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ALPHA6 BETA2 SUBUNIT CONTAINING NICOTINIC ACETYLCHOLINE RECEPTOR
CONTRIBUTIONS TO ABUSE-RELATED EFFECTS OF NICOTINE AND ALCOHOL

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of
Philosophy at Virginia Commonwealth University

by

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Acknowledgement

The work in this dissertation would not have been possible without the immense support of many people. I would like to express my sincere gratitude to my advisor, Dr. Darlene H. Brunzell, for her mentorship, patience, and encouragement. She has always pushed me to be the best and has helped me to become the scientist I have always dreamed of being. I am grateful for the rest of my committee members, Drs. Keith Shelton, Michael Miles, Joseph Porter, and Michelle Block, who contributed to the development and completion of my dissertation work. I consider myself lucky to have been able to receive valuable insight and advice from these exceptional scientists. I would like to thank all of my lab mates over the years, especially Jennifer, Shawn, Lindsay, and Claire. They made daily life in the lab way more fun than I thought it could be and were always there to lend a hand. I would not have made it through graduate school without them and I will always cherish their friendships. Thanks to all of our collaborators, Drs. Matthew Banks, Keith Shelton, Ryan Drenan, Michael McIntosh. My research greatly benefited from their expertise and assistance in carrying out my experiments and interpreting data. I would like to thank the Department of Pharmacology and Toxicology, especially to Drs. William Dewey and Hamid Akbarali, for accepting me into the department and providing the students with a thriving academic environment. I cannot thank all of my friends and family enough for their support. To my best friend and partner in life, Jon Haynie, without whom I'd be lost. He has been my rock and has never stopped supporting and encouraging me in everything that I do. And he has taught

me to enjoy life and not take it so seriously all the time. To my sisters, my dad, and my step-dad, their love and support is unwavering. Finally, and most importantly, I would not have made it this far in life without the continuous support and guidance from my mother, Marjorie Beck. She has taught me to never give up on my dreams and inspires me to be the best version of myself. For that, I am forever grateful.

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Clarification of Contributions

Other than the contributions stated below, all of the experiments performed in this dissertation, other than cited work, is my own work.

Chapter 2

Dr. Matthew L. Banks assisted with conducting microdialysis experiments and helped with analysis and interpretation of the data. Amy Johnson also provided technical assistance with the microdialysis experiments. Megan White assisted with brain sectioning for cannula placement confirmations. Dr. Ryan M. Drenan provided the α 6L9'S mice. Dr. J. Michael McIntosh provided the α -conotoxin MII.

Chapter 3

Shawn M. Anderson performed the basal anxiety experiments in α 6L9'S and α 6KO mice and contributed to data analysis and interpretation. Jennifer Lee helped perform the basal anxiety studies in α 4L9'A mice. Dr. Ryan M. Drenan provided the α 6L9'S mice. Dr. Andrew Tapper provided the L9'A mice.

Chapter 4

Dr. Ryan M. Drenan provided the α 6L9'S mice.

Chapter 5

Dr. Keith L. Shelton performed the blood ethanol concentration (BEC) analysis to assess the correlation of BEC with estimations of ethanol consumption (g/kg).

Chapter 6

Megan White assisted with EtOH self-administration, rotorod, and locomotor activity experiments.

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List of Abbreviations

5-HT	Serotonin
α 2KO	alpha2 null mutant mouse
α 3 β 2*nAChRs	alpha3 and beta2 containing nAChRs
α 3 β 4*nAChRs	alpha3 and beta4 containing nAChRs
α 4 β 2*nAChRs	alpha4 and beta2 containing nAChRs
α 4 α 5 β 2*nAChRs	alpha4, alpha5 and beta2 containing nAChRs
α 4 α 6 β 2*nAChRs	alpha4, alpha6 and beta2 containing nAChRs
α 4KO	alpha4 null mutant mouse
α 4L9'A	alpha4 gain-of-function mouse
α 4L9'S	alpha4 gain-of-function mouse
α 5KO	alpha5 null mutant mouse
α 5*nAChRs	alpha5 nAChRs
(non- α 4) α 6 β 2*nAChRs	non-alpha4, alpha6, and beta2 subunit containing nAChRs
α 6 β 2*nAChRs	alpha6 and beta2 subunit containing nAChRs
α 6HET	alpha6 heterozygous mouse
α 6KO	alpha6 null mutant mouse
α 6L9'S	alpha6 gain-of-function mouse

α 6L9'S- α 4KO alpha6 gain-of-function mouse with the α 4 null mutation
 α 7 nAChRs alpha7 nAChRs
 α 7KO alpha7 null mutant mouse
 α -Ctx MII alpha-conotoxin MII
 α -Ctx PIA alpha-conotoxin PIA
ACh Acetylcholine
AChE Acetylcholinesterase
aCSF Artificial cerebrospinal fluid
ADE Alcohol deprivation effect
ANOVA Analysis of variance
ATP Adenosine triphosphate
AUD Alcohol use disorder
 β 2*nAChRs beta2 subunit containing nAChRs
 β 2KO beta2 null mutant mouse
 β 3KO beta3 null mutant mouse
 β 4KO beta4 null mutant mouse
 β 4*nAChRs beta4 containing nAChRs
cAMP Cyclic adenosine monophosphate
CHRNA3 gene encoding the alpha3 nicotinic subunit
CHRNA4 gene encoding the alpha4 nicotinic subunit
CHRNA5 gene encoding the alpha5 nicotinic subunit
CHRNA6 gene encoding the alpha6 nicotinic subunit
CHRNB2 gene encoding the beta2 nicotinic subunit

CHRNB3 gene encoding the beta3 nicotinic subunit
 CHRNB4 gene encoding the beta4 nicotinic subunit
 CINs Cholinergic interneurons
 CNS Central nervous system
 CPA Conditioned place aversion
 CPD Cigarettes per day
 CPP Conditioned place preference
 DA Dopamine
 DAT Dopamine transporter
 DH β E Dihydro-beta-erythrodine
 DID Drinking-in-the-dark
 DSM-IV Diagnostic and Statistical Manual of Mental Disorders, 4th Edition
 DSM-V Diagnostic and Statistical Manual of Mental Disorders, 5th Edition
 e-cigarette Electronic cigarette
 EPM Elevated plus maze
 EtOH Ethanol
 FR Fixed ratio schedule of reinforcement
 FSCV Fast scan cyclic voltammetry
 FTND Fagerström Test for Nicotine Dependence
 FTQ Fagerström Tolerance Questionnaire
 GABA γ -amino butyric acid
 g/kg Grams per kilogram
 GWAS Genome wide association study

HPLC High performance liquid chromatography
i.c.v. Intracerebroventricular
i.p. Intraperitoneal
i.v. Intravenous
LC Locus coeruleus
LDTn Laterodorsal tegmental nuclei
LORR Loss of righting reflex
mA Milliamp
mg/kg Milligrams per kilogram
min Minute
MLA Methyllycaconitine
NAc Nucleus accumbens
nAChRs Nicotinic acetylcholine receptors
nM Nanomoles
NE Norepinephrine
NIC Nicotine
PFA Paraformaldehyde
PFC Prefrontal cortex
PPn Pedunculopontine tegmental nucleus
PPTg Pedunculopontine tegmental nucleus
QTL Quantitative trait loci
SAC Saccharin
SAL Saline

SEM Standard error of the mean
SNP Single nucleotide polymorphism
VEH aCSF or saline vehicle
VCU Virginia Commonwealth University
VR Variable ratio
VTA Ventral tegmental area
WT Wild type

Abstract

ALPHA6 BETA2 SUBUNIT CONTAINING NICOTINIC ACETYLCHOLINE RECEPTOR CONTRIBUTIONS TO ABUSE-RELATED EFFECTS OF NICOTINE AND ALCOHOL

By Alexandra McIver Stafford, B.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2017.

Major Director, Darlene H. Brunzell, Ph.D., Associate Professor, Pharmacology and Toxicology

Pharmacotherapies for tobacco and alcohol cessation are only modestly successful, so it is important to better understand mechanisms underlying their use and abuse. The overarching goal of this research is to assess $\alpha 6\beta 2$ subunit containing nicotinic acetylcholine receptor ($\alpha 6\beta 2$ *nAChR; *denotes possible assembly with other subunits) contributions to abuse-related effects of nicotine and alcohol. In the absence of $\alpha 6\beta 2$ *nAChR-selective agonists, $\alpha 6\beta 2$ *nAChR gain-of-function ($\alpha 6L9'S$) mice provide a tool for selective activation of $\alpha 6\beta 2$ *nAChRs. Using the $\alpha 6L9'S$ mice together with nicotine doses sub-threshold for stimulation of native nAChRs, these studies tested the hypothesis that activation of $\alpha 6\beta 2$ *nAChRs is sufficient to promote neurochemical and behavioral effects relevant to nicotine addiction. Intracranial infusions of an $\alpha 6\beta 2$ *nAChR-selective antagonist further tested the neuroanatomical locus of $\alpha 6\beta 2$ *nAChR contributions to mesolimbic dopamine (DA) release and nicotine reward behavior. Our *in vivo*

microdialysis and nicotine conditioned place preference (CPP) studies reveal that stimulation of $\alpha 6\beta 2^*$ nAChRs on ventral tegmental area (VTA) DA neurons, as well as on DA terminals in the nucleus accumbens (NAc) shell support nicotine reward. VTA $\alpha 6\beta 2^*$ nAChR stimulation is required for elevated basal NAc DA levels in $\alpha 6L9^*S$ mice, who also show elevated nicotine CPP. These studies also showed elevated anxiety-like behavior in $\alpha 6L9^*S$ mice, but no change in $\alpha 6$ subunit null mutant ($\alpha 6KO$) mice to suggest that elevated cholinergic tone at $\alpha 6\beta 2^*$ nAChRs promotes anxiety-like behavior. To better define the molecular make-up of $\alpha 6\beta 2^*$ nAChRs supporting nicotine reward and anxiety-like behavior, these studies crossed $\alpha 6L9^*S$ to $\alpha 4$ subunit knockout mice to differentiate (non- $\alpha 4$) $\alpha 6\beta 2^*$ and $\alpha 4\alpha 6\beta 2^*$ nAChR contributions. (non- $\alpha 4$) $\alpha 6\beta 2^*$ nAChRs appear to promote nicotine reward behavior, while the $\alpha 6\beta 2^*$ nAChR subtype that regulates anxiety-like behavior depends on the anxiety assay. Finally, these studies developed a mouse model of oral operant ethanol (EtOH) self-administration and assessed EtOH reinforcement in $\alpha 6$ heterozygous ($\alpha 6HET$) and $\alpha 6KO$ mice to characterize the role of $\alpha 6\beta 2^*$ nAChRs in EtOH reinforcement. EtOH self-administration was similar to wild type mice in $\alpha 6KO$ mice, but not $\alpha 6HET$ mice, suggesting that expression of $\alpha 6\beta 2^*$ nAChRs modulates EtOH reinforcement. Together, these preclinical studies implicate $\alpha 6\beta 2^*$ nAChRs in various abuse-related effects of nicotine and alcohol, identifying this receptor as a potential therapeutic target for treatment of dependence.

Chapter 1 – Introduction

The prevalence of tobacco dependence is higher than for any other abused substance (U.S. Department of Health and Human Services, 2010), with tobacco use being the leading preventable cause of death worldwide (WHO, 2015). Smokers report multiple factors that contribute to their tobacco use, including pleasure and anxiety relief received from smoking. Nicotine, a primary addictive component in tobacco, exerts its behavioral and physiological effects via nicotinic acetylcholine receptors (nAChRs) that are normally activated by the endogenous neurotransmitter acetylcholine (ACh). Like nicotine, basal cholinergic signaling itself is known to modulate behaviors relevant to addiction (Avena and Rada, 2012; Hoebel *et al*, 2007; Lanca *et al*, 2000; Rada *et al*, 2001; Xiao *et al*, 2016). Alcohol abuse is also a significant health concern, causing 5.9% of all deaths worldwide (WHO, 2014). Nicotine is commonly co-abused with alcohol, with as many as 96% of alcoholics being smokers; thus, it is likely that nAChRs also mediate phenotypes relevant to alcohol use and dependence. There are a multitude of nAChR subtypes based on subunit composition that display different expression patterns in the brain where they regulate the activity of neurons to ultimately affect behavior. The development of nAChR subtype-selective ligands and various nAChR subunit knockout and transgenic mice have greatly enhanced our understanding of how nAChRs contribute to complex behaviors, including those relevant to addiction. Using these pharmacological and genetic techniques, the preclinical studies described in this dissertation aimed to characterize $\alpha 6\beta 2$

subunit containing nAChR ($\alpha 6\beta 2^*$ nAChR, * indicates possible assembly with other subunits) contributions to abuse-related behavioral and neurochemical effects of nicotine and alcohol, two of the most common legal abused drugs in the United States. Compared to other $\beta 2^*$ nAChRs, $\alpha 6\beta 2^*$ nAChRs are more selectively expressed in catecholaminergic nuclei in the brain (Champtiaux *et al*, 2002; Klink *et al*, 2001; Le Novere *et al*, 1996). Important to these studies, $\alpha 6\beta 2^*$ nAChRs are enriched in the mesolimbic dopamine (DA) pathway on dopamine (DA) neuron cell bodies in the ventral tegmental area (VTA) and on DA projection terminals in the nucleus accumbens (NAc). Given the restricted expression pattern of $\alpha 6\beta 2^*$ nAChRs, especially in brain regions associated with the effects of most abused drugs, we believe that this nAChR subtype is a promising potential therapeutic target for nicotine and alcohol addiction.

Tobacco use and nicotine dependence

Tobacco dependence is a substantial health problem worldwide. Nearly one billion people across the world use tobacco (Ng *et al*, 2014). There are many different types of tobacco products, all which contain nicotine. Tobacco can be consumed using smokeless tobacco products (e.g. gum, snus, chewing or dipping tobacco, lozenges), combustible tobacco products (e.g. cigarettes, cigars), water pipes, and more recently, electronic cigarettes (e-cigarettes). Cigarettes are the most commonly used tobacco product, representing over 90% of tobacco use (Giovino, 2007). Approximately one-third of people who try smoking go on to become daily smokers (USDHHS, 1994) and only around 10% of smokers are successful at quitting (CDC, 2002). Thus, it is important to understand the mechanisms that underlie nicotine addiction in order to develop novel pharmacotherapies for smoking cessation.

Tobacco use disorder, as described by the *Diagnostic and Statistical Manual of Mental Disorders*, Fifth Edition (DSM-V), is a problematic pattern of tobacco use that leads to clinically

significant impairment or distress as manifested by at least two of the criteria listed in the DSM-V occurring within a 12 month period (American Psychiatric Association, 2013). In addition to the DSM-V criteria, the degree of tobacco dependence can be characterized using the Fagerström Tolerance Questionnaire (FTQ) (Fagerstrom, 1978; Fagerstrom and Schneider, 1989), along with the modified version called the Fagerström Test for Nicotine Dependence (FTND) (Heatherton *et al*, 1991). There are also other measures of tobacco dependence that address limitations of the DSM-V and FTQ/FTND and consider dependence to be comprised of multiple phenotypes. These measures include the Heaviness of Smoking Index (Heatherton *et al*, 1989), Hooked on Nicotine Checklist (DiFranza *et al*, 2002), Cigarette Dependence Scale (Etter, 2005; Etter *et al*, 2003), Wisconsin Inventory of Smoking Dependence Motives (Piper *et al*, 2006), and Nicotine Dependence Syndrome Scale (Shiffman and Sayette, 2005; Shiffman *et al*, 2004). For detailed descriptions of these measures, see U.S. Department of Health and Human Services, 2010.

Tobacco products contain more than 4,000 chemicals, some of which may contribute to tobacco dependence. Many studies have implicated nicotine as a potent addictive component in tobacco. Intravenous nicotine and smoking produce similar subjective and physiological effects, and nicotine has repeatedly been shown to serve as a positive reinforcer, as animals and humans will self-administer nicotine. Smokers self-administering nicotine report rewarding sensations, such as euphoria, comfort, “liking”, reduced negative mood, and reduced pain. These positive effects are also accompanied with negative ones, such as tension and jitteriness (Cohen and George, 2013; Harvey *et al*, 2004; Henningfield and Goldberg, 1983; Perkins *et al*, 1994; Rose *et al*, 2010b; Sofuoglu *et al*, 2008). Chronic nicotine consumption can eventually lead to physiological dependence, in which nicotine tolerance develops and cessation produces withdrawal symptoms (Benowitz, 1988).

