).

Effects of mecamylamine and nicotine on ethanol drinking patterns.

To determine if mecamylamine, a nicotinic antagonist, and nicotine, an agonist could affect ethanol intake differently, we measured the pattern of alcohol drinking in mice pre-injected with each drug. Ethanol intake was measured in fifteen minute intervals over the course of two hours. Figure II-4A illustrates ethanol intake in two separate groups of mice that received either saline/nicotine or saline/mecamylamine pre-injections. Data from each group were normalized to their average saline values per 15 minute interval so comparisons could be made between groups. Pre-injection of 0.5 mg/kg nicotine decreased ethanol

intake during the first hour of drinking (Fig. II-4A). Conversely, 3 mg/kg mecamylamine reduced ethanol intake predominantly during the second hour of the DID assay. Figure II-4B illustrates average interval intake in the first hour compared to the second hour of the DID assay. One Way ANOVA indicated a significant effect of nicotine on average interval intake in the first hour compared to saline (Fig. II-4B). Actual values from the first hour are 0.502 g/kg/interval after saline injection compared to 0.159 g/kg/interval after nicotine ($F_{1,46} = 14.5$, $p<0.001$). Mecamylamine significantly inhibited ethanol intake in the second hour of the assay (0.688 g/kg/interval after saline compared to 0.33 mg/kg/interval after mecamylamine, $F_{1,54} = 11.0$, $p<0.01$).

Effects of mecamylamine and nicotine on ethanol-induced VTA DAergic neuron c-Fos expression.

To gain mechanistic insight into how nicotinic antagonists and agonists may influence ethanol intake, we analyzed expression of the immediate early gene, c-Fos, as a measure of neuronal activation (Cole et al., 1989) in tyrosine hydroxylase (TH) positive neurons of the VTA via immunohistochemistry. The number of c-Fos, TH double positive cells in VTA was counted in mice that received mecamylamine or nicotine prior to an i.p. injection of 2.0 g/kg ethanol (Fig. II-5). A single ethanol exposure significantly increased the number of double positive cells in VTA compared to saline injection (Fig. II-5a, C, $p<0.01$, independent two-sample Student's t test). Pre-injection of 3 mg/kg mecamylamine 45 minutes prior to ethanol injection significantly reduced the

number of c-Fos/TH positive cells compared to a saline pre-injection (Fig. II-5c, $p<0.05$).

To determine how nicotine may effect ethanol-induced c-Fos expression, we injected mice with 0.5 mg/kg nicotine, followed by either a saline or 2.0 g/kg ethanol injection. In the absence of ethanol, nicotine significantly increased the number of VTA c-Fos/TH double positive neurons compared to saline injected animals (Fig. II-5b, d, $p<0.01$). Ethanol exposure after the initial nicotine injection did not significantly increase or decrease the number of double positive neurons compared to nicotine alone (Fig. II-5d, $p>0.05$).

II.D. DISCUSSION

Previously, the nonspecific nicotinic receptor antagonist, mecamylamine, has been shown to reduce ethanol intake in rats that have learned to drink ethanol through at least two week training with increasing concentration of free or limited access ethanol (Blomqvist et al. 1996; Le et al. 2000). In addition, mecamylamine has been reported to reduce the subjective euphoria of ethanol in humans (Blomqvist et al., 1996; Chi and de Wit, 2003; Le et al., 2000). To our knowledge, this is the first report that nAChR blockade reduces ethanol consumption in mice during the DID paradigm, a model of binge drinking where C57BL/6J mice consume alcohol until intoxicated. Mecamylamine dose-dependently reduced alcohol intake and this also led to a significant reduction in blood-ethanol concentration suggesting that mecamylamine was not inhibiting

the metabolism of ethanol. In addition, sucrose intake was not reduced indicating specificity for alcohol consumption and not a general effect on reward signaling. Reduction of ethanol intake by mecamylamine was mediated by blockade of neuronal nAChRs expressed in the CNS because the non-specific nAChR antagonist, hexamethonium, did not significantly alter alcohol consumption. Prior studies indicate that mecamylamine delivered systemically or directly into the VTA blocks elevation of ethanol-mediated dopamine release in the nucleus accumbens (Blomqvist et al., 1993; Blomqvist et al., 1997). Thus, it is likely that mecamylamine is reducing ethanol intake via a similar mechanism in the DID assay. Although there have been reports that high doses of mecamylamine can non-competitively inhibit NMDA receptors (Fu et al., 2008; O'Dell and Christensen, 1988), we observe a decrease in the volume of ethanol consumption at doses as low as 0.5 mg/kg suggesting that mecamylamine is acting via blockade of neuronal nAChRs.

Because of the vast array of nAChR subtypes expressed in the CNS, identifying the specific composition of receptors involved in ethanol reinforcement is a difficult, but important question.   High affinity α4β2* and low affinity α7 nAChRs are two of the most abundant nicotinic receptors in the CNS and could represent potential candidates for at least partially mediating ethanol reward, α4β2 in particular since these receptors have been clearly implicated in nicotine dependence (Picciotto et al., 1998; Tapper et al., 2004).   However, the α4β2 selective and α7 selective antagonists DHβE and MLA, respectively, both of

which readily cross the blood-brain barrier, failed to significantly reduce ethanol intake. These data support prior studies that have shown little effect of these compounds on both operant responding, ethanol-mediated dopamine release in nucleus accumbens, and ethanol self-administration in rats (Le et al., 2000; Soderpalm et al., 2000). The straightforward interpretation of these data would be that α4β2 and α7 nAChRs are not involved in alcohol self-administration. However, caution in this interpretation is warranted especially in regard to higher affinity heteromeric nicotinic receptors that could contain α4β2 in addition to a third or even fourth subunit that may render them relatively insensitive to DHβE (Salminen et al., 2004).

Interestingly, acute exposure to nicotine dose dependently reduced alcohol intake in the DID paradigm. This is in opposition to at least one previous study that indicates that nicotine can enhance ethanol intake in rats in a restricted access drinking assay (Smith et al., 1999). The most likely difference between studies is that our DID assay utilized mice from the C57BL/6J strain which are high alcohol preferring animals; whereas Smith et al.'s study utilized rats that needed to be given low doses of ethanol for weeks before voluntary drinking was established. Throughout the adaptation period, where rats learned to drink increasing alcohol doses that produced robust blood ethanol concentrations, they were exposed to nicotine daily. Thus, chronic nicotine enhanced ethanol consumption, while our study illustrates that acute nicotine in

naïve mice reduces ethanol intake. It will be interesting to determine the effect of chronic nicotine exposure on consumption in the DID assay.

Our results indicate that cytisine can also reduce ethanol drinking. While nicotine is a full agonist, cytisine is known to be a full agonist for β4* nAChRs and a partial α4β2 agonist (Mineur et al., 2007; Picciotto et al., 1995). The α4β2 selective partial agonist, varenicline is a derivative of cytisine and recently has been shown to inhibit alcohol intake and seeking in rats (Coe et al., 2005; Steensland et al., 2007). Based on these observations, cytisine may also be a candidate compound for alcohol cessation.

Because of the complexity of nAChR subunit composition, as well as the robust expression patterns of nAChRs throughout the CNS, it is not so surprising that blocking nAChRs (i.e., with mecamylamine) and activating them with agonist can both reduce ethanol intake. However, could both classes of compounds impact the same ethanol reward circuit to impact voluntary ethanol intake? Based on multiple studies indicating that nAChRs rapidly desensitize after a single nicotine exposure, often for prolonged periods of time (Mansvelder et al., 2002; Pidoplichko et al., 1997), it is possible that an acute injection of nicotine or cytisine prior to ethanol exposure desensitizes the relevant nAChR subtype precluding activation of circuits involved in voluntary drinking. Thus, blocking nAChRs with an antagonist or desensitizing nAChRs with pre-exposure to agonists would both reduce alcohol consumption.

Alternatively, ethanol intake may be reduced by the nAChR agonists because the agonists themselves elevate nucleus accumbens dopamine release, thereby increasing dopamine signaling prior to ethanol drinking (Marubio et al., 2003; Picciotto et al., 1998). The dopamine reuptake blocker GBR 12909 has been shown to also reduce ethanol intake in the DID paradigm, presumably via a similar mechanism (Kamdar et al., 2007) but this compound was also shown to decrease sugar water intake. Our results argue against a common reward pathway because nicotine and cytisine reduced ethanol intake without affecting sucrose drinking suggesting that nicotinic receptor activation is involved in alcohol/nicotine reward specifically.

Interestingly, mecamylamine and nicotine differentially modulate alcohol drinking patterns. Mecamylamine reduced ethanol intake predominantly in the second hour of the DID assay; whereas nicotine reduced intake during the first hour, perhaps indicating independent mechanisms of action for each compound. Although drinking patterns may be explained by differences in the pharmacokinetics of each drug and how readily they cross the blood brain barrier. Nicotine is known to permeate the brain on the order of seconds (Lockman et al., 2005), while mecamylamine likely has a longer latency to reach effective concentrations in the CNS (Young et al., 2001).

In summary our data indicate that nAChRs are involved in acute ethanol drinking until intoxication. Identification of the specific nAChR subtypes involved

in this behavior should lead to novel therapeutic targets that could be used to prevent binge drinking.

Table 1 DID experiments

Experiment #	Type	Number of mice used	Drug injected	Dose (mg/kg)	Drinking solution utilized in each DID experiment
1	DID	9	Mecamylamine	0.5	20% Ethanol
2a	DID	7	Mecamylamine	1	20% Ethanol
2b	DID		Mecamylamine	3	20% Ethanol
3	DID	6	Mecamylamine	1	10% Sucrose
4	DID	6	Mecamylamine	6	10% Sucrose
5a	BEC	5	Mecamylamine	1	20% Ethanol
5b	BEC	6	Saline		20% Ethanol
6a	DID	8	Hexamethonium	1	20% Ethanol
6b	DID		Hexamethonium	3	20% Ethanol
7a	DID	8	DHβE	1	20% Ethanol
7b	DID		DHβE	3	20% Ethanol
8a	DID	7	MLA	5	20% Ethanol
8b	DID		MLA	10	20% Ethanol
9a	DID	7	Nicotine	0.25	20% Ethanol
9b	DID		Nicotine	0.5	20% Ethanol
10a	DID	8	Nicotine	0.25	10% Sucrose
10b	DID		Nicotine	0.5	10% Sucrose
11	DID	9	Cytisine	1	20% Ethanol
12	DID	4	Cytisine	3	20% Ethanol
13a	DID	8	Cytisine	1	10% Sucrose
13b	DID		Cytisine	3	10% Sucrose
14	DID pattern	6	Nicotine	0.5	20% Ethanol
15	DID pattern	7	Mecamylamine	3	20% Ethanol

Different letters (a and b) refer to the same group of mice used for that experiment number except for experiment 5 (BEC)
DID drinking in the dark, *BEC* blood ethanol concentration

Table II-1. DID experiments.
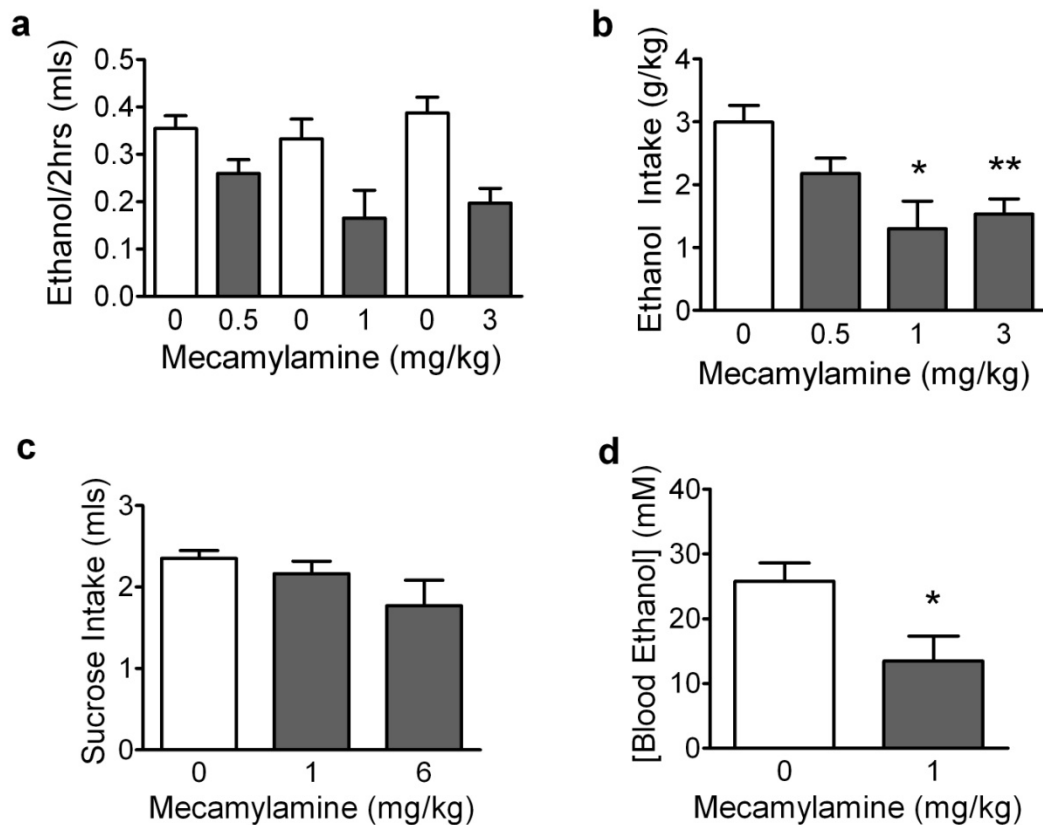
Figure II-1. Mecamylamine dose dependently reduces ethanol DID. A) Total ethanol drinking volume (mls ethanol solution) over the course of two hours starting two hours after lights off. Immediately prior to introduction of the ethanol solution into each individual cage, mice were injected i.p. with either 0 (saline), 0.5, 1.0, or 3.0, mg/kg mecamylamine. One group of animals was used for the 0.5 mg/kg dose; whereas a second group of animals was used for the 1 and 3 mg/kg doses (see methods). B) Bar graph representation of total ethanol intake over the two hour DID assay (g/kg) for the three mecamylamine doses. C) Total 10 % sucrose volume intake (mls) after an i.p. injection of 0 (saline), 1 or 6 mg/kg mecamylamine. Mice had access to 10 % sucrose for two hours during the dark cycle starting two hours after lights out. D) Blood ethanol concentration (mM) in mice given an i.p. preinjection of saline (n = 5) or 1 mg/kg mecamylamine (n = 6) immediately prior to an alcohol bottle. Blood was isolated immediately after the two hour drinking session. Data presented as mean +/- SEM. * $p<0.05$, *** $p<0.001$ compared to same group saline controls, One-Way or Repeated Measure ANOVA, Tukey post-hoc (see Methods and Results section for details).
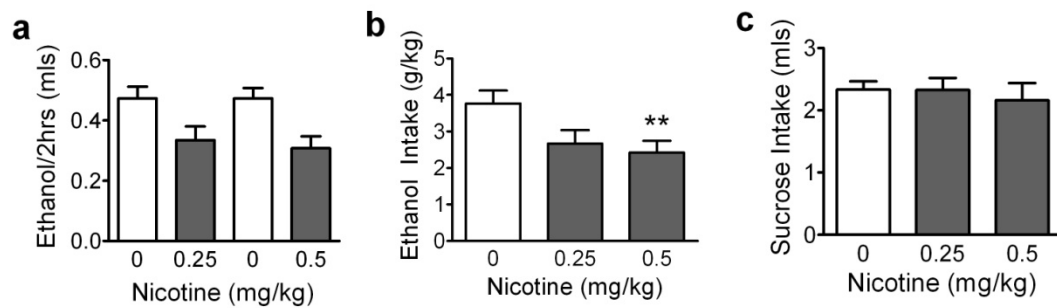
Figure II-2. Nicotine reduces ethanol DID. A) The effect of a preinjection of nicotine on ethanol drinking volume is shown. One group of mice were used for both drug concentrations (n = 7) B) Ethanol intake (g/kg) from (A). C) Average effect of a preinjection of nicotine on sucrose intake. Data are presented as mean +/- SEM. * $p<0.05$, ** $p<0.01$ compared to same group saline controls, Repeated Measures ANOVA, Tukey post-hoc.
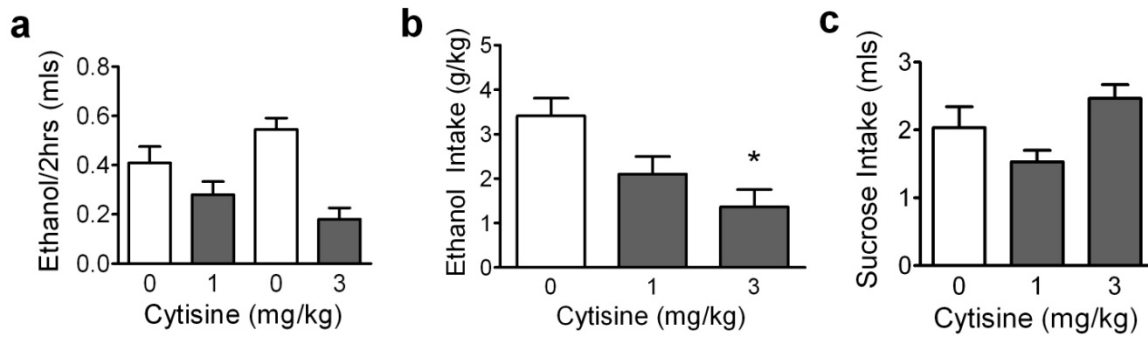
Figure II-3. Cytisine reduces ethanol DID. A) Total volume of ethanol intake after saline, 1, or 3 mg/kg cytisine pre-injection. Separate groups of animals were used for each dose. B) Ethanol intake (g/kg) from (A). Asterisk indicates significance compared to within group intake after a saline pre-injection. C) Effect of saline, 1, or 3 mg/kg cytisine on sucrose intake. * $p<0.05$ compared to same group saline controls, One-Way ANOVA, Tukey post hoc.
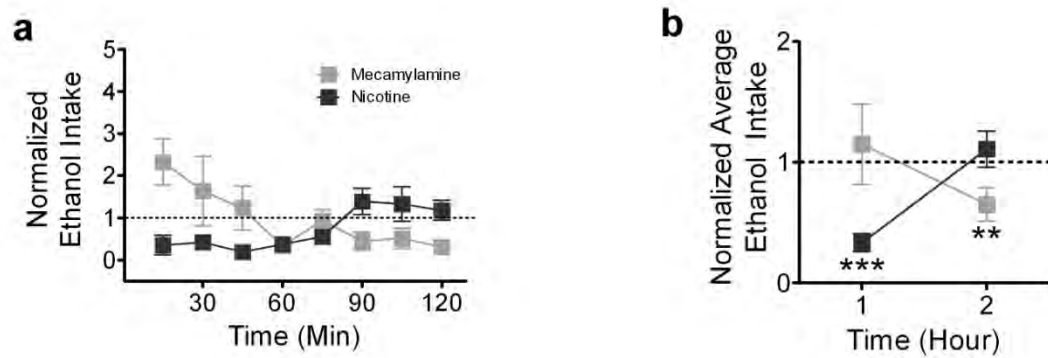
Figure II-4. Mecamylamine and nicotine differentially affect DID ethanol drinking pattern. A) Normalized drinking bouts in two separate groups of mice that received saline/3 mg/kg mecamylamine or saline/0.5 mg/kg nicotine. Dotted line represents the normalized saline value for each group. B) Average 15 minute bout during the first and second hour of the DID assay in the two groups of animals. ** $p<0.01$, *** $p<0.001$ compared to same group saline controls, One-Way ANOVA, Tukey post hoc.
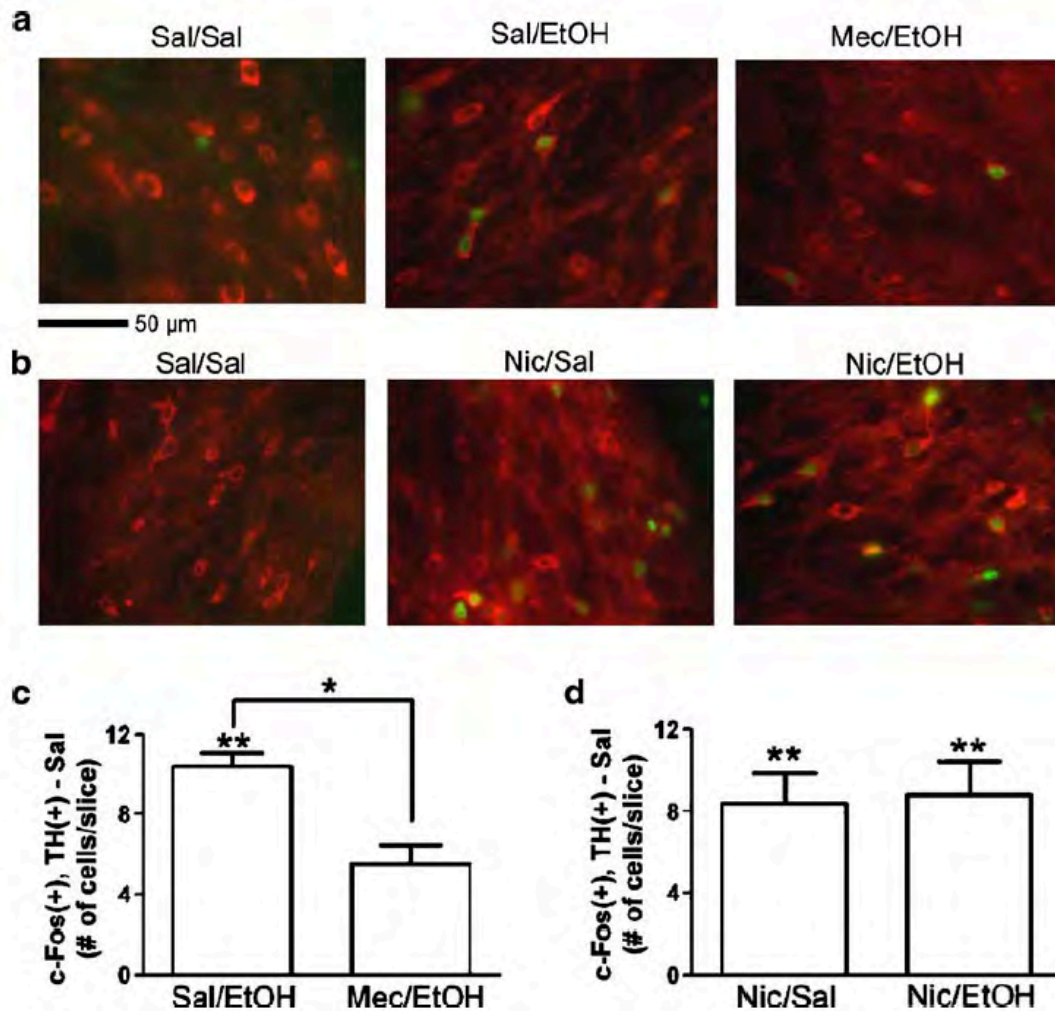
Figure II-5. Mecamylamine and nicotine exhibit distinct effects on ethanol-induced VTA DAergic neuron activation. A) Representative images depicting VTA slices from mice receiving two saline injections (left), saline followed by a 2.0 g/kg ethanol injection (middle), or 3.0 mg/kg mecamylamine followed by a 2.0 g/kg ethanol injection (right). Slices are fluorescently double-labeled with anti-tyrosine hydroxylase (red) and anti-c-Fos (green). B) Representative images depicting VTA slices from mice receiving saline injections (left), 0.5 mg/kg nicotine followed by saline (middle), or 0.5 mg/kg nicotine followed by 2.0 g/kg ethanol (right). C) Average number of c-Fos positive, TH positive cells per slice from mice treated as in A. D) Average number of c-Fos positive, TH positive cells per slice from mice treated as in b. Baseline c-Fos positive, TH positive cells from saline-injected control mice were subtracted from each value. Cells were counted from 23 to 33 VTA slices per mouse. Three mice per treatment were used for analysis. Asterisks directly above each bar indicate significance from saline-treated control mice. *$p<0.05$, **$p<0.01$

Preface to Chapter III

This chapter is unpublished, in preparation for submission.

Author contributions

Hendrickson LM, Tapper AR designed experiments.

Hendrickson LM, Derner M performed experiments.

Hendrickson LM performed imaging of c-Fos immunostaining and data analysis.

Hendrickson LM wrote the manuscript.

CHAPTER III

Alpha4* nAChRs play a critical role in ethanol reward

III.A. INTRODUCTION

Alcohol abuse is the third largest cause of preventable mortality in the world (CDC, 2004). Similar to all drugs of abuse, ethanol activates the mesocorticolimbic dopamine reward pathway resulting in a release of dopamine (DA) in the nucleus accumbens (NAc) (Soderpalm et al., 2009). While it is clear how many addictive drugs including nicotine and cocaine activate this pathway, it is unclear how ethanol modulates DA release. Systemic ethanol has been shown to increase acetylcholine (ACh) concentrations in the ventral tegmental area (VTA), presumably activating nicotinic acetylcholine receptors (nAChRs) in this area (Ericson et al., 2003) and several studies in the past two decades have shown that ethanol-induced activation of the mesolimbic dopamine system may be modulated by nAChRs. In rats, ethanol mediated DA elevation in NAc is inhibited by either direct infusion into the VTA or systemic administration of the non-competitive nicotinic receptor antagonist, mecamylamine (Blomqvist et al., 1993; Blomqvist et al., 1996; Ericson et al., 2003) suggesting nAChRs specifically within the VTA are important for ethanol reinforcement. Additionally, blocking VTA nAChRs decreases ethanol self-administration in high-ethanol-preferring rats (Blomqvist et al., 1996). Recently, using a restricted access ethanol-drinking paradigm termed 'Drinking-in-the-dark' (DID) as a model of

binge drinking, our lab has demonstrated that nAChRs are involved in acute voluntary ethanol drinking to intoxication in C57BL/6J mice. However, as multiple nAChR subtypes exist throughout the VTA, an important goal of alcohol research is to identify which subtype(s) modulate ethanol reinforcement.

Neuronal nAChRs are ligand gated cation channels that are activated by the endogenous neurotransmitter, ACh, as well as the addictive component of tobacco smoke, nicotine. Currently, 12 neuronal nAChR subunits have been identified (α2-10 and β2-4). The majority of high affinity nAChRs are heteromeric pentamers consisting of α and β subunits. Thus, multiple receptor subtypes with varying subunit compositions, and electrophysiological properties exist (Albuquerque et al., 2009).

Indeed, many neuronal nAChR subtypes are expressed throughout the VTA in both DAergic neurons projecting to striatum and in local GABAergic interneurons (Klink et al., 2001; Wooltorton et al., 2003). How does ethanol interact with these receptors? Interestingly, systemic ethanol has been shown to increase ACh concentrations in the VTA, presumably, activating nAChRs in this area (Ericson et al., 2003). In addition, ethanol can directly modulate nAChR activity depending on the subtype of nicotinic receptor expressed (Forman and Zhou, 2000; Zhou et al., 2000; Zuo et al., 2002). Because a variety of subtypes exist in these nuclei, an emerging goal of alcohol research is focused on identifying the nicotinic receptor subtype(s) that underlie ethanol reward.

One of the highest affinity and widely expressed nAChR subtypes in the brain are those that contain the α4 subunit, termed α4* nAChRs (Grady et al., 2007; Klink et al., 2001). These receptors have been shown to be necessary and sufficient for nicotine addiction (Tapper et al., 2004) and interestingly nicotine dependence and alcoholism have very high rates of comorbidity (Bobo, 1992; Miller and Gold, 1998). Additionally, to our knowledge, the role of α4* nAChRs has not been thoroughly investigated in mouse models of voluntary ethanol consumption or ethanol conditioned place preference.

III.B. MATERIALS AND METHODS

Animals. Adult (8-10 week) male α4 KO mice and their wild-type (WT) litter-mates, as well as heterozygous Leu9′Ala KI mice and their WT litter-mates, all bred on site, were used.   The genetic engineering of both α4 KO and Leu9′Ala mouse lines have been described previously (Ross et al., 2000; Tapper et al., 2004). Both lines have been back-crossed to a C57BL/6J background > nine generations.   For consumption experiments, mice were individually housed on a reversed 12 h light/ 12 h dark cycle (lights on 10 PM, off 10 AM) with *ad libitum* access to food and water (except during experiments as described below). For all other experiments, mice were group housed in the colony room on a standard light/dark cycle with *ad libitum* access to food and water. All experiments were conducted in accordance with the guidelines for care and use of laboratory animals provided by the National Research Council(National Research Council,

1996), as well as with an approved animal protocol from the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Drugs and drinking solutions. Ethanol drinking solutions were prepared from 190 proof absolute anhydrous ethanol (95 % ethanol, 5 % water; Pharmco-Aaper) diluted to 2 %, 5 %, 10 % or 20 % ethanol (v/v) using tap water. Sucrose (EMD) was dissolved in tap water to make a 10 % (w/v) concentration. Saccharin sodium salt hydrate (Sigma-Aldrich,) was dissolved in tap water to a final concentration of 10 mM. Quinine was dissolved in tap water to a final concentration of 0.1 mM.

Drinking in the dark (DID). Ethanol consumption was measured using a DID procedure as previously described (Hendrickson et al., 2009). Animals were singly housed in experimental chambers for 1 week prior to the beginning of the DID sessions. The mice received a 15-ml graduated cylinder water bottle fitted with a one-hole rubber stopper with a stainless steel double-ball-bearing sipper tube which was sealed with parafilm to prevent leakage. In week one, for the first four nights, two hours after the lights were off, the water bottle was removed and replaced with a 2 % ethanol bottle, and mice were allowed to drink for two hours. This procedure was repeated the following weeks, with the concentrations increasing to 5 %, 10 % and 20 % on weeks 2, 3, and 4, respectively. Therefore, each concentration of ethanol was seen for 4 consecutive nights, with 3 nights off

in between each ethanol concentration. The amount of ethanol consumed was recorded immediately after each two-hour session and converted to g/kg per each animal's ethanol consumption and body weight. In a separate group of mice, saccharine, quinine and sucrose intake was measured, in a similar assay but mice only drank each solution for two nights.

Ethanol metabolism. Prior to an ethanol injection, blood was obtained from the tail vein (~30 uL each time point) to provide a zero point for each animal. After a 2 g/kg i.p. injection of ethanol, blood samples were taken at intervals of 30, 60, 90, and 120 min. Blood was collected in heparinized capillary tubes, centrifuged at 1500Xg for 5 minutes and blood analyzed using an alcohol oxidase-based assay. Blood ethanol concentrations were measured on a GM7 Micro-Stat Analyzer (Analox Instruments Ltd.)

Immunohistochemistry. Adult (8-10 weeks), male, α4 KO mice and their WT litter-mates, as well as heterozygous Leu9′Ala KI mice and their WT litter-mates were injected i.p. with saline for three days prior to the start of the experiment to habituate them to handling and to reduce c-Fos activation due to stress. In the α4 KO and WT group, two groups of three mice from each genotype were used in each of the following conditions: an i.p. injection of saline or an i.p. injection of 2.0 g/kg ethanol. In the Leu9′Ala and WT group, three groups of three mice were used per genotype in each of the following conditions: an i.p. injection of saline,

0.5 g/kg ethanol or 2.0 g/kg ethanol. Ninety minutes after the injection, mice were deeply anesthetized with sodium pentobarbital (200 mg/kg, i.p.) and perfused transcardially with 10 ml of 0.1 M phosphate-buffered saline (PBS) followed by 10 ml of 4 % paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Brains were removed and post-fixed for 2 h with the same fixative and cryoprotected in sodium phosphate buffer containing 30 % sucrose until brains sank. VTA serial coronal sections (20 µm) were cut on a microtome (Leica Microsystems, Inc.) and collected into a 24-well tissue culture plate containing 1 X PBS. Slices containing VTA were collected between -2.8 mm and -4.04 mm from bregma. After rinsing sections in PBS twice for 5 min, they were treated with 0.2 % Triton X-100 PBS (PBST) for 5 min followed by incubation in 2 % BSA/PBS for 30 min. Sections were washed with PBS once and then incubated in a cocktail of primary antibodies for tyrosine hydroxylase (TH, mouse monoclonal, 1:250 dilution, Santa Cruz Biotechnology) and c-Fos (rabbit polyclonal, 1:400 dilution, Santa Cruz Biotechnology) in 2 % BSA/PBS overnight at 4°C. The sections were then washed with PBS three times for 5 min followed by incubation in secondary fluorescent labeled antibodies (goat anti- rabbit Alexa Fluor® 488 and goat anti-mouse Alexa Fluor® 594, 1:300 dilutions, Molecular Probes, Inc.) at room temperature in the dark for 30 min. After washing with PBS 5 times for 5 min/wash, sections were mounted on slides using VECTASHIELD® Mounting Medium (Vector Laboratories, Inc.). The number of positive neurons was counted under a fluorescence microscope (Carl Zeiss MicroImaging, Inc.) at

a magnification of 400X. The intensity of fluorescence was quantified using a computer-associated image analyzer (Axiovision Release 4.6). Neurons were counted as signal-positive if intensities were at least 2 times higher than that of the average value of background (sections stained without primary antibodies). Image analysis was performed by an individual blind to drug treatment.

Conditioned Place Preference (CPP). The conditioning and testing apparatus (Med Associates) used contains two white chambers (6.6" x 5.0") with a door separating the two sides. The floors of the chambers are interchangeable; one a 'ROD' floor and the other a 'MESH' floor. The apparatus is placed inside a sound attenuation chamber equipped with a fan and corner mounted lights. Infrared photo beams record the movement and activity of the mouse in each chamber and data was collected by MED-PC software. The CPP experiment consists of one habituation session, eight training sessions and one testing session. On the habituation day, one side of the chamber has the ROD floor and the other side has the MESH floor. The mice are allowed free access to both sides of the chamber for 30 min and the time spent on each floor during the 30 min session is recorded. For training, mice are assigned to an ethanol (+) and saline (-) floor combination: ROD+ and MESH- or MESH+ and ROD-. In the first training session, mice are injected with saline and placed in the chamber with both sides containing the saline-paired floor (i.e. for the ROD+ group, the mice are injected with saline and placed on mesh floors). The mice are allowed free access to

both sides for 5 min. The following day, mice are injected with ethanol (α4 KO

and WT receive 2 g/kg while Leu9′Ala and WT receive 0.5 g/kg) and placed in

the chamber for 5 min with both sides containing the ethanol-paired floors (i.e. for

the ROD+ group, the mice are injected with ethanol and placed on rod floors).

Daily training session alternate between the ethanol-paired and saline-paired

floors for eight sessions such that the mice experience the ethanol-paired floor

on the ninth day. On the test day, mice are placed in the chamber with one side

ROD floors and one side MESH floors for 30 min and the time spent on each

floor is recorded. A difference score is calculated by time spent on the ethanol-

paired floor on the Test Day - Habituation Day such that a positive number

indicates that more time was spent on the ethanol-paired floor after training.

Data Analysis. Data were analyzed using One-way or Two-way ANOVAs with

genotype and treatment as variables followed by Bonferroni *post hoc* tests. Data

were analyzed using Graphpad software (Graphpad Software, Inc.).   Results

were considered significant at $p<0.05$. All data are expressed as means ±

standard errors of means (SEM).

III.C. RESULTS

α4 KO mice consume less ethanol than WT mice

To determine if α4* nAChRs modulate voluntary ethanol consumption, we

measured baseline ethanol intake in α4 KO and WT mice using the DID assay at

4 different concentrations of ethanol (2 %, 5 %, 10 %, and 20 % ethanol, Fig. III-1A). Two-way ANOVA revealed a significant main effect of treatment ($F_{3,60}$ = 103.6, $p<0.0001$), genotype ($F_{1,60}$ = 36.25, $p<0.0001$) and a significant treatment x genotype interaction ($F_{3,60}$ = 11.37, $p<0.0001$). The *post hoc* test indicates that α4 KO mice drank significantly less 10 % and 20 % ethanol compared to WT mice (Fig. III-1A, $p<0.001$ for both concentrations, consumption of 2 % and 5 % was not statistically different).

Because ethanol has both taste and caloric value, we measured intake of one tastant solution, quinine, and one caloric solution, sucrose, in α4 KO and WT mice (Fig. III-2A). We found no significant difference in intake between the two genotypes for any of the solutions tested. Furthermore, there is no difference in the metabolism of an i.p. injection of 2 g/kg ethanol in α4 KO and WT mice (Fig. III-2C).

Voluntary ethanol consumption in Leu9′Ala mice does not differ from WT

We measured voluntary 2 %, 5 %, 10 %, and 20 % ethanol intake in Leu9′Ala mice and their WT littermates using the same assay described above (Fig. III-1B). Surprisingly, we saw no significant differences between ethanol consumption of Leu9′Ala and WT mice in any concentration tested. Additionally, there were no differences in quinine or sucrose intake or ethanol metabolism between Leu9′Ala and WT mice (Fig. III-2B, 2C).

Ethanol activates dopaminergic neurons of posterior VTA by an α4* nAChR dependent mechanism

Previously, using c-Fos as a marker for neuronal activation and tyrosine hydroxylase (TH) as a marker for dopaminergic (DAergic) neurons, we have shown that ethanol activates DAergic neurons of the posterior VTA and that these activated neurons express higher levels of the α4, α6 and β3 nAChR subunit mRNA (Hendrickson et al., 2010). To determine if α4* nAChRs are necessary for this activation, we injected α4 KO and WT mice with 2 g/kg ethanol and examined the VTA for c-Fos expression within TH-immunopositive neurons 90 min later (Fig. III-3). Two-way ANOVA indicated that ethanol had no effect on c-Fos expression of TH neurons of the anterior VTA in either genotype (Fig. III-3C). However, in the posterior VTA, there was a significant main effect of genotype ($F_{1,8}$ = 8.15, $p<0.05$), treatment ($F_{1,8}$ = 12.28, $p<0.01$) and a significant genotype x treatment interaction ($F_{1,8}$ = 13.25, $p<0.01$). Bonferroni post-test indicates that WT mice injected with 2 g/kg ethanol had significantly higher expression of c-Fos compared to α4 KO mice injected with 2 g/kg ethanol (Fig. III-3C, $p<0.01$). One-way ANOVAs also indicated that WT mice treated with 2 g/kg ethanol had significantly increased c-Fos expression compared to a saline injection (Fig. III-3A, 3C, $p<0.05$) in the posterior VTA whereas the same dose of ethanol had no effect in α4 KO mice (Fig. III-3B, 3C, $p> 0.05$).

Because of the lack of ethanol induced c-Fos expression in α4KO mice, we sought to determine if α4* nAChRs are sufficient for ethanol activation of DAergic neurons. We injected hypersensitive Leu9′Ala and WT mice with two concentrations of ethanol, the rewarding dose of 2 g/kg and a sub-threshold dose

of 0.5 g/kg and analyzed their brains for c-Fos expression in TH-immunopositive neurons. In the anterior VTA, there was no significant expression of c-Fos in either genotype, after both doses of ethanol (Fig. III-4E). In the posterior VTA, Two-Way ANOVA revealed a significant main effect of treatment ($F_{2,14} = 22.01$, $p<0.001$), genotype ($F_{1,14} = 11.65$, $p<0.01$) and a significant treatment x genotype interaction ($F_{2,14} = 8.97$, $p<0.01$). A Bonferroni post-test indicates that Leu9′Ala mice treated with 0.5 g/kg had significantly increased c-Fos expression compared to WT mice (Fig. III-4B, 4E, $p<0.0001$). Additionally, one-way ANOVAs revealed that Leu9′Ala mice treated with 0.5 g/kg and 2 g/kg ethanol had significantly increased c-Fos expression compared to a saline injection ($p<0.01$ for both doses) and WT mice treated with 2 g/kg had significantly increased c-Fos expression compared to both saline and 0.5 g/kg treated WT mice ($p<0.001$ for both, Fig. III-4E).

α4* nAChRs are critical for ethanol reward

Since α4 KO mice drink less ethanol than WT mice and are resistant to ethanol activation of DAergic neurons of the posterior VTA, we hypothesized that they would also be resistant to ethanol reward. To assess ethanol reward, we measured ethanol conditioned place preference (CPP) in α4 KO and WT mice using the known rewarding dose of ethanol, 2 g/kg (Fig. III-5A). There was a significant main effect of treatment ($F_{1,22} = 20.27$, $p<0.001$) but not genotype and there was no interaction between the two. Bonferroni post-test indicates that after training, WT mice spent significantly more time on the ethanol-paired floor

compared to the saline paired floor (Fig. III-5A, $p<0.001$) while the time spent on the ethanol or saline paired floors did not significantly differ for α4 KO mice after training ($p>0.05$). These data suggest that α4* nAChRs are necessary for ethanol induced CPP, and thus of ethanol reward.

Based on the c-Fos experiments above, we hypothesized that Leu9′Ala mice may have a lower threshold for ethanol reward. To test this, we used the CPP assay, but lowered the dose of ethanol to 0.5 g/kg, the same dose that significantly increased DAergic neuron activation in the posterior VTA of Leu9′Ala mice, but had no effect in WT mice (Fig. III-4A, 4B, 4E). Two-way ANOVA indicated a significant main effect of treatment ($F_{1,26}$ = 9.92, $p<0.01$) but not of genotype and there was no significant interaction between the two. Bonferroni post-test revealed a significant difference between the time spent in the 0.5 g/kg ethanol and saline paired chambers of Leu9′Ala mice, but not of WT mice (Fig. III-5B, $p<0.05$).

III.D. DISCUSSION

Since a variety of nAChR subtypes are expressed in the mesolimbic reward circuitry (Wooltorton et al., 2003), an emerging goal of alcohol research is focused on identifying the nicotinic receptor subtype(s) that may underlie ethanol reward. Attempts have been made to identify possible nAChR subtypes that may be involved, however, most studies to date have used pharmacological approaches and have had mixed results. For example, the nonspecific

antagonist, mecamylamine, when injected systemically or locally within the VTA, blocks ethanol self-administration and locomotor stimulation (i.e., sensitization) in rats (Blomqvist et al., 1992; Ericson et al., 1998) as well as reduced voluntary ethanol intake in mice (Hendrickson et al., 2009). Both methyllycaconitine citrate (MLA) and dihydro-β-erythroidine (DHβE), α7 and β2* nAChR specific antagonists, respectively, failed to block ethanol consumption in rats (Le et al., 2000) and mice (Hendrickson et al., 2009) or ethanol-induced dopamine overflow in the NAc (Ericson et al., 2003; Larsson et al., 2002).

Here, we used two complementary genetically modified nAChR mouse models to investigate the role of α4* nAChRs in ethanol reward. Utilizing a voluntary ethanol drinking assay, we have identified α4* nAChRs as being necessary for voluntary ethanol intake. In the absence of α4* nAChRs, mice consume significantly less 10 % and 20 % ethanol compared to their WT littermates. Importantly, α4 KO mice consume the same amount of sucrose and quinine as WT mice indicating that the difference in ethanol intake was specific for the drug and not a more general effect on caloric/taste reward or drinking volume.

Surprisingly, ethanol consumption did not significantly differ between Leu9′Ala and WT littermate mice at any concentration of ethanol tested. However, the current number of mice used in the assay remains low and the large error bars warrant further investigation, especially that of 10 % and 20 % ethanol.

We hypothesized that α4* nAChRs are necessary for ethanol-induced activation of the mesolimbic dopamine pathway. The lack of these receptors in the α4 KO mice would result in less ethanol consumption than in WT mice, as we saw. In addition, our data measuring c-Fos induction in ventral midbrain, as a marker for neuronal activation, supports this hypothesis. Similar to previous studies, an acute injection of the rewarding dose of ethanol, 2 g/kg, activated tyrosine hydroxylase (TH) positive neurons of the posterior VTA of WT mice; whereas this same dose in α4 KO mice resulted in no activation. Next, we showed that the sub-reward threshold dose of 0.5 g/kg ethanol significantly activated the posterior VTA of the hypersensitive Leu9′Ala mice, but had no effect in WT mice. Taken together, our data suggest that activation of α4* nAChRs mediate ethanol-induced activation of the posterior VTA, a key region of the mesolimbic dopamine reward pathway.

Additionally, the rewarding properties of ethanol were absent in α4 KO mice as measured by the place preference assay. This result is consistent with previous work indicating that ethanol place preference is expressed through a VTA dependent mechanism (Bechtholt and Cunningham, 2005) and that DAergic neuron activity is sufficient for CPP (Tsai et al., 2009). Our CPP results are less likely to be affected by differences in learning between WT and α4 KO mice because, in the passive avoidance paradigm, both genotypes learned to avoid the dark chamber after it was paired with a 2 mA shock (data not shown). Furthermore, the hypersensitive Leu9′Ala mice conditioned a place preference to

an extremely low dose of ethanol, 0.5 g/kg, suggesting that activation of α4* nAChRs modulates ethanol induced reward.

While nAChRs are known to play a primary role in nicotine dependence, to our knowledge, this is the first study firmly implicating α4* nAChRs in modulating the reinforcing effects of alcohol. Our data indicate that expression of α4* nAChRs is necessary for voluntary consumption of 10 % and 20 % ethanol, ethanol-induced activation of the posterior VTA and ethanol reward. Additionally, our results highlight the importance of α4* nAChRs as possible targets for alcohol cessation therapeutics.
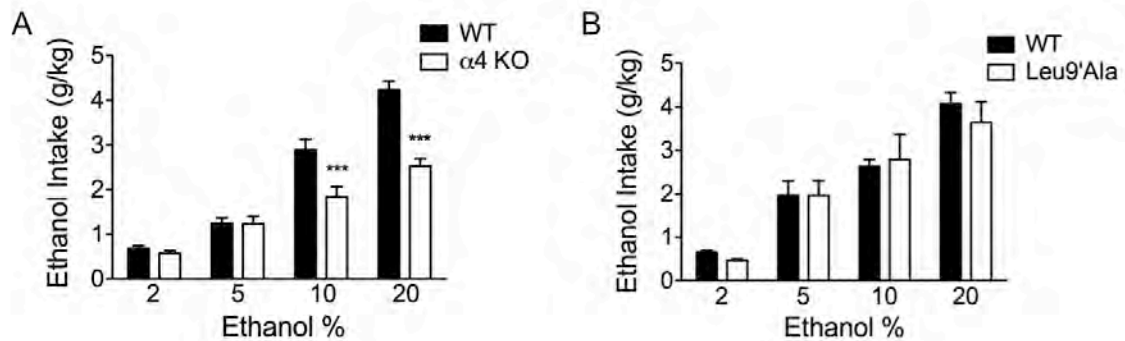
Figure III-1. Voluntary ethanol intake in a 2h limited access assay in α4 KO and Leu9′Ala nAChR mouse models. A) Ethanol intake in g/kg (± SEM) of α4 KO mice was not significantly different from WT litter-mate mice at lower concentrations of ethanol (2 % and 5 %) but was significantly lower than WT mice at higher concentrations of ethanol (10 % and 20 %) (n = 8/genotype). B) Leu9′Ala mice consumed similar amounts of ethanol at all concentrations tested compared to WT litter-mate mice (n = 4-8/genotype). ***$p<0.001$
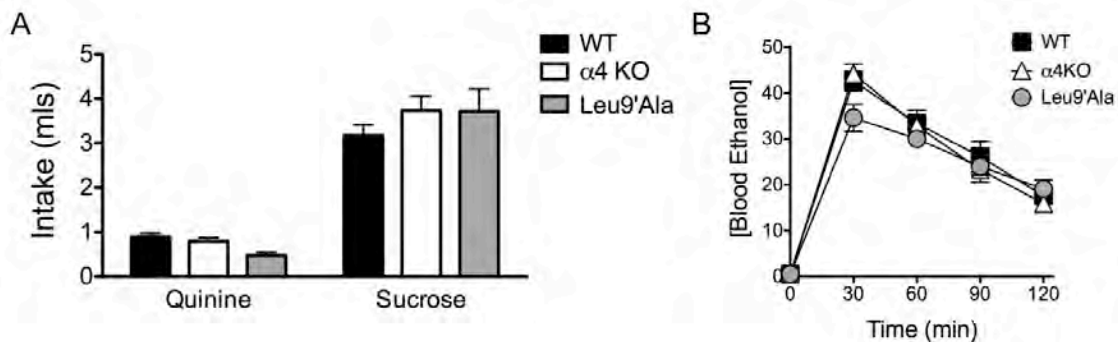
Figure III-2. α4 KO and Leu9′Ala mice have no differences in taste, caloric or metabolic controls. A) WT, α4 KO and Leu9′Ala mice consume similar volumes (mls ± SEM) over a two hour period of the bitter solution quinine, as well as the sweet, caloric solution, sucrose (n = 8-19/genotype/solution). B) WT, α4 KO and Leu9′Ala have similar peak blood ethanol concentration (in mM) and ethanol metabolism after challenge with 2 g/kg i.p. injection of ethanol (n = 4/genotype).