Nicotine is an alkaloid found in tobacco plants, which are part of the nightshade family (*Solanaceae*). Nicotine gets its name from the tobacco plant *Nicotiana tabacum*, which is named after the French ambassador Jean Nicot de Villemain, who sent tobacco to Paris in 1560. At the time, smoking tobacco was believed to protect against various ailments and diseases, such as pain, cancer, and respiratory problems (Kell *et al*, 1965). The North American Indians were the first to introduce tobacco smoke enemas, which were used for artificial respiration and treatment of gastrointestinal ailments (Jones, 1827; Nordenskiold, 1929; Hurt *et al*, 1996). In 1828, Wilhelm Heinrich Posselt and Karl Ludwig Reimann first isolated nicotine from the leaves of tobacco plants (Posselt and Reimann, 1828; Henningfield and Zeller, 2006). Melsens then described the chemical formula of nicotine in 1843 (Melsens, 1843), and Adolf Pinner and Richard Wolffenstein discovered its structure in 1893 (Pinner and Wolffenstein, 1891; Pinner, 1893a,b). In 1904, Amé Pictet and A. Rotschy were the first to synthesize nicotine (Pictet and Rotschy, 1904). By the late 17th century, nicotine was not only used in tobacco products for smoking, it was also used as an insecticide, acting as an antiherbivore chemical. The use of nicotine as an insecticide declined in the 1980s when insecticides that were cheaper and reportedly less harmful to humans became available (Ujváry, 1999). As per the EPA, nicotine is no longer available as a pesticide in the US (USEPA, 2009). However, most insecticides are still indirect agonists of nicotinic and muscarinic acetylcholine receptors, acting as inhibitors of acetylcholinesterase, which breaks down ACh. Similar to nicotine, neonicotinoids are nAChR agonists that are also currently used as insecticides (Tomizawa and Casida, 2005). Over the past few decades, neonicotinoids have come under scrutiny as they have been linked to adverse environmental effects such as honey-bee colony collapse (e.g. Gill *et al*, 2012). As a result, some countries have restricted their use (Cressey, 2013).

A cigarette contains approximately 10-14 mg of nicotine and smokers intake between 1-1.5 mg of nicotine per cigarette (Benowitz, 1988; Benowitz and Jacob, 1984; Jarvis *et al*, 2001; Kozlowski *et al*, 1998). The total amount of nicotine intake ranges from about 0.1-1 mg/kg/day in smokers (Benowitz *et al*, 1984). When tobacco is smoked, nicotine enters the blood stream in tens of seconds, primarily through the lungs (Benowitz *et al*, 2009). Of all methods of nicotine delivery, smoking produces the highest peak blood concentration of nicotine and the most rapid rate of nicotine absorption, reaching the brain within 20 seconds and peak blood concentration within 5 minutes (Benowitz *et al*, 2009). This rapid increase of nicotine levels after smoking contributes to a more intense effect of nicotine compared to other routes of administration and allows the smoker to titrate their dose of nicotine to achieve the desired effect, making smoking the most reinforcing form of tobacco use and driving the development of dependence (Benowitz *et al*, 2009; Henningfield and Keenan, 1993)

While cigarettes are the most commonly used tobacco product (Giovino, 2007), waterpipe and e-cigarette use has become increasingly prevalent (Pepper and Eissenberg, 2014). Depending on variables such as puff topography and certain device characteristics, e-cigarettes can deliver nicotine in quantities much less or much greater compared to cigarettes (Shihadeh and Eissenberg, 2015). E-cigarettes may have less abuse liability compared to cigarettes, as e-cigarettes were found to be less reinforcing compared to cigarettes using a multiple choice procedure (Vansickel *et al*, 2012) and appeared less addictive than cigarettes in multiple tests of dependence (Etter and Eissenberg, 2015). Cigarettes and waterpipes deliver similar amounts of nicotine, as plasma nicotine concentration did not differ when comparing a single waterpipe use episode to a single cigarette smoked. In addition, subjective effects for waterpipe and cigarette

smoking were similar in magnitude, but lasted longer for waterpipe, suggesting that waterpipe smoking can produce dependence (Cobb *et al*, 2011).

In the brain, nicotine binds to nAChRs to exert a wide variety of behavioral and physiological effects that can ultimately lead to nicotine dependence. An abundance of data exists demonstrating that activity at diverse nAChR subtypes is critical for nicotine's addictive properties, but there is still much to be explored regarding the precise molecular make up of nAChR subtypes that support tobacco use and the mechanisms both upstream and downstream of nAChR regulation that contribute to the abuse-related effects of nicotine. In addition, the current smoking cessation therapies available (e.g. nicotine replacement therapy, bupropion, varenicline) are only modestly effective with a success rate of 20-25% (Gonzales *et al*, 2006), warranting further research in order to identify novel therapeutic targets for smoking cessation.

Alcohol use and dependence and its co-abuse with nicotine

As with nicotine, alcohol abuse is a significant global health concern, ranking among the top five risk factors for disease, disability, and death. In the US, the 12-month prevalence of alcohol use disorder (AUD) is 13.9% and the lifetime prevalence is 29.1% (Grant *et al*, 2015). In addition to its vast health consequences, alcohol abuse also poses a great economic burden; in the US alone, it costs \$366 billion per year (Chatterjee and Bartlett, 2010). According to the DSM-V, AUD is defined as a problematic pattern of alcohol use leading to clinically significant impairment or distress, as manifested by at least two of the criteria listed by the DSM-V occurring within a 12-month period (American Psychiatric Association, 2013).

There is a high prevalence of comorbid alcohol and nicotine abuse. In fact, nicotine and alcohol are the most common co-abused drugs (Sussman *et al*, 2011). As many as 96% of alcoholics also smoke tobacco (Barrantes *et al*, 1995; Marks *et al*, 1997; Miller and Gold, 1998).

Having an AUD decreases successful smoking cessation attempts and increases smoking relapse rates (Hymowitz *et al*, 1997; Kahler *et al*, 2010; Toll *et al*, 2012; Weinberger *et al*, 2013).

Tobacco smokers are more likely to binge drink, consume more alcohol, and are more likely to meet DSM-V criteria for an AUD compared to non-smokers (Britt and Bonci, 2013; Carmody *et al*, 1985; DiFranza and Guerrera, 1990; McKee and Weinberger, 2013). Smoking is also associated with increased alcohol dependence, increased alcohol withdrawal syndrome, increased binge drinking, and decreased rates of alcohol cessation (Chiappetta *et al*, 2014; McKee *et al*, 2013). Nicotine even increases drinking in non-dependent humans (Barrett *et al*, 2006; Harrison and McKee, 2008; Kouri *et al*, 2004) and rodents (Alen *et al*, 2009; Hauser *et al*, 2012; Le *et al*, 2010; Le *et al*, 2003; Olausson *et al*, 2001). Studies have also shown that nicotine enhances alcohol reinforcement in humans (McKee *et al*, 2013) and in rodent models of self-administration (Doyon *et al*, 2013a). Overall, levels of alcohol use are higher in smokers and smoking is more common in individuals with an AUD (Craig and Van Natta, 1977; Dawson, 2000; Schorling *et al*, 1994).

Alcohol is known to interact directly or indirectly with different types of molecules, such as enzymes and ion channels, to exert its behavioral and physiological effects. Alcohol can bind to alcohol dehydrogenase (Ramaswamy *et al*, 1994; Rosell *et al*, 2003; Svensson *et al*, 2000), and it can also enhance adenylyl cyclase production of adenosine 3',5'-monophosphate (cAMP) from adenosine triphosphate (ATP) (Yoshimura *et al*, 2006; Yoshimura and Tabakoff, 1995).

Alcohol is thought to modulate opioidergic transmission at multiple levels, including biosynthesis, release, and degradation of opioid peptides, as well as binding of endogenous opioid ligands (Mendez and Morales-Mulia, 2008). Alcohol interacts with potassium channels, including G protein-activated inwardly rectifying channels, large-conductance calcium-activated

channels, and Shaw2 voltage-gated channels (Covarrubias and Rubin, 1993; Dopico *et al*, 1998; Kobayashi *et al*, 1999; Lewohl *et al*, 1999). Several classes of ligand-gated ion channels are also affected by alcohol. Alcohol can inhibit NMDA receptors and enhance GABA_A and glycine receptor function (Dildy-Mayfield *et al*, 1996; Mascia *et al*, 1996). In addition, n-alcohols can inhibit GABA_C receptors (Mihic and Harris, 1996), and short-chain alcohols enhance nAChR function, while long-chain alcohols block nAChRs (Borghese *et al*, 2003; Godden *et al*, 2001).

Given the high rate of nicotine and alcohol co-abuse, it is likely that nAChRs, the primary molecular targets of nicotine, may contribute to the abusive properties of alcohol as well; an accumulation of evidence (to be discussed in detail below) suggests that this is the case. Relatively speaking, however, the contribution of nAChRs to the abuse-related effects of alcohol is not well understood. Thus, research into the role of nAChRs in alcohol's addictive properties is greatly warranted. The success rate of medications available for the treatment of AUDs (disulfiram, naltrexone, and acamprosate) is around 30% at the highest (Chatterjee *et al*, 2010; Spanagel, 2009), so identifying novel therapeutic targets for the treatment of AUDs is important; nAChRs are a promising candidate for the development of these treatments. In fact, varenicline, which is a FDA-approved smoking cessation aid with high therapeutic efficacy targeting $\alpha 7$ - and $\beta 2$ *nAChRs (Gonzales *et al*, 2006; Jorenby *et al*, 2006), is effective in reducing some abuse-related effects of EtOH. Varenicline reduces EtOH intake and self-administration in rodents (Bito-Onon *et al*, 2011; Feduccia *et al*, 2014; Hendrickson *et al*, 2010; Kamens *et al*, 2010b; Santos *et al*, 2013; Steensland *et al*, 2007) and it decreases EtOH self-administration and craving after a priming dose of EtOH in humans (McKee *et al*, 2009). Varenicline alters EtOH's reinforcing and rewarding in the absence of nicotine, suggesting that endogenous cholinergic activity regulates these effects.

Nicotinic acetylcholine receptors

nAChRs, which are endogenously targeted by ACh, are the primary target of nicotine and other exogenous nicotinic agonists and antagonists. These receptors belong to the superfamily of cysteine-loop ligand-gated ion channels that also includes γ -amino butyric acid (GABA), 5-hydroxytryptamine (5-HT), and glycine receptors. nAChRs were first purified from the electric organ of the *Torpedo* fish where they were easily discovered, as they make up 40% of the protein content. The discovery of α -bungarotoxin, a component of krait snake venom, which binds to nAChRs to promote paralysis at the neuromuscular junction, aided in the purification of nAChRs. α -bungarotoxin was used on affinity columns to isolate nAChRs from the electric organs (Albuquerque *et al*, 2009).

nAChRs can be separated into two categories: muscle and neuronal nAChRs. Muscle nAChRs are composed of α 1, β 1, δ , γ , and ϵ subunits that assemble in a pentameric arrangement around a central pore. There are two main types of muscle nAChRs, including the embryonic $(\alpha$ 1)₂ β 1 $\delta\gamma$ nAChR and the adult $(\alpha$ 1)₂ β 1 $\delta\epsilon$ nAChR, which are located at the neuromuscular junction (Mishina *et al*, 1986). Neuronal nAChRs, which are the focus of this dissertation, are also pentamers but are made up of either five identical α subunits to make homopentameric nAChRs or five α and β subunits to make heteropentameric combinations arranged around a central pore. To date, nine α subunits (α 2-10) and three β subunits (β 2-4) have been identified to yield a wide variety of receptor subtypes expressed in the peripheral and central nervous system (CNS). All nAChR subunits are expressed in mammals, with the exception of α 8, which has only been found in avian tissue. Homomeric nAChRs are composed of α 7- α 9 subunits. Heteromeric nAChRs are composed of a combination of α and β subunits, including α 2-6 and β 2-4. More recently, researchers have discovered that α 9 and α 10 also assemble together to form

a heteromeric nAChR (Elgoyhen *et al*, 2001; Lustig *et al*, 2001; Sgard *et al*, 2002). Ligands bind to homomeric nAChRs at the interface between α subunits, while they bind to heteromeric nAChRs at the interface between α and β subunits. $\alpha 5$ and $\beta 3$ are accessory subunits, meaning they do not participate in ligand binding (Dani and Bertrand, 2007; Gotti *et al*, 2009). Instead, these two accessory subunits contribute to the receptor's channel permeability, binding affinity, desensitization, sensitivity to allosteric modulators, and sensitivity to up-regulation (Kuryatov *et al*, 2008; Moroni *et al*, 2008; Moroni *et al*, 2006; Tapia *et al*, 2007). Moreover, it has been shown that the presence of $\beta 3$ is important for the formation of $\alpha 6\beta 2$ *nAChRs and the loss of $\beta 3$ alters the assembly, degradation, and trafficking of the receptor (Gotti *et al*, 2006).

nAChR subunits have a hydrophilic extracellular NH₂-terminal domain that acts as the ligand binding site. This extracellular domain is followed by three hydrophobic transmembrane domains (M1-M3), an intracellular loop, and finally a fourth hydrophobic transmembrane domain (M4) with an extracellular COOH-terminal sequence. The M2 domain lines the pore, the M1 and M3 domains surrounds the M2 domain, separating it from the lipid bilayer of the cell membrane, and the M4 domain is the most exposed to the lipid bilayer. The M2 domain is important for establishing the ion gate, receptor selectivity, and channel conductivity. When the receptor is unbound, hydrophobic residues in helices of the M2 domains of all five subunits form a barrier, blocking the passage of ions through the pore. When a ligand binds to the receptor, the M2 domains rotate to open the channel; this torque is transferred from the ligand binding site in the extracellular NH₂-terminal domain through interactions between residues in this extracellular domain, including the Cys-loop and linker region between M2 and M3. More specifically, residues in the Cys-loop interact with residues in the linker region, acting as a pivot around which the M2 domain rotates. Mutations in the M2 domain that substitute hydrophobic residues

with hydrophilic residues increases channel permeability. In addition, the M4 domain undergoes the greatest structural change during channel opening due to its proximity to the lipid bilayer, where it has fewer contacts with other proteins. This movement may be functionally relevant, as a conserved cysteine residue seems to be involved in receptor aggregation and interaction with cholesterol and other lipid molecules (Albuquerque *et al*, 2009; Gotti *et al*, 2009). For an illustration of the subunit and receptor, see Fig. 1.1.

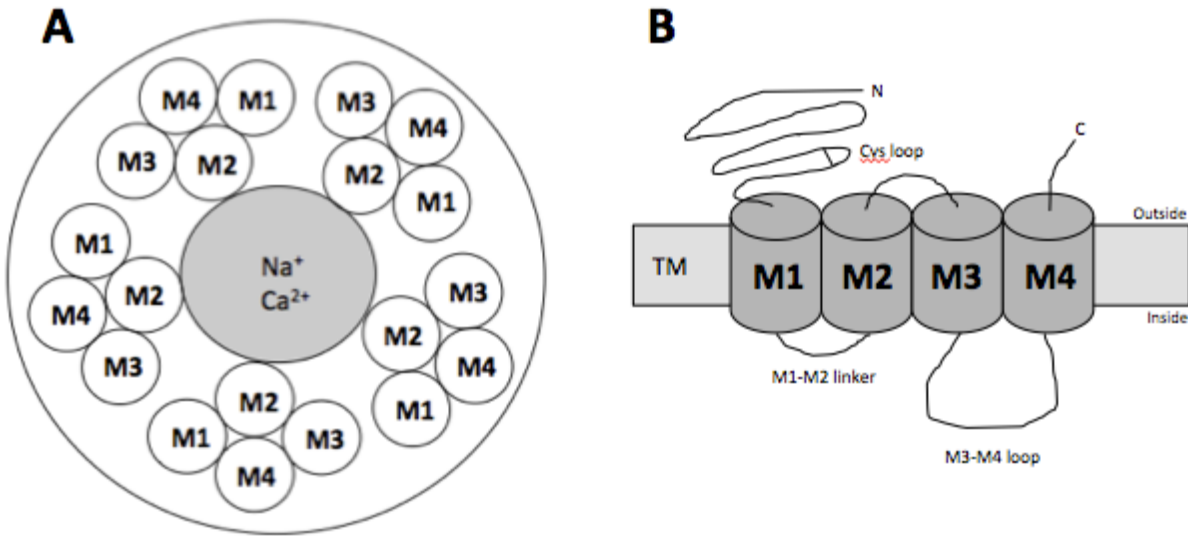


Figure 1.1 – Structure of nicotinic acetylcholine receptors (nAChRs). a) Illustration of nAChR subunits arranged around a cation-conducting pore. Each subunit is made up of four transmembrane domains (M1-M4). b) Representation of a single nAChR subunit, which is made up of a NH_2 -terminal extracellular domain containing a cys-loop, four transmembrane domains (M1-M4), a linker between M1 and M2, a cytoplasmic domain (M3-M4 loop), and an extracellular COOH-terminal domain. The ligand binding site is located in the NH_2 -terminal extracellular domain.

nAChRs can exist in three conformational states: resting, open, or desensitized. In the absence of agonist, the receptor is in the resting state with the channel closed, blocking the passage of cations. When an agonist binds to the receptor, it undergoes a conformational change and transitions to the open state, allowing sodium and calcium to flow through the channel down their electrochemical gradient into the cell. This can lead to depolarization of neurons, facilitation of neurotransmitter release, or activation of downstream signaling cascades. Prolonged exposure to agonist causes the receptor to transition into the desensitized state, where agonist is bound, but the channel is closed and cations cannot pass through the pore (Gotti *et al*, 2009). Desensitization of nAChRs by ACh is thought to be prohibited by acetylcholinesterase, the degradative enzyme of ACh (Brown *et al*, 1936; Katz and Thesleff, 1957; Thesleff, 1955). Nicotine can either mimic or block ACh signaling at nAChRs to exert its effects, which are likely stronger compared to ACh, as there is no enzymatic breakdown of nicotine. That is, nicotine affects certain behaviors and physiological processes through activation of nAChRs, while it affects others by inhibition via desensitization of the receptor (Picciotto *et al*, 2008). Micromolar concentrations of nicotine activate and subsequently desensitize most nAChRs, while nanomolar concentrations preferentially desensitize most nAChRs without first activating them (Fenster *et al*, 1997; Grady *et al*, 2012; Kuryatov and Lindstrom, 2011; Lester and Dani, 1995; Lu *et al*, 1999; Mansvelder *et al*, 2002; Pidoplichko *et al*, 1997). Studies have shown that brain concentrations of nicotine peak at the micromolar level (1-3 μ M), but are likely maintained on a nanomolar scale (200-450 nM) throughout the day (Rose *et al*, 2010a). Concentrations of nicotine achieved in smokers initially activate nAChRs on midbrain DA neurons to increase neuronal activity. Upon prolonged exposure to these low levels of nicotine, which mimics the steady low levels of nicotine maintained throughout the day in smokers, nAChRs are

desensitized, resulting in a reduction of DA neuron activity (Pidoplichko *et al*, 1997). This phenomenon could explain why the first cigarette of the day is the most pleasureable (Dani and Heinemann, 1996), and may further explain some aspects of tolerance to nicotine's rewarding effects upon repeated nicotine exposure.