A WT: 2 g/kg ethanol

B α4 KO: 2 g/kg ethanol

C
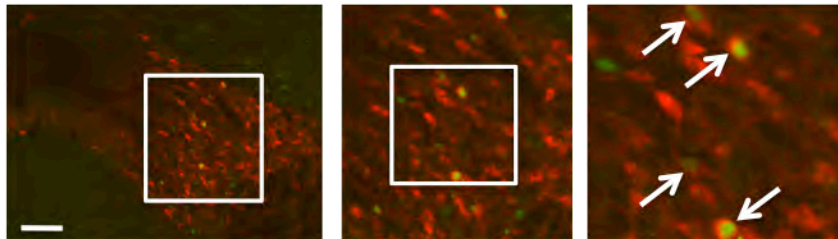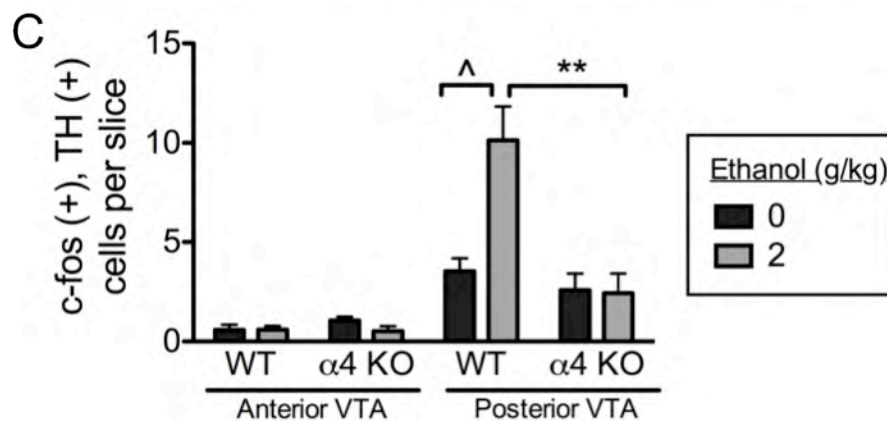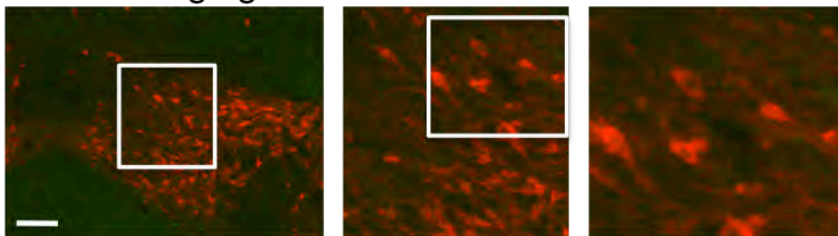
Figure III-3. Ethanol induced c-Fos activation in TH positive neurons is dependent on α4* nAChRs. Representative photomicrographs illustrating midbrain sections of the posterior VTA from A) WT mice and B) α4 KO mice injected with 2 g/kg ethanol. Sections are immunolabeled for TH (red) and c-Fos (green). White boxes delineate slice regions that are magnified in the adjacent photomicrographs. Scale bar = 100 µm. C) Number of TH (+) c-Fos (+) neurons per slice taken from mice given an i.p. injection of 2 g/kg ethanol. 48 slices/treatment/mouse were analyzed, n = 3 mice/treatment. One-way ANOVA and Bonferroni post-test comparing saline to ethanol treatment in WT mice was used, $^{\wedge}p<0.05$. Two-way ANOVA and Bonferroni post-test comparing treatments in WT and α4 KO mice was also used, $^{**}p<0.01$.
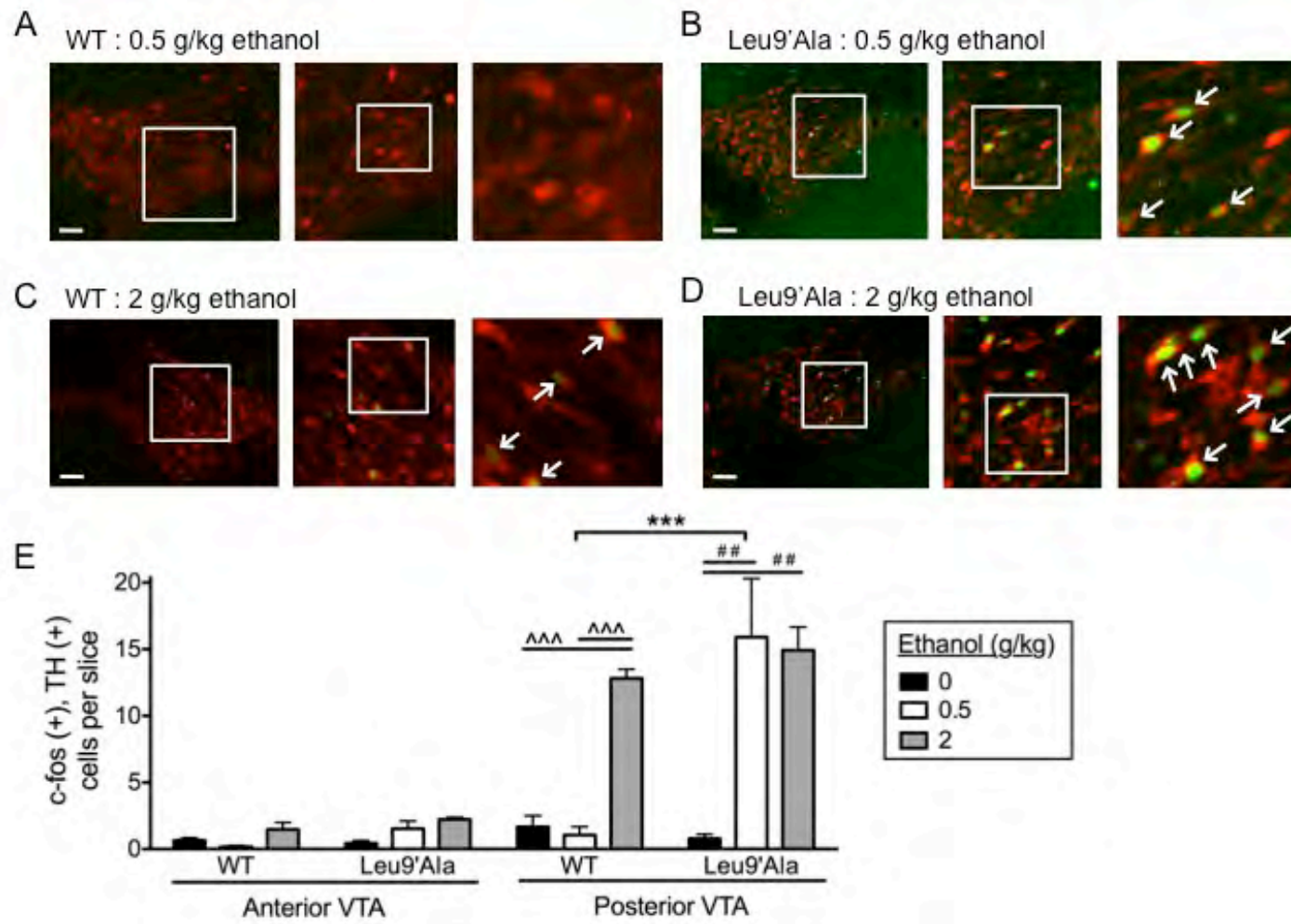
Figure III-4.

Figure III-4. α4* nAChRs are sufficient for ethanol induced activation of DAergic neurons. Representative photomicrographs illustrating midbrain sections of the posterior VTA from A) WT mice and B) Leu9′Ala mice injected with 0.5 g/kg ethanol as well as C) WT mice and D) Leu9′Ala mice injected with 2 g/kg ethanol. Sections are immunolabeled for TH (red) and c-Fos (green). White boxes delineate slice regions that are magnified in the adjacent photomicrographs. White arrowheads point to neurons that are TH (+) c-Fos (+). Scale bar = 100 μm. E) Number of TH (+) c-Fos (+) neurons per slice taken from mice given an i.p. injection of 0.5 g/kg or 2 g/kg ethanol. 48 slices/treatment/mouse were analyzed, n = 3 mice/treatment. One-way ANOVA and Bonferroni post-test comparing saline to ethanol treatments in WT mice or Leu9′Ala mice was used, ^^^$p<0.001$ for WT mice, ##$p<0.01$ for Leu9′Ala mice. Two-way ANOVA and Bonferroni post-test comparing treatments in WT and α4 KO mice was also used, ***$p<0.001$.
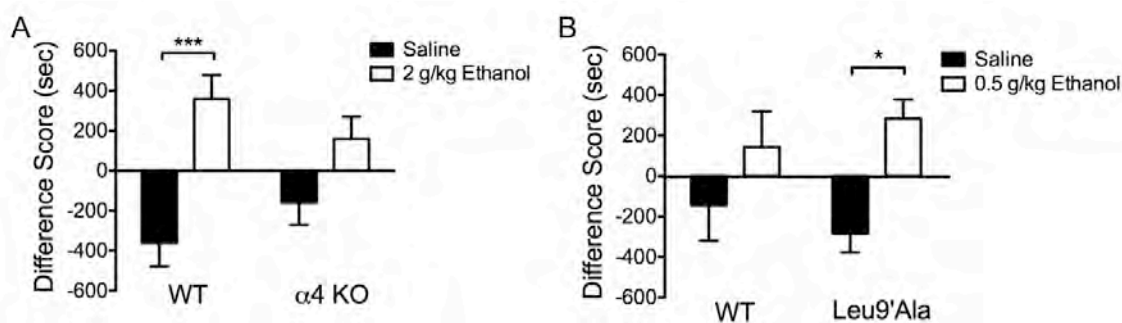
Figure III-5. α4* nAChRs are critical for ethanol reward. A) After 4 training sessions with 2 g/kg ethanol, WT mice spent significantly more time on the ethanol-paired floor compared to the saline-paired floor. Difference score (in seconds) calculated as the difference in time spent on each floor on the test day compared to the habituation day. In the α4 KO mice, the time spent on the ethanol-paired floor did not differ from the time spent on the saline-paired floor (n = 8/genotype). B) After 4 training sessions with the low dose of ethanol, 0.5 g/kg, Leu9'Ala mice spent significantly more time on the ethanol paired floor compared to the saline paired floor while there was no significant difference in the WT mice (n = 8/genotype). Two-way ANOVA and Bonferroni post-test comparing treatments in WT and α4 KO or WT and Leu9'Ala mice was used, *$p<0.05$, ***$p<0.001$.

Preface to Chapter IV

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Author Contributions:

Hendrickson LM, Gardner PD, Tapper AR designed experiments.

Hendrickson LM performed all experiments except LCM & qRT-PCR.

Zhao-Shea R performed LCM & qRT-PCR experiments and imaging.

Pang X assisted in surgeries.

Hendrickson LM, Tapper AR wrote the paper.

CHAPTER IV

Activation of α4* nAChRs is necessary and sufficient for varenicline-induced

reduction of alcohol consumption.

IV.A. INTRODUCTION

Complications from alcoholism are responsible for up to 1.8 million deaths per year making it the third largest cause of preventable mortality in the world (W.H.O., 2004). Despite large costs to society, very few therapeutics that successfully aid in curbing alcohol consumption are available, highlighting the need to identify new molecular targets and treatments for alcoholism. Recently, varenicline, a neuronal nicotinic acetylcholine receptor (nAChR) partial agonist, currently FDA approved as a smoking cessation aid (Coe et al., 2005; Jorenby et al., 2006), was shown to reduce drinking in alcohol preferring rats (Steensland et al., 2007) and in a group of heavy-drinking smokers (McKee et al., 2009), suggesting that nAChRs may represent novel therapeutic targets for reducing alcohol consumption.

Ethanol is reinforcing, at least in part, by its propensity to activate dopaminergic (DAergic) neurons within the ventral tegmental area (VTA) (Brodie and Appel, 1998; Brodie et al., 1999), a key region of the mesocorticolimbic DA system, resulting in DA release in the nucleus accumbens (NAc), a phenomenon widely associated with drug reinforcement (Soderpalm et al., 2009). Several nAChR subunit genes are expressed throughout the mesocorticolimbic DA

system particularly in DAergic neurons of the VTA. Neuronal nAChRs are ligand-gated, pentameric cation channels normally activated by the endogenous neurotransmitter, acetylcholine (ACh). Twelve mammalian genes encoding neuronal nAChR subunits have been identified (α2-α10, β2-β4) which form either hetero- or homomeric receptors yielding a vast array of nAChR subtypes with distinct pharmacological and biophysical properties (Albuquerque et al., 2009). Although ethanol is not a direct agonist of nAChRs, alcohol increases ACh release into the VTA (Ericson et al., 2003) and potentiates the response of high affinity nAChR subtypes to ACh (Zuo et al., 2002). Mecamylamine, a non-selective nAChR antagonist, either directly infused into the VTA or delivered systemically, decreases ethanol mediated DA release in the NAc (Blomqvist et al., 1997; Larsson et al., 2002) and also decreases self-administration in rodents (Ericson et al., 1998; Hendrickson et al., 2009). Thus, nAChR activation modulates alcohol consumption and reinforcement.

While varenicline was designed as a selective α4β2* nAChR partial agonist (Coe et al., 2005; Jorenby et al., 2006), it is also a partial agonist at α3β2* and α6* nAChR subtypes, and a full agonist at α3β4* and α7 nAChR subtypes (Mihalak et al., 2006). Varenicline has also been shown to reduce alcohol consumption in knockout (KO) mice lacking expression of either α7 or β2* nAChRs (Kamens et al., 2010). Thus, the nAChR subtype(s) that varenicline targets to reduce alcohol consumption is unknown. The goal of the present study was to localize and identify nAChR subtypes expressed in the VTA that may be

involved in the response to alcohol and to determine if they play a role in the molecular mechanism by which varenicline reduces alcohol consumption.

IV.B MATERIALS AND METHODS

Animals. Adult (8-10 week), male C57BL/6J mice bred in house were used for immunohistochemistry, gene expression, and brain infusion experiments (n = 46). For consumption experiments, adult, male α4 knock-out (α4 KO) mice and their wild type (WT) litter mates (n = 45), as well as heterozygous Leu9′Ala knock-in mice and their WT litter mates (n = 42), all bred on site, were used. The genetic engineering of both α4 KO and Leu9′Ala mouse lines have been described previously (Ross et al., 2000; Tapper et al., 2004). Both lines have been back-crossed to a C57BL/6J background for at least nine generations. C57BL/6J mice were group housed four mice/cage and given food and water *ad libitum*. For consumption experiments, mice were individually housed on a reversed 12 h light/dark cycle (lights on 10 PM, off 10 AM) with *ad libitum* access to food and water (except during experiments as described below). All experiments were conducted in accordance with the guidelines for care and use of laboratory animals provided by the National Research Council (National Research Council, 1996), as well as with an approved animal protocol from the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Drugs and drinking solutions. Ethanol drinking solutions were prepared from 190 proof absolute anhydrous ethanol (Pharmco-Aaper) diluted to 2 % or 20 % ethanol (v/v) using tap water. Sucrose was dissolved in tap water to make a 10 % (w/v) concentration. Varenicline tartrate, a gift from Pfizer, and ethanol were dissolved in 0.9 % saline and administered via intraperitoneal (i.p.) injection at the indicated doses. For infusion of drug into the brain, varenicline was dissolved in artificial cerebrospinal fluid (aCSF containing, in mM: 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 25 D-glucose). For immunohistochemistry and behavioral experiments, varenicline doses were chosen based on previous studies of varenicline effects on nicotine self-administration and DA turnover in addition to predicted therapeutic concentrations achieved in smokers' brains (O'Connor et al., 2010; Rollema et al., 2010). Varenicline concentrations are reported as freebase.

Immunohistochemistry. Adult (8-10 weeks), male, C57BL/6J mice were injected i.p. with saline for three days prior to the start of the experiment to habituate them to handling and to reduce c-Fos activation due to stress. Four groups of three mice were used in each of the following conditions: an i.p. injection of saline followed by an i.p. injection of saline, an i.p. injection of saline, followed by a 2.0 g/kg ethanol injection, an i.p. injection of 0.3 mg/kg varenicline followed by a saline injection, and 0.3 mg/kg varenicline i.p. injection followed by a 2.0 g/kg ethanol injection. The time between the first and second injections was 15 min.

Ninety minutes after the second injection, all mice were deeply anesthetized with sodium pentobarbital (200 mg/kg, i.p.) and perfused transcardially with 10 ml of 0.1 M phosphate-buffered saline (PBS) followed by 10 ml of 4 % paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Brains were removed and post-fixed for 2 h with the same fixative and cryoprotected in sodium phosphate buffer containing 30 % sucrose until brains sank. VTA serial coronal sections (20 μm) were cut on a microtome (Leica Microsystems Inc.) and collected into a 24-well tissue culture plate containing 1 X PBS. Slices containing VTA were collected between -2.8 mm and -4.04 mm from bregma. After rinsing sections in PBS twice for 5 min, they were treated with 0.2 % Triton X-100 PBS (PBST) for 5 min followed by incubation in 2 % BSA/PBS for 30 min. Sections were washed with PBS once and then incubated in a cocktail of primary antibodies for tyrosine hydroxylase (TH mouse monoclonal, 1:250 dilution, Santa Cruz Biotechnology) and c-Fos (rabbit polyclonal, 1:400 dilution, Santa Cruz Biotechnology) in 2 % BSA/PBS overnight at 4°C. The sections were then washed with PBS three times for 5 min followed by incubation in secondary fluorescent labeled antibodies (goat anti-rabbit Alexa Fluor® 488 and goat anti-mouse Alexa Fluor® 594, 1:300 dilutions, Molecular Probes, Inc.) at room temperature in the dark for 30 min. After washing with PBS 5 times for 5 min/wash, sections were mounted on slides using VECTASHIELD® Mounting Medium (Vector Laboratories, Inc.). The number of positive neurons was counted under a fluorescence microscope (Carl Zeiss MicroImaging Inc.) at a

magnification of 400X. The intensity of fluorescence was quantified using a computer-associated image analyzer (Axiovision Rel. 4.6). Neurons were counted as signal-positive if intensities were at least 2 times higher than that of the average value of background (sections stained without primary antibodies). Image analysis was performed by an individual blind to drug treatment.

Laser Capture Microdissection (LCM). Adult, male C57BL/6J mice were i.p. injected with saline for three days prior to the start of the experiment to habituate them to handling and to reduce c-Fos activation due to stress. On the experiment day, mice were i.p. injected with 2.0 g/kg ethanol and decapitated 90 min later. The brain was removed, snap-frozen in dry ice-cooled 2-methylbutane (-60° C) and stored at -80° C. Coronal serial sections (10 µm) of the VTA were cut using a cryostat (Leica Microsystems Inc.) and mounted on pre-cleaned glass slides (Fisher Scientific). The sections were immediately placed in a slide box on dry ice until completion of sectioning followed by storage at -80°C. A quick immunofluorescence double-staining protocol for TH and c-Fos was used to identify TH and c-Fos immunopositive neurons. First, frozen sections were allowed to thaw for 30 seconds then immediately fixed in cold acetone for 4 min. Slides were then washed in PBS, incubated with a cocktail of primary antibodies for mouse anti-TH and rabbit anti-c-Fos (Santa Cruz Biotechnology, Inc., 1:50 dilutions) for 10 min, washed in PBS once followed by incubation in secondary fluorescent-labeled antibodies (Molecular Probes, Inc., a cocktail of goat anti-

mouse Alex Fluor 594® and goat anti-rabbit Alex Fluor 488®, 1:100 dilution) for 10 min. The slides were washed in PBS once, then subsequently dehydrated in a graded ethanol series (for 30 s each in 70 % ethanol, 95 % ethanol, 100 % ethanol, and once for 5 min in xylene). Slides were allowed to dry for 5 min. All antibodies were diluted in DEPC-treated PBS containing 2 % BSA and 0.2 % Triton X-100. All ethanol solutions and xylene were prepared fresh to preserve RNA integrity.

The Veritas^{TM} Microdissection System Model 704 (Arcturus Bioscience, Inc.) was used for LCM. Approximately 800 to 1400 TH-immunopositive neurons (including separate pools of c-Fos-immunopositive and c-Fos-immunonegative) were cut from the VTA in each animal. Five to seven different mice were used per treatment. Neurons were captured on CapSure® Macro LCM caps (Arcturus Bioscience, Inc.) for mRNA isolation.

Real-time PCR. Total RNA was extracted from individual replicate samples using a Micro Scale RNA Isolation Kit (Ambion, Inc.). RNA samples extracted from DAergic neurons were reverse-transcribed into cDNA using TaqMan® Gene Expression Cells-to-CT™ Kit (Ambion, Inc.). PCR reactions were set up in 10-µl reaction volumes using TaqMan Gene Expression Assays (ABI, Inc.). GAPDH was used as an internal control gene to normalize gene expression levels. PCR was performed using an ABI PRISM 7500 Sequence Detection System. Negative controls with no reverse transcriptase were performed for all Taqman Assays. All

reactions were performed in triplicate. Relative amplicon quantification was calculated as the difference between Ct values of GAPDH and that of the gene of interest. Relative gene expression differences between c-Fos immunopositive neurons and c-Fos immunonegative neurons were calculated using the $2^{-\Delta\Delta Ct}$ method.

Drinking in the Dark (DID). Ethanol consumption was measured using a similar Drinking in the Dark (DID) procedure as previously described (Hendrickson et al., 2009). Animals were singly housed in experimental chambers for 1 week prior to the beginning of the DID sessions. The mice received a 15-ml graduated water bottle fitted with a one-hole rubber stopper with a stainless steel double-ball-bearing sipper tube which was sealed with Parafilm to prevent leakage. For the first three nights, two hours after the lights were off, mice were i.p. injected with saline immediately before their water bottle was replaced with the ethanol bottle (2 % or 20 %), and allowed to drink for two hours. This procedure was used to acclimatize the mice to the experimental conditions and allow them to reach a baseline of ethanol intake prior to drug administration. On the fourth night, the mice received their first dose of drug just prior to placement of the ethanol bottle. The amount of ethanol consumed was recorded after each two-hour session and converted to g/kg per each animal's ethanol consumption and body weight. The mice were given 2 days of rest (no injections or ethanol) and then began the saline injection/ethanol consumption assay for two to three days or until a stable

ethanol intake was reached. Once the baseline returned, a second, higher dose of drug was administered prior to the ethanol bottle being placed in the cage. In this design, all mice in one group drink a single concentration of ethanol throughout the experiment, but receive two doses of drug, 4-5 days apart, with the lower concentration of drug first. The baseline value immediately prior to the first drug exposure is shown in all figures. There was no significant difference in baseline ethanol intake between doses in any of the experiments (data not shown). For control experiments, mice received 10 % sucrose for two hours instead of ethanol.

Cannula surgeries. C57BL/6J mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) (VEDCO). The surgical area was shaved and disinfected. Mice were placed in a stereotaxic frame (Stoelting Co) with mouse adaptor and a small incision was cut in the scalp to expose the skull. Using bregma and lambda as landmarks, the skull was leveled in the coronal and sagittal planes. For cannula placement, holes were drilled in the skull at the anteroposterior (AP, in reference to bregma) and mediolateral (ML) coordinates that correspond to either the anterior VTA (-2.6 mm AP, ± 0.5 mm ML) or posterior VTA (-3.4 mm AP, ± 0.5 mm ML) based on "The Mouse Brain in Stereotaxic Coordinates" (Paxinos and Franklin, 2000). Stainless steel unilateral guide cannula (-4.0 mm dorsal ventral, + 0.5 mm ML, Plastics One) with dummy cannula in place, were inserted into the brain and secured to the skull with

cerebond (Plastics One). Mice were allowed to recover for at least 3 days before behavioral testing.