The effect of nicotine on the brain and resulting behavioral outputs is based on activity at a wide variety of nAChR subtypes that have differential expression patterns, as well as unique pharmacological (e.g. varying affinities and potencies of ligands) and functional properties. nAChRs are distributed widely throughout the brain, including areas associated with addiction (Dani *et al*, 2007; Dani and Harris, 2005; Dani *et al*, 1996; De Biasi and Dani, 2011; Leslie *et al*, 2013). nAChRs act as neuromodulators, being expressed on many different neurons that release a variety of neurotransmitters. nAChRs are located all along the neuron on preterminal, presynaptic, postsynaptic, axonal, dendritic, and soma regions. Preterminal and presynaptic nAChRs modulate neurotransmitter release, while postsynaptic and nonsynaptic nAChRs are involved in neuronal excitation and participate in the modulation of circuits and enzymatic processes. Through these neuronal mechanisms, nAChRs have a wide variety of functions in the CNS, being involved in learning, memory, attention, development, etc. (Albuquerque *et al*, 2009; Dani *et al*, 2007). These functions are largely based on subtype and vary depending on brain region.

Nicotine has varying potencies and affinities at the different nAChR subtypes. Nicotine is the most potent nicotinic agonist and has the highest affinity at $\beta 2^*$ nAChRs compared to other nAChR subtypes (Grady *et al*, 2010; Pauly *et al*, 1991; Whiting and Lindstrom, 1988; Xiao and Kellar, 2004). $\beta 2^*$ nAChRs are the most common nAChR subtype in the brain and are critical for many of nicotine's addictive properties, including reward, reinforcement, and anxiety relief (for

review, see Brunzell *et al*, 2015). The $\beta 2$ subunit primarily assembles with $\alpha 4$ and/or $\alpha 6$ to make up three subclasses of $\beta 2$ *nAChRs: $\alpha 4\beta 2$ *nAChRs, $\alpha 4\alpha 6\beta 2$ *nAChRs, and (non- $\alpha 4$) $\alpha 6\beta 2$ *nAChRs. The $\alpha 4\alpha 6\beta 2$ *nAChRs have the highest sensitivity to nicotine (Exley *et al*, 2008; Kuryatov *et al*, 2011; Liu *et al*, 2012; Salminen *et al*, 2007; Salminen *et al*, 2004), and are persistently activated in the VTA at concentrations of nicotine (300 nM) similar to those achieved by smokers that typically desensitize other nAChRs (Liu *et al*, 2012). α -conotoxin MII (α -Ctx MII)-insensitive $\alpha 4\beta 2$ *nAChRs are widely expressed in the brain, while α -Ctx MII-sensitive $\alpha 6\beta 2$ *nAChRs are more selectively expressed in catecholaminergic nuclei (Champtiaux *et al*, 2002; Klink *et al*, 2001; Le Novere *et al*, 1996). This dissertation aims to investigate the subunit make-up of $\beta 2$ *nAChRs, especially those expressed in the mesolimbic DA pathway, that modulate abuse-related effects of nicotine and alcohol.

*Studying the function of $\beta 2$ *nAChRs: genetic and pharmacological tools*

The availability of various nAChR subunit knockout and transgenic mice and nAChR subtype-selective ligands are widely utilized for the study of the function of nAChR subtypes. Studies in nAChR subunit knockout and transgenic mice, where the nAChR subunit of interest is silenced or mutated, are useful to elucidate the role of specific nAChR subtypes. The $\alpha 3$ -7, $\alpha 9$, $\beta 2$ -4 subunits have all been knocked out in mice, and all but the $\alpha 3$ KO mice are viable and grossly normal (Gotti and Clementi, 2004). There are drawbacks to using such mice; in the presence of a loss- or gain-of-function mutation, potential developmental adaptations may occur that can lead to functional compensation by other nAChR subtypes, which can confound interpretation of results. nAChR subtype-selective agonists and antagonists are also used to study the function of nAChRs. Use of these selective ligands complement studies in nAChR subunit knockout and

transgenic mice and can provide insight into whether behavioral or physiological changes observed in genetically engineered mice are a result of developmental adaptation/functional compensation. However, while there are ligands selective for various nAChR subtypes, they are not necessarily specific, as many have affinity at other nAChRs.

Studies in $\beta 2$ KO mice (Picciotto *et al*, 1995) provide valuable insight into the function of $\beta 2$ *nAChRs but, these data should be carefully interpreted due to the limitations of using knockout mice discussed above. While $\beta 2$ KO mice are grossly normal, development of the visual system is altered (Bansal *et al*, 2000; Rossi *et al*, 2001). Other studies commonly use DH β E, a $\beta 2$ -selective antagonist, and A-85380, a $\beta 2$ -selective agonist, as pharmacological tools to elucidate the function of $\beta 2$ *nAChRs. As mentioned above, nAChR subtype-selective ligands circumvent potential compensatory mechanisms that may occur in knockout or transgenic mice, but it should be noted that while DH β E and A-85380 are selective for $\beta 2$ *nAChRs, they are not necessarily specific, as they may have affinity for other nAChR subtypes. While these tools have provided great insight into $\beta 2$ *nAChR function, they are incapable of differentiating the various $\beta 2$ *nAChR subtypes.

The studies in this dissertation aim to investigate $\alpha 6\beta 2$ *nAChR contributions to abuse-related effects of nicotine and alcohol using genetic and pharmacological tools, including the $\alpha 6\beta 2$ *nAChR-selective antagonist, α -Ctx MII, as well as $\alpha 6$ KO and $\alpha 6\beta 2$ *nAChR gain-of-function ($\alpha 6L9$ 'S) mice. $\alpha 6$ KO mice (Champtiaux *et al*, 2002) are grossly normal, exhibiting no neurological or behavioral deficits. Specifically, there are no developmental alterations in $\alpha 6$ KO mouse visual or dopaminergic systems, where $\alpha 6\beta 2$ *nAChRs are highly expressed. However, $\alpha 6$ KO mice exhibit an increase in $\alpha 4\beta 2$ *nAChRs as shown by epibatidine binding studies (Champtiaux *et al*, 2003), indicating potential functional compensation that may confound

interpretations of results generated using these $\alpha 6\text{KO}$ mice. The $\alpha 6\beta 2^*\text{nAChR}$ -selective antagonist, $\alpha\text{-Ctx MII}$ (Cartier *et al*, 1996), is also available to study the effects of $\alpha 6\beta 2^*\text{nAChR}$ loss-of-function and complement knockout studies. It should be noted that $\alpha\text{-Ctx MII}$ also binds to $\alpha 3\beta 2^*\text{nAChRs}$, which are expressed in some brain areas where $\alpha 6\beta 2^*\text{nAChRs}$ are located. The binding affinity of $\alpha\text{-Ctx MII}$ for $\alpha 3\beta 2^*\text{nAChRs}$ ($K_i=50$ nM) is approximately 50-fold lower compared to $\alpha 6\beta 2^*\text{nAChRs}$ ($K_i=1.1$ nM), and $\alpha\text{-Ctx MII}$ is slightly more potent at $\alpha 6\beta 2^*\text{nAChRs}$ ($\text{IC}_{50}=0.39$ nM) compared to $\alpha 3\beta 2^*\text{nAChRs}$ ($\text{IC}_{50}=0.5\text{-}2.2$ nM) (Gotti *et al*, 2006). There are other conotoxin derivatives, such as $\alpha\text{-Ctx PIA}$ (Dowell *et al*, 2003), that are selective for $\alpha 6\beta 2^*$ versus $\alpha 3\beta 2^*\text{nAChRs}$ ($\text{IC}_{50}=0.69$ nM and 74.2 nM, respectively) (Gotti *et al*, 2006). More recently, another $\alpha 6\beta 2^*\text{nAChR}$ -selective antagonist, r-bPiDI, was developed (Beckmann *et al*, 2015). Unlike $\alpha\text{-Ctx MII}$, which must be administered intracranially as it does not cross the blood brain barrier, r-bPiDI crosses the blood brain barrier, allowing for systemic administration. Studies in this dissertation utilized $\alpha 6\text{KO}$ mice and intracranial infusions of $\alpha\text{-Ctx MII}$ in combination with behavioral and neurochemical techniques to elucidate functions of $\alpha 6\beta 2^*\text{nAChRs}$.

$\alpha 6\text{KO}$ mice and $\alpha 6\beta 2^*\text{nAChR}$ -selective antagonists used to reduce $\alpha 6\beta 2^*\text{nAChR}$ function have been valuable in investigating the function of $\alpha 6\beta 2^*\text{nAChRs}$, but there are limited tools available to activate this nAChR subtype. To date, development of $\alpha 6\beta 2^*\text{nAChR}$ -selective agonists has been unsuccessful due to poor function of $\alpha 6\beta 2^*\text{nAChRs}$ in heterologous expression systems (Drenan *et al*, 2008). As result, Drenan *et al* developed an $\alpha 6\beta 2^*\text{nAChR}$ gain-of-function mouse strain (Drenan *et al*, 2008), which can be used as an alternative strategy to selectively activate $\alpha 6\beta 2^*\text{nAChRs}$ in the absence of a selective agonist. These BAC transgenic

mice ($\alpha 6L9'S$) possess a single leucine to serine point mutation at the 9' position of the M2 pore-forming region of the $\alpha 6$ subunit. This mutation renders the $\alpha 6\beta 2^*nAChRs$ hypersensitive to ACh and nicotine, so that sub-threshold concentrations of these agonists are able to selectively activate $\alpha 6\beta 2^*nAChRs$ in the absence of activation of other nAChRs that don't respond to these low concentrations of ACh and nicotine. For example, sub-threshold concentrations of ACh and nicotine are able to increase striatal DA in $\alpha 6L9'S$ synaptosome preparations and activate VTA DA neurons in $\alpha 6L9'S$ midbrain slices compared to WT littermates (Cohen *et al*, 2012; Drenan *et al*, 2010; Drenan *et al*, 2008; Engle *et al*, 2013; Powers *et al*, 2013; Wang *et al*, 2014b); these *in vitro* effects are blocked by α -Ctx MII, confirming that this hypersensitivity is mediated by $\alpha 6\beta 2^*nAChRs$. Behaviorally, these gain-of-function $\alpha 6L9'S$ mice exhibit locomotor hyperactivity at baseline and in response to doses of nicotine that have no effect in WT mice (Berry *et al*, 2015; Cohen *et al*, 2012; Drenan *et al*, 2010; Drenan *et al*, 2008). $\alpha 6L9'S$ mice show normal expression of $\alpha 6\beta 2^*nAChRs$, with normal localization and intensity of [^{125}I]- α -Ctx MII. To confirm this, [^{125}I]-epibatidine binding coupled with inhibition by unlabeled α -Ctx MII revealed normal levels and localization of $\alpha 6\beta 2^*$ and $\alpha 4\beta 2^*nAChRs$ (Drenan *et al*, 2008). Further, $\alpha 6L9'S$ DAT levels are similar to or higher than in WT mice and DA turnover is unchanged (Drenan *et al*, 2010; Wang *et al*, 2014). Complementary to genetic and pharmacological tools that inactivate $\alpha 6\beta 2^*nAChRs$, the studies described in this dissertation also used $\alpha 6L9'S$ gain-of-function mice as a tool to selectively activate $\alpha 6\beta 2^*nAChRs$ *in vivo* using doses of nicotine sub-threshold to effect WT mice, allowing function isolation of this nAChR subtype.

Nicotinic contributions to tobacco and alcohol dependence in humans

Human studies suggest that $\beta 2^*nAChRs$ contribute to tobacco use and dependence. PET and SPECT imaging studies show that nicotine binds to about 88% of the $\beta 2^*nAChRs$ in brains of smokers after a single cigarette (Brody *et al*, 2006) and that smoking to satiety (2.4 cigarettes on average) results in a prolonged period of $\beta 2^*nAChR$ occupancy (Esterlis *et al*, 2010).

Postmortem studies show an up-regulation of $\alpha 4\beta 2^*nAChR$ numbers in the brains of smokers (Benwell *et al*, 1988; Breese *et al*, 1997). Imaging studies have also shown $\alpha 4\beta 2^*nAChR$ up-regulation in various brain regions (e.g. striatum, cerebellum, cortex, midbrain, corpus callosum) in smokers (Brody *et al*, 2013; Cosgrove *et al*, 2009; Mamede *et al*, 2007; Mukhin *et al*, 2008; Staley *et al*, 2006; Wullner *et al*, 2008). In addition, decreased $\alpha 4\beta 2^*nAChRs$ expression in the brains of smokers has been associated with better smoking cessation outcomes (Brody *et al*, 2014). Further, the $\beta 2^*nAChR$ partial agonist varenicline promotes smoking cessation by reducing craving, withdrawal, and pleasurable effects of smoking (Cahill *et al*, 2014; Ebbert, 2013; Fagerstrom and Hughes, 2008; Gonzales *et al*, 2006; Jorenby *et al*, 2006).

Genome-wide associated studies (GWAS) have failed to provide convincing data implicating polymorphisms in the gene encoding the $\beta 2$ subunit (CHRNA2) in the risk for tobacco dependence. However, candidate gene studies have identified CHRNA2 polymorphisms that are associated with the subjective effects of nicotine, FTND scores, and cessation therapy outcomes (Conti *et al*, 2008; Ehringer *et al*, 2007; Heatherton *et al*, 1991; Hoft *et al*, 2011; King *et al*, 2012; Perkins *et al*, 2009; Wessel *et al*, 2010). $\beta 2$ primarily assembles with the $\alpha 4$ and $\alpha 6$ subunits in brain areas associated with nicotine addiction. Not surprisingly, candidate gene studies have shown that variation in the genes that encode the $\alpha 4$ and $\alpha 6$ subunits (CHRNA4 and CHRNA6, respectively) is linked to measures of tobacco dependence. Multiple CHRNA4

polymorphisms are associated with greater FTND scores, cigarettes per day (CPD), and DSM-IV symptoms (Han *et al*, 2011; Kamens *et al*, 2013; Li *et al*, 2005; Saccone *et al*, 2009; Saccone *et al*, 2010; Saccone *et al*, 2007; Voineskos *et al*, 2007). These gene variants are also linked to greater subjective effects and better smoking cessation outcomes (Hoft *et al*, 2011; Hutchison *et al*, 2007). In addition, linkage analysis has revealed rare CHRNA4 variants are protective against tobacco dependence. These protective variants are also associated with altered $\beta 2$ *nAChR binding and increased expression and function of $\alpha 4\beta 2$ *nAChRs in the brain (McClure-Begley *et al*, 2014; Xie *et al*, 2011). In regards to CHRNA6, genetic variation is associated with smoking initiation, initial sensitivity to nicotine, and positive subjective effects that predict vulnerability to smoking (Thorgeirsson *et al*, 2010; Zeiger *et al*, 2008). One polymorphism located upstream of the CHRNA6-CHRNA3 gene cluster has also been associated with risk for developing nicotine dependence (Culverhouse *et al*, 2014; Hoft *et al*, 2009; Saccone *et al*, 2009; Saccone *et al*, 2010; Saccone *et al*, 2007; Stevens *et al*, 2008; Thorgeirsson *et al*, 2010; Wang *et al*, 2014a).

While GWAS studies were unsuccessful in identifying CHRNA3 polymorphisms associated with nicotine dependence, they have identified single nucleotide polymorphisms (SNPs) within the CHRNA3-CHRNA5-CHRNA4 gene cluster, encoding the $\alpha 3$, $\alpha 5$, and $\beta 4$ subunits, that are associated with nicotine dependence (Berrettini *et al*, 2008; Bierut *et al*, 2008; Saccone *et al*, 2009; Thorgeirsson *et al*, 2010). Additional candidate gene studies and meta-analyses identified SNPs in this gene cluster that are associated with nicotine dependence (Chen *et al*, 2009; Keskitalo *et al*, 2009; Munafò *et al*, 2012), smoking initiation (Grucza *et al*, 2010; Schlaepfer *et al*, 2008; Sherva *et al*, 2008), and heavy smoking (Liu *et al*, 2010; Stevens *et al*, 2008).

Genetic variation in the nicotinic subunits has also been associated with alcohol use and dependence. SNPs in CHRNA2 are linked to the initial subjective response to alcohol (Ehringer *et al*, 2007). Frequency of binge drinking in young adults is associated with SNPs in CHRNA4 (Coon *et al*, 2014), and variation in the CHRNA3-CHRNA6 gene cluster have been linked to heavy alcohol consumption (Hoft *et al*, 2009). Polymorphisms in the CHRNA3-CHRNA5-CHRNA4 gene cluster different from ones associated with nicotine dependence have been associated with alcohol dependence as defined by the DSM-IV (Wang *et al*, 2009). Level of response (LR) to alcohol, which is defined by an individual's subjective and physiological response to a given dose and blood level of alcohol (Enoch, 2014), has also been associated with two SNPs within this gene cluster (Joslyn *et al*, 2008; Saccone *et al*, 2007). In addition, SNPs in the CHRNA3-CHRNA5-CHRNA4 gene cluster that are associated with nicotine dependence have also been shown to be a risk factor for early initiation of alcohol use and frequency of binge drinking in adolescence (Lubke *et al*, 2012; Schlaepfer *et al*, 2008).

Neuroanatomy of nAChRs and their function in the mesolimbic dopamine pathway

The mesolimbic DA pathway is made up of DA neurons that originate in the VTA and project to the NAc, where DA is released. As observed with most other drugs of abuse, nicotine increases DA levels in the NAc (Di Chiara and Imperato, 1988). The NAc is divided into two main regions known as the NAc core and NAc shell. Intravenous nicotine administration results in an initial preferential increase in NAc shell DA release (Pontieri *et al*, 1996). Upon repeated noncontingent injections of nicotine, DA release in the NAc core becomes sensitized, while it remains unchanged in the shell (Benwell and Balfour, 1992; Cadoni and Di Chiara, 2000). This sensitization in the core is also observed with nicotine self-administration in rats (Lecca *et al*, 2006). These studies suggest that nicotine initially engages the NAc shell to promote its

reinforcing effects, while the NAc core comes into play during the later stages of dependence, such as cue-induced reinstatement of nicotine seeking (D'Souza and Markou, 2014).

In humans, PET imaging has revealed that smoking reduces DA receptor availability in the ventral striatum, reflecting an increase in DA release. The magnitude of DA release in the ventral striatum is related to reduction of craving and withdrawal symptoms, suggesting a role for striatal DA transmission in nicotine dependence (Barrett *et al*, 2004; Brody *et al*, 2009; Brody *et al*, 2004; Le Foll *et al*, 2014). Moreover, baseline striatal DA tone appears to be a risk factor for addiction (Volkow *et al*, 2012). In regards to smoking, the results are inconsistent. Several SPECT studies using various DA receptor ligands observed no differences in baseline striatal DA receptor availability (Staley *et al*, 2001; Yang *et al*, 2006; Yang *et al*, 2008). However, these studies were admittedly underpowered. Several PET studies with larger sample sizes observed lower baseline DA receptor availability in the striatum and putamen of smokers (Brown *et al*, 2012; Fehr *et al*, 2008).

In rodents, electrophysiological and microdialysis studies have shown that nicotine stimulates VTA DA neuron firing (Calabresi *et al*, 1989; Grenhoff *et al*, 1986; Keath *et al*, 2007; Mansvelder *et al*, 2002; Picciotto *et al*, 1998; Pidoplichko *et al*, 1997; Pidoplichko *et al*, 2004; Zhang *et al*, 2009) and DA release in the NAc (Di Chiara *et al*, 1988; Picciotto *et al*, 1998; Zhang *et al*, 2009). Nicotine results in a persistent increase of NAc DA release from terminals of VTA DA neurons despite rapid desensitization of nAChRs. To explain this conundrum, one study demonstrated that nAChR subtypes with different desensitization properties regulate GABAergic and glutamatergic inputs to the VTA. Nicotine is thought to enhance glutamatergic input to DA neurons through $\alpha 7$ nAChR activation, while $\beta 2^*$ nAChRs on GABA neurons are desensitized, reducing inhibition of DA neurons. This results in a net excitation of DA neurons,

producing prolonged DA release seen in response to nicotine (Mansvelder *et al*, 2002).

Stimulation of the mesolimbic DA pathway is thought to underlie the abuse-related effects of nicotine in rodents. This is supported by data showing that lesions of VTA DA projections to the NAc attenuate nicotine self-administration (Corrigall *et al*, 1992) and nicotine conditioned place preference (CPP) in rats (Sellings *et al*, 2008).

A variety of nAChR subtypes are expressed in the mesolimbic DA pathway. Many of the nAChR subunits are expressed in the VTA, but the $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs have been identified as the two main populations located on VTA DA neuron soma (Champtiaux *et al*, 2003; Drenan *et al*, 2008; Gotti *et al*, 2010; Klink *et al*, 2001; Zoli *et al*, 2002). The $\alpha 5$ accessory subunit is also highly expressed in the VTA; about half of the $\alpha 4\beta 2^*$ nAChRs expressed in the VTA assemble with the $\alpha 5$ subunit (Chatterjee *et al*, 2013). Other nAChRs expressed in the VTA include $\alpha 7$ and $\alpha 3^*$ nAChRs. In the NAc, there are four main populations of nAChRs expressed on DA terminals. These include the $\alpha 4\beta 2^*$, $\alpha 4\alpha 5\beta 2^*$, $\alpha 6\beta 2^*$, and $\alpha 4\alpha 6\beta 2^*$ nAChRs. According to one study, there is very little $\alpha 5$ expression on these terminals (Salminen *et al*, 2004). $\alpha 7$ nAChRs are also expressed on cortical and thalamic inputs to the NAc (Champtiaux *et al*, 2003; Kaiser and Wonnacott, 2000; Marchi *et al*, 2002; Rousseau *et al*, 2005).

Nucleus Accumbens

Ventral Tegmental Area

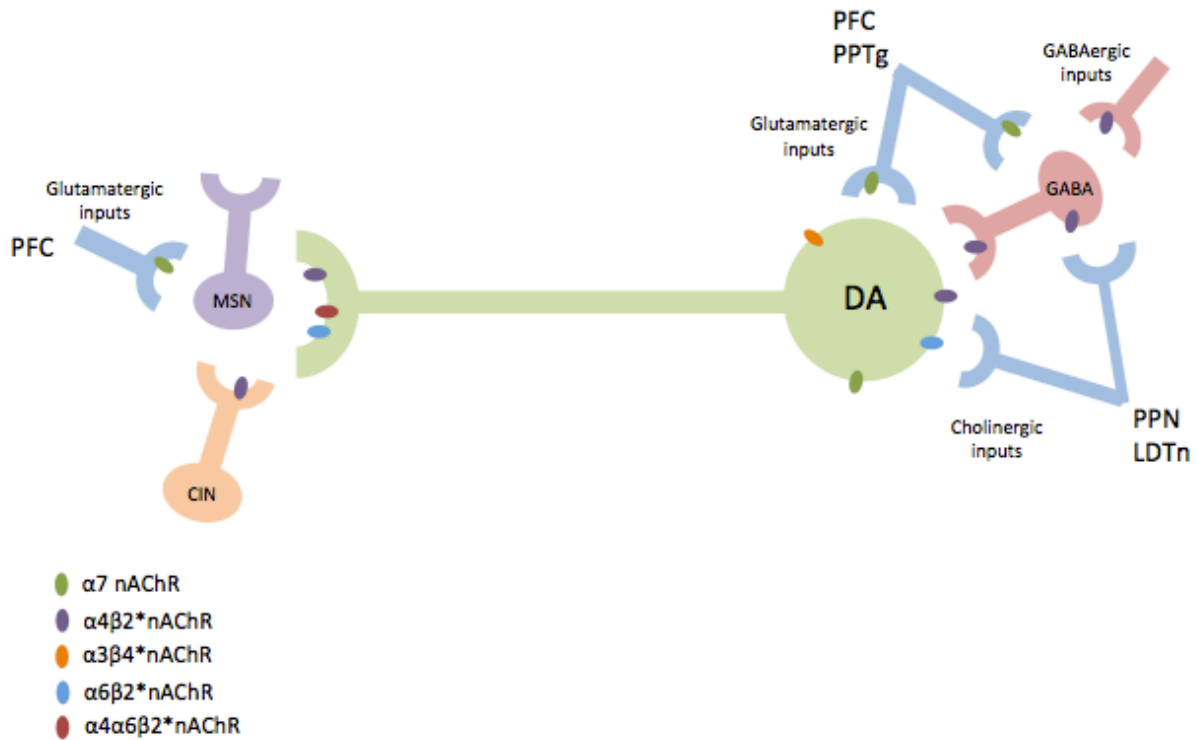


Figure 1.2 – Diagram of the mesolimbic dopamine (DA) pathway and its expression of nicotinic acetylcholine receptors (nAChRs). The mesolimbic DA pathway consists of DA neurons that originate in the ventral tegmental area (VTA) and project to the nucleus accumbens (NAc). These neurons synapse with medium spiny neurons (MSN) in the NAc. The medium spiny neurons receive glutamatergic input from the prefrontal cortex (PFC) and cholinergic input from interneurons (CIN). In VTA, the DA neurons receive glutamatergic input from the PFC and pedunculopontine tegmental nucleus (PPTg), GABAergic input from GABA interneurons, and cholinergic input from the pedunculopontine (PPn) and laterodorsal tegmental nuclei (LDTn).

In humans, mesolimbic $\beta 2^*nAChRs$ appear to play a role in nicotine dependence, as this nAChR subtype is up-regulated in the striatum and midbrain of smokers (Cosgrove *et al*, 2009; Mukhin *et al*, 2008; Staley *et al*, 2006). $\beta 2^*nAChR$ availability is correlated with the urge to smoke to relieve withdrawal symptoms during early abstinence (Staley *et al*, 2006). In rodent studies, $\beta 2^*nAChRs$ are critical for nicotine-stimulated mesolimbic DA neuron activity. Nicotine desensitizes GABAergic $\beta 2^*nAChRs$ to reduce the inhibitory drive on DA neurons in the VTA (Mansvelder *et al*, 2002). Nicotine-stimulated VTA DA neuron firing is ablated in $\beta 2$ null mutant ($\beta 2KO$) mouse slice preparations (Picciotto *et al*, 1998). Spontaneous VTA DA neuron firing is also absent in anesthetized $\beta 2KO$ mice and re-expression of $\beta 2$ restores neuron firing (Maskos *et al*, 2005). Nicotine does not stimulate NAc DA release in $\beta 2KO$ mice (Champtiaux *et al*, 2003; Grady *et al*, 2002; Picciotto *et al*, 1998; Salminen *et al*, 2004) and it has been shown that ACh released from cholinergic interneurons acts via $\beta 2^*nAChRs$ on NAc DA terminals, maintaining a high probability of action potential-evoked DA release (Threlfell *et al*, 2012).

Studies in this dissertation seek to determine $\beta 2^*nAChR$ subtypes involved in regulation of mesolimbic DA activity. The $\alpha 4$ and $\alpha 6$ subunits, which assemble with $\beta 2$, are important for mesolimbic DA activity. Inward currents of DA neurons in the VTA and nicotine-stimulated DA release is blunted in $\alpha 4$ null mutant ($\alpha 4KO$) mice (Champtiaux *et al*, 2003; Exley *et al*, 2011; Liu *et al*, 2012; Marubio *et al*, 2003; Zhao-Shea *et al*, 2011); re-expression of the $\alpha 4$ subunit in the VTA restores nicotinic control of NAc DA release and nicotine-stimulated increases in VTA DA neuron firing (Exley *et al*, 2011). $\alpha 4\beta 2^*nAChR$ gain-of-function mice with a single point mutation of the $\alpha 4$ subunit ($\alpha 4L9'A$) that renders their $\alpha 4\beta 2^*nAChRs$ hypersensitive to nicotine

exhibit enhanced activation of DA neurons, suggesting that $\alpha 4\beta 2$ *nAChR stimulation is sufficient for this effect (Liu *et al*, 2012; Tapper *et al*, 2004; Zhao-Shea *et al*, 2011).

In regards to $\alpha 6\beta 2$ *nAChRs, this nicotinic subtype has been shown to be primarily responsible for DA release in the NAc (Exley *et al*, 2008). Further, nicotine-stimulated striatal or NAc DA release is reduced in $\alpha 6$ null mutant ($\alpha 6$ KO) mice or upon administration of α -Ctx MII (Azam and McIntosh, 2005; Champtiaux *et al*, 2003; Grady *et al*, 2002; Kulak *et al*, 1997; Salminen *et al*, 2007; Salminen *et al*, 2004). In the VTA, ACh and nicotine fail to increase DA neuron firing in $\alpha 6$ KO mice or following local infusion of α -Ctx MII (Champtiaux *et al*, 2003; Liu *et al*, 2012; Zhao-Shea *et al*, 2011). In $\alpha 4L9$ 'A mice, α -Ctx MII infused into the VTA blocked the enhancement of VTA DA neuron activation in the response to physiologically relevant concentrations of nicotine, implicating $\alpha 4\alpha 6\beta 2$ *nAChRs in the control of nicotine-stimulated DA neuron activity (Liu *et al*, 2012; Zhao-Shea *et al*, 2011). Infusion of α -Ctx MII or α -Ctx PIA in the VTA reduces nicotine-stimulated DA release in the NAc (Gotti *et al*, 2010). In addition, α -Ctx MII robustly attenuates evoked phasic DA release in the NAc core of rats (Wickham *et al*, 2013). $\alpha 6\beta 2$ *nAChR gain-of-function mice with a single point mutation in the M2 pore-forming region of the $\alpha 6$ subunit ($\alpha 6L9$ 'S) are hypersensitive to ACh and nicotine, as VTA DA neuron activation and striatal or NAc DA release is augmented and left-shifted *in vitro* compared to wild type (WT) mice, suggesting that stimulation of $\alpha 6\beta 2$ *nAChRs is sufficient for mesolimbic DA neurons activity (Cohen *et al*, 2012; Drenan *et al*, 2010; Drenan *et al*, 2008; Engle *et al*, 2013; Powers *et al*, 2013; Wang *et al*, 2014b). This enhancement of DA neuron activity requires the $\alpha 4$ subunit, further suggesting that $\alpha 4\alpha 6\beta 2$ *nAChRs regulate DA neuron firing and DA release (Drenan *et al*, 2010; Engle *et al*, 2013).

$\alpha 7$ nAChRs appear to also be involved in nicotine's effects on the mesolimbic pathway. Nicotine enhances glutamatergic input to VTA DA neurons, resulting in excitation of these DA neurons (Mansvelder *et al*, 2002). Further, intra-VTA infusion of an $\alpha 7$ nAChR-selective antagonist, methyllycaconitine (MLA) prevents nicotine-elicited increases in NAc DA release (Schilstrom *et al*, 1998). For a summary of preclinical studies investigating nAChR contributions to mesolimbic DA activity, see Table 1.1.

Table 1.1 – Nicotinic acetylcholine receptor (nAChR) contributions to activity of the mesolimbic dopamine (DA) pathway

Subunit	Manipulation	Behavioral Outcome	Reference
$\beta 2$	KO	Evoked DA release blocked Nicotine-elicited DA release blocked Blocks ACh- and nicotine-stimulated DA neuron firing	Zhou et al. 2001 Picciotto et al. 1998; Grady et al. 2002; Champtiaux et al. 2003; Salminen et al. 2004 Picciotto et al. 1998; Champtiaux et al. 2003; Maskos et al. 2005
	DH β E	Evoked DA release blocked	Zhou et al. 2001; Rice and Cragg, 2004
$\alpha 4$	KO	Blocks ACh- and nicotine-stimulated DA neuron firing Evoked DA release blocked Blunted nicotine-stimulated DA release	Champtiaux et al. 2003; Marubio et al. 2003; Exley et al. 2011; Zhao-Shea et al. 2011; Liu et al. 2012 Exley et al. 2011 Champtiaux et al. 2003; Marubio et al. 2003; McGranahan et al. 2011
	$\alpha 4L9A$	Hypersensitive to nicotine-stimulated DA neuron firing	Tapper et al. 2004; Zhao-Shea et al. 2011; Liu et al. 2012
$\alpha 6$	KO	Blocks ACh- and nicotine-stimulated DA neuron firing Blunted nicotine-stimulated DA release Blocks evoked DA release	Liu et al. 2012 Champtiaux et al. 2003 Exley et al. 2011
	$\alpha 6L9S$	Hypersensitive ACh- and nicotine-stimulated DA neuron firing Hypersensitive DA release	Drenan et al. 2008; Engle et al. 2013; Powers et al. 2013 Drenan et al. 2008, 2010; Cohen et al. 2012
	α -CTX MII	Blocks ACh- and nicotine-stimulated DA neuron firing Blunted nicotine-stimulated DA release Blocks evoked DA release	Champtiaux et al. 2003; Zhao-Shea et al. 2011; Liu et al. 2012 Kulak et al. 1997; Grady et al. 2002; Champtiaux et al. 2003; Salminen et al. 2004, 2007; Azam and McIntosh et al. 2005; Gotti et al. 2010 Wickham et al. 2013
$\alpha 7$	MLA	Blocks nicotine-elicited DA release	Schilstrom et al. 1998

Abbreviations: acetylcholine (ACh); dopamine (DA)

Nicotinic regulation of nicotine reward and reinforcement

Many people use tobacco due to nicotine's pleasurable effects. An accumulation of evidence highly implicates $\beta 2^*nAChRs$ in rodent models of reward and reinforcement. $\beta 2KO$ mice do not self-administer nicotine intravenously (i.v) or intracranially in the VTA (Besson *et al*, 2006; Maskos *et al*, 2005; Picciotto *et al*, 1998; Pons *et al*, 2008), suggesting that $\beta 2^*nAChRs$ are important for nicotine reinforcement. Tail vein i.v. nicotine self-administration consisted of only one session (Pons *et al*, 2008), while jugular i.v. (Picciotto *et al*, 1998) and intracranial self-administration (Besson *et al*, 2006; Maskos *et al*, 2005) was conducted over multiple sessions, modeling initiation and maintenance of nicotine self-administration, respectively. $\beta 2KO$ mice do not express nicotine CPP either (Walters *et al*, 2006), indicating that $\beta 2^*nAChRs$ are also critical for nicotine reward. As nicotine is administered acutely during CPP, this likely models the initial rewarding properties of nicotine that often predict later risk for dependence (for review, see de Wit and Phillips, 2012). In addition, nicotine does not enhance conditioned reinforcement in $\beta 2KO$ mice as it does in WT mice (Brunzell *et al*, 2006). $\beta 2KO$ mice also fail to exhibit nicotine-stimulated locomotor activation (King *et al*, 2004b), which like reward and reinforcement is also a DA-dependent behavior.