Intra-VTA infusions and DID. Two hours after the lights were turned off, mice were anesthetized with 2 % Isoflurane via a nose cone adaptor at a flow rate of 800 ml/L. Once anesthetized, an infusion cannula designed to reach -4.5 mm below the skull was inserted into the guide cannula and vehicle, 10 pmol, 100 pmol, or 1000 pmol varenicline was infused at a rate of 1 µl/min for 45 s and a total volume of 0.75 µl. Immediately after infusion, mice were placed back into their home cages and monitored until awake, ~45 s. During this time, the home cage water bottle was removed and replaced with a bottle containing 20 % ethanol, which was left in place for 2 hours as previously described for the DID experiments. Before the drug exposure day, mice were infused with vehicle daily until a stable baseline of ethanol intake was reached. After completion of behavioral experiments, mice were culled and brains were removed and frozen on dry ice. Coronal sections (20 µm) were cut with a cryostat (Leica Microsystems Inc.). Sections were mounted and stained with neutral red (Sigma) to visualize cannula placement. A total of 6 mice were excluded from analysis due to incorrect cannula placement.

Data Analysis. Data were analyzed using Two-Way ANOVA with drug treatment and either genotype or brain region as variables as indicated followed by Bonferroni post

hoc tests. Data were analyzed using Graphpad software (Graphpad Software, Inc.). Paired *t*-tests were used to analyze fold expression of qRT-PCR data. Results were considered significant at *p*<0.05. All data are expressed as means ± standard errors of means (SEM).

IV.C. RESULTS

Varenicline and alcohol activate DAergic neurons within the posterior VTA

Recent evidence suggests that the VTA is not a homogeneous brain structure but is divided into distinct subregions (Ikemoto, 2007; Shabat-Simon et al., 2008). Thus, using c-Fos expression as a marker of neuronal activation and TH as a marker of DAergic neurons of the VTA, we examined the activating effects of varenicline and ethanol alone and in combination throughout the VTA. The VTA was divided into two distinct regions, anterior and posterior, using known neuroanatomical landmarks and stereotaxic coordinates based on Paxinos and Franklin (Paxinos and Franklin, 2000) and previous publications (Shabat-Simon et al., 2008). C57BL/6J mice were injected i.p. with drug(s) and their brains were examined for c-Fos expression within TH-immunopositive neurons 90 min later. Overall, there was a significant main effect of brain region ($F_{1,20}$ = 166.5, *p*<0.001), treatment ($F_{3,20}$ = 49.21, *p*<0.001), and a significant brain region × treatment interaction ($F_{3,20}$ = 28.25, *p*<0.001). Mice treated with 0.3 mg/kg varenicline followed by a saline injection exhibited an increase in the number of TH and c-Fos double immunopositive neurons, which were restricted

to the posterior VTA (Fig. IV-1A, 1B). Mice i.p. injected with saline, followed by 2 g/kg ethanol also displayed a dramatic increase in the number of TH/c-Fos double-immunopositive neurons within the posterior but not anterior VTA (IV-Fig. 1A, 1B). Finally, mice i.p. injected with 0.3 mg/kg varenicline followed by 2.0 g/kg ethanol also exhibited a significant increase in the number of TH/ c-Fos double-immunopositive neurons restricted to the posterior VTA (Fig. IV-1A, 1B). Bonferroni post tests revealed that each treatment condition was significantly different from control injections (saline/saline) within the posterior VTA (Fig. IV-1B, p<0.001). There were no significant effects of treatment on the number of TH/c-Fos double-immunopositive neurons within the anterior VTA when compared to saline injection. These results are consistent with the finding that the VTA can indeed be divided into distinct regions and that ethanol and varenicline predominantly activate DAergic neurons within the posterior VTA.

Differential expression of nAChR subunits in alcohol-activated posterior VTA neurons.

To gain insight into potential nicotinic receptor subunits that may be involved in alcohol activation of posterior VTA DAergic neurons, we challenged C57BL/6J mice with 2.0 g/kg ethanol and used LCM and qRT-PCR to analyze nicotinic receptor gene expression in activated, TH-, c-Fos-immunopositive neurons compared to non-activated, TH-immunopositive, c-Fos-immunonegative neurons. mRNA from the two groups of neurons, (Fig. IV-2A) only within the posterior VTA was isolated and reverse transcribed. Neuronal nAChR subunit

gene expression was then analyzed using qRT-PCR. In TH-, c-Fos-immunopositive neurons, the order of expression for nicotinic receptor subunit genes, from highest to lowest expression, was: α4 > α6 > β3 > β2 > α7 > α3 > α5. For TH-immunopositive, c-Fos-immunonegative neurons, the order of expression was: α4 > β3 > β2 > α6 > α7 > α3 > α5 (Table IV-1). A paired *t*-test showed significantly higher levels of expression of the α4 (t = 2.24, df = 4, $p<0.05$), α6 (t = 4.06, df = 3, $p<0.05$), and β3 (t = 4.28, df = 4, $p<0.01$) nAChR subunit genes as well as c-Fos (t = 2.69, df = 3, $p<0.05$) in the c-Fos-immunopositive neurons compared to c-Fos-immunonegative neurons (Fig. IV-2B, Table IV-1). These results indicate specific nAChR subtypes (α4α6β3*) may be important for modulating alcohol activation of posterior VTA DAergic neurons.

Role of α4* nAChRs in varenicline-induced reduction of alcohol consumption

Because (1) the specific role of α4* nAChR in alcohol consumption has not been described and (2) α4 gene expression was higher in DAergic neurons activated by ethanol within posterior VTA, we tested the hypothesis that varenicline may reduce alcohol consumption via these receptors. First, we examined the effects of 0.1 mg/kg and 0.3 mg/kg varenicline on 2 % alcohol intake in mice that do not express α4* nAChRs (α4 KO), and their WT litter mates (Fig. IV-3A). There was a significant main effect of treatment ($F_{2,45}$ = 8.0, $p<0.001$) but not genotype and there was no significant interaction between these two factors. Both doses of varenicline significantly decreased 2 % ethanol

consumption in WT mice compared to saline ($p<0.01$ for both doses), but did not reduce consumption in α4 KO mice (Fig. IV-3A). This experiment was repeated in a separate group of animals with the same doses of varenicline, but using a higher concentration of alcohol, 20 %. Two-way ANOVA revealed a significant main effect of treatment ($F_{2,64} = 5.31$, $p<0.01$), genotype ($F_{1,64} = 12.11$, $p<0.001$), and a significant treatment × genotype interaction ($F_{2,64} = 3.19$, $p<0.05$). Whereas WT and α4 KO mice consumed similar baseline quantities of 2 % alcohol, baseline 20 % alcohol consumption after saline injection was significantly less in α4 KO mice compared to WT ($p<0.01$, Fig. IV-3B). Varenicline significantly decreased 20 % ethanol consumption in WT animals (Fig. IV-3B) and post hoc tests indicated that alcohol consumption after each varenicline dose was significantly lower compared to consumption after saline injection ($p<0.01$ for both doses). In α4 KO mice, alcohol consumption after either varenicline dose was not significantly different compared to consumption after saline. Thus, these data suggest that expression of α4* nAChRs is necessary for varenicline-induced reduction of ethanol consumption.

To determine whether activation of α4* nAChRs by varenicline was sufficient to decrease ethanol intake, we analyzed the effect of low doses of drug on alcohol consumption in mice expressing a single point mutation, Leu9′Ala, that renders α4* nAChRs hypersensitive to agonist (Tapper et al., 2004). Leu9′Ala and WT littermate mice were challenged with i.p. injections of 0.0125 mg/kg and 0.05 mg/kg varenicline prior to receiving a 2 % ethanol bottle. There

was a significant main effect of treatment ($F_{2,55}$ = 6.86, $p<0.01$) and genotype ($F_{1,55}$ = 6.14, $p<0.05$) and a significant treatment × genotype interaction ($F_{2,55}$ = 3.74, $p<0.05$). Surprisingly, both low doses of varenicline significantly decreased 2 % ethanol intake in Leu9′Ala (0.0125 mg/kg and 0.05 mg/kg compared to saline, $p<0.01$ and $p<0.001$, respectively) but not WT mice (Fig. IV-3C). However, higher doses (0.1 mg/kg and 0.3 mg/kg) of varenicline did significantly reduce consumption in WT mice (Fig. IV-3C, D, $p<0.05$, and $p<0.01$ comparing 0.1 mg/kg and 0.3 mg/kg varenicline to saline, respectively). Similar results were obtained when this experiment was repeated and the concentration of alcohol was increased to 20 %. There was a main effect of treatment ($F_{2,40}$ = 6.73, $p<0.01$) and genotype ($F_{1,40}$ = 22.65) but no significant interaction. Varenicline significantly decreased ethanol consumption in Leu9′Ala but not WT mice when challenged with low doses of the drug, and each dose of varenicline was significantly different compared to a saline injection (Fig. IV-3D, $p<0.01$ for both doses). Together, these data suggest that selective activation of α4* nAChRs by varenicline is sufficient for reduction of alcohol consumption. Importantly, varenicline did not significantly reduce 10 % sucrose consumption in any of the genotypes, indicating that the effect of varenicline was specific for alcohol intake (Fig. IV-3E).

Varenicline infusion into the anterior and posterior VTA

To determine if varenicline reduction of alcohol consumption was mediated by drug action in the posterior VTA, we selectively infused the drug into

either the anterior or posterior VTA of C57BL/6J mice and measured alcohol consumption. Guide cannula were implanted into either brain region (see Methods) and vehicle, 10, 100, or 1000 pmol varenicline was infused into the VTA prior to presentation of a 20 % alcohol bottle. Figures IV-4A and IV-4B depict the location of the guide cannula within each mouse brain from the two groups of animals. Two-way ANOVA revealed a significant main effect of treatment ($F_{3,47}$ = 3.13, $p<0.05$) and brain region ($F_{1,45}$ = 4.88, $p<0.05$) and a significant treatment × brain region interaction ($F_{3,47}$ = 4.39, $p<0.01$). When infused into the anterior VTA, varenicline did not significantly reduce alcohol consumption compared to vehicle infusion (Fig. IV-4C). However, 10, 100, and 1000 pmol varenicline, when infused into the posterior VTA, significantly reduced alcohol consumption (Fig. IV-4D, $p<0.01$, $p<0.05$, and $p<0.01$ respectively). These data indicate that infusion of varenicline into the posterior VTA is sufficient to reduce alcohol consumption.

IV.D. DISCUSSION

Because alcohol is one of the most commonly abused psychoactive drugs in the world resulting in significant health consequences, it is critical to identify novel therapies and molecular targets to treat alcoholism. The nAChR partial agonist varenicline is an FDA approved smoking cessation aid that may hold promise as a treatment for alcoholism (Coe et al., 2005; Gonzales et al., 2006). For example, varenicline reduces alcohol consumption and seeking in rats

(Steensland et al., 2007) and also significantly reduces alcohol consumption in heavy drinking smokers (McKee et al., 2009). Although much is known about how varenicline may reduce nicotine dependence, much less is known regarding the brain regions and nAChR subtypes that varenicline may target to reduce alcohol consumption.

Varenicline-induced activation of the posterior VTA reduces alcohol consumption.

Our data indicate that varenicline and ethanol interact in the VTA. Indeed, much emphasis has been placed on the VTA because of its central importance in the mesocorticolimbic reward pathway (Funk et al., 2006). Alcohol activates DAergic neurons within this region, ultimately increasing DA release in the NAc driving dependence (Brodie and Appel, 1998; Brodie et al., 1999; Mansvelder et al., 2002; Pidoplichko et al., 1997). Mounting evidence indicates that the VTA is not a homogeneous brain region; rather it can be anatomically and functionally divided into at least two brain regions, the anterior and posterior VTA (Ikemoto, 2007; Shabat-Simon et al., 2008). Although both regions of the VTA contain predominantly two subtypes of neurons, DAergic projection neurons and local GABAergic interneurons, studies have shown that neurons within the anterior and posterior VTA project to distinct regions of striatum and also may respond differently to drugs of abuse (Ikemoto, 2007; Shabat-Simon et al., 2008; Zangen et al., 2006). The predominant VTA subregion that is critical for alcohol's action in the VTA is unclear. Previous studies indicate that the anterior VTA is an

important modulator of alcohol intake (Ericson et al., 2008; Moore and Boehm, 2009) while others find a role for the posterior VTA (Linsenbardt and Boehm, 2009). Additionally, local infusion of ethanol into the anterior VTA does not increase NAc DA output (Ericson et al., 2003; Lof et al., 2007a) while local infusion of ethanol to the posterior VTA is sufficient for increased DA release in the NAc (Ding et al., 2009). Our data are in line with previous work highlighting the importance of the posterior VTA in alcohol-mediated activation of DAergic neurons. Injection of 2.0 g/kg alcohol, a dose that conditions a place preference in C57BL/6J mice (i.e., a rewarding dose) (Cunningham et al., 2003) activated DAergic neurons predominantly in the posterior VTA. Varenicline also activated DAergic neurons selectively in this region and infusion of varenicline directly into the posterior, but not anterior, VTA reduced alcohol consumption, suggesting that the posterior VTA, specifically, could be a neuroanatomical substrate where both drugs interact. Thus, our data support previous studies indicating that rats will self-administer nicotine or ethanol directly into the posterior, but not anterior, VTA (Ikemoto et al., 2006; Rodd et al., 2004; Rodd-Henricks et al., 2000).

Activation of DAergic neurons by alcohol at least partially mediates the rewarding properties of the drug. Interestingly, varenicline also activates these neurons and this reduces alcohol consumption. Although the precise mechanism of this effect is unknown, one possibility is that activation of DAergic neurons by varenicline precludes further activation by alcohol. At the molecular level varenicline may serve to occupy or desensitize nAChRs necessary for alcohol

activation of DAergic neurons thereby reducing DA release in the NAc and decreasing consumption. Supporting this idea, varenicline has been shown to reduce alcohol stimulated NAc DA release in rats (Ericson et al., 2009). This would also be consistent with the mechanism by which varenicline is thought to reduce nicotine reinforcement (Rollema et al., 2007a).

The role of nAChRs in alcohol consumption.

Reduction of alcohol consumption by varenicline indicates that nAChRs may play a critical role in the reinforcing properties of ethanol. Several studies have utilized various nicotinic agonists and antagonists to implicate nAChR activation as potentially important for alcohol consumption. The non-specific nAChR antagonist, mecamylamine, either delivered systemically or selectively into the VTA, reduces alcohol intake and blocks alcohol-mediated DA release in NAc (Blomqvist et al., 1993; Blomqvist et al., 1997; Blomqvist et al., 1996; Hendrickson et al., 2009). However, specific nicotinic receptor subtypes involved in alcohol consumption are unclear. Previous studies have demonstrated that the selective α4β2* nAChR competitive antagonist, DHβE, fails to modulate alcohol consumption suggesting that this subtype may not be involved in the response to alcohol (Hendrickson et al., 2009; Le et al., 2000). The α7 selective antagonist, methyllycaconitine, also does not reduce alcohol intake (Hendrickson et al., 2009). To gain insight into nAChR subtypes that may influence alcohol consumption, and be targeted by varenicline, we compared nAChR subunit gene expression between posterior VTA DAergic neurons that were activated by

alcohol and posterior VTA DAergic neurons not activated by alcohol. We found that DAergic neurons that were activated by alcohol express higher levels of α4, α6, and β3 nAChR subunit transcripts. Although this difference in mRNA expression does not necessarily translate into protein and/or assembled receptor expression, these data suggest that α4α6β3* nAChRs may be involved in alcohol consumption. Our results are consistent with previous studies indicating that the α6/β3* nAChR selective antagonist α-conotoxin MII, when infused into the VTA, can reduce alcohol consumption and block alcohol-mediated DA release in NAc (Jerlhag et al., 2006; Larsson et al., 2004; Lof et al., 2007b). Importantly, a significant portion of α6β3* nAChRs also contain the α4 subunit and these receptors represent one of the highest affinity nAChRs identified in the brain thus far (Salminen et al., 2007; Salminen et al., 2004).

Activation of α4* nAChRs are critical for varenicline-induced reduction of alcohol consumption.

The role of α4* nAChRs in varenicline reduction of alcohol consumption has not been examined previously. We used two complementary genetic nAChR mouse models, one lacking the gene encoding the α4 subunit, *CHRNA4* (α4 KO) (Ross et al., 2000) and another that has a single point mutation, Leu9′Ala, within the endogenous *CHRNA4* exon 5 resulting in α4* nAChRs that are hypersensitive to agonist (Leu9′Ala) (Fonck et al., 2005; Tapper et al., 2004). Varenicline reduced consumption of both a low and high dose of alcohol in WT mice but did not significantly reduce consumption in α4 KO mice indicating that

expression of α4* nAChRs is necessary for the effects of the drug. In contrast to the effects of varenicline in the KO animals, low doses of varenicline that had little effect on consumption in WT mice dramatically reduced ethanol intake in Leu9′Ala mice suggesting that activation of α4* nAChRs is also sufficient for varenicline effects on alcohol consumption. Importantly, varenicline did not reduce sucrose intake indicating that the effects the drug were specific for alcohol consumption and did not dampen general reward signaling. Because varenicline may also target α7 as well as other nAChR subtypes in addition to α4β2* nAChRs, the mechanism by which varenicline reduces alcohol consumption is an open question (Mihalak et al., 2006; Papke et al., 2010). A recent study demonstrated that varenicline reduced ethanol consumption in mice lacking either α7 or β2* nAChRs similar to WT (Kamens et al., 2010) indicating that expression of these receptors is not necessary for varenicline's effects. As discussed above, our data suggest that α4α6β3* nAChRs mediate varenicline's effects on alcohol consumption, although it is expected that this subtype should also contain the β2 subunit (Grady et al., 2007). Thus, varenicline reduction of ethanol intake in β2 KO mice may occur because of compensatory mechanisms in nAChR expression or subunit composition. Alternatively, we cannot rule out the possibility that higher doses of varenicline than used in our study may reduce alcohol consumption by a non-α4* nAChR dependent mechanism. However, we expect that the doses we used would result in concentrations of varenicline more selective for high affinity nAChRs. For example, 0.1 mg/kg varenicline is

predicted to yield a brain concentration of 38 nM (Rollema et al., 2009). This concentration is predicted to be within the range experienced by smokers taking therapeutic doses of varenicline (Rollema et al., 2010). Similar doses also reduce nicotine self-administration in rats without impacting food reinforcement unlike higher doses (O'Connor et al., 2010). In addition, this range of varenicline dose also increases DA turnover in rat NAc (Rollema et al., 2010) consistent with our data illustrating that 0.3 mg/kg varenicline activates DAergic neurons.

Taken together, our results demonstrate that ethanol and varenicline selectively activate DAergic neurons within the posterior VTA and that activation of α4* nAChRs is necessary and sufficient for varenicline-induced reduction of ethanol consumption. Our data combined with recent clinical studies indicate that varenicline could potentially be a therapeutic candidate for the treatment of alcoholism.

Table 1. Comparison of nAChR subunit gene expression (-dCt) of c-Fos-immunopositive and c-Fos-immunonegative dopaminergic neurons in pVTA with acute ethanol (2 g/kg) injection

nAChR subunit	α3	α4*	α5	α6*	α7	β2	β3**	c-*Fos***
TH(+) / c-Fos (+)	-8.12±0.42	-2.51±0.33	-9.54±0.46	-4.42±0.44	-7.05±0.41	-4.89±0.42	-4.84±0.42	-4.51±0.68
TH(+) / c-Fos(-)	-7.97±0.61	-3.70±0.31	-9.66±0.55	-5.71±0.30	-7.83±0.38	-5.47±0.37	-5.43±0.34	-7.38±0.30

*$p < 0.05$, **$p < 0.01$, paired T-test

Table IV-1. Quantitative gene expression of nAChR subunit genes in pVTA DAergic neurons either activated or not activated by 2.0 g/kg ethanol (TH(+)/c-Fos(+) or TH(+)/c-Fos(-), respectively). Values represent the negative change in threshold cycle (-DC$_t$) compared to GAPDH.
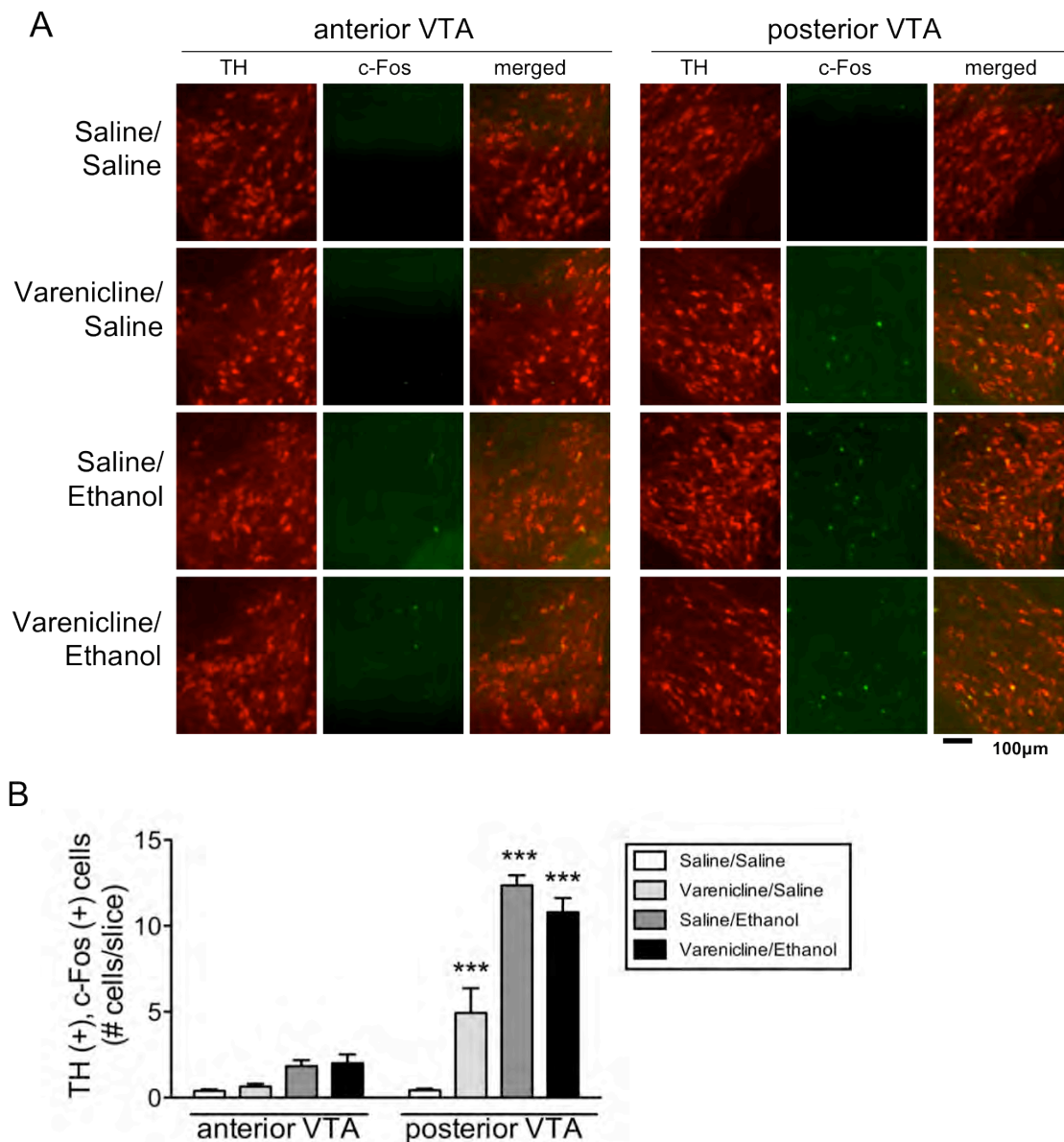
Figure IV-1. Varenicline activates DAergic neurons within the posterior VTA. A) Representative images of coronal sections from the anterior (left) and posterior (right) VTA. Sections were isolated from C57BL/6J mice injected with saline, 0.3 mg/kg varenicline, 2 g/kg ethanol, or both drugs. Sections were immunolabeled to detect TH expression (red, left columns) and c-Fos expression (green, middle columns). Merged images are represented in the right column. White arrowheads mark c-Fos positive TH neurons. B) Quantification of the number of TH-, c-Fos immunopositive (TH(+), c-Fos(+)) neurons within each region of the VTA after each drug treatment (34-51 slices analyzed per region, n = 3 mice/treatment). * $p<0.05$, *** $p<0.001$.
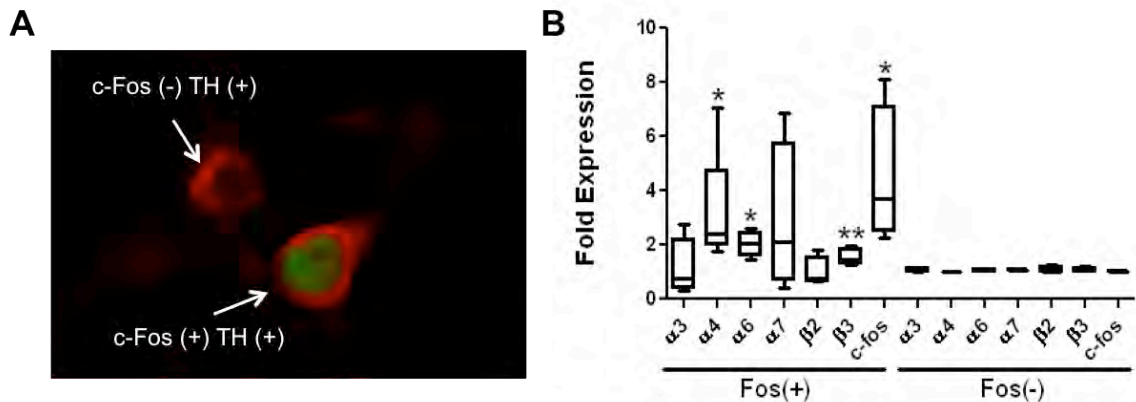
Figure IV-2. Differential nAChR subunit gene expression in alcohol-activated versus non-activated DAergic neurons. A) Immunofluorescence image of coronal midbrain slice from C57BL/6J mice challenged with 2.0 g/kg ethanol. Sections were immunolabeled to detect TH (red) expression to visualize DAergic neurons and c-Fos (green) expression to identify neurons activated by ethanol. Arrows point to two different cells, one, a TH immunopositive c-Fos immunonegative neuron (TH(+), c-Fos(-)) and the other a TH-, c-Fos immunopositive neuron (TH(+), c-Fos (+)). B) Fold change of nAChR subunit gene expression, as measured by qRT-PCR, in TH-, c-Fos immunopositive (left) neurons compared to TH immunopositive, c-Fos immunonegative neurons (right). Three mice per treatment group were used. Neurons were captured via LCM. TH-, c-Fos immunopositive neurons n = 2,298 and TH immunopositive, c-Fos immunonegative n = 3,645. * $p<0.05$, ** $p<0.01$, paired T-test.
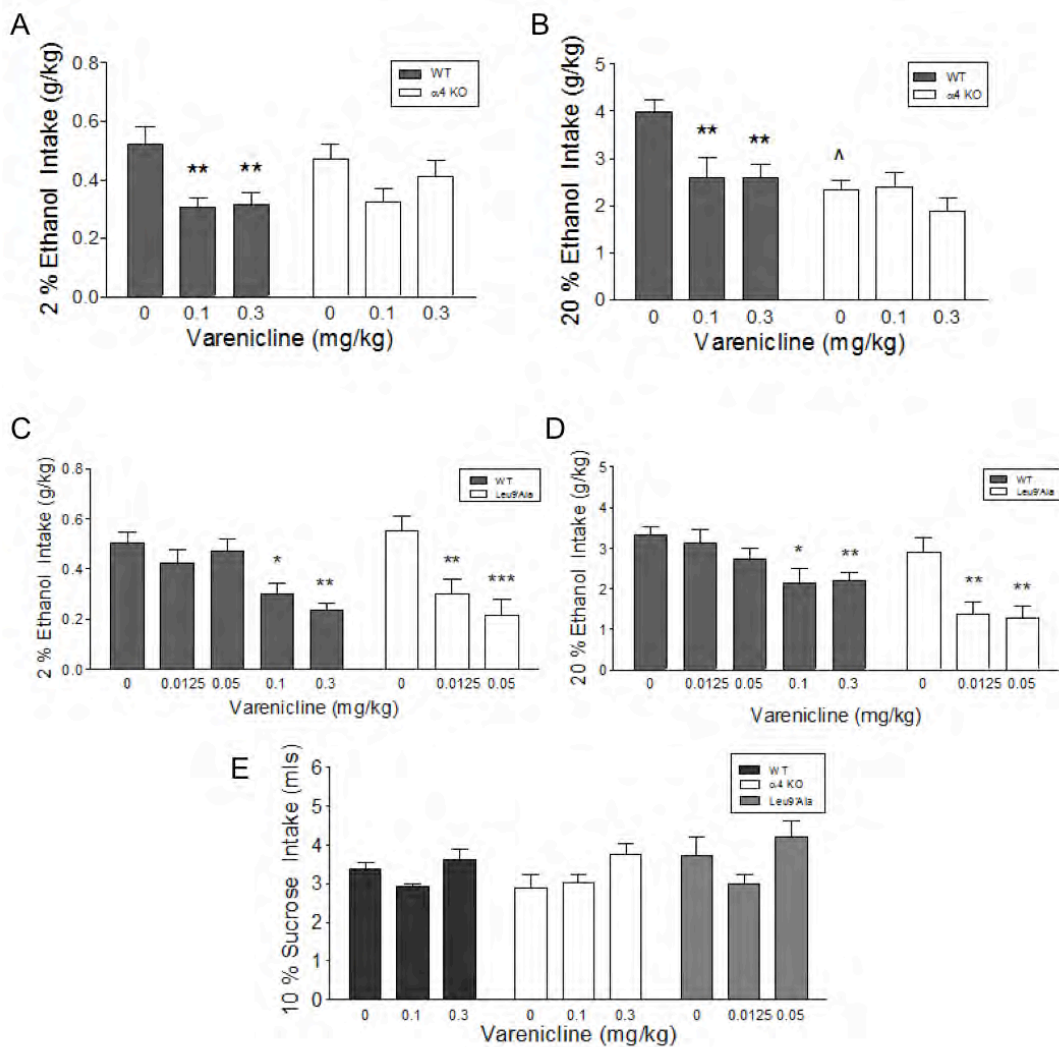
Figure IV-3. Alcohol intake after varenicline treatment in α4 KO, Leu9′Ala and WT mice. A) 2 % ethanol intake in WT and α4 KO mice after saline or varenicline treatment (n = 8-9 / genotype). B) 20 % ethanol intake in WT and α4 KO mice after saline or varenicline treatment (n = 10-12 / genotype). C) Effect of saline and varenicline on 2 % ethanol intake in WT and Leu9′Ala (n = 10-11 / genotype). D) Effect of saline and varenicline on 20 % ethanol intake in Leu9′Ala and Leu9′Ala WT mice (n = 6-9 / genotype). E) Effect of varenicline treatment on 10 % sucrose intake (n = 5-11 / genotype) in WT, α4 KO, and Leu9′Ala mice. * $p<0.05$, ** $p<0.01$, ^ $p<0.01$ (α4 KO compared to WT after saline injection)
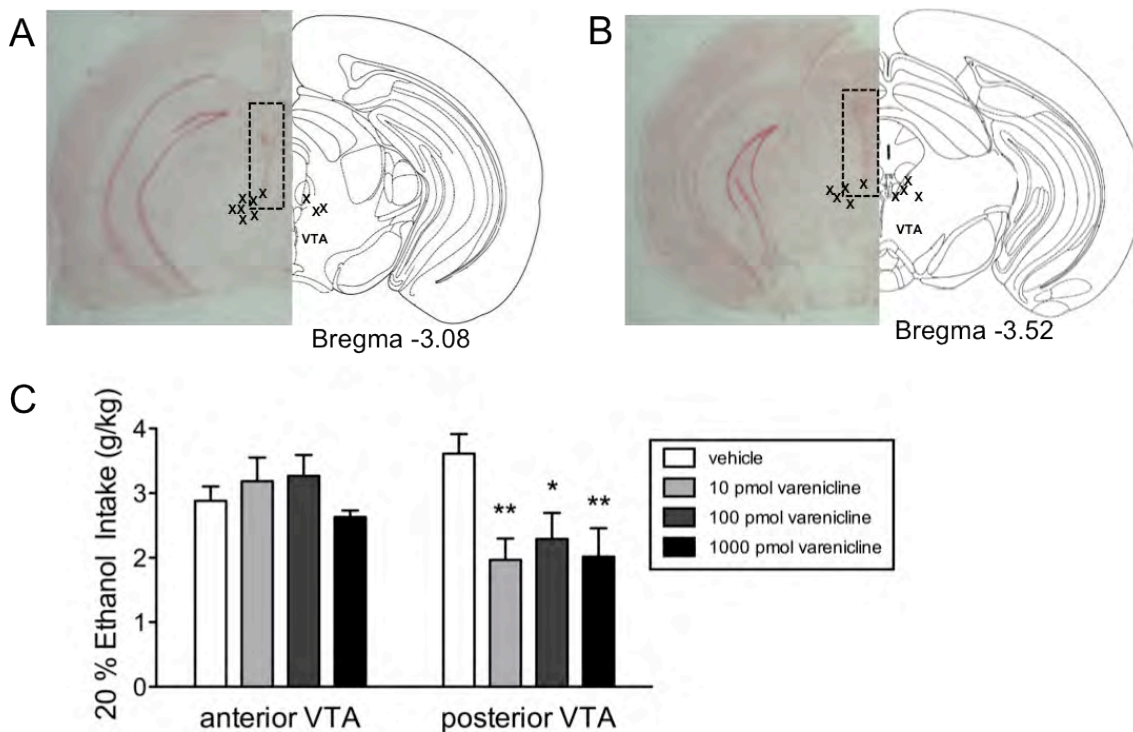
Figure IV-4. Alcohol intake after varenicline infusion into anterior or posterior VTA. A) Left, representative neutral red stained coronal brain slice from a mouse with a guide cannula placed just dorsal to the anterior VTA (dotted box). Right, schematic diagram of anterior VTA -3.08 mm from bregma (adapted from (Paxinos and Franklin, 2000)). X's represent guide cannula placements within the anterior VTA. B) Left, representative neutral red stained coronal brain slice from a mouse with a guide cannula placed just dorsal to the posterior VTA (dotted box). Right, schematic diagram of posterior VTA at bregma -3.52. X's represent guide cannula placements within the posterior VTA. C) 20 % ethanol intake after infusion of aCSF, 10 pmol, or 100 pmol varenicline in either the anterior or posterior VTA (n = 9-10 / brain region). * $p<0.05$, ** $p<0.01$, Bonferroni post-test.

Preface to Chapter V

This chapter has been published separately in:

Author Contributions:

Hendrickson LM, Tapper AR designed experiments.

Hendrickson LM performed experiments.

Hendrickson LM wrote the paper.

Tapper AR, Gardner PD edited the paper.

CHAPTER V

Nicotinic acetylcholine receptors containing the α4 subunit are critical for

nicotine-induced reduction of acute voluntary ethanol consumption.

V.A. INTRODUCTION

Similar to all drugs of abuse, ethanol administration causes an increase of

dopamine (DA) release in the nucleus accumbens (NAc). This increase in DA

release is thought to underlie the rewarding or reinforcing properties of the drug

(Soderpalm et al., 2009). Although ethanol has been found to modulate several

receptors and ion channels, emerging evidence indicates that nicotinic

acetylcholine receptors (nAChRs) may play a role in ethanol-induced accumbal

DA release and ethanol reinforcement (Soderpalm et al., 2000). Pre-application

of mecamylamine, a nAChR antagonist, either by direct infusion into the ventral

tegmental area (VTA) or an intraperitoneal (i.p.) injection, decreases ethanol-

induced DA release in the NAc (Blomqvist et al., 1997; Larsson et al., 2002) as

well as decreases ethanol self-administration in rats (Ericson et al., 1998) and

ethanol voluntary intake in mice (Hendrickson et al., 2009). Thus, nAChRs are

now a focus of intense investigations as molecular targets for not only nicotine

addiction, but alcoholism as well.

For example, the nAChR partial agonist varenicline is an FDA approved

smoking cessation aid that has also shown promise to reduce ethanol

consumption in several rodent models and one clinical model (Hendrickson et al.,

2010; Kamens et al., 2010; McKee et al., 2009; Steensland et al., 2007). The molecular mechanism by which varenicline acts to decrease nicotine intake is well understood, while on the other hand, until recently, its role in decreasing ethanol intake was not entirely clear due to the vast array of nAChR subtypes it may act upon.

Neuronal nAChRs are pentameric, ligand-gated cation channels that are activated by the endogenous neurotransmitter acetylcholine (ACh) as well as exogenous agonists such as nicotine. To date, twelve mammalian neuronal nAChR subunits have been identified ($\alpha2$-$\alpha10$, $\beta2$-$\beta4$), which can combine to form either hetero- or homomeric receptors, producing multiple functional subtypes of receptors, each with distinct pharmacological and biophysical properties (Albuquerque et al., 2009).

Given the large number of nAChR subtypes with which varenicline may interact (Mihalak et al., 2006), we sought to identify which nAChR subtype underlies varenicline's effect on ethanol intake. Because varenicline was designed to be a partial agonist of nAChRs containing $\alpha4$ and $\beta2$ subunits (designated as $\alpha4\beta2$*) (Coe et al., 2005; Jorenby et al., 2006) we examined the effect of the drug on ethanol intake in $\alpha4$ KO and $\alpha4$ hypersensitive mice. We found that activation of $\alpha4$* nAChRs was necessary and sufficient for varenicline-induced reduction of voluntary ethanol intake in a limited access ethanol-drinking assay (Hendrickson et al., 2010). Here, we extend our previous findings and

present further evidence for the activation of α4* nAChRs in modulating the rewarding effects of ethanol.

V.B. MATERIALS AND METHODS

Animals. Adult (8-10 week) male α4 knock-out (α4 KO) mice and their wild type (WT) litter mates, as well as heterozygous Leu9′Ala knock-in mice and their WT litter mates, all bred on site, were used. The genetic engineering of both α4 KO and Leu9′Ala mouse lines have been described previously (Ross et al., 2000; Tapper et al., 2004). Both lines have been back-crossed to a C57BL/6J background > nine generations. Mice were individually housed on a reversed 12 h light/dark cycle (lights on 10 PM, off 10 AM) with *ad libitum* access to food and water (except during experiments as described below). All experiments were conducted in accordance with the guidelines for care and use of laboratory animals provided by the National Research Council (National Research Council, 1996), as well as with an approved animal protocol from the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Drugs and drinking solutions. Ethanol drinking solutions were prepared from 190 proof absolute anhydrous ethanol (Pharmco-Aaper) diluted to 20 % ethanol (v/v) using tap water. Nicotine hydrogen bitartrate (Sigma-Aldrich), was dissolved in 0.9 % saline and was administered via intraperitoneal (i.p.) injections at the indicated doses. Nicotine concentrations are reported as nicotine base.

Drinking in the dark (DID). Ethanol consumption was measured using a DID procedure as previously described (Hendrickson et al., 2010). Animals were singly housed in experimental chambers for 1 week prior to the beginning of the DID sessions. The mice received a 15-ml graduated cylinder water bottle fitted with a one-hole rubber stopper with a stainless steel double-ball-bearing sipper tube, which was sealed with Parafilm to prevent leakage. For the first three nights, two hours after the lights were off, mice were i.p. injected with saline immediately before their water bottle was replaced with the 20 % ethanol bottle, and allowed to drink for two hours. This procedure was used to acclimatize the mice to the experimental conditions and allow them to reach a baseline of ethanol intake prior to drug administration. On the fourth night, the mice received their first dose of drug immediately before placement of the ethanol bottle. The amount of ethanol consumed was recorded immediately after each two-hour session and converted to g/kg per each animal's ethanol consumption and body weight. The mice were given 2 days of rest (no injections or ethanol) and then began the saline injection/ethanol consumption assay for two to three days until a stable ethanol intake was reached. Once the baseline returned, a second, higher dose of drug was administered prior to the ethanol bottle being placed in the cage. In this design, all mice in one group drink a single concentration of ethanol throughout the experiment, but receive two doses of drug, 4-5 days apart, with the lower concentration of drug first.

Data Analysis. The effect of nicotine on ethanol intake was compared to ethanol intake after a saline injection from the previous day using a Two-way ANOVA followed by Bonferroni *post hoc* tests. Data were analyzed using Graphpad software (Graphpad Software, Inc.). Results were considered significant at $p<0.05$. All data are expressed as means ± standard errors of means (SEM).

V.C. RESULTS

α4* nAChRs are necessary for nicotine-induced reduction of ethanol consumption.

To examine the role of α4* nAChRs in baseline ethanol intake and after a pre-injection of nicotine, we used α4 KO and WT mice in the Drinking in the Dark (DID) assay (Rhodes et al., 2005) (Fig. V-1). Two-way ANOVA revealed that there was a significant main effect of treatment ($F_{2,72} = 7.77$, $p<0.001$) but not genotype and a significant treatment x genotype interaction ($F_{2,72} = 4.57$, $p<0.05$). Similar to our previously published observations (Hendrickson et al., 2009), we again saw that nicotine dose dependently decreased voluntary ethanol intake in WT mice at doses of 0.25 mg/kg and 0.5 mg/kg nicotine compared to a saline injection ($p<0.05$, $p<0.001$, respectively). In the α4 KO mice, neither dose of nicotine had any significant effect on ethanol intake, indicating that α4* nAChRs are necessary for the nicotine-induced effect on ethanol drinking. In addition to nicotine having no effect in decreasing ethanol intake in the α4 KO mice, there was also a significant reduction in baseline ethanol consumption after

a saline injection when compared to WT mice (Fig. V-1, $p<0.001$) suggesting that α4* nAChRs may also be involved in generalized ethanol consumption.

Activation of α4* nAChRs is sufficient for nicotine-induced reduction of ethanol consumption.

To determine if activation of α4* nAChRs was sufficient for nicotine-induced reduction of ethanol consumption, we used the hypersensitive Leu9′Ala mice in the 20 % ethanol DID assay (Fig. V-2). In this experiment however, the concentration of nicotine was lowered such that the dose used would have no effect on WT nAChRs, but would selectively activate mutant Leu9′Ala α4* nAChRs (Tapper et al., 2004). Two-way ANOVA showed a significant main effect of genotype ($F_{1,28}$ = 4.56, $p<0.05$) but not treatment and there was no significant interaction between these two factors. A low dose of nicotine (0.05 mg/kg) significantly decreased ethanol intake in Leu9′Ala mice, but had no effect on WT mice (Fig. V-2, $p<0.05$). These data indicate that selective activation of α4* nAChRs is sufficient for nicotine-induced reduction of ethanol consumption.

V.D. DISCUSSION

It is widely accepted that α4β2* nAChRs are critical for mediating the rewarding properties of nicotine and nicotine addiction (Picciotto et al., 1998; Tapper et al., 2004). It is also known that nicotine and alcohol are often co-abused and that 70–75 % of alcoholics are also dependent on nicotine (Bobo and Husten, 2000; Room, 2004), suggesting a functional interaction between

these drugs. Varenicline, acting as a partial agonist of α4β2* nAChRs, is a successful smoking cessation aid and has also been shown to attenuate acute ethanol-induced DA release as well as reduce ethanol craving in smokers (Ericson et al., 2009; McKee et al., 2009).

Previously, several labs including our own have shown that varenicline can decrease ethanol intake in several rodent models (Hendrickson et al., 2010; Kamens et al., 2010; Steensland et al., 2007). Because of the pharmacological promiscuity of varenicline (Mihalak et al., 2006), we sought to identify the nAChR subtype responsible for varenicline-induced reduction of alcohol consumption. While most of the previous studies primarily used pharmacology alone, our study combined pharmacology with genetically modified nAChR knock-out and knock-in mice. We showed that both varenicline and ethanol can activate DAergic neurons of the posterior VTA and that these activated neurons express higher levels of the α4, α6 and β3 nAChR subunit gene transcripts. Additionally, we showed that nAChRs containing the α4 subunit, and possibly the α4α6β2β3 subtype, are critical for varenicline's effect on drinking (Hendrickson et al., 2010).

It is likely that the mechanism of action underlying varenicline's ability to decrease ethanol intake is similar to how it is thought to decrease nicotine use. That is, acting as a partial agonist, varenicline selectively binds and partially activates the nAChRs that modulate ethanol intake (i.e., α4* nAChRs) thus occupying or desensitizing the relevant receptors, precluding any further activation by ethanol. Additionally, the selective activation of α4* nAChRs

themselves may act to increase DA release in the NAc such that ethanol has no further enhancing effect.

In line with this idea, here we show that the nAChR full agonist nicotine also decreases ethanol consumption in an α4* nAChR-dependent mechanism. Nicotine reduced ethanol consumption in WT mice but had no effect in α4 KO mice implying that expression of α4* nAChRs is necessary for nicotine-induced reduction of ethanol consumption. In contrast, low doses of nicotine that have no effect in WT mice significantly reduced ethanol intake in the Leu9′Ala hypersensitive mice indicating that the selective activation of α4* nAChRs is sufficient for the effect on alcohol consumption.

One common argument against a role for α4β2* nAChRs in alcohol consumption is that the selective competitive antagonist, dihydro-β-erythroidine (DHβE), fails to reduce ethanol intake in both rats and mice at several different concentrations (Hendrickson et al., 2009; Le et al., 2000). However, caution should be used in interpreting these data for several reasons. First, the ability of DHβE to block α4β2* nAChRs depends on the stoichiometry of the target receptor population (Moroni et al., 2006; Salminen et al., 2004), which is unknown in vivo. And second, the subunit composition of nAChRs involved in alcohol reward, while likely containing α4 and β2 subunits, has not been fully elucidated. For example, higher affinity α4β2* heteromeric nicotinic receptors that contain additional α or β subunits may be insensitive to DHβE (Salminen et al., 2004).

Taken together, our results show that the selective activation of α 4* nAChRs is necessary and sufficient to reduce acute ethanol consumption. This result also further supports the hypothesis that selective agonists or partial agonists of α 4* nAChRs may be valuable therapeutics for the treatment of alcoholism.
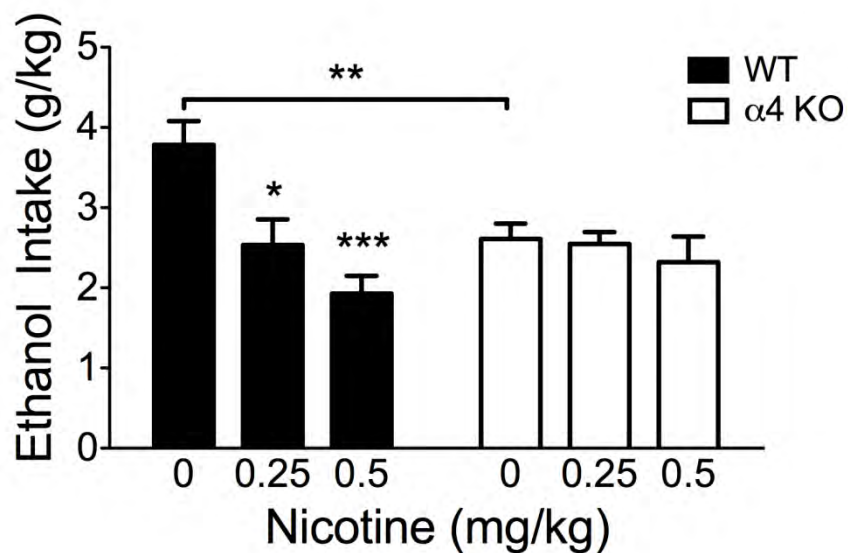
Figure V-1. Alcohol intake after nicotine treatment in α4 KO and WT mice. A pre-injection of nicotine (0.25 mg/kg and 0.5 mg/kg) dose dependently decreased 20 % ethanol intake in WT mice compared to a saline injection, but had no effect on α4 KO mice (n = 9–18/genotype). Additionally, α4 KO mice consumed significantly less 20 % ethanol compared to WT mice after a saline injection. *$p<0.05$, **$p<0.01$, ***$p<0.001$.
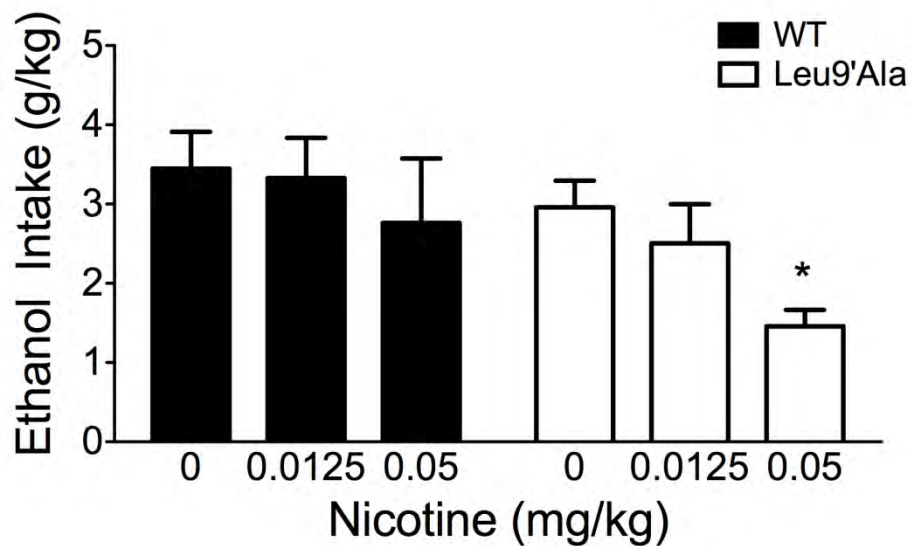
Figure V-2. Alcohol intake in Leu9′Ala and WT mice after nicotine pre-treatment. Low doses of nicotine (0.0125 mg/kg and 0.05 mg/kg) did not significantly decrease 20 % ethanol intake in WT mice compared to a saline injection. Low dose nicotine (0.05 mg/kg) did significantly decrease 20 % ethanol intake in the hypersensitive Leu9′Ala mice compared to a saline injection (n = 5-12 /genotype). *$p<0.05$.