More specifically, $\beta 2^*nAChRs$ expressed in the mesolimbic DA pathway are important for nicotine's abuse-related effects. Local infusion of the $\beta 2$ -selective antagonist, dihydro- β -erythroidine (DH β E), into the VTA reduces nicotine self-administration, while infusion into the NAc has no effect (Corrigall *et al*, 1994). Moreover, lentiviral re-expression of the $\beta 2$ subunit in the VTA of $\beta 2KO$ mice rescues nicotine self-administration (Pons *et al*, 2008). $\beta 2$ transgenic mice expressing $\beta 2^*nAChRs$ only in VTA neurons show a restoration of nicotine-stimulated

locomotor activity after seven days of nicotine administration compared to $\beta 2$ KO mice (Mineur *et al*, 2009).

Studies in this dissertation aim to investigate subtypes of $\beta 2$ *nAChRs that regulate nicotine reward. $\beta 2$ primarily assembles with the $\alpha 4$ and $\alpha 6$ subunits, which are also involved in the rewarding and reinforcing effects of nicotine. $\alpha 4$ KO mice do not express nicotine CPP (McGranahan *et al*, 2011; Sanjakdar *et al*, 2015); but see Cahir *et al*, 2011) and do not self-administer nicotine i.v. (tail vein) or intracranially into the VTA (Exley *et al*, 2011; Pons *et al*, 2008). Further, selectively deleting the $\alpha 4$ subunit from DA neurons is sufficient to block nicotine reward (McGranahan *et al*, 2011). However, for jugular i.v. nicotine self-administration, $\alpha 4$ KO mice do not differ from WT mice (Cahir *et al*, 2011). $\alpha 4\beta 2$ *nAChR gain-of-function ($\alpha 4L9'A$) mice exhibit leftward shifts in nicotine CPP (Tapper *et al*, 2004) and $\alpha 4$ -S248F mice, who are also more sensitive to low dose nicotine, show leftward shifts in jugular i.v. nicotine self-administration (Cahir *et al*, 2011). Like $\alpha 4$ KO mice, $\alpha 6$ KO mice also show reductions in i.v. (tail vein) or VTA intracranial nicotine self-administration (Exley *et al*, 2011; Pons *et al*, 2008). $\alpha 6$ KO mice also exhibit rightward shifts in the dose response curve for nicotine CPP (Sanjakdar *et al*, 2015). Local infusion of the $\alpha 6\beta 2$ *nAChR-selective antagonist α -Ctx MII into the VTA (Gotti *et al*, 2010) or NAc shell (Brunzell *et al*, 2010) decreases nicotine self-administration. The latter finding in the NAc shell is in contrast to another study showing that infusion of DH β E, which inhibits both $\alpha 4\beta 2$ * and $\alpha 6\beta 2$ *nAChRs, has no effect on self-administration when infused into the NAc (Corrigall *et al*, 1994). Intracerebroventricular (i.c.v.) (Jackson *et al*, 2009) or local NAc infusion of α -Ctx MII (Sanjakdar *et al*, 2015) blocks nicotine CPP. α -Ctx MII also decreases locomotor activity when infused into the VTA (Gotti *et al*, 2010), but not when infused i.c.v. (Jackson *et al*, 2009) or locally in the NAc shell (Brunzell *et al*,

2010). Moreover, basal and nicotine-stimulated locomotor activity is enhanced in $\alpha 6L9$ 'S mice, an effect blocked by D_1 (SCH 23390) and D_2 (sulpiride) receptor antagonism (Berry *et al*, 2015; Cohen *et al*, 2012; Drenan *et al*, 2010; Drenan *et al*, 2008). This response appears to require the $\alpha 4$ subunit, as knocking out the $\alpha 4$ subunit in $\alpha 6L9$ 'S mice reverses this locomotor effect (Drenan *et al*, 2010).

Initial studies indicated that $\alpha 7$ nAChRs do not play a critical role in nicotine reward and reinforcement (Pons *et al*, 2008; Walters *et al*, 2006). However, more recent data suggests that $\alpha 7$ nAChRs act in opposition to $\beta 2$ *nAChRs. That is, inhibiting $\alpha 7$ nAChRs enhances nicotine reward and reinforcement, while stimulating $\alpha 7$ nAChRs reduces nicotine's rewarding and reinforcing effects (Brunzell and McIntosh, 2012; Harenza *et al*, 2014). Local infusion of an $\alpha 7$ nAChR-selective antagonist, α -conotoxin ArIB [V11L, V16D] into the NAc or anterior cingulate cortex promotes nicotine self-administration, producing dramatic increases in active lever presses and breakpoints during a progressive ratio schedule of reinforcement (Brunzell *et al*, 2012). Results from studies assessing the effects of MLA, another $\alpha 7$ nAChR-selective antagonist, on nicotine self-administration are conflicting (Grottick *et al*, 2000; Markou and Paterson, 2001), perhaps due to the fact that MLA also acts at $\alpha 6\beta 2$ *nAChRs (Mogg *et al*, 2002). $\alpha 7$ null mutant ($\alpha 7$ KO) mice show leftward shifts in nicotine CPP (Harenza *et al*, 2014). Consistently, $\alpha 7$ -selective agonists inhibit nicotine reward and reinforcement, blocking nicotine CPP (Harenza *et al*, 2014) and reducing nicotine-self-administration (Brunzell *et al*, 2012). However, $\alpha 7$ KO mice show reductions in oral nicotine self-administration in a two bottle choice paradigm after 40 days of exposure. These conflicting results suggest that $\alpha 7$ nAChRs may play different roles in the initiation versus maintenance of nicotine self-administration (Levin *et al*, 2009; Pons *et al*, 2008).

Other nAChR subunits also play a role in nicotine reward and reinforcement. $\alpha 2$ null mutant ($\alpha 2$ KO) mice exhibit increases in nicotine self-administration (Lotfipour *et al*, 2013). When the accessory $\alpha 5$ subunit is genetically deleted in mice, nicotine self-administration is markedly increased compared to WT mice. Nicotine self-administration is normalized to WT levels when $\alpha 5$ is re-expressed in the medial habenula (Fowler *et al*, 2011). Overexpression of the $\beta 4$ subunit produces reductions in nicotine intake, which is rescued by expression of the $\alpha 5$ variant, D398N (Frahm *et al*, 2011; Morel *et al*, 2014; Tammimaki *et al*, 2012). Thus, it appears that $\beta 4$ *nAChRs and $\alpha 5$ *nAChRs may work against each other or that assembly of $\alpha 3\beta 4$ *nAChRs with the $\alpha 5$ subunit may change how this subtype regulates nicotine intake. For a summary of the rodent data implicating nAChRs in nicotine reward and reinforcement, see Table 1.2.

Table 1.2 – Nicotinic acetylcholine receptor (nAChR) contributions to nicotine reward and reinforcement

Subunit	Manipulation	Behavioral Outcome	Reference
$\beta 2$	KO	CPP blocked Self-administration blocked Conditioned reinforcement blocked Locomotor activation blocked	Walters et al. 2006 Picciotto et al. 1998; Maskos et al. 2005; Besson et al. 2006; Pons et al. 2008 Brunzell et al. 2006 King et al. 2004
	DH β E	CPP blocked Self-administration blocked	Walters et al. 2006 Corrigall et al. 1994
$\alpha 2$	KO	Increased self-administration	Lotfipour et al. 2013
$\alpha 4$	KO	CPP blocked CPP unchanged Self-administration blocked	McGranahan et al. 2011; Sanjakdar et al. 2015 Cahir et al. 2011 Pons et al. 2008; Exley et al. 2011
	$\alpha 4L9'A$	Enhanced nicotine CPP	Tapper et al. 2004
	$\alpha 4-S248F$	Enhanced self-administration	Cahir et al. 2011
$\alpha 5$	KO	Increased self-administration	Fowler et al. 2011
$\alpha 6$	KO	CPP right-shifted Self-administration reduced	Sanjakdar et al. 2015 Pons et al. 2008; Exley et al. 2011
	α -CTX MII	CPP blocked Self-administration blocked	Jackson et al. 2009; Sanjakdar et al. 2015 Brunzell et al. 2010; Gotti et al. 2010
$\alpha 7$	KO	CPP unaffected Leftward shift in CPP Self-administration unaffected Chronic oral nicotine intake decreased	Walters et al. 2006 Harenza et al. 2014 Pons et al. 2008 Levin et al. 2009
	MLA	Self-administration unaffected Self-administration blocked	Grottick et al. 2000 Markou and Paterson 2001
	α -CTX ArIB	Self-administration increased	Brunzell et al. 2012
	PHA-543613	CPP blocked	Harenza et al. 2014
	PNU-282987	Self-administration blocked	Brunzell et al. 2012

Abbreviations: conditioned place preference (CPP)

Nicotinic modulation of anxiety-like behavior

In addition to the pleasure obtained from smoking, smokers also report that they smoke to relieve anxiety. Stress contributes to escalation of smoking and can precipitate relapse (Shiffman *et al*, 1997; Skara *et al*, 2001), and smokers experience more intense anxiety compared to non-smokers (Fidler and West, 2009; Parrott, 1999; Perkins and Grobe, 1992). There is a significant correlation between smoking and anxiety disorders, such as panic disorder, phobias, and post-traumatic stress disorder (Gilbert *et al*, 2008; Grillon *et al*, 2007; John *et al*, 2004; McCabe *et al*, 2004; Morissette *et al*, 2006; Tsuda *et al*, 1996; Vujanovic *et al*, 2010).

It has been suggested that expression of $\beta 2$ *nAChRs contributes to anxiety phenotypes (Picciotto *et al*, 2015). Chronic nicotine exposure produces an up-regulation of the $\alpha 4\beta 2$ *nAChRs in mice (Even *et al*, 2008; Metaxas *et al*, 2010; Nashmi *et al*, 2007; Nuutinen *et al*, 2005; Pakkanen *et al*, 2005; Pauly *et al*, 1996; Perez *et al*, 2008; Pietila *et al*, 1998; Sparks and Pauly, 1999; Turner *et al*, 2011; Xiao *et al*, 2009), rats (Abdulla *et al*, 1996; Barrantes *et al*, 1995; Collins *et al*, 1990; el-Bizri and Clarke, 1994; Flores *et al*, 1997; Nguyen *et al*, 2003, 2004; Perez *et al*, 2008; Walsh *et al*, 2008; Wang *et al*, 2007; Yates *et al*, 1995), non-human primates (Kassiou *et al*, 2001; McCallum *et al*, 2006; Perez *et al*, 2012; Perez *et al*, 2013a; Slotkin *et al*, 2002) and human smokers (Benwell *et al*, 1988; Breese *et al*, 1997; Brody *et al*, 2013; Cosgrove *et al*, 2009; Mamede *et al*, 2007; Metaxas *et al*, 2010; Mukhin *et al*, 2008; Staley *et al*, 2006; Staley *et al*, 2005; Wullner *et al*, 2008) who have higher overall levels of anxiety. In contrast, most of the evidence suggest that $\alpha 6\beta 2$ *nAChRs are down-regulated after chronic nicotine (Perez *et al*, 2008; Perez *et al*, 2012; Perez *et al*, 2013a, b); but see Parker *et al*, 2004). It should be noted that expression of $\alpha 6\beta 2$ *nAChRs in response to chronic nicotine depends on whether $\alpha 4$ is present, where $\alpha 4\alpha 6\beta 2$ *nAChRs are down-regulated and (non-

$\alpha 4\beta 2$ *nAChRs are up-regulated in the striatum (Baker *et al*, 2013; Lomazzo *et al*, 2011; Metaxas *et al*, 2010; Moretti *et al*, 2010; Perez *et al*, 2008; Perez *et al*, 2013a; Xiao *et al*, 2009).

In support of the theory that expression of $\beta 2$ *nAChRs regulated anxiety-like behavior, preclinical rodent studies report that the $\beta 2$ *nAChR-selective antagonist, DH β E and $\beta 2$ *nAChR partial agonists, varenicline, ABT-089, and sazetidine, produce decreases in anxiety-like behavior in the conditioned inhibition, marble burying, light-dark, and elevated plus maze (EPM) tasks (Anderson and Brunzell, 2012, 2015; Hussmann *et al*, 2014; Turner *et al*, 2010; Yohn *et al*, 2014). Low doses of nicotine (0.01 and 0.032 mg/kg, i.p.) mimic this anxiolytic effect, indicating that desensitization by these low doses may promote decreases in anxiety-like behavior (Anderson *et al*, 2012). Conversely, high doses of nicotine (0.5 and 1.0 mg/kg, i.p.) increase anxiety-like behavior (Anderson *et al*, 2015; File *et al*, 1998), an effect that is blunted in $\beta 2$ KO mice. High doses of the $\beta 2$ -selective agonist, 5I-A85380 also produce an anxiogenic-like phenotype (Anderson *et al*, 2015). These findings suggest that inactivation of $\beta 2$ *nAChRs, presumably via desensitization, promotes anxiolysis, while stimulation of $\beta 2$ *nAChRs promotes anxiogenesis.

The subtype of $\beta 2$ *nAChRs that contribute to anxiety-like behavior is a topic of question in this dissertation. $\alpha 4\beta 2$ *nAChRs have previously been implicated in anxiety-like behavior. A low dose of nicotine (0.01 mg/kg, i.p.) loses its anxiolytic effect in the EPM test when the $\alpha 4$ subunit is genetically deleted in VTA DA neurons (McGranahan *et al*, 2011), suggesting that $\alpha 4\beta 2$ *nAChRs expressed in the VTA are required for nicotine's anxiolytic properties. $\alpha 4$ KO mice show elevated levels of basal anxiety-like behavior (Ross *et al*, 2000), suggesting that inhibition of $\alpha 4\beta 2$ *nAChRs promotes anxiety-like behavior. Interestingly, $\alpha 4\beta 2$ *nAChR gain-of-function ($\alpha 4L9'S$) mice also show increases in basal anxiety-like behavior in the EPM task

(Labarca *et al*, 2001). Based on these data in $\alpha 4$ KO and $\alpha 4$ L9'S mice, it is not clear whether activation or inhibition of $\alpha 4\beta 2$ *nAChRs promotes anxiety-like behavior. For $\alpha 6\beta 2$ *nAChRs, no studies have investigated their contribution to basal or nicotine-associated anxiety-like behavior. However, one study has shown that i.c.v. infusion of α -Ctx MII blocks nicotine withdrawal-induced anxiety-like behavior (Jackson *et al*, 2009), suggesting that $\alpha 6\beta 2$ *nAChRs regulate anxiety-like behavior in response to withdrawal from nicotine.

Other nAChR subtypes have also been implicated in anxiety-like behavior. $\beta 3$ ($\beta 3$ KO) and $\beta 4$ null mutant ($\beta 4$ KO) mice exhibit reductions in basal anxiety-like behavior in the EPM, light-dark, and prepulse inhibition tasks compared to WT mice (Booker *et al*, 2007; Salas *et al*, 2003; Semenova *et al*, 2012), suggesting that nAChRs containing the $\beta 3$ and $\beta 4$ subunits promote anxiety-like behavior. $\alpha 7$ KO mice are similar to WT mice in the open field, EPM, and light-dark tasks, but MLA infused locally into the hippocampus reduces nicotine-induced anxiogenic effects in the social interaction task (Tucci *et al*, 2003). Systemic PNU-282987, an $\alpha 7$ nAChR-selective agonist, increases anxiety-like behavior (Pandya and Yakel, 2013). These data suggest that inhibition of $\alpha 7$ nAChRs reduces anxiety-like behavior. See table 1.3 for a summary of the studies implicating nAChRs in anxiety-like behavior.