CHAPTER VI:

DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

The neurobiological mechanisms underlying alcohol consumption and the development of alcohol addiction encompasses a broad set of questions with the most basic being, why do people drink? (Morikawa and Morrisett, 2010). The effects of ethanol in the brain are varied and depend on the rate and amount of ethanol consumed. Ethanol consumption produces a wide array of intoxicated states that alter behavioral and cognitive functions including euphoria, impaired memory, sedation, slurred speech, impaired balance and in severe cases, coma or death. Despite (or possibly because of) these symptoms, alcohol use progresses in some people to the point of alcoholism, and withdrawal from chronic ethanol consumption produces a "negative affective state" resulting in dysphoria, anhedonia, and depression, which may persist for a prolonged period of time (Koob, 2009; Trevisan et al., 1998). Thus, some would answer the proposed question by stating that, "We drink because we like the state of being drunk and we keep drinking because we want to avoid the affective symptoms of withdrawal" (Morikawa and Morrisett, 2010).

Alcoholism is the result of several interactions between a number of neural mechanisms that modulate the rewarding effects of ethanol, the acute sensitivity to ethanol, the tolerance to and dependence on ethanol and the desire to continue to consume ethanol (Lovinger and Crabbe, 2005). Thus, one way to

elucidate the molecular mechanisms that modulate alcoholism is to break the disease into distinct stages that can be modeled and manipulated in the laboratory setting. This approach, in combination with genetic mouse models and pharmacology, has allowed for the careful examination of the initial stage of alcoholism, the binge/intoxication stage.

While it is clear that most drugs of abuse act to increase extracellular dopamine levels in the NAc (Wise and Rompre, 1989), the molecular mechanisms mediating this process vary depending on the molecular target each drug acts on (Gonzales et al., 2004). The molecular targets that mediate the rewarding properties of most drugs of abuse including cocaine, amphetamine, heroin and nicotine have been well established for some time; however, the molecular mechanisms by which ethanol acts to mediate reward have not been fully elucidated (Gonzales et al., 2004). In this thesis, I have examined the role of nAChRs, known molecular targets for nicotine addiction, in mediating the initial rewarding properties of alcohol addiction. Using a mouse model of voluntary ethanol consumption, in combination with nAChR pharmacology, I have provided further evidence for the role of nAChRs in mediating the initial rewarding properties of ethanol (Chapter II). I have also shown, for the first time, that a specific nAChR subtype, those that contain the α4 subunit, mediate voluntary ethanol consumption and reward (Chapter III) and also that α4* nAChRs are potential molecular targets for the development of alcohol use disorder treatments (Chapter IV and V).

VI.A. nAChRs IN ETHANOL REWARD: ARE THEY INHERENTLY CRITICAL?

Considerable evidence over the past two decades has implicated nAChRs as common molecular targets for the interactions of ethanol and nicotine in the brain. In 2005, a paper was published describing a new ethanol self-administration paradigm in which mice reliably drink to pharmacologically significant BECs without lengthy training periods, or water or food restriction, called Drinking–In-The-Dark (DID) (Rhodes et al., 2005). In this assay, WT mice consume ~ 4 g/kg ethanol in a 2 hour period which increases BEC > 1.0 mg/ml (Rhodes et al., 2005), and has been shown to produce behavioral intoxication as measured by impairment in rotarod and balance beam tests (Rhodes et al., 2007). For example, we found that the BEC of male WT mice after a 2h drinking session was 25 mM (Fig. II-1 D) which is equivalent to 1.15 mg/ml. This high level of ethanol intake that occurs over a relatively short period of time is within the range of the recently proposed definition of human binge drinking (Courtney and Polich, 2009; Lowery et al., 2010). Using this assay, we found that ethanol intake was significantly and dose dependently attenuated in WT mice after an i.p. injection of the nAChR antagonist mecamylamine (Fig. II-1. B). While this was not the first study to show that mecamylamine can reduce ethanol intake in rodents, previous reports used ethanol consumption assays in which the rodents consumed ethanol for at least 2 weeks prior to treatment (Blomqvist et al., 1996; Le et al., 2000). Prior studies indicate that mecamylamine delivered systemically

or directly into the VTA attenuates ethanol-induced dopamine release in the NAc (Blomqvist et al., 1993; Blomqvist et al., 1997) and mecamylamine has also been reported to reduce the subjective euphoria of ethanol in humans (Chi and de Wit, 2003). Thus, it is likely that mecamylamine reduces voluntary ethanol consumption via a similar mechanism in the DID assay. Additionally, while some reports indicate that high doses of mecamylamine can non-competitively inhibit NMDA receptors, we saw a significant decrease in the amount of ethanol consumed at doses as low as 0.5 g/kg suggesting that mecamylamine is acting to reduce ethanol intake via blockade of nAChRs. As an additional control, we tested the nAChR antagonist hexamethonium, which does not cross the blood brain barrier, and found that peripheral nAChRs did not affect voluntary ethanol consumption. Therefore, this was the first report, to our knowledge, to show that blocking nAChRs in the CNS reduced acute ethanol intake in a model of voluntary ethanol consumption.

Interestingly, we also found that the nAChR full agonist nicotine and the selective, partial agonist cytisine both dose dependently decreased acute ethanol consumption in the DID assay (Fig. II-2 and II-3). This result seemed to be in opposition to one previous study that found that nicotine can enhance ethanol intake in rats (Smith et al., 1999). However one important difference between the two studies, among others, was the mode of nicotine administration, chronic in the Smith et al. study and acute in ours. An obvious question then became, how can both blocking nAChRs with mecamylamine and activating them with nicotine

and cytisine decrease ethanol consumption? We hypothesized that the two actions (blocking and activating) result in the same overall net effect. That is, while mecamylamine blocks the nAChR to prevent activation, an acute single exposure of nicotine or cytisine will initially activate nAChRs which then become desensitized, often for prolonged periods of time (Mansvelder et al., 2002; Pidoplichko et al., 1997). Thus, nAChR desensitization would prevent any further activation of the relevant nAChRs by ethanol. This was further supported by our data showing that 1) mecamylamine blocks the ethanol-induced c-Fos activity of DA neurons in the VTA (Fig. II-5. C) and 2) nicotine activates DA neurons of the VTA to the same extent as nicotine pre-treatment followed by ethanol (Fig. II-5. D).

Taken together, these data suggest that nAChRs are inherently critical for voluntary ethanol consumption as well as ethanol-induced activation of the mesolimbic DA pathway. Furthermore, these data indicate that nicotine and ethanol may activate similar pathways in the VTA, or possibly act on the same nAChR subtype. One intriguing follow up to our initial findings would be to determine the effects of chronic nicotine exposure on daily ethanol consumption in WT mice using the DID assay (similar to Smith et al., 1999).

VI.B. SUBTYPE SPECIFICITY: A ROLE FOR α4* nAChRs IN ETHANOL REWARD

Because of the vast array of nAChR subtypes expressed within the VTA, an important goal of alcohol research is identifying which of these subtypes most directly modulates ethanol reinforcement. Using pharmacology, we investigated the role of two of the most abundant nAChR subtypes found in the brain, the low affinity α7 and high affinity α4β2 nAChRs in mediating voluntary ethanol consumption (Chapter II). We found that the nAChR antagonists DHβE, selective for α4β2 nAChRs and MLA, selective for α7 nAChRs, failed to significantly reduce ethanol intake in WT mice in the DID assay. Previous studies have shown that similar doses of DHβE as used in our study (1 mg/kg - 3.2 mg/kg) are able to block the locomotor activating and discriminative stimulus effect of nicotine in rats (Stolerman et al., 1997) as well as attenuate nicotine self-administration (Corrigall et al., 1994). Thus, because of the evidence for α4β2* nAChRs modulating nicotine addiction (Picciotto et al., 1998; Tapper et al., 2004) and the high co-abuse of nicotine and ethanol (Falk et al., 2006), we were surprised that DHβE had no effect on the ethanol intake of WT mice in our assay. However, this result is consistent with previous findings in the field that DHβE also has little effect on operant ethanol responding, ethanol-mediated DA release in the NAc and ethanol self-administration in rats (Le et al., 2000; Soderpalm et al., 2000). Importantly, one caveat to these DHβE studies is the finding that higher affinity nAChRs that contain the α4β2 subunits in addition to a third or

fourth subunit are relatively insensitive to DHβE (Salminen et al., 2004). Therefore, it would be interesting to see if a pre-injection of DHβE could block the nicotine-induced reduction of ethanol consumption in WT mice. If the nAChR subtype responsible for modulating nicotine-induced reduction of ethanol intake contains subunits in addition to α4β2, then I would expect that a pre-injection of DHβE would have no effect.

One of the most widely expressed, high affinity nAChR subtypes in the mammalian brain are α4* nAChRs, which have been demonstrated to be critical for behaviors associated with nicotine dependence including reward, sensitization and tolerance (Tapper et al., 2004). Therefore, we were interested to see if α4* nAChRs are also critical for ethanol reward. Until recently, studies had only utilized pharmacology to address this question, mostly in high alcohol preferring rats or WT mice. Due to the diversity of nAChR subtypes in the brain, and the limited pharmacological compounds selective for specific nAChRs (such as DHβE as discussed above), we took a genetic approach to test our hypothesis.

Using mice that lack expression of the α4 subunit, α4 KO, and a line of mice that have α4* nAChRs that are hypersensitive to agonist, Leu9′Ala, we tested the necessity of α4* nAChRs in voluntary ethanol consumption, ethanol-induced activity of DAergic neurons in the VTA, and ethanol conditioned place preference (Chapter III). We found that α4 KO mice consumed significantly less 10 % and 20 % ethanol compared to WT mice in the DID assay. Therefore,

these data suggest that while WT mice voluntarily consume ethanol to the point of intoxication, mice lacking expression of the high affinity α4* nAChRs drink significantly less, presumably because ethanol is not rewarding to these mice. Importantly, WT and α4 KO mice consumed similar amounts of quinine and sucrose, indicating that the two genotypes have similar aversion to bitter tasting solutions and that the WT mice were not consuming the ethanol for caloric value (Fig. III-2A). Furthermore, α4 KO and WT mice had similar peak BEC after a 2 g/kg i.p. injection of ethanol and similar rates of ethanol clearance, indicating that there were no differences in the metabolism of ethanol between the groups (Fig. III-2B).

The finding that α4 KO mice voluntarily consume less ethanol compared to WT mice has been repeated in our lab by multiple researchers, over several years, in at least three generations of mice, and is therefore quite robust. Although the result that α4 KO mice consume less ethanol than WT mice is intriguing on its own, it does not necessarily mean that ethanol is not rewarding to them. Recent studies indicate that ethanol may indirectly activate nAChRs and, in turn, DAergic neurons, by increasing acetylcholine concentrations in ventral midbrain (Ericson et al., 2003). Using c-Fos as a measure of gross neuronal activation (Cole et al., 1989), and to further elucidate the circuitry involved in mediating the rewarding properties of ethanol, we analyzed the VTA of α4 KO and WT mice for ethanol-induced activation of the immediate early gene, c-Fos. In WT mice, at the rewarding dose of ethanol (2 g/kg, see below),

the DAergic neurons of the posterior VTA were significantly activated whereas this same dose of ethanol produced no activation in the VTA of α4 KO mice (Fig. III-3). Since ethanol did not induce c-Fos expression in α4 KO mice, our data strongly suggest that α4* nAChRs are necessary for activation of the mesolimbic pathway by alcohol.

Because c-Fos induction by ethanol occurred in a part of the DAergic mesolimbic reward pathway, this prompted us to directly compare the rewarding effects of ethanol in WT and α4 KO mice. Using the conditioned place preference assay, previous studies have shown that 2 g/kg ethanol is the rewarding dose in many mouse strains, including C57BL/6J (Boyce-Rustay and Holmes, 2006; Cunningham et al., 2006). This assay is used as an alternative to classic drug self-administration paradigms and is considered a direct measure of drug reward (Bardo and Bevins, 2000). Using this paradigm, 2 g/kg ethanol conditioned a place preference to the chamber containing the ethanol-paired floor in WT mice but not in the α4 KO mice (Fig. III-5A). These results are consistent with previous work indicating that ethanol place preference is expressed through a VTA dependent mechanism (Bechtholt and Cunningham, 2005). Taken together, our data indicate that expression of α4* nAChRs is necessary for voluntary ethanol intake, ethanol-induced activation of the mesolimbic DA pathway, and ethanol reward.

The hypersensitive Leu9'Ala nAChR mouse serves as a nice complement to the α4 KO mouse, because it allows for ethanol reward to be analyzed in

response to the selective activation of α4* nAChRs. While we saw no significant difference in baseline ethanol intake in these mice, this is a result that has not been as consistent as the α4 KO result, as evidenced by the larger error bars in this group (Fig. III-1B). We have seen in the past, and a trend in the current example, that the Leu9'Ala mice also consume less ethanol than WT mice. We hypothesized that these mice consume less ethanol for the opposite reason as the α4 KO mice; i.e., that the Leu9'Ala mice experience ethanol reward at a lower threshold compared to WT mice, similar to results with nicotine (Tapper et al., 2004). This result was perplexing for some time, that is, until we analyzed the brains of Leu9'Ala mice and saw that very low doses of ethanol (0.5 g/kg), that have no effect in WT mice, significantly activated the DAergic neurons of the posterior VTA (Fig. III-4). Our hypothesis was confirmed when the same low dose of ethanol conditioned a place preference in the hypersensitive Leu9'Ala mice, but not WT mice (Fig. III-5B). Thus, these data suggest that ethanol mediated activation of α4* nAChRs is critical for ethanol reward.

While analyzing brains of WT or Leu9'Ala mice that had been injected with ethanol and immunolabeled for TH and c-Fos, our lab discovered that nicotine (Zhao-Shea et al., 2011) and ethanol (Figs. III-3, III-4) both activate DAergic neurons almost exclusively within the posterior VTA. We hypothesized that those neurons activated by ethanol differentially expressed nAChR subunit genes that were important for mediating ethanol reward. To gain insight into nAChR subtypes that may influence alcohol consumption, we compared nAChR

subunit gene expression between posterior VTA DAergic neurons that were activated by alcohol and posterior VTA DAergic neurons not activated by alcohol. Interestingly, we found that DAergic neurons that were activated by alcohol express higher levels of α4, α6, and β3 nAChR subunit transcripts (Table IV-1; Fig IV-2B). These data suggest that α4α6β3* nAChRs may be involved in alcohol consumption. Our results are consistent with previous studies indicating that the α6/β3* nAChR selective antagonist α-conotoxin MII, when infused into the VTA, can reduce alcohol consumption and block alcohol-mediated DA release in NAc (Jerlhag et al., 2006; Lof et al., 2007b). Importantly, a significant portion of α6β3* nAChRs also contain the α4 subunit and these receptors represent one of the highest affinity nAChRs identified in the brain thus far (Salminen et al., 2007; Salminen et al., 2004). Notably, the α4α6β2β3 subtype is insensitive to DHβE (Salminen et al., 2004). Thus, this experiment provides insight to the discrepancy regarding the DHβE results, as discussed previously.

VI.C. α4* nAChRs AS MOLECULAR TARGETS FOR ALCOHOL CESSATION

Currently, only three medications are approved for the treatment of alcohol use disorders and these medications have only proven useful for 20 % -30 % of patients who use them. Although a large body of evidence exists implicating nAChRs as potential targets for mediating ethanol reward, the lack of nAChR-selective as well as nAChR-subtype selective ligands has slowed progress in this field (Chatterjee and Bartlett, 2010). Additionally, once a ligand is discovered,

there are many obstacles in the road to developing new targets for ethanol cessation from the bench to the clinic, a process that can take up to 15 years or more (Chatterjee and Bartlett, 2010).

However, drugs already on the market for treating nicotine addiction can go directly into clinical research trials to fast track the development of novel medications for the treatment of alcoholism. Thus, recent studies have found that the smoking cessation drug varenicline, an $\alpha4\beta2^*$ partial agonist clinically approved as a smoking cessation therapeutic (Coe et al., 2005; Gonzales et al., 2006; Steensland et al., 2007; Tonstad et al., 2006), can reduce both ethanol intake and seeking in rats (Steensland et al., 2007). However, while varenicline was designed to be a partial agonist of $\alpha4\beta2^*$ nAChRs, the precise nAChR subtype that varenicline acts on to modulate ethanol consumption was unknown.

In our study, we found that varenicline reduced consumption of both a low and high dose of alcohol in WT mice but did not significantly reduce consumption in $\alpha4$ KO mice, indicating that $\alpha4^*$ nAChRs are necessary for the effects of the drug (Fig. IV-3A, B). In addition, low doses of varenicline that had little effect on consumption in WT mice dramatically reduced ethanol intake in Leu9'Ala mice suggesting that activation of $\alpha4^*$ nAChRs are also sufficient for varenicline effects on alcohol consumption (Fig. IV-3C, D). Importantly, varenicline did not reduce sucrose intake indicating that the effects of the drug were specific for alcohol consumption and did not dampen general reward signaling (Fig. IV-3D).

Because higher doses of varenicline than used in our study may also

target α7 as well as other nAChR subtypes, in addition to α4β2* nAChRs, the mechanism by which varenicline reduces alcohol consumption is an open question (Mihalak et al., 2006; Papke et al.). A recent study demonstrated that varenicline reduced ethanol consumption in mice lacking either α7 or β2* nAChRs similar to WT (Kamens et al., 2010) indicating that expression of these receptors is not necessary for varenicline's effects. As discussed above, our data suggest that α4α6β3* nAChRs may mediate varenicline's effects on alcohol consumption, although it is expected that this subtype should also contain the β2 subunit (Grady et al., 2007). Thus, varenicline reduction of ethanol intake in β2 KO mice may occur because of compensatory mechanisms in nAChR expression or subunit composition.

Interestingly, varenicline, similar to nicotine and ethanol, also activated DAergic neurons selectively in the posterior VTA (Fig. IV-1). Furthermore, an infusion of varenicline directly into the posterior, but not anterior, VTA reduced alcohol consumption (Fig. IV-4). This suggests that the posterior VTA, specifically, could be a neuroanatomical substrate where both drugs interact. Thus, using the α4 KO and Leu9'Ala mouse models, we were the first to show that varenicline-induced reduction of ethanol consumption in mice is dependent on the activation of α4* nAChRs and that the posterior VTA is a site of action for mediating its effect.

Indeed, both the nicotine and alcohol addiction fields have now focused on not only selective-nAChR antagonists, but on selective partial agonists as well.

Partial agonists are thought to assist in smoking cessation by binding to and partially activating the specific nAChRs that mediate nicotine addiction (Rollema et al., 2007b). The partial agonist activity at these receptors would cause a moderate, sustained release of DA in the NAc, which would counteract the low DA levels experienced during withdrawal and help to relieve craving. Furthermore, by binding the high affinity nAChRs, the partial agonist then acts as an antagonist by preventing nicotine from binding and fully activating the receptor, thus removing the enhanced DA transmission in the NAc experienced while smoking, essentially removing the reward experienced by smoking (Rollema et al., 2007b). A similar mechanism is expected to be occurring in the brains of alcohol-addicted individuals as well as those who are dependent on both drugs (Crunelle et al., 2010).

Additionally, our follow up study (Chapter V) provides further evidence for a role of α4* nAChRs in mediating both nicotine and ethanol reward. Similar to our previous study (Chapter II), we found that nicotine decreased ethanol intake in WT mice in the DID assay; however this same dose of nicotine had no effect in the α4 KO mice (Fig. V-1). Furthermore, low doses of nicotine, that had no effect in WT mice, significantly reduced ethanol intake in the hypersensitive Leu9'Ala mice (Fig. V-2). Taken together, these studies imply that a combination approach, of both activating and blocking nAChRs will be an optimal strategy for reduction of the rewarding behaviors and craving symptoms of alcohol addiction.

VI.D. EVIDENCE FOR ADDITIONAL nAChR SUBTYPES IN ETHANOL REWARD

Since a variety of nAChR subtypes are expressed in the mesolimbic reward circuitry (Wooltorton et al., 2003), an emerging goal of alcohol research is focused on identifying the nicotinic receptor subtype(s) that may underlie ethanol reinforcement. In an effort to tease out individual nAChR subunits in ethanol-related behaviors, most work has utilized pharmacology. For example, both MLA and DHβE, an α7 and β2* nAChR specific antagonist, respectively, fail to block ethanol-induced locomotion (Larsson et al., 2002), reduce ethanol consumption (Le et al., 2000), or block ethanol-induced dopamine overflow in nucleus accumbens (Ericson et al., 2003; Larsson et al., 2002). However, the α3β2*, β3*, and α6* subunit specific antagonist, α-conotoxin MII, does inhibit these behaviors (Larsson et al., 2004). These data suggest that α7 and β2* nAChRs, the two most common CNS nicotinic receptors, may not have a critical role in ethanol-mediated striatal dopamine release. However, a recent study found that varenicline, a partial agonist of α4β2* nAChRs, can reduce both ethanol intake and seeking in rats (Steensland et al., 2007). While the varenicline data and α-conotoxin MII data are seemingly at odds, there is evidence that varenicline is not purely an α4β2* nAChR partial agonist, but can also activate α6* nAChRs, as well as other subtypes (Mihalak et al., 2006).

Interestingly, our result, that DAergic neurons activated by ethanol have higher gene expression of the α4, α6, and β3 subunits correlates nicely with

studies that found that blocking the α6β3* nAChR subtype with the peptide antagonist α-conotoxin MII decreases alcohol consumption and blocks alcohol-mediated DA release in the NAc (Jerlhag et al., 2006; Larsson et al., 2004; Lof et al., 2007b). Furthermore, the α6 subunit is very specifically expressed in the catecholaminergic system (Le Novere et al., 1996), making it an attractive target. While our data support the idea that α4* nAChRs are critical for ethanol reinforcement, recent data indicates that 1) over half of α-conotoxin MII-sensitive receptors contain the α4 subunit and 2) these receptors are most sensitive to acetylcholine-induced striatal dopamine release (Grady et al., 2007; Salminen et al., 2007). Therefore, it is likely that α4α6β3* mediate activation of the mesolimbic pathway by ethanol. Thus, it would certainly be interesting to investigate the role of α6* nAChRs in baseline ethanol consumption as well as ethanol conditioned place preference, preferably by use of the α6 KO mouse as opposed to pharmacology alone (Champtiaux et al., 2003; Champtiaux et al., 2002; Pons et al., 2008).

Recently, human genetic association studies have implicated the genes encoding the α5 (CHRNA5), α3 (CHRNA3) and β4 (CHRNB4) nAChR subunits as playing a critical role in the development of nicotine and alcohol dependence (Joslyn et al., 2008; Saccone et al., 2009; Wang et al., 2009). Additionally, one study showed that a new compound that is a partial agonist of α3β4* nAChRs, CP-601932, selectively decreased ethanol but not sucrose consumption and operant self-administration after long-term exposure to the drug (Chatterjee et al.,

2011). However, this compound is also a low-efficacy α4β2* nAChR partial agonist, so conclusions from this study are unclear. Thus, because of the limited pharmacological compounds that cross the blood brain barrier and are also selective for α3β4* nAChRs, it is difficult to determine the role of these nAChR subtypes. Therefore, more careful genetic and pharmacological experiments are necessary to determine the role of α3β4* nAChRs in ethanol reward.