Table 1.3 – Nicotinic acetylcholine receptor (nAChR) contributions to anxiety-like behavior

Subunit	Manipulation	Behavioral Outcome	Reference
β2	KO	Blunted nicotine-induced anxiogenesis (light-dark)	Anderson and Brunzell, 2015
	DHβE	Anxiolytic (EPM; marble burying)	Anderson and Brunzell 2012
	Varenicline	Anxiolytic (marble burying; hypophagia)	Turner et al. 2010; Hussman et al. 2014
	ABT-089	Anxiogenic (hypophagia)	Yohn et al. 2014
	5I-A85380	Anxiogenic (light-dark; EPM)	Anderson and Brunzell, 2015
β3	KO	Decreased anxiety levels (EPM)	Booker et al. 2007
β4	KO	Decreased anxiety levels (EPM; light-dark)	Salas et al. 2003; Semenova et al. 2012
α4	KO	Nicotine-stimulated anxiolysis blocked (EPM)	McGranahan et al. 2011
	α4L9'S	Anxiogenic (EPM; mirrored chamber)	Labarca et al. 2001
	Sazetidine	Anxiolytic (hypophagia)	Hussman et al. 2014
α6	α-Ctx MII	Decreased nicotine withdrawal-induced anxiety-like behavior	Jackson et al. 2009
α7	KO	Anxiety-like behavior unaffected (EPM; light-dark; open field)	Salas et al. 2007; Jackson et al. 2008
	MLA	Reversed nicotine-induced anxiogenesis	Tucci et al. 2003
	PNU-282987	Increased anxiety levels	Pandya et al. 2013

Abbreviations: elevated plus maze (EPM)

Nicotinic contributions to abuse-related effects of ethanol

nAChRs have also been implicated in abuse-related effects of EtOH. Studies investigating the role of $\beta 2^*$ nAChRs in the behavioral and neurochemical effects of EtOH have generated conflicting results. DH β E or genetic knockdown of the $\beta 2$ subunit has no effect on EtOH consumption and preference (Dawson *et al*, 2013; Kamens *et al*, 2010a; Kuzmin *et al*, 2009; Tolu *et al*, 2017), self-administration (Kuzmin *et al*, 2009), EtOH-induced VTA DA neuron firing (Tolu *et al*, 2017), or NAc DA release (Ericson *et al*, 2003; Larsson *et al*, 2002), but it does attenuate the sedative effect of EtOH (Dawson *et al*, 2013). However, varenicline, which acts as a partial agonist at $\beta 2^*$ nAChRs, does reduce ethanol consumption (Feduccia *et al*, 2014; Hendrickson *et al*, 2010; Kamens *et al*, 2010b; Santos *et al*, 2013; Steensland *et al*, 2007) and self-administration in rodents (Bito-Onon *et al*, 2011; Steensland *et al*, 2007), while enhancing the ataxic and sedative effects of EtOH (Kamens *et al*, 2010b). Varenicline does not have an effect on EtOH-associated NAc DA release however (Ericson *et al*, 2009; Feduccia *et al*, 2014).

One goal of this dissertation is to investigate $\beta 2^*$ nAChR subtypes that modulate EtOH reinforcement. Previous data implicate that $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs are involved in abuse-related effects of EtOH. $\alpha 4$ KO mice consume less EtOH and show reduced preference for EtOH compared to WT mice (Hendrickson *et al*, 2010; Liu *et al*, 2013a), while $\alpha 4L9^*A$ mice exhibit increases in EtOH preference (Liu *et al*, 2013a). EtOH-induced VTA DA neuron activation is also reduced in $\alpha 4$ KO mice and enhanced in $\alpha 4L9^*A$ mice compared to WT mice (Liu *et al*, 2013a). Intra-VTA α -Ctx MII results in reductions of EtOH consumption and preference in mice and rats (Larsson *et al*, 2004), as well as reductions in EtOH self-administration (Kuzmin *et al*, 2009) and EtOH-associated conditioned reinforcement in rats (Lof *et al*, 2007). However, systemic DH β E has no effect on self-administration (Kuzmin *et al*, 2009) and it does not reduce

conditioned reinforcement when infused into the VTA (Lof *et al*, 2007). In contrast to the studies using α -Ctx MII to inhibit $\alpha 6\beta 2$ nAChRs, $\alpha 6$ KO mice show no differences compared to WT mice for measures of EtOH consumption and preference (Guildford *et al*, 2016; Kamens *et al*, 2012). But, $\alpha 6$ KO mice do show a loss of reward sensitivity at high doses of EtOH in CPP (Guildford *et al*, 2016). $\alpha 6$ L9'S mice show increases in EtOH consumption and CPP compared to WT mice (Powers *et al*, 2013). Consistent with these results, VTA infusion of α -Ctx MII blocks EtOH-induced DA release in mice (Larsson *et al*, 2004) and intra-VTA α -Ctx MII or $\alpha 6$ genetic deletion reduces EtOH-induced activation of VTA DA neurons (Liu *et al*, 2013b).

Other nAChR subunits have been tested for involvement in EtOH's effects. In one study, $\alpha 7$ KO mice consumed less EtOH compared to WT mice (Kamens *et al*, 2010a). However, antagonizing $\alpha 7$ nAChRs has no effect on operant EtOH self-administration (Kuzmin *et al*, 2009) or EtOH-elicited DA release (Larsson *et al*, 2002). $\beta 3$ KO mice show similar levels of EtOH intake as WT mice. This mutation does not affect EtOH-induced sedation or ataxia either, showing no differences in the loss of righting reflex (LORR) and balance beam tests compared to WT mice (Kamens *et al*, 2012). $\alpha 5$ null mutant mice ($\alpha 5$ KO) consume similar amounts of EtOH as WT mice in the DID paradigm, but they are more sensitive to EtOH's sedative and ataxic effects, showing slower LORR recovery and decreases in rotarod performance compared to WT mice (Santos *et al*, 2013). Ethanol activation of VTA DA neurons is decreased in $\beta 4$ KO mice, while they consumed more alcohol compared to WT mice in a two-bottle choice procedure (Tolu *et al*, 2017). For a summary of the data demonstrating the role of nAChRs in ethanol's addictive effects, see Table 1.4.

Table 1.4 – Nicotinic acetylcholine receptor (nAChR) contributions to EtOH-induced mesolimbic activity and associated intake, reward, and reinforcement

Subunit	Manipulation	Behavioral Outcome	Reference
$\beta 2$	KO	No change in EtOH intake (two-bottle choice; intermittent access) or preference No effect on EtOH-associated conditioned reinforcement Decreased EtOH-induced sedation (LORR) Decreased EtOH-induced VTA DA neuron firing	Kamens et al. 2010a; Dawson et al. 2013 Lof et al. 2007 Dawson et al. 2013 Tolu et al. 2017
	DH β E	No effect on EtOH operant self-administration Decreased EtOH-induced hypnosis (LORR) No effect on EtOH-elicited DA release	Le et al. 2000; Kuzmin et al. 2009 Dawson et al. 2013 Larsson et al. 2002; Ericson et al. 2003
	Varenicline	Reduced EtOH intake (two-bottle choice; DID) Reduced EtOH operant self-administration Increased EtOH-induced sedation and ataxia (LORR; balance beam; dowel test) No effect on EtOH-elicited DA release	Steensland et al. 2007; Hendrickson et al. 2010; Kamens et al. 2010a; Santos et al. 2013; Feduccia et al. 2014 Steensland et al. 2007; Bito-Onon et al. 2011 Kamens et al. 2010b Ericson et al. 2009; Feduccia et al. 2014
$\beta 3$	KO	No change in EtOH intake (two-bottle choice) or EtOH-induced sedation and ataxia (LORR; balance beam)	Kamens et al. 2012
$\beta 4$	KO	Increased EtOH intake (two-bottle choice)	Tolu et al. 2017
$\alpha 4$	KO	Reduced EtOH intake (DID) EtOH CPP blocked Reduced EtOH-stimulation of DA neurons	Hendrickson et al. 2010 Liu et al. 2013a Liu et al. 2013a
	L9A	Enhanced EtOH CPP Hypersensitive EtOH-stimulation of DA neurons	Liu et al. 2013a Liu et al. 2013a
$\alpha 5$	KO	No change in EtOH intake (DID) Increased EtOH-induced sedation and ataxia (LORR; rotarod)	Santos et al. 2013 Santos et al. 2013
$\alpha 6$	KO	No change in EtOH intake or preference (two-bottle choice; DID) Increased EtOH-induced sedation Reduced EtOH-stimulation of DA neurons	Kamens et al. 2012; Guildford et al. 2016 Kamens et al. 2012 Liu et al. 2013b
	$\alpha 6$ L9'S	Increased EtOH intake (DID) and enhanced EtOH CPP	Powers et al. 2013
	α -CTX MII	Reduced EtOH operant self-administration Reduced EtOH intake and preference (two-bottle choice) EtOH-associated conditioned reinforcement Reduced EtOH-stimulation of DA neurons Reduced EtOH-induced DA release	Kuzmin et al. 2009 Larsson et al. 2004 Lof et al. 2007 Liu et al. 2013b Larsson et al. 2004
$\alpha 7$	KO	Reduced EtOH intake (two-bottle choice)	Kamens et al. 2010a
	MLA	No effect on EtOH operant self-administration No effect on EtOH-elicited DA release	Kuzmin et al. 2009 Larsson et al. 2002

Abbreviations: ethanol (EtOH); drinking-in-the-dark (DID); loss of righting reflex (LORR); dopamine (DA); conditioned place preference (CPP)

Experimental aims

Overall, the goal of these studies is to build on our knowledge related to $\alpha 6\beta 2$ *nAChR contributions to abuse-related effects of nicotine and alcohol. This will be accomplished using pharmacological and genetic manipulations in various mouse behavioral and neurochemical models, including: 1) *in vivo* microdialysis in awake animals to quantify NAc DA release, 2) nicotine CPP to measure reward, 3) multiple tests of anxiety, including the open field, light-dark, and EPM tests, and 4) EtOH self-administration to measure EtOH reinforcement.

Specific Aim 1 will test the overall hypothesis that stimulation of mesolimbic $\alpha 6\beta 2$ *nAChRs promotes nicotine reward and NAc DA release using nicotine CPP and *in vivo* microdialysis in WT and $\alpha 6L9$ 'S mice. These studies will assess the effects of acute nicotine exposure, which is relevant to addiction, as responses to initial exposure are known to predict risk for later dependence (for review, see de Wit *et al*, 2012). Given *in vitro* studies showing that $\alpha 6L9$ 'S $\alpha 6\beta 2$ *nAChRs respond to sub-threshold concentrations of nicotine that aren't effective at native nAChRs (Berry *et al*, 2015; Cohen *et al*, 2012; Drenan *et al*, 2010; Drenan *et al*, 2008; Engle *et al*, 2013; Powers *et al*, 2013; Wang *et al*, 2014b), we reason that we can use lower doses of nicotine *in vivo* to selectively activate $\alpha 6\beta 2$ *nAChRs, isolating their function in $\alpha 6L9$ 'S mice. Chapter 2 of this dissertation will describe the results from the microdialysis and CPP experiments in Specific Aim 1.

First, we will assess basal NAc DA levels using *in vivo* microdialysis in awake, behaving WT and $\alpha 6L9$ 'S mice. We expect that $\alpha 6L9$ 'S mice will exhibit elevated basal NAc DA levels compared to WT mice to corroborate previous *in vitro* studies (Cohen *et al*, 2012; Wang *et al*, 2014b), suggesting that stimulation of $\alpha 6\beta 2$ *nAChRs promotes NAc DA release. As NAc DA release is associated with nicotine's rewarding and locomotor stimulating properties, we will

next compare nicotine CPP and locomotor activity in a second group of WT and $\alpha 6L9'S$ mice. As with NAc DA release, we predict that $\alpha 6L9'S$ mice will exhibit a leftward shift in the nicotine CPP and locomotor activity dose response curves compared to WT mice to suggest that stimulation of $\alpha 6\beta 2*nAChRs$ also promotes nicotine reward and locomotor activity.

To expand on these initial studies, we seek to determine neuroanatomical loci where $\alpha 6\beta 2*nAChRs$ promote nicotine reward. To accomplish this goal, we will administer local NAc shell infusions of saline or artificial cerebrospinal fluid (aCSF) vehicle (VEH) or the $\alpha 6\beta 2*nAChR$ -selective antagonist, α -Ctx MII, prior to nicotine CPP training in a third group of WT and $\alpha 6L9'S$ mice. Based on studies reporting that nicotine induces an initial preferential increase in DA in the NAc shell to drive nicotine reward (Balfour, 2015), we predict that local infusion of α -Ctx MII into the NAc shell during CPP training will block the acquisition of nicotine CPP in WT and $\alpha 6L9'S$ mice. Consistent with our lab's previous nicotine self-administration studies (Brunzell *et al*, 2010), this would suggest that $\alpha 6\beta 2*nAChRs$ located on terminals in the NAc shell are involved in nicotine's rewarding properties.

Specific Aim 2 will assess $\alpha 6\beta 2*nAChR$ contributions to anxiety-like behavior. Chapter 3 will describe results from these studies where $\alpha 6L9'S$ and $\alpha 6KO$ mice, and their WT counterparts, will be tested in a series of behavioral assays that assess anxiety-like behavior. These assays, including the open field, light-dark, and EPM assays, exploit a rodent's opposing drives to explore novel areas and avoid brightly lit, open areas where they might be vulnerable to predators. Levels of anxiety-like behavior are determined by the ability of an experimental manipulation (e.g. genotype, drug) to alter behaviors in aversive areas, such as the center zone of the open field arena, the light chamber of the light-dark apparatus, and the open arms of the EPM, with increases in behavior indicating anxiolysis and decreases indicating anxiogenesis. We

will also evaluate locomotor activity as a control experiment to ensure that any effect observed in the anxiety assays is not an artifact of altered locomotion. An accumulation of evidence suggests that inhibition of $\beta 2$ *nAChRs promotes anxiolytic effects (Anderson *et al*, 2012, 2015; Hussmann *et al*, 2014; Turner *et al*, 2010; Yohn *et al*, 2014). Based on data reporting persistent activation of $\alpha 4\alpha 6\beta 2$ *nAChRs by low concentrations of nicotine that typically desensitize other nAChRs (Liu *et al*, 2012), we hypothesize that activation of $\alpha 6\beta 2$ *nAChRs promotes angiogenesis. Thus, we predict that $\alpha 6L9$ 'S mice will exhibit elevated anxiety-like behavior, while $\alpha 6KO$ mice will show decreases in these measures.

The goal of Specific Aim 3 is to differentiate the contributions of $\alpha 4\alpha 6\beta 2$ *nAChRs and (non- $\alpha 4$) $\alpha 6\beta 2$ *nAChRs to nicotine reward and anxiety-like behavior in mice. Chapter 4 will describe the results from these experiments in which WT, $\alpha 6L9$ 'S, $\alpha 4KO$, and $\alpha 6L9$ 'S mice with an $\alpha 4$ null mutation ($\alpha 6L9$ 'S- $\alpha 4KO$) will be compared at a range of i.p. nicotine doses in nicotine CPP, as well as in behavioral assays of anxiety-like behavior including the open field and light-dark assays. We will also evaluate locomotor activity. Based on previous data demonstrating that $\alpha 4\alpha 6\beta 2$ *nAChRs regulate mesolimbic activity and associated locomotor activity (Drenan *et al*, 2010; Engle *et al*, 2013; Liu *et al*, 2012; Zhao-Shea *et al*, 2011), we predict that the enhancement of nicotine CPP and anxiety-like behavior in $\alpha 6L9$ 'S mice will be at least partially reversed in $\alpha 6L9$ 'S- $\alpha 4KO$ mice, which would demonstrate that $\alpha 6$ assembles with $\alpha 4$ to promote behaviors relevant to nicotine addiction (reward and anxiety-like behavior in this case). It is unclear whether nicotine reward and anxiety-like behavior will return to WT levels to suggest that $\alpha 4\alpha 6\beta 2$ *nAChRs are solely responsible for $\alpha 6\beta 2$ *nAChR-mediated effects, or if $\alpha 6L9$ 'S- $\alpha 4KO$ mice will show intermediate levels of nicotine CPP and anxiety-like

behavior between WT and $\alpha 6\beta 2$ mice to suggest that these effects are modulated by both (non- $\alpha 4$) $\alpha 6\beta 2$ *nAChRs and $\alpha 4\alpha 6\beta 2$ *nAChRs.

Finally, Specific Aim 4 focuses on developing a mouse model of oral, operant EtOH self-administration to utilize to test the hypothesis that $\alpha 6\beta 2$ *nAChRs contribute to EtOH reinforcement in mice. Chapter 5 will describe the development of this mouse model of oral, operant EtOH self-administration, and Chapter 6 will describe studies utilizing this model in WT mice, mice with a 50% reduction in the $\alpha 6$ subunit ($\alpha 6$ HET), and $\alpha 6$ KO mice. We will also test these mice in rotorod and locomotor activity tests following 2 g/kg i.p. EtOH to assess possible genotype effects on sensitivity to EtOH. Based on evidence that α -Ctx MII blocks EtOH self-administration in rats (Kuzmin *et al*, 2009), we predict that $\alpha 6$ KO mice will fail to show EtOH reinforcement compared to WT mice to implicate $\alpha 6\beta 2$ *nAChRs in EtOH's reinforcing properties. It is less clear whether $\alpha 6$ HET mice will show EtOH reinforcement to indicate whether full expression of $\alpha 6\beta 2$ *nAChRs is required for EtOH reinforcement.

The mechanisms underlying nicotine and alcohol use and dependence have yet to be fully understood. As a consequence of our incomplete understanding of these substance use disorders, treatments have only proved modestly successful. Along with previous studies by our lab and others, the studies conducted in this dissertation will provide further insight into $\alpha 6\beta 2$ *nAChR contributions to the abuse-related effects of nicotine and alcohol.

Chapter 2 – Activation of $\alpha 6\beta 2$ subunit containing nicotinic acetylcholine receptors promotes in vivo nucleus accumbens dopamine release and nicotine reward

INTRODUCTION

Tobacco use remains the leading preventable cause of death worldwide (WHO, 2015). The psychoactive properties of nicotine support the use of tobacco products such as cigarettes, e-cigarettes, chew, and snus (NIDA, 2017). Nicotine elicits mesolimbic dopamine (DA) release from terminals in the nucleus accumbens (NAc) (Di Chiara *et al*, 1988), a process that supports nicotine reward and reinforcement (Corrigall *et al*, 1992; Sellings *et al*, 2008). α -Conotoxin MII (α -Ctx MII)-sensitive $\alpha 6\beta 2$ subunit containing nicotinic acetylcholine receptors ($\alpha 6\beta 2$ *nAChRs, *denotes possible assembly with other subunits) have the highest known sensitivity to nicotine and acetylcholine (ACh) (Exley *et al*, 2008; Kuryatov *et al*, 2011; Liu *et al*, 2012; Salminen *et al*, 2007; Salminen *et al*, 2004) and are selectively expressed in catecholaminergic nuclei of the brain, being particularly enriched on mesolimbic DA neurons (Champtiaux *et al*, 2002; Klink *et al*, 2001; Le Novere *et al*, 1996) where they promote neuronal activation and DA release (Champtiaux *et al*, 2003; Exley *et al*, 2011; Gotti *et al*, 2010; Grady *et al*, 2002; Kulak *et al*, 1997; Liu *et al*, 2012; Salminen *et al*, 2007; Salminen *et al*, 2004; Wickham *et al*, 2013; Zhao-Shea *et al*, 2011). Due to their high sensitivity and restricted expression pattern, $\alpha 6\beta 2$ *nAChRs may provide a more selective therapeutic target for smoking

cessation. In fact, studies utilizing selective antagonists and null mutant strategies demonstrate that mesolimbic $\alpha 6\beta 2$ *nAChRs are necessary for nicotine reward and reinforcement (Brunzell *et al*, 2010; Exley *et al*, 2011; Gotti *et al*, 2010; Pons *et al*, 2008; Sanjakdar *et al*, 2015).

Due to poor function in heterologous expression systems however, little progress has been made to identify $\alpha 6\beta 2$ *nAChR-selective agonists that can demonstrate behaviors and physiological effects for which $\alpha 6\beta 2$ *nAChRs are sufficient (Drenan *et al*, 2008). The development of $\alpha 6\beta 2$ *nAChR gain-of-function ($\alpha 6L9$ 'S) mice has provided a complementary research approach to null mutant strategies to test the sufficiency of $\alpha 6\beta 2$ *nAChRs. $\alpha 6L9$ 'S mice possess a leucine to serine single point mutation in the M2 pore-forming region of the $\alpha 6$ subunit that renders their $\alpha 6\beta 2$ *nAChRs hypersensitive to ACh and nicotine (Drenan *et al*, 2008); nicotine doses that are sub-threshold for activation of native nAChRs can selectively activate $\alpha 6\beta 2$ *nAChRs, allowing for assessment of $\alpha 6\beta 2$ *nAChR function in isolation of other nAChRs. *In vitro* slice studies reveal that the $\alpha 6\beta 2$ *nAChR gain-of-function mutation produces leftward shifts in ACh- and nicotine-stimulated VTA DA neuron activation and striatal DA release (Cohen *et al*, 2012; Drenan *et al*, 2010; Drenan *et al*, 2008; Engle *et al*, 2013; Powers *et al*, 2013; Wang *et al*, 2014b). Further, these mice exhibit locomotor hyperactivity in response to nicotine, which is blocked by D₁ (SCH 23390) and D₂ (sulpiride) receptor antagonists (Berry *et al*, 2015; Cohen *et al*, 2012; Drenan *et al*, 2010; Drenan *et al*, 2008). The DA-dependent nature of this effect is supported by previous data showing that locomotor activation by nicotine requires $\beta 2$ *nAChR-associated DA release (King *et al*, 2004b). These data suggest that activation of $\alpha 6\beta 2$ *nAChRs is sufficient for mesolimbic DA neuron activity, however, no studies to date have assessed this *in vivo* or determined if $\alpha 6\beta 2$ *nAChR activity is sufficient to support nicotine reward. Being that cholinergic tone from pedunculo pontine and laterodorsal

tegmental projections to the VTA (Blaha *et al*, 1996; Lanca *et al*, 2000; Lester *et al*, 2008; Xiao *et al*, 2016) and striatal cholinergic interneurons (Berlanga *et al*, 2003; Cachope *et al*, 2012; Rice *et al*, 2004; Threlfell *et al*, 2012; Zhang and Sulzer, 2004; Zhou *et al*, 2001) support DA release and drug reward and reinforcement, we hypothesized that elevated cholinergic activity at $\alpha 6\beta 2^*$ nAChRs in $\alpha 6L9^*$ S mice would promote *in vivo* NAc DA release and associated nicotine reward behavior.

The present microdialysis experiment demonstrates that NAc DA release is significantly elevated in awake behaving $\alpha 6L9^*$ S mice compared to WT littermates and demonstrate that doses sub-threshold to produce nicotine CPP in WT mice support nicotine reward behavior in $\alpha 6L9^*$ S mice. To identify neuroanatomical loci within the mesolimbic DA pathway where $\alpha 6\beta 2^*$ nAChRs support nicotine reward behavior, we expanded on previous findings (Sanjakdar *et al*, 2015) to demonstrate that local antagonism of $\alpha 6\beta 2^*$ nAChRs in the NAc shell subdivision blocked nicotine CPP in WT mice. In $\alpha 6L9^*$ S mice, however, which express global elevations in $\alpha 6\beta 2^*$ nAChR sensitivity, local antagonism of VTA $\alpha 6\beta 2^*$ nAChRs was required to block nicotine CPP. We further report that VTA infusion of α -Ctx MII significantly attenuated NAc DA release in these mice.

MATERIALS AND METHODS

Subjects

A total of 256 adult, male C57BL/6J wild type (WT) and $\alpha 6\beta 2^*$ nAChR gain-of-function mice ($\alpha 6L9^*$ S) backcrossed > 10 generations on a C57BL/6J background were used in these studies. A single allele for the $\alpha 6L9^*$ S transgene produces the hypersensitive $\alpha 6\beta 2^*$ nAChR phenotype (Drenan *et al*, 2008) so that breedings to WT mice resulted in 50% $\alpha 6L9^*$ S and 50% WT

offspring. Mice were housed in polycarbonate cages with Teklad corncob bedding (catalog number 7092) in a temperature- and humidity-controlled vivarium on a 12 h light/dark cycle (lights on at 6:00 a.m.). Mice had access to food (Teklad LM-485 Mouse/Rat Sterilizable Diet, catalog number 7012) and water *ad libitum*. Experimental protocols were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. All animals were treated according to NIH Guidelines for the Care and Use of Laboratory Animals.

Drugs

Nicotine hydrogen tartrate salt (Sigma-Aldrich, St. Louis, MO) and (-)-Cocaine HCl (National Institute on Drug Abuse Drug Supply Program, Bethesda, Maryland) were dissolved in 0.9% sterile saline vehicle (SAL). Injection volumes were 0.1 ml/30 g for nicotine and 0.3 ml/30 g for cocaine. Nicotine doses are expressed by free base weight and cocaine doses are expressed by salt weight. α -Ctx MII was synthesized as previously described (Cartier *et al*, 1996). Intra-cerebral infusions of α -Ctx MII were dissolved in SAL or artificial cerebrospinal fluid (aCSF) vehicle (VEH) and administered at 0.5 μ l/min for a total volume of 0.25 μ l per side.

***In vivo* microdialysis in awake, behaving mice**

WT ($n=4$) and $\alpha 6L9'S$ ($n=4$) mice were anesthetized with 2.5% isoflurane and 3 liter/min of oxygen for surgical implantation of a unilateral guide cannula (CXG-4, Eicom, San Diego, CA) targeting the NAc (+1.5 mm AP, +/-0.5 mm Lat, -4.0 DV in reference to bregma). The surgical area was shaved and cleaned with betadine (Purdue Products, Stamford, CT) and 70% ethanol. Guide cannulae were secured with dental cement anchored to the skull with jeweler's screws. To determine if $\alpha 6L9'S$ mice show enhanced NAc DA release *in vivo*, a microdialysis probe with an artificial cellulose cuprophan membrane (5 mm long, CX-I-4-1, Eicom) designed to extend 1

mm beyond the guide into the NAc was carefully inserted into the guide cannula of awake, gently restrained mice. Probes were connected to a two-channel liquid swivel (TCS-2-23, Eicom) with Teflon tubing (JT-10, Eicom) and perfused with aCSF (147 mM NaCl, 2.8 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂) at 1.0 µl/min.

Dialysate samples were collected at 15 min intervals into a 50 µl injector loop using an online autoinjector (EAS-20s, Eicom) and immediately analyzed for DA concentrations by high-pressure liquid chromatography (HPLC) coupled to electrochemical detection (HTEC-500, Eicom). Mobile phase consisted of 1.5% methanol (EMD, Gibbstown, NJ, USA), 100 mM phosphate buffer (Sigma Chemicals, St. Louis, MO, USA), 500 mg/L 1-decane sodium sulfonate (TCI America, Montgomeryville, PA, USA), and 50 mg/L EDTA-2Na⁺ (Dojindo Laboratories, Kumamoto, Japan). DA was separated using a C₁₈-reverse phase column (PP-ODS II, Eicom) and detected using a graphite working electrode and a Ag⁺ vs. AgCl reference electrode with an applied potential of +450 mV. DA was identified according to the characteristic standard solution retention time, and concentrations were quantified by comparison with peak heights of the standard concentration curve generated prior to each test. Once DA stabilized, three samples were collected to quantify basal DA. At the end of each test, mice received 20 mg/kg cocaine intraperitoneal (i.p.), and three additional samples were collected to assess sampling site sensitivity.

To specifically assess VTA $\alpha 6\beta 2$ *nAChR contributions to NAc DA release, a separate cohort of $\alpha 6L9$ 'S mice were additionally implanted with 26-gauge bilateral guide cannula that enabled infusion of the $\alpha 6\beta 2$ *nAChR-selective antagonist, α -CTX MII into the VTA (-3.4 mm AP, ± 0.5 mm Lat, -2.9 mm DV, with 1.25 mm infusion cannula projection) prior to collection of NAc dialysate. Initial studies implanted the guide cannula at -3.4 mm DV, but most of these

subjects were not viable after surgery. Raising the guide cannula up to -2.9 mm DV with a longer infusion cannula projecting to -3.4 mm for micro-infusions increased viability after surgery. A microdialysis probe was inserted into the NAc guide cannula as described above. Once DA stabilized, three baseline samples were collected, followed by intra-VTA infusions of aCSF ($n=4$) or 10 pmol α -Ctx MII ($n=5$) whereupon 3 additional dialysate samples were collected for comparison to baseline DA levels. All microdialysis data were quantified with eDAQ PowerChrom software (eDAQ, Colorado Springs, CO).

Unbiased Nicotine Conditioned Place Preference (CPP)

$\alpha 6\beta 2$ 'S mice ($n=13-20$ /dose) were compared to their WT littermates ($n=14-19$ /dose) to determine if $\alpha 6\beta 2$ *nAChR gain-of-function would shift nicotine place conditioning. CPP was conducted in Med Associates mouse place conditioning chambers (Med Associates, St. Albans, VT). The CPP apparatus consisted of two unique but equally preferred conditioning chambers with distinct floors (parallel bars or grid) and walls (black or striped) separated by retractable doors and a small neutral (grey) chamber with Plexiglas floor. CPP training took place twice a day between the hours of 10:00 a.m. and 12:00 p.m. and 1:00 p.m. and 3:00 p.m., with baseline and testing taking place at an intermediate timepoint on the day prior to and following training, respectively. During baseline, mice received i.p. SAL prior to being placed in the neutral chamber with doors retracted to allow free exploration of the apparatus for 15 min. The following 3 days during the a.m. training session, mice received i.p. SAL prior to 30 min confinement in the saline-paired chamber. During the p.m. session, mice received i.p. nicotine (0, 0.03 or 0.1 mg/kg) prior to 30 min of confinement in the opposite, nicotine-paired conditioning chamber. Control mice received i.p. SAL prior to exposure to both chambers. Assignment of conditioning chamber was counterbalanced with mice showing overall similar preferences across

chambers. During test, mice received i.p. SAL and were placed in the chamber as during baseline and allowed to explore the entire apparatus for 15 min. Photobeams detected movement and data were recorded using Med-PC IV software (Med Associates, St. Albans, VT).

Nicotine CPP following NAc shell or VTA infusion of α -Ctx MII

To assess the neuroanatomical location of $\alpha 6\beta 2^*$ nAChR contributions to nicotine reward, a separate cohort of mice received local infusions of 10 pmol α -Ctx MII or VEH prior to the p.m. nicotine conditioning sessions as described above; all mice received a VEH infusion during the a.m. session. Mice were surgically implanted with a 26-gauge bilateral guide cannula (Plastics One, Roanoke, VA) targeting the NAc shell (+1.5 mm AP, \pm 0.5 mm Lat, -4.25 DV with 0.5 mm infusion cannula projection) or VTA as described above. Infusions were delivered to awake, gently restrained mice via a micro infusion pump (PHD 2000, Harvard Apparatus, Holliston, MA) through an internal cannula attached to Hamilton syringes and PE 20 tubing (Stoelting, Wood Dale, IL). A 2 min post-infusion wait period allowed for drug diffusion and prevented backflow through the guide cannula.

Contextual Threat Conditioning

To assess if genotypic differences in nicotine CPP were due to generalized changes in contextual learning, contextual threat conditioning was conducted in mouse operant conditioning chambers (Med Associates, St. Albans, VT). Training occurred during a 5.5 min session with WT ($n=14$) and $\alpha 6L9^*S$ ($n=13$) mice placed in the chamber for 2 min before presentation of a 30 s light plus tone cue that terminated with a 2 s, 0.5 mA footshock. This sequence was repeated followed by a 30 s post-shock period. The next day, mice were placed in the conditioning chamber for 5.5 min in the absence of the cue and footshock to test for context-specific freezing. Freezing (absence of

voluntary movement except for respiration) was measured using Anymaze tracking software (Stoelting, Wood Dale, IL) and confirmed by a blind experimenter. Freezing during the first 2 minutes of the training session (before any footshock) was compared to freezing during the corresponding first 2 minutes of the test session (24h after Pavlovian fear conditioning) to assess whether mice learned to associate the context with the previous footshocks received during training.

Histology

Confirmation of cannula placement was confirmed following behavioral and neurochemistry assays. Mice were trans-cardially perfused with 4% paraformaldehyde (PFA) with brains removed and post-fixed in PFA for 24 h followed by storage in 30% sucrose until sectioned. 20 μ m coronal sections were collected using a Cryostat (Leica, Buffalo Grove, IL). Slices were mounted on Colorfrost Plus positively charged microscope slides (VWR, Radnor, PA) and stained with cresyl violet.

Statistical Analysis

All statistical analyses were performed using SPSS. Basal DA levels were averaged across the three timepoints and compared between genotypes using a two-tailed t-test. CPP data were analyzed using a 2x3 (genotype x nicotine dose) ANOVA, followed by post hoc two-tailed t-tests. For contextual threat conditioning, percent time freezing during the pre-shock period (first 2 min) was compared between the training and test sessions and was analyzed using a 2x2 (genotype x session) repeated measures ANOVA. Locomotor data collected during CPP were analyzed using a 2x2x3 (CPP training session x genotype x nicotine dose) repeated measures ANOVA, followed by post hoc paired t-tests. For NAc shell infusion CPP experiments, data

were analyzed using a 2x3 (infusion dose x nicotine dose) ANOVA, followed by post hoc two-tailed t-tests. For the VTA infusion CPP experiments, data were analyzed using a 2x2 (infusion dose x nicotine dose) ANOVA, followed by post hoc two-tailed t-tests. DA levels in response to VTA α -Ctx MII were averaged across the three timepoints and compared to VEH controls using a two-tailed t-test. The criterion for significance was set at $p < 0.05$. Mice with off-target guide cannula placement or data points more than 2 standard deviations from the mean were considered outliers and excluded from analysis.

RESULTS

***In vivo* basal NAc DA levels and nicotine CPP are augmented in α 6L9'S mice**

Previous *in vitro* studies report that ACh-stimulated VTA DA neuron firing and ACh-elicited or electrically evoked DA release is enhanced in α 6L9'S striatal slices or synaptosomes (Cohen *et al*, 2012; Drenan *et al*, 2010; Drenan *et al*, 2008; Engle *et al*, 2013; Powers *et al*, 2013; Wang *et al*, 2014b). In support of these *in vitro* findings, we observed significantly elevated basal levels of NAc DA in awake, behaving α 6L9'S mice compared to WT controls ($t_6 = -2.67$, $p = 0.04$; Figure 2.1a). 20 mg/kg cocaine increased NAc DA levels similarly in WT and α 6L9S mice, indicating tissue sampling site sensitivity (Figure 1A, inset). These *in vivo* data provide further evidence that ACh activation of α 6 β 2*nAChRs is sufficient to enhance basal NAc DA tone.