In line with the genetic association studies, a recent study found that α5* nAChRs within the medial habenula (MHb) - interpeduncular nucleus (IPN) pathway are necessary to inhibit the motivational signal that controls nicotine intake (Fowler et al., 2011). In the absence of α5* nAChRs within the MHb, mice had similar levels of nicotine reward, but had significantly increased nicotine intake (Fowler et al., 2011). Although α5 nAChR subunit expression is low in the VTA, it is quite high in the habenulo-interpeduncular pathway (Marks et al., 1992), which is known to regulate the avoidance of noxious substances (Donovick et al., 1970). Interestingly, the lateral habenula (LHb) provides inhibitory control to the DAergic neurons of the VTA, is also activated by aversive stimuli, and is thought to be the source of negative motivational signals in the brain (Matsumoto and Hikosaka, 2007). Although the nAChR subunit composition of the LHb remains unclear, it would be interesting to test α5 null mutant mice in our ethanol DID assay to see if they have escalated ethanol intake similar to the nicotine studies and in ethanol place preference to see if they have altered ethanol reward.

VI.E. FUTURE DIRECTIONS

In this thesis, a combination of behavioral assays, pharmacology and genetically modified nAChR mouse models have been used to ask a series of questions; are nAChRs inherently critical for ethanol reward? If so, which subtype of nAChR is necessary for the rewarding properties of alcohol? And are they sufficient for ethanol reward?

Using pharmacology, we found that blocking or activating nAChRs in WT mice decreases voluntary ethanol intake. Using α4 KO and α4 Leu9'Ala KI mice, we determined that activation of α4* nAChRs are critical for ethanol reward, as well as necessary and sufficient for varenicline- and nicotine-induced reduction of ethanol consumption. Despite the work discussed in this thesis, two main questions remain: 1) exactly which nAChR subtype modulates ethanol consumption and reward and 2) which brain region(s) are necessary and sufficient.

In continuation of Chapter III, the next immediate steps to take will be to further elucidate the specific nAChR subtypes responsible for the modulation of ethanol activation of the VTA. The first step should try to block ethanol-induced c-Fos activation of the posterior VTA by pre-infusions of the α6β3* nAChR antagonist, α -conotoxin MII directly to the posterior VTA in WT and Leu9'Ala mice. In WT mice, if α-conotoxin MII attenuates c-Fos expression induced by the rewarding dose of ethaonol (2 g/kg), then one can conclude that α6β3* nAChRs modulate ethanol activation of DAergic VTA neurons. However, in the Leu9'Ala

mice, if α-conotoxin MII blocks activation of a low dose of ethanol (0.5 g/kg), one can conclude that the nAChR subtype responsible contains α4 in addition to α6 and β3 nAChR subuits.

Next, to further illuminate the nAChR subtypes that modulate ethanol reward, one could try to block ethanol conditioned place preference by infusing α-conotoxin MII to the posterior VTA via cannula implants just prior to the ethanol training sessions. This would first be done in WT mice to see if blockade of α6β3* nAChRs has an effect on ethanol reward, then in combination with the Leu9'Ala mice.

In addition to the continuation of experiments using the α4 KO and Leu9'Ala KI mice, our lab has begun to investigate ethanol reward in the α6 KO mice. The first steps will be to characterize voluntary ethanol intake using the DID assay, then to analyze the posterior VTA for ethanol-induced c-Fos expression and finally to see if α6* nAChRs are necessary for ethanol reward using the place preference assay. The α6 KO mice will also be crossed to the Leu9'Ala and α4 KO mouse lines for further investigation.

To address the second question, one important next step would be to identify the brain region that mediates ethanol reward. While the evidence is quite strong that the posterior VTA is a critical region, further techniques and experiments are necessary to verify this. There are several recently developed strategies to re-express, with regional specificity, nAChR subunits on KO backgrounds. Localized re-expression of functional nAChRs in the posterior VTA

that rescue the behaviors absent in the α4 KO mice, would be sufficient to denote

a role for the posterior VTA in ethanol reward.

APPENDIX 1.

This chapter has been published separately in:

Identification of a BK channel auxiliary protein controlling molecular and behavioral tolerance to alcohol. Martin GE, Hendrickson LM, Penta KL, Friesen RM, Pietrzykowski AZ, Tapper AR*, Treistman SN*. Proc Natl Acad Sci U S A. 2008 Nov 11;105(45):17543-8. Epub 2008 Nov 3. * Authors contributed equally

Author Contributions:

Linzy Hendrickson, Gilles Martin, Andrew Tapper designed experiments

Gilles Martin, Andrew Tapper, Linzy, Hendrickson, Krista, Penta, Ryan, Friesen, Andrzej, Pietrzykowski, Andrew Tapper performed experiments

Gilles Martin, Andrew Tapper, Steven Treistman wrote the paper

My role in this project was to design and coordinate the behavioral experiments including the Drinking in the Dark (DID) and Blood Ethanol Concentration (BEC) assays, but not including activity monitoring. Specifically, I oversaw and assisted Krista Penta in the creation of Figures A1-4D, 5A, 5B and provided data for Figures A1-5C, 5D.

A1.A. INTRODUCTION

Alcohol abuse is the third largest cause of preventable mortality in the world. Tolerance, described as the gradual loss of drug effectiveness over time, is a hallmark of abused drugs. This phenomenon is particularly important in the response to acute alcohol because the degree of tolerance exhibited by a naïve subject can predict the likelihood to develop alcohol abuse (Chrostek and Szmitkowski, 1998; Erwin et al., 1980; Fillmore et al., 2005; Heath et al., 1999). Thus, identifying the mechanistic and molecular underpinnings of tolerance is essential for understanding the pathophysiology of alcoholism, as well as determining potential therapeutic targets for alcohol abuse. The neurobiology of tolerance is thought to involve several types of adaptation, ranging from alteration in membrane lipid composition (Yuan et al., 2008) to neuroadaptive changes in target proteins (Crews et al., 1996; Woodward et al., 2006).

In recent years, large conductance calcium- and voltage-gated potassium (BK) channels have emerged as one of the key targets of ethanol action, yet their role in the physiological and behavioral response to alcohol are unknown. Invertebrate studies suggest that BK channels may be important for the development of tolerance to ethanol (Cowmeadow et al., 2005; Davies et al., 2003). In mammals, BK channels exist as a complex formed by the association of the pore-forming α subunit with the auxiliary β subunit. The α subunit is encoded by only one gene (slo) with several splice variants (STREX, P27, insertless, etc.), whereas the β subunit is the product of four distinct genes (β1-

β4). BK α subunits, unlike β, form functional BK channels (Coetzee et al., 1999; Kaczorowski et al., 1996; Vergara et al., 1998). BK α subunit expression is robust and widespread throughout the brain, with particularly high levels in the neo-, olfactory and hippocampal cortices, striatum, habenula and cerebellum (Brenner et al., 2000; Chang et al., 1997; Gribkoff et al., 2001; Kaczorowski et al., 1996). Other prominent sites for BK α are thalamus and amygdala, and to a lesser degree, the brain stem, and spinal cord (Chang et al., 1997). In contrast, the β4 subunit, although highly expressed, appears to be restricted to specific brain regions like the lateral hypothalamus, the purkinje layer and the striatum (Brenner et al., 2000; Chang et al., 1997). Whereas β1 expression is found at low levels in brain, β2 and β3 do not appear to be expressed in the central nervous system (Uebele et al., 2000; Wallner et al., 1999). In previous work, we showed that low EtOH concentrations (10 – 50 mM) potentiated BK channel open probability in a number of brain regions (hypothalamo-hypophyseal axis and nucleus accumbens) (Dopico et al., 1996; Martin et al., 2004; Pietrzykowski et al., 2004). Recently, we also reported that EtOH effects depend on BK channel subunit composition in ventral striatum. We found that αβ4 BK channels were potentiated by EtOH, whereas αβ1 channels were not (Martin et al., 2004). In the present study, we tested the hypothesis that BK subunit composition can control the degree and duration of ethanol sensitivity and, because of robust expression in striatum, a brain region implicated in addiction, we predicted that differences in BK subunit expression can translate into altered ethanol-induced

behaviors. We focused on the β4 subunit because of our previous work that indicates it is widely expressed in ventral striatum and co-assembles with BK α to form functional, ethanol sensitive channels in the soma of MSNs.

A1.B. RESULTS

We transfected HEK-293 cells with α alone or in combination with β4, and recorded BK single channel activity in cell-attached patch clamp mode for 20 sec every minute for up to 20 min. 50 mM EtOH, a concentration known to strongly influence channel activity (Martin et al., 2004), increased αβ4 BK channel open probability (Fig. A1-1A; middle trace EtOH 3 min) compared to control (Fig. A1-1A; top trace Control). This effect persisted up to 8 min after the start of EtOH exposure (Fig. A1-1A bottom trace). The lack of tolerance was not voltage-dependent because we observed a similar phenomenon when large (+150 mV) depolarizing voltage steps evoked outward BK currents on 3 additional patches (data not shown). In five patches (two from HEK cells and three from freshly isolated neurons), we found that BK channel activity remained potentiated (about 3.4 fold) for up to 14 min (the longest tested) after the beginning of EtOH perfusion compared to control values (data not shown). A ceiling effect that would distort the magnitude of EtOH's effects is very unlikely as we systematically set BK channel NPo to low values before exposing cells to the drug. Furthermore, even in presence of EtOH, BK channels typically spent only a small fraction of their total open time in the second (Fig. A1-1A; O2 middle trace) or third open states (Fig. A1-1C; O3 middle trace), indicating that

EtOH had not maxed out BK channel activity. In control experiments, we measured BK channel activity for up to 15 minutes in the absence of ethanol and found no change in baseline (data not shown). On average (n = 8), 50 mM EtOH increased BK channel activity by about 2.5 fold (Fig. A1-1B).

We examined the effects of 50 mM EtOH on BK channels consisting of only α subunits. We found that EtOH effects on both inward (Fig. A1-1C) and outward (data not shown) currents were similar. Thus, under these conditions, 50 mM EtOH also boosted channel activity compared to control. In contrast to its effect on αβ4 BK channels, EtOH potentiation disappeared minutes after the beginning of alcohol exposure, demonstrating acute tolerance of the response (Fig. A1-1C lower trace EtOH 7 min). When averaged over 8 patches, EtOH initially increased α BK channel activity by about 3 fold (Fig. A1-1D, light shaded box), before returning to control levels (Fig. A1-1D; darker shaded box). To evaluate whether EtOH potentiation was voltage dependent, we tested EtOH's effect on both α and αβ4 BK activity by recording macroscopic currents in whole cell mode at multiple potentials. Plotting current amplitude (normalized to I_{max}, typically observed around +150 mV) *vs*. voltage revealed that EtOH increases both α and αβ4 BK current amplitude over a range of potentials (data not shown), indicated it was not voltage-dependent.

From previous work, we know that the BK β4 subunit is coexpressed with the α subunit in rat ventral striatum MSNs (Martin et al., 2004), and that these channels are dose-dependently potentiated by EtOH. RT-PCR amplification of

β4 mRNA confirms that this subunit is robustly expressed in mouse striatum along with much lower levels of the β1 subunit (Fig. A1-2A). Therefore, we hypothesized that MSNs from WT mice should functionally express αβ4 BK channels and that they should be potentiated by EtOH, mirroring αβ4 BK activity in heterologous expression studies. Indeed, the WT BK channel response to 50 mM EtOH was very similar to that observed with αβ4 BK channels in HEK293 cells: EtOH potentiated BK channel activity (Fig. A1-2B, middle trace and Fig. A1-2C, light shaded box) and this was sustained throughout the recording session (Fig. A1-2B, bottom trace and Fig. A1-2C, darker shaded box). To determine if this persistent EtOH mediated channel potentiation was dependent on β4 expression, we recorded from BK channels in MSNs isolated from mice that do not express KCNMb4, the gene encoding the β4 subunit (β4 KO) (Gribkoff et al., 2001). Interestingly, MSNs from KO mice exhibited BK channel activity that was potentiated by EtOH but rapidly returned to control levels (Fig. A1-2D and A1-2E, 4/5 neurons), indicating that, in the absence of β4, acute tolerance develops. This effect mirrored what we found when BK α subunit alone was expressed in HEK293 cells (Fig. A1-1D and A1-1E).

To better understand the physiological role of BK β4 subunit expression on neuronal excitability, we evoked APs in WT and β4 KO mice using whole cell patch clamp recordings in striatal slice, and freshly isolated MSNs. Similar to previous reports from hippocampal neurons (Brenner et al., 2005), the number of APs evoked by current injection was increased in β4 KO MSNs compared to WT

(Fig. A1-3A). Since BKs contribute to determining MSNs AP patterning in WT mice, we tested the idea that EtOH-mediated modulation of BK channels should alter the excitability of these neurons, and that this effect should show little tolerance. In contrast, in KO mice where most BK channels are presumably composed of α subunits only, we expected to see a transient effect of EtOH on AP patterning. In WT mice, 50 mM EtOH markedly decreased the number of APs 2 min after EtOH perfusion (Fig. A1-3B; middle trace, left panel) and this effect persisted 8 min after the start of EtOH application, indicating a lack of tolerance. In β4 KO MSNs, the number of APs was also reduced 2 minutes after EtOH perfusion. However, unlike WT responses, significant tolerance developed to EtOH induced suppression of excitability within the 8 minute EtOH exposure (Fig. A1-3B, middle panel, bottom trace). In freshly isolated MSNs from KO mice (n = 3, Fig. A1-3B; right panel), EtOH similarly transiently reduced the number of APs, mirroring results obtained in slices. This latter experiment demonstrates that EtOH effects on MSN spike patterning are intrinsic to these neurons. On average, 2 min after EtOH exposure, the number of evoked APs in β4 KO MSNs decreased by 60% of control compared to 40 % for neurons from WT mice (Fig. A1-3C). While the number of APs in KO mice was almost back to control level after 8 min exposure, it was smaller in WT mice compared to the 2 min time point (Fig. A1-3C). The development of tolerance (or its absence) is also shown in the inset of Fig. A1-3C as the ratio of APs at 8 min over the number of APs 2 min after EtOH exposure. In WT mice, the ratio was below 1 (broken line, Fig. A1-

3C; inset) while it was significantly higher in MSNs from KO mice (~ 2, p < 0.001).

To further establish a functional a link between β4 expression and the development of tolerance of spike patterning, we exposed β4 KO neurons in slice and dissociated culture to 100 nM charybdotoxin (ChTx), known to block α and αβ1 BK, the two BK channel subtypes found in these KO mice. In both slices (n = 2) and freshly isolated MSNs (n = 4), not only did 50 mM EtOH fail to decrease spiking, but it slightly increased it (n = 6; Fig. A1-3D). Fig. A1-3E shows the average number of APs before (open circles) and during EtOH exposure in the presence of 100 nM ChTx. We also tested the effect of 1.5 μM tetrandrine, a blocker of αβ4 BK channels. Tetrandrine completely prevented EtOH from altering excitability of WT MSNs. The number of APs remained unchanged up to 3 min after EtOH exposure (data not shown), confirming that EtOH effects are mediated by αβ4 BK.

Because striatum is a brain region known to be involved in both the motivational and locomotor properties of drugs of abuse (for reviews see (Everitt and Robbins, 2005; Hyman et al., 2006)) we wondered if the stark difference in physiology at the single channel and whole cell levels could also be observed in the behavioral response to EtOH. Thus, we challenged β4 KO and WT mice with 2g/kg EtOH once a day and monitored their ambulatory activity 5, 10 and 15 min after injection. Following EtOH injection on day 1, WT mice showed a marked (70%) reduction of their activity levels 5 min after injection (Fig. A1-4A; black

square symbols, $F_{1,22} = 25.0$, $p < 0.001$) compared to a saline injection. Activity

remained depressed when monitored over the following 10 and 15 min time

blocks. On day 4, the response of WT mice to EtOH, 5 and 10 min after injection

were comparable to that of Day 1, and only showed tolerance at the 15 min time

point (Fig. A1-4A; open squares, $F_{1,22} = 7.36$, $p < 0.05$ Day 1 compared to Day

4). However, in sharp contrast to WT mice, ambulatory activity in KO mice

displayed rapid tolerance to the locomotor suppressing effects of EtOH. Thus,

on day 1, although EtOH significantly depressed locomotor activity (Fig. A1-4B;

Day 1) 5 minutes after injection compared to control ($F_{1,22} = 9.27$, $p < 0.01$), when

tested at 10 and at 15 minutes after EtOH injection on day 1, ambulatory activity

had returned to control levels (Fig. A1-4B; filled squares). In the same KO mice,

by the fourth day of ethanol challenge, nearly complete tolerance to ethanol-

induced hypolocomotion was observed. In addition, we compared summed

locomotor activity 15 min after EtOH injection on days 1, 4, 7 and 10 between β4

and WT mice. On Day 1 (first injection), the activity of both WT and KO mice

decreased (Fig. A1-4C). However, by the fourth injection day (Day 4), complete

tolerance to ethanol-induced hypolocomotion developed in β4 KO mice (Fig. A1-

4C; Day 4 significance between genotypes, $F_{1,22} = 7.1$, $p < 0.05$), whereas

suppression remained evident in WT mice through the 10th day of injection (Fig.

A1-4C). The difference in acute locomotor tolerance could not be explained by a

difference in the pharmacokinetics of ethanol between β4 KO and WT mice

because the peak blood ethanol concentration (BEC), as well as the clearance

rate of ethanol after an i.p. injection of 2 g/kg was identical in the two genotypes (Fig. A1-4D).

Because rapid tolerance is predictive of increased alcohol consumption, we compared voluntary ethanol intake in WT and β4 KO mice, utilizing a restricted access EtOH self-administration paradigm termed "drinking in the dark" (Rhodes et al., 2005; Rhodes et al., 2007). This assay produces robust EtOH intake in C57BL/6J mice, the background strain of the β4 KOs. Remarkably, β4 KO mice consumed significantly greater levels of EtOH compared to consumption in WT mice during each of the first 3 nights of the assay (Fig. A1-5A). In addition, the ethanol intake averaged over four nights was significantly higher in β4 KO mice (Fig. A1-5B, $F_{1,81}$ = 19.7, p < 0.001). We measured blood alcohol levels (BAL) immediately following ethanol exposure on night 2 of the DID assay, where we observed the largest difference in intake between genotypes. As expected, BALs of KO mice (26.62 ± 7.53; n = 4) were much higher than that of WT mice (5.85 ± 0.53 mM, n = 4). Water intake between WT and KO mice was not different (Fig. A1-5C), suggesting that changes in ethanol consumption were not due to differences in drinking volume. Importantly, there was also no significant difference between genotypes in sucrose intake, indicating that changes in ethanol drinking were specific for the drug (Fig. A1-5D). WT and KO mice had similar aversion for quinine, suggesting that the difference of EtOH intake was not due to aversive taste (Fig. A1-5D; n = 5).

A1.C. DISCUSSION

Our results suggest remarkable parallels in the effects of the BK β4 subunit on acute alcohol tolerance at the level of single channel recording, spike patterning, and behavioral studies. The development of tolerance was apparent within a few minutes at each level of analysis in β4 KO but not WT mice.

The bridge between molecular events and behavioral outcome is always difficult to establish. We believe that our finding of β4-dependent tolerance at the single channel and action potential levels is a compelling candidate for mediating effects we see on behavior (locomotor tolerance and alcohol consumption). Because the β4 subunit is expressed in a number of brain regions (Gribkoff et al., 2001; Uebele et al., 2000), we cannot rule out that regions outside the striatum may participate in the changes in ethanol-related behavior. However, our focus on striatum is based, in part, on the known role this circuitry plays in these behavioral outcomes. Ethanol, via both direct and indirect activation of DAergic neurons in the ventral tegmental area (Blomqvist et al., 1993; Ericson et al., 1998; Larsson et al., 2002; Okamoto et al., 2006), increases dopamine release in the striatum, which is associated with both the motivational and locomotor properties of most abused drugs. In addition, our data are consistent with recent reports in *c-elegans* indicating a role for BK channels in depression of locomotor effects of alcohol (Kaczorowski et al., 1996). MSNs make up ~95 % of neurons in striatum and receive inputs from DAergic neurons in the VTA and substantia

nigra pars compacta. MSNs express BK channels consisting of α and β4 subunits that are potentiated by ethanol, an effect that develops little tolerance in response to acute alcohol. In the absence of the β4 subunit, the rate of tolerance to ethanol potentiation is dramatically enhanced at both the single channel and whole cell level. This is associated with an increase in the rate of tolerance to locomotor suppression elicited by both acute and chronic ethanol exposure in β4 KO mice compared to WT mice. The fact that β4 KO mice also self-administer more alcohol than WT animals, corroborates the important role β4 subunit expression has on alcohol tolerance. This dramatic difference in tolerance and alcohol consumption is specific for ethanol because β4 KO mice consume equal amounts of water, quinine, and sucrose solution compared to WT mice. In addition, the pharmacokinetics of ethanol does not differ between genotypes.

At the macroscopic level, the influence of the BK channel in shaping APs in MSNs of the dorsal striatum is not surprising. Studies carried out in CA1 pyramidal neurons from the hippocampus (Gu et al., 2007; Shao et al., 1999), dorsal vagal neurons (Pedarzani et al., 2000), lateral amygdala (Faber and Sah, 2002) and purkinje cells (Edgerton and Reinhart, 2003; Sausbier et al., 2004), report that toxin-mediated BK channel blockade widens APs, suggesting that BK channels facilitate repolarization. In striatal interneurons and MSNs, BK contributions to shaping APs has also been reported (Bennett et al., 2000). Interestingly, our data showing that spike frequency is significantly higher in β4 KO mice compared to WT mice, is consistent with a recent study in CA1

neurons by Brenner et al (2005) (Brenner et al., 2005) with the same knockout animals. The similarity with the Brenner study confirms the role of β4 subunit mRNA, since it is highly expressed in both striatum and hippocampus (Behrens et al., 2000). Although MSNs express other channels involved in shaping APs and neuronal excitability, the effects of EtOH on MSN excitability are likely mediated by BK channels. First, delayed-rectifier and rapidly inactivating I_A K^+ channels, the two other main potassium channels activated by depolarization in striatal MSNs, have been shown in other preparations to be insensitive to 50 mM EtOH (Camacho-Nasi and Treistman, 1986), the concentration tested here. Additionally, tolerance to EtOH-mediated effects of AP patterns in MSNs observed in KO mice occurs over a similar time course to tolerance to EtOH-mediated potentiation of BK single channel activity in the same mice. Finally, the effects of ethanol on AP spike patterns are inhibited by charybdotoxin in β4 KO mice suggesting a BKα dependent mode of drug action.

Our data indicate that the BK β4 subunit controls tolerance to alcohol at both the molecular and behavioral levels. Since a dramatic association between tolerance to alcohol and the propensity to develop alcoholism exists, our data suggest that the gene encoding the BK channel β4 subunit, KCNMB4 should be evaluated as a candidate gene for susceptibility to alcohol abuse and alcoholism.

A1.D. MATERIAL AND METHODS

Cell culture: Our methods are essentially the same as previously published (Feinberg-Zadek and Treistman, 2007). Briefly, α BK channels were derived from stable cell lines (Tseng-Crank et al., 1996), a gift from Peter Ahring (Ahring et al., 1997). αβ4 channels were derived from cell lines stably expressing α and transiently expressing β4.

Slice preparation and freshly isolated striatal neurons: This is described in detail in Martin et al. (Martin et al., 2002). Briefly, mouse brains were sliced (350 μm) using a Vibratome 3000 (USA) and incubated for up to 6 h at room temp (20-22°C) in a gassed (95% O_2 and 5% CO_2) saline solution. Following enzymatic digestion with protease XIV (1 mg/ml), the tissue was mechanically triturated using fire-polished Pasteur pipettes, and cells were plated into a 35 mm Petri dish.

Electrophysiological recording: Single-cell cell-attached patch clamp recording used standard methods (Hamill et al., 1981). We pulled patch electrodes from 1.5 mm OD borosilicate capillary glass (Warner Instrument, CT) to a resistance of 4-6 MΩ. The recording pipette solution was (in mM): 130 K_2MeSO_4, 2 $MgCl_2$, 2 $CaCl_2$, 15 HEPES. We set sampling rate and low-pass filter at 10 and 2 kHz, respectively, using an EPC10 double amplifier (HEKA Electronics, Germany). Voltage and current were digitized and stored using PatchMaster 2.1 (HEKA Electronics, Germany). We recorded BK activity for 20 sec, every minute, three

times to ensure a stable baseline. We averaged the open probability of these three controls, and all subsequent NPo values were expressed relative to this average. Drugs were applied and BK channel activity was recorded in successive blocks of 20 sec, every minute, for up to 10 min. Data were expressed as mean ± SEM (with the number of cells or patches in parentheses). For whole cell recording in slices, MSNs were visually identified, and characterized electrophysiologically. Series resistance (Rs) was monitored throughout experiments. Recordings showing Rs changes of more than 15-20% were discarded. We used MultiClamp 700 B and EPC10 double amplifiers, at a rate of 20 kHz, to record APs. Voltage and current traces were acquired and analyzed with pClamp 10 (Molecular Devices; CA USA) and FitMaster 2.15 (HEKA Elektronik, Germany) software packages.

Charybdotoxin treatment: To ensure we recorded exclusively $\alpha\beta_4$ channels in HEK-293 cells and in WT striatal neurons, we added low concentrations of charybdotoxin (ChTx), a toxin that rapidly and selectively inhibits activity of α and $\alpha\beta1$ BK channels at 100 nM (Behrens et al., 2000). Calculation of the steady-state channel activity, NPo. We used all-points amplitude histograms to calculate BK activity, determined from the product of the total number of functional channels present in the membrane patch (*N*) and the probability that a particular channel was open under steady-state conditions (P_o). Calculations of NPo were performed with TAC analysis software (Bruxton Inc, OR, USA). NPo ratios generated from the first ethanol exposure were used for normalization of the

data.   BK activity was measured as NPo ratio percent ((NPo ethanol / NPo control) X 100).

Behavioral experiments: Male and female C57BL/6J mice (Jackson Laboratory) between 8-14 weeks of age were housed 3-4 animals per cage until the start of each experiment. For drinking in the dark, mice purchased from Jackson Labs were habituated to BNRI colony rooms for at least 2 weeks and the DID procedure room for at least 1 wk prior to the start of experiments. Mice used for locomotor studies were bred and raised at the BNRI. β4 KO mice were back-crossed at least ten generations to the C57BL/6J strain. We kept mice on a standard 12 h light/dark cycle with lights on at 7:00am and off at 7:00pm, and given food and water *ad libitum*. We conducted all experiments in accordance with the guidelines for care and use of animals provided by the National Institute of Health, as well as with an approved animal protocol from the Institutional Animal Care and Use Committee of the UMass Medical School.

Drugs and drinking solutions: Ethanol solution was prepared from 190 proof absolute anhydrous ethanol (Pharmco-Aaper brand, Brookfield, CT) diluted to 10 % ethanol (v/v) using tap water. Sucrose (EMD) and quinine hydrochloride (Sigma-Aldrich, St. Louis, MO) was dissolved in tap water to make a 10 % (w/v) and 1 mM concentration solution, respectively.

Drinking in the dark (DID): Two hours after lights out, water bottles were removed and replaced with 10 % ethanol bottles and left in place for two hours. Control animals had water replaced with another water bottle or 10 % sucrose or 1 mM

quinine solution. An empty cage was set up and a water bottle was replaced with ethanol to control for evaporation.

Activity Monitoring: Locomotor activity was measured by a photobeam system (San Diego Instruments). Mice were placed in activity cages and allowed to habituate for 90 minutes prior to first i.p. injection of either saline or ethanol (2 g/kg, 20 % v/v with saline, 10 ml/g body weight).

Ethanol metabolism: Prior to an ethanol injection, blood was obtained from the tail vein (~30uL each time point) to provide a zero point. After a 2 g/kg i.p. injection of EtOH, blood samples were taken at 30, 60, 90, and 120 min. For BEC measurements after DID, mice were culled immediately after a 2 hr EtOH exposure on night 2 and trunk blood was collected in heparinized capillary tubes. Blood was centrifuged (1500Xg for 5 min) and analyzed using an alcohol oxidase-based assay. BECs were measured on a GM7 Micro-Stat Analyzer (Analox Inst Ltd.)
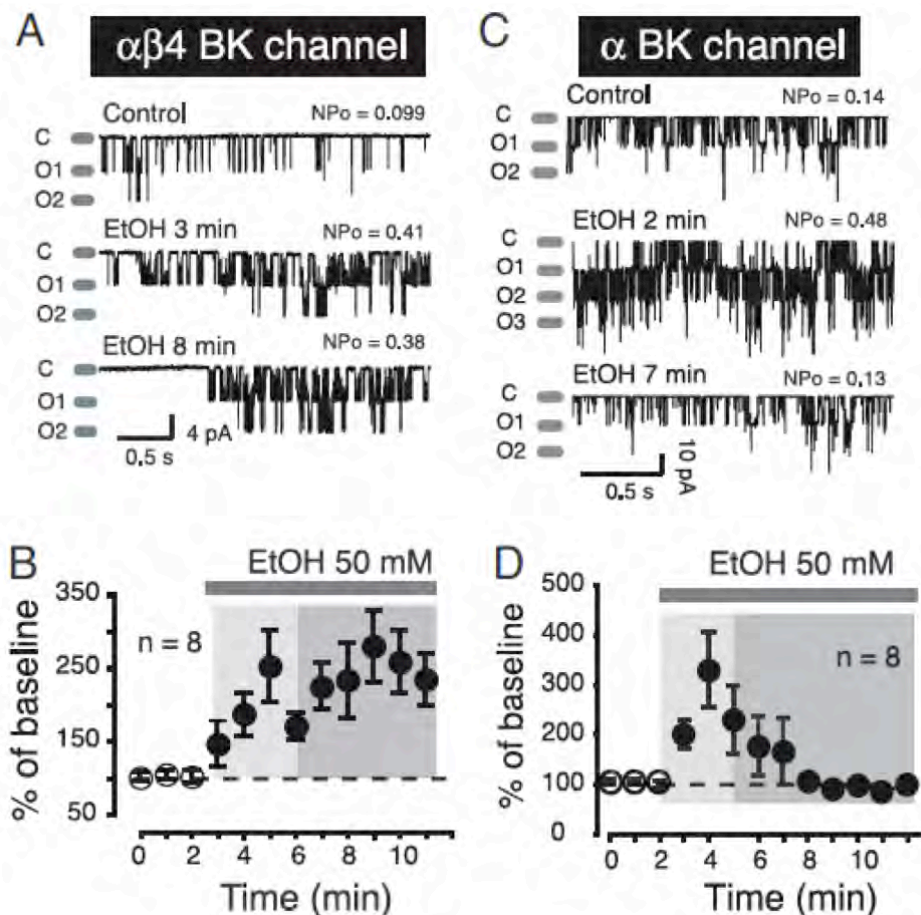
Fig. A1-1. BK β4 subunit influences tolerance: A) αβ4 BK Single channel activity recorded at hyperpolarized potentials from HEK293 cells. B) EtOH's effects on αβ4 BK channel activity averaged over several cells (n values indicated in graphs). Panel C shows α BK single channel activity before (control) and during EtOH exposure. D) Magnitude of EtOH's effects on α BK activity averaged over several cells (n values are indicated in graphs). Representative traces of BK activity before (control) and during EtOH exposure (EtOH). 'C' and 'O' refer to BK closed and open states, respectively. NPo indicates BK channel open probability under each experimental condition. In graphs (B and D) lightly shaded areas indicate where BK channel potentiation typically occurs, while the darker shaded areas show where tolerance is observed. EtOH effects are expressed as % of baseline.
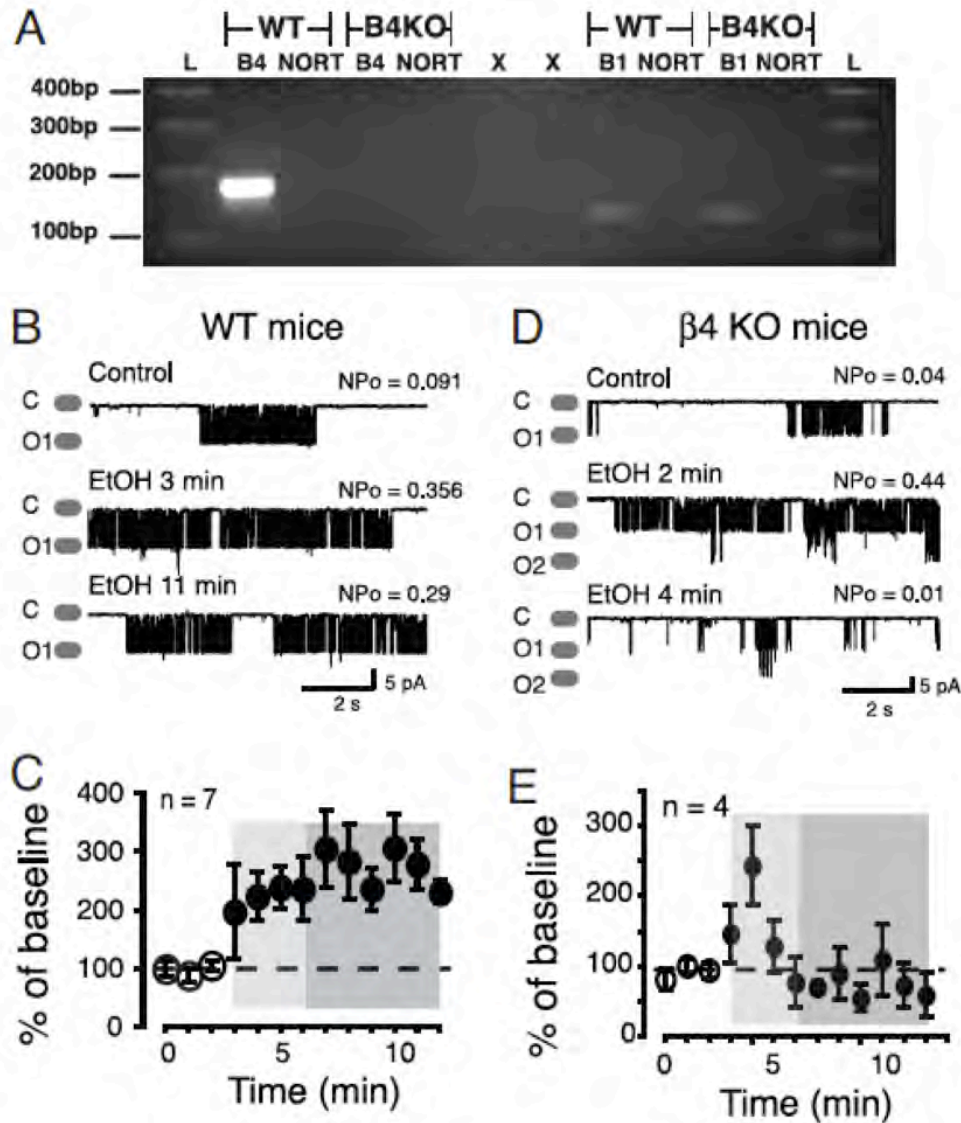
Fig. A1-2: β4 subunit controls tolerance of BK single channel activity in freshly isolated striatal MSNs. A) DNA agarose gel shows that only β4 expression is lacking in striatum isolated from β4 KO mice; whereas β1 mRNA is present in both WT and KO animals. "L" denotes the 100 bp marker on molecular weight ladder. Base pair number is indicated in the left hand margin. "B1" and "B4" refer to BK β1 and β4 subunits, respectively. "NORT" are negative controls with omitted reverse transcriptase and "X" indicates columns where no material was loaded. Single channel activity recorded from striatal MSNs acutely

isolated from WT (B and C) or β4 KO (D and E) mice, respectively. B and D) Representative traces of BK activity from WT and KO mice, before (control) and during EtOH exposure (EtOH). 'C' and 'O' refer to closed and open states. NPo indicates BK channel open probability. C and E) graphs show magnitude of EtOH's effects averaged over several cells (n values are indicated in graphs). Lightly shaded areas indicate where BK channel potentiation typically occurs, while the darker shaded areas show where tolerance is observed. EtOH effects are expressed as % of baseline.
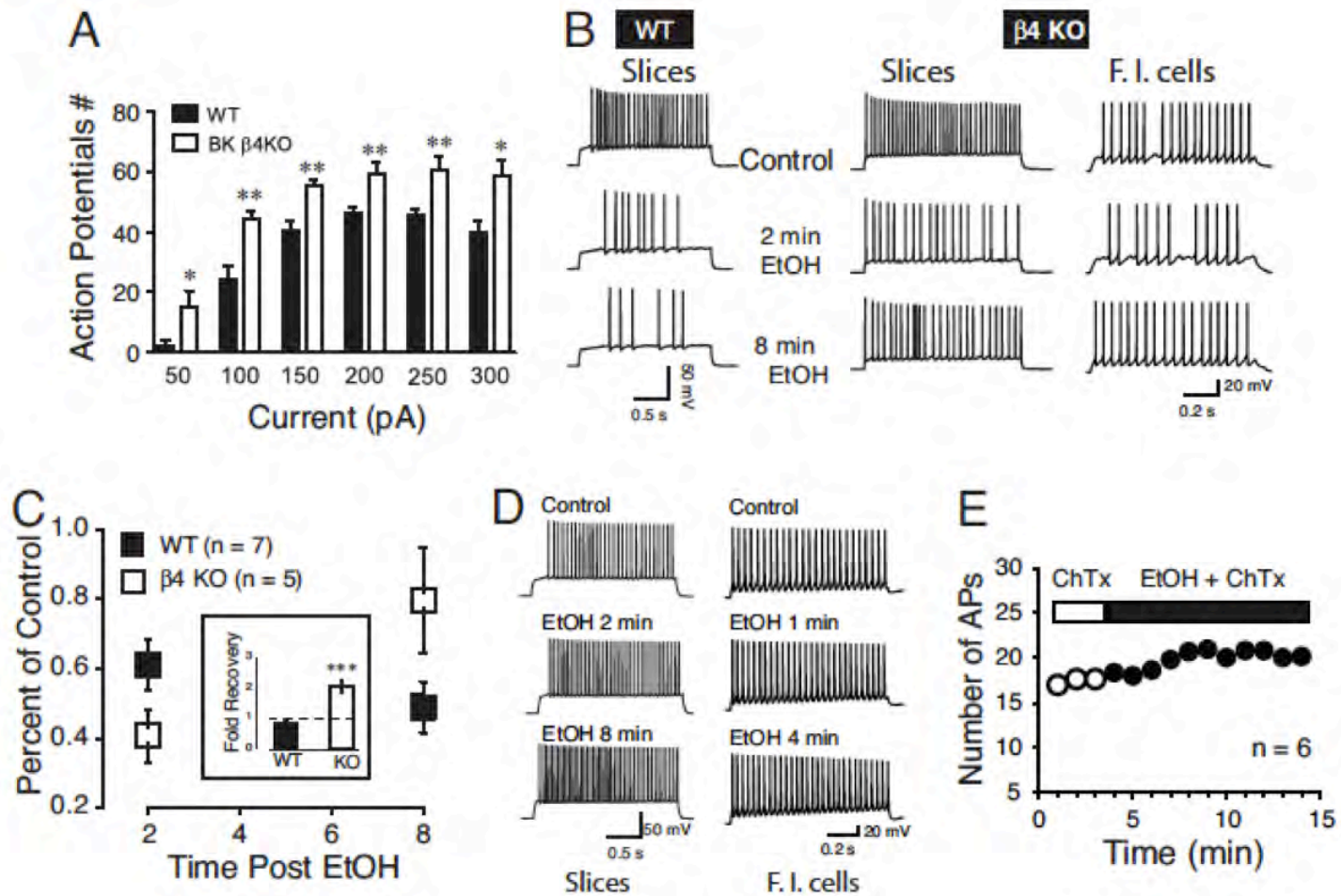
Fig. A1-3.

Fig. A1-3: **EtOH-mediated decrease of MSN excitability exhibits tolerance in KO, but not WT mice.** A) Number of APs recorded from WT (filled columns) and KO (open columns) MSNs following a series of incremental (50 pA) current steps (50 to 300 pA). B) Representative action potential trains evoked in a slice preparation (Slices) by a single 100 pA current step in WT (left panel) and KO (middle panel) mice before (control) and after 50 mM EtOH exposure (2 or 8 min). Two min after EtOH, the number of APs is smaller in both WT and KO mice. While KO mice MSN excitability partially recovers 8 min after EtOH exposure (bottom trace; right panel), WT neuronal excitability remains depressed (left panel, bottom trace). Results obtained in slices were reproduced on freshly isolated neurons from KO mice (right panel; β4 KO / F.I. cells). C) Averaged change in action potential number recorded in MSNs in slices and freshly isolated after 2 or 8 minutes EtOH exposure, presented as percent of control before EtOH exposure in MSNs from WT and β4 KO striatal slices. 5/7 neurons were ethanol sensitive and developed tolerance in β4 KO MSNs, whereas 7/9 MSNs from WT were ethanol sensitive and did not develop tolerance (*$p < 0.05$). The inset shows the ratio of AP number at 2 and 8 min reported as fold recovery from ethanol; value below the broken bar indicates a further decrease of APs number at 8 min compared to 2 min EtOH (solid column; WT), while value above the line indicate a recovery (KO); $F_{1,10} = 27.6$, $p < 0.001$. D) 100 nM ChTx blocks EtOH effects on striatal MSNs AP patterns in slices (left panel) and freshly isolated neurons (F.I cells, right panel). E) Number of APs measured every minute before (open circles) and during 50 mM EtOH exposure (solid circles) in presence of 100 nM ChTx. Data from slices and freshly isolated MSNs were combined.
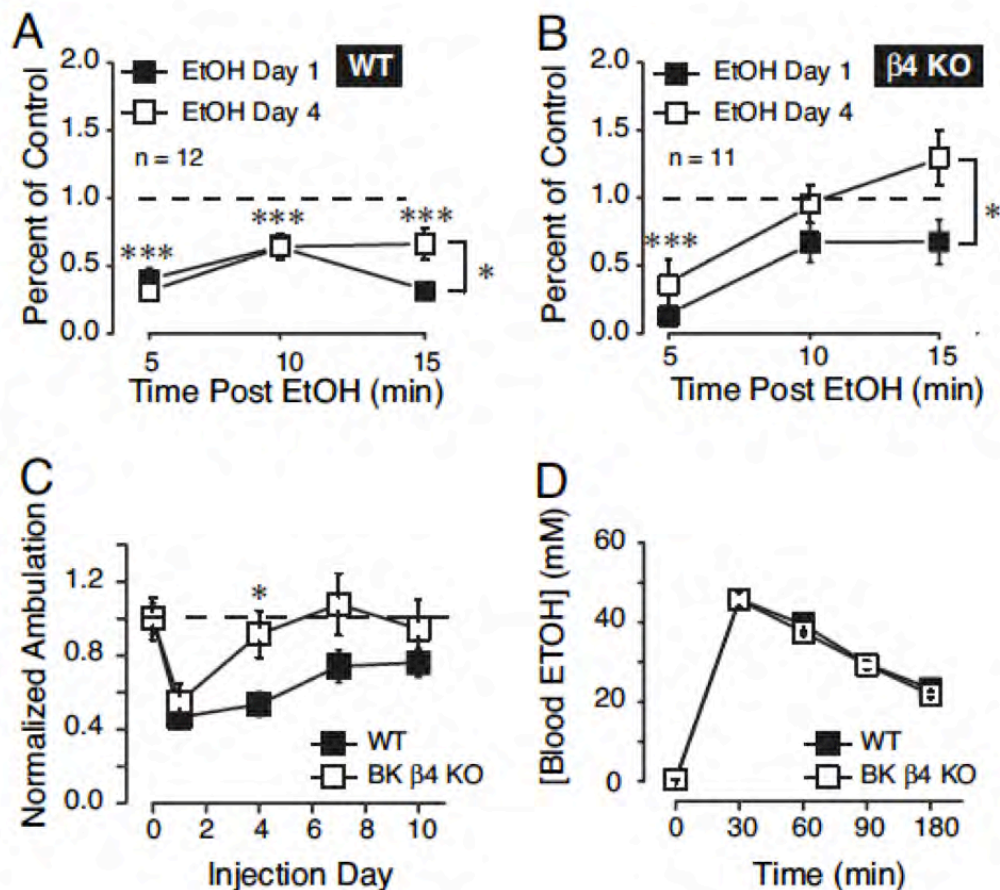
Fig. A1-4: **Effects of EtOH on locomotor activity in WT and β4 KO mice.** A) Ambulatory activity of WT mice 5, 10 and 15 min after a 2 g/kg i.p. EtOH injection on day 0, 1 and 4. Each data point represents 5 minutes summed activity. Asterisks above symbols indicate significant locomotor activity on both day 1 and 4 of EtOH injection at each time point (5, 10 and 15 min) compared to saline injection baseline (represented by the dashed line). Asterisk next to bracket indicates difference between day 1 and 4 at 15 min time point only. B) Effects of the same EtOH dose at the same time points in KO mice. C) Fifteen minute summed activity immediately after a daily ethanol injection. Note the rapid development of tolerance for KO mice upon repeated injections of 2 g/kg EtOH, compared to WT mice. D) Blood alcohol concentration in WT and KO mice before (time 0) and at 30 min after a single i.p. injection of 2 g/kg ethanol. One-way ANOVA, Tukey post-hoc, * $p < 0.05$, *** $p < 0.001$.
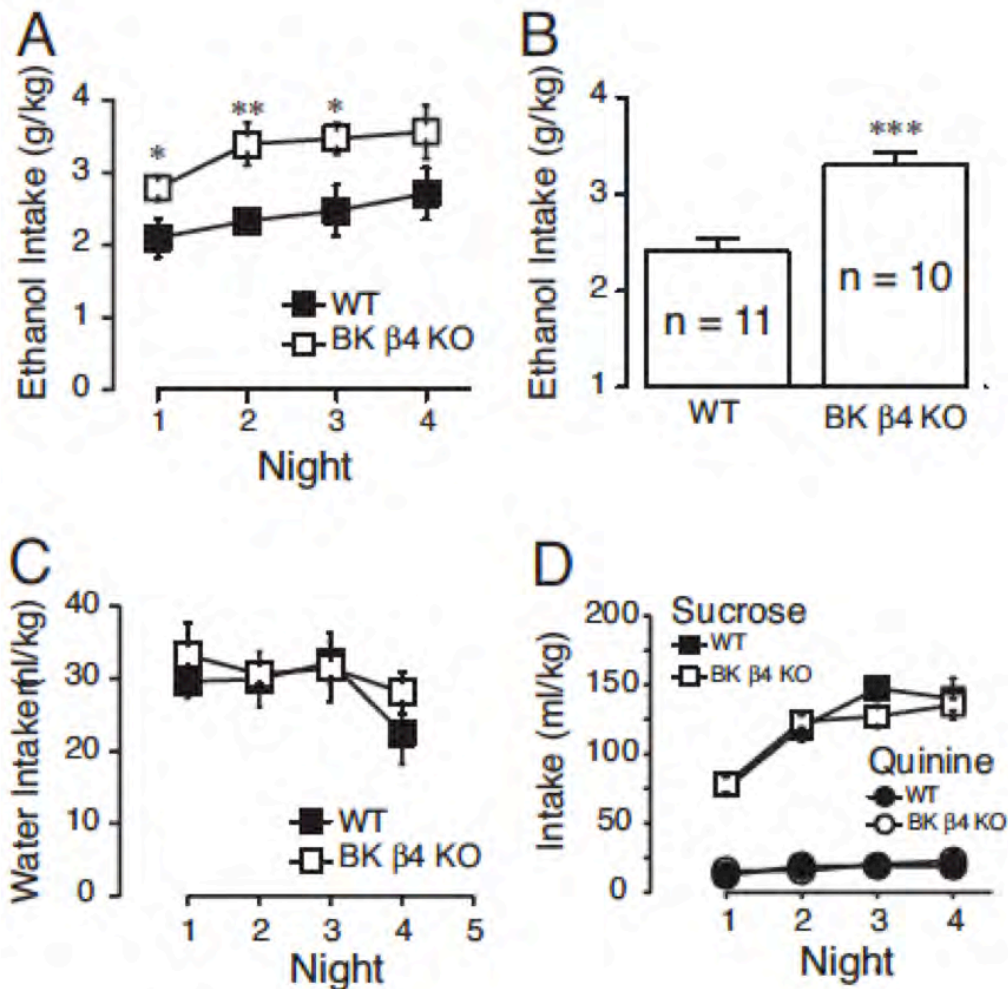
Fig. A1-5: **EtOH consumption is higher in β4 KO.** A) EtOH consumption of WT (black squares) and β4 KO mice (white squares) using a restricted access single bottle self-administration assay. Measurements were taken every day for four days for two hours after lights were turned off. B) Average daily EtOH intake in WT and KO mice. C and D illustrate water, sucrose and quinine intake of WT (black squares and circles) and KO (white squares and circles) mice, respectively. No significant difference was observed in either condition. One Way ANOVA, Tukey post-hoc, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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