To determine if enhanced function of α 6 β 2*nAChRs also supports nicotine reward behavior, α 6L9'S mice were compared to WT littermates using an unbiased nicotine CPP task (Figure 2.1b). A significant interaction of genotype by nicotine dose ($F_{2,97} = 3.75$, $p = 0.027$) revealed a leftward shift in the amount of time α 6L9'S mice spent in the nicotine-paired chamber; α 6L9'S mice showed nicotine CPP at 0.03 mg/kg ($t_{37} = -4.32$, $p < 0.001$) and 0.1 mg/kg

i.p. nicotine ($t_{31}=-4.28, p<0.001$), while WT mice showed nicotine CPP only at 0.1 mg/kg i.p. nicotine ($t_{30}=-2.196, p=0.036$), as shown previously (Brunzell *et al*, 2009a; Mineur *et al*, 2009). These findings suggests that $\alpha6\beta2$ *nAChR stimulation promotes nicotine reward, but it is possible that $\alpha6L9$ 'S mice showed elevated contextual learning rather than a sensitization to nicotine reward per se. To test this latter possibility, mice were tested using a Pavlovian threat conditioning assay. For contextual threat conditioning (Figure 2.1c), there was a main effect of session ($F_{1,25}=37.089, p<0.001$), where % time freezing in a chamber under the same context where mice had previously received 2 mild footshocks was greater during the first 2 minutes of the test compared to the first 2 minutes of the training, indicating that the mice learned to associate the context with the footshocks. There was no main effect of genotype ($F_{1,25}=1.772, p=0.195$) and no interaction of session by genotype ($F_{1,25}=2.322, p=0.140$) to suggest that genotype had no impact on threat conditioning. Thus, elevated nicotine contextual reward learning did not appear to generalize to contextual threat learning.

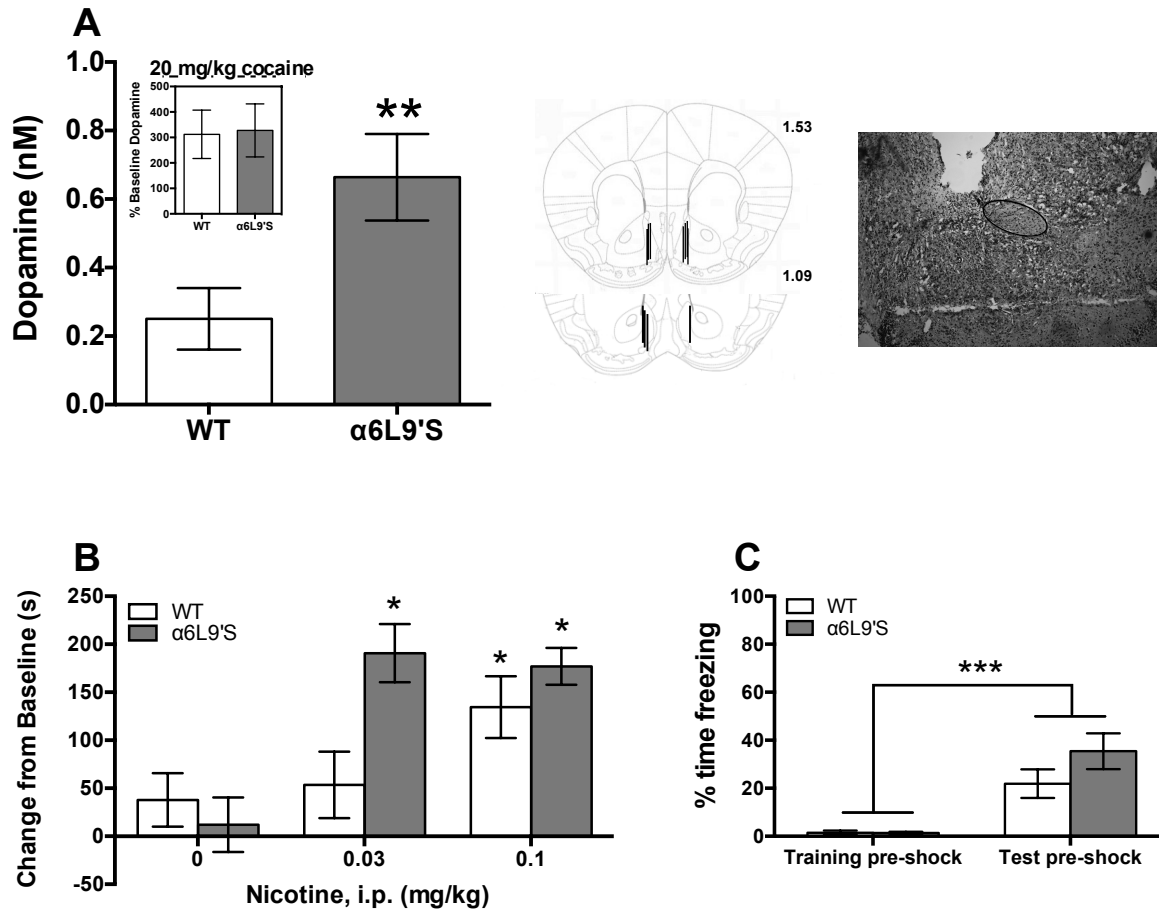


Figure 2.1 – *In vivo* basal nucleus accumbens (NAc) dopamine (DA) levels and nicotine reward behavior, but not contextual threat conditioning, are augmented in α6L9'S mice. a) Left, Basal NAc DA levels in WT ($n=4$) and α6L9'S mice ($n=4$) (inset represents % baseline DA in response to 20 mg/kg i.p. cocaine). Right schematic diagram of the NAc shell 1.09 to 1.5 from Bregma (adapted from Paxinos and Franklin) and representative photomicrograph depicting the tip of the guide cannula from which the 1 mm long microdialysis probe membrane protruded (4X magnification). Black lines on schematic diagram represent 1 mm long microdialysis probe membrane placements within the NAc and the black circle on photomicrograph highlights the anterior commissure. **b)** Nicotine CPP expressed as change from baseline time spent in the nicotine-paired chamber for saline-injected (0 mg/kg nicotine-WT, $n=14$ and α6L9'S, $n=20$) and nicotine-injected mice (0.03 mg/kg-WT, $n=19$ and α6L9'S, $n=19$; 0.1 mg/kg-WT, $n=18$ and α6L9'S, $n=13$). **c)** Contextual threat conditioning expressed as percent time freezing during the training and test session for the 2 min time period corresponding to the time period before first footshock during training (pre-shock) in WT ($n=14$) and α6L9'S mice ($n=13$). ** $p<0.05$ vs. WT; * $p<0.05$ vs. saline of same genotype; *** $p<0.001$ vs. training pre-shock. Data are expressed as mean \pm SEM.

α 6L9'S mice exhibit hyperactive nicotine-stimulated locomotor activity

In addition to nicotine reward behavior, α 6L9'S mice displayed an enhanced locomotor response to nicotine. We compared locomotor activity within-subject during a.m. (saline-paired) versus p.m. (nicotine-paired) CPP training sessions. A 2x2x3 repeated measures ANOVA revealed a 3-way interaction of CPP training session by genotype by nicotine dose ($F_{2,30}=17.493, p<0.001$). Consistent with previous data (Berry *et al*, 2015; Cohen *et al*, 2012; Drenan *et al*, 2010; Drenan *et al*, 2008), nicotine stimulated locomotor activity in α 6L9'S (Figure 2.2b), but not WT mice (Figure 2.2a) at 0.03 ($t_5=-4.17, p=0.009$) and 0.1 mg/kg i.p. nicotine ($t_4=-6.922, p=0.002$). We did not observe differences in locomotor activity of α 6L9'S compared to WT littermates that received saline injections.

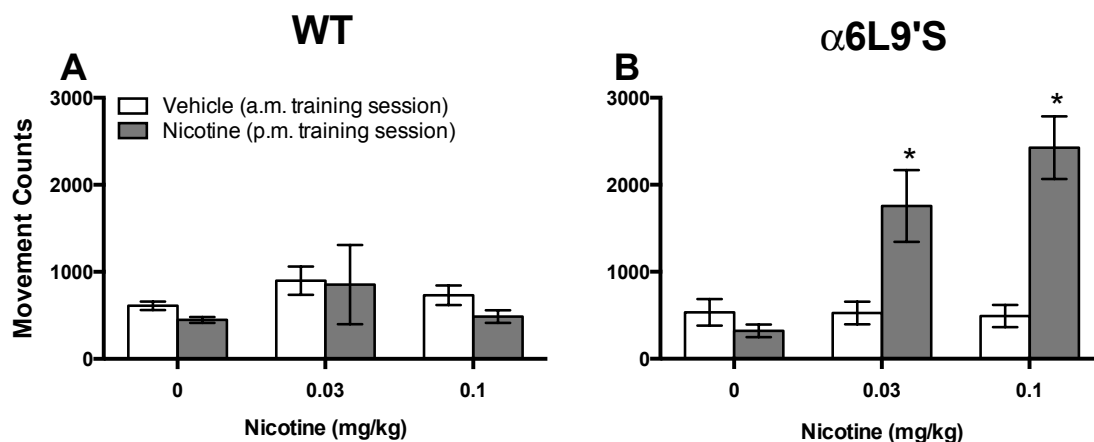


Figure 2.2 – α6L9'S mice exhibit enhanced nicotine-stimulated locomotor activity. a) Locomotor activity expressed as movement counts in WT mice following 0 mg/kg ($n=9$), 0.03 mg/kg ($n=4$), and 0.1 mg/kg nicotine ($n=7$) compared within-subject to the saline-paired a.m. CPP training sessions. **b)** Locomotor activity expressed as movement counts in α6L9'S mice following 0 mg/kg ($n=5$), 0.03 mg/kg ($n=6$), and 0.1 mg/kg nicotine ($n=5$) compared within-subject to the saline-paired a.m. CPP training sessions. * $p<0.01$ vs. vehicle (a.m. training session). Data are expressed as mean \pm SEM.

Nicotine CPP is blocked by intra-NAc shell α -Ctx MII in WT, but not α 6L9'S mice

To investigate the neuroanatomical loci of α 6 β 2*nAChR contributions to nicotine reward behavior, WT and α 6L9'S mice received intra-NAc shell infusions of VEH or the α 6 β 2*nAChR-selective antagonist, α -Ctx MII prior to systemic nicotine injection during CPP training. Given that WT and α 6L9'S behaved differently in the above CPP task, we analyzed the effect of intra-NAc shell α -Ctx MII on WT and α 6 L9'S nicotine CPP separately. In WT mice, there was a significant interaction of α -Ctx MII infusion by nicotine dose ($F_{3,48}=3.818, p=0.016$). Intra-NAc shell infusion of α -Ctx MII significantly reduced nicotine CPP at 0.1 mg/kg i.p. nicotine ($t_{11}=2.53, p=0.03$), a rewarding dose in WT mice (Figure 2.3a), suggesting that α 6 β 2*nAChRs on terminals in the NAc shell promote nicotine reward. α -Ctx MII also induced an apparent conditioned place aversion at 0.03 mg/kg i.p. nicotine ($t_{10}=2.81, p=0.02$), however, α -Ctx MII did not appear to be aversive on its own since mice receiving i.p. saline did not show reductions in preference for the chamber paired with α -Ctx MII infusion. By contrast, intra-NAc shell α -Ctx MII did not impact nicotine CPP in α 6L9'S mice (Figure 2.3b), suggesting that α 6 β 2*nAChRs elsewhere in the brain were driving nicotine reward behavior of α 6L9'S mice.

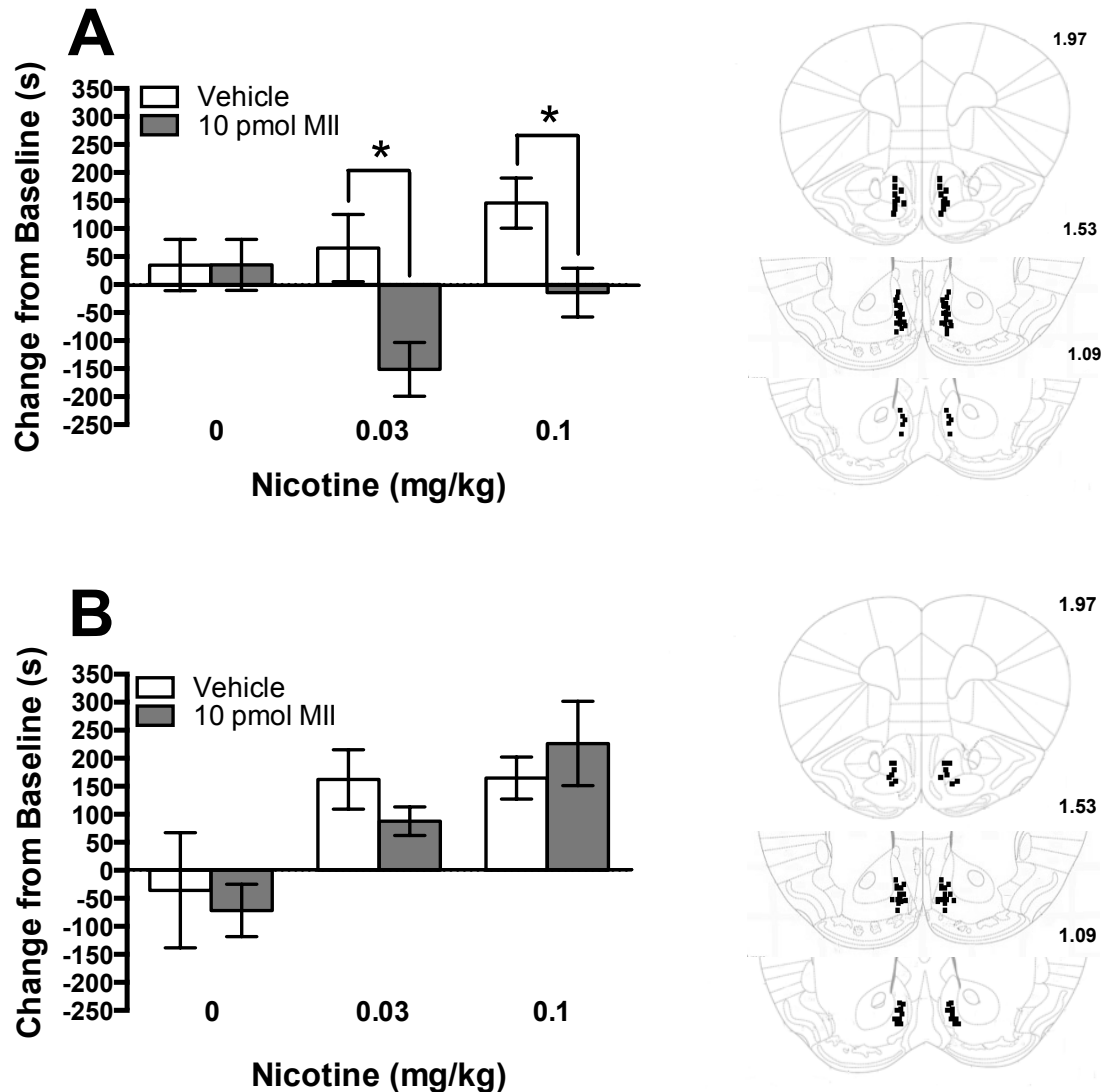


Figure 2.3 – Nucleus accumbens (NAc) shell α -Conotoxin MII (α -Ctx MII) blocks nicotine CPP in WT, but not α 6L9'S mice. a) Left, Nicotine CPP with NAc shell saline vehicle (VEH) or α -Ctx MII pretreatment expressed as change from baseline time spent in the nicotine-paired chamber in WT mice at 0 mg/kg (VEH: $n=9$; α -Ctx MII: $n=10$), 0.03 mg/kg (VEH: $n=6$; α -Ctx MII: $n=6$), and 0.1 mg/kg i.p. nicotine (VEH: $n=7$; α -Ctx MII: $n=6$). Right, schematic diagram of the NAc shell 1.09 to 1.97 from Bregma (adapted from Paxinos and Franklin). Black dots represent guide cannula placements within the NAc shell. **b)** Left, Nicotine CPP with NAc shell VEH or α -Ctx MII pretreatment expressed as change from baseline time spent in the nicotine-paired chamber in α 6L9'S mice at 0 mg/kg (VEH: $n=6$; α -Ctx MII: $n=8$), 0.03 mg/kg (VEH: $n=10$; α -Ctx MII: $n=8$), and 0.1 mg/kg i.p. nicotine (VEH: $n=5$; α -Ctx MII: $n=4$). Right, schematic diagram of the NAc shell 1.09 to 1.97 from Bregma (adapted from Paxinos and Franklin). Black dots represent guide cannula placements. * $p<0.05$ vs. VEH. Data are expressed as mean \pm SEM.

Intra-VTA α -Ctx MII blocks enhanced nicotine reward behavior and attenuates elevated basal NAc DA release in α 6L9'S mice

To determine if VTA α 6 β 2*nAChRs promote enhanced nicotine reward, α 6L9'S mice received intra-VTA infusions of VEH or α -Ctx MII prior to systemic nicotine injection during CPP training. A 2x2 ANOVA revealed a significant interaction of α -Ctx MII infusion by nicotine dose ($F_{1,26}=4.238, p=0.05$). Intra-VTA α -Ctx MII blocked nicotine CPP at 0.03 mg/kg i.p. nicotine in a naïve cohort of α 6L9'S mice ($t_{14}=3.228, p=0.006$; Figure 2.4a), suggesting that α 6 β 2*nAChRs in the VTA support enhanced nicotine reward. Data are not shown for WT VTA-infused mice due to a failure of vehicle-infused subjects to show nicotine CPP, precluding assessment of intra-VTA α -Ctx MII effect on nicotine CPP.

To determine if VTA α 6 β 2*nAChRs also modulate enhanced α 6L9'S basal NAc DA release, α 6L9'S mice received intra-VTA infusions of aCSF or α -Ctx MII during microdialysis. Intra-VTA infusion of α -Ctx MII similarly attenuated NAc DA levels compared to vehicle controls ($t_7=3.317, p=0.01$; Figure 2.4b), suggesting that, like nicotine reward, stimulation of α 6 β 2*nAChRs on VTA DA neuron soma promotes NAc DA release.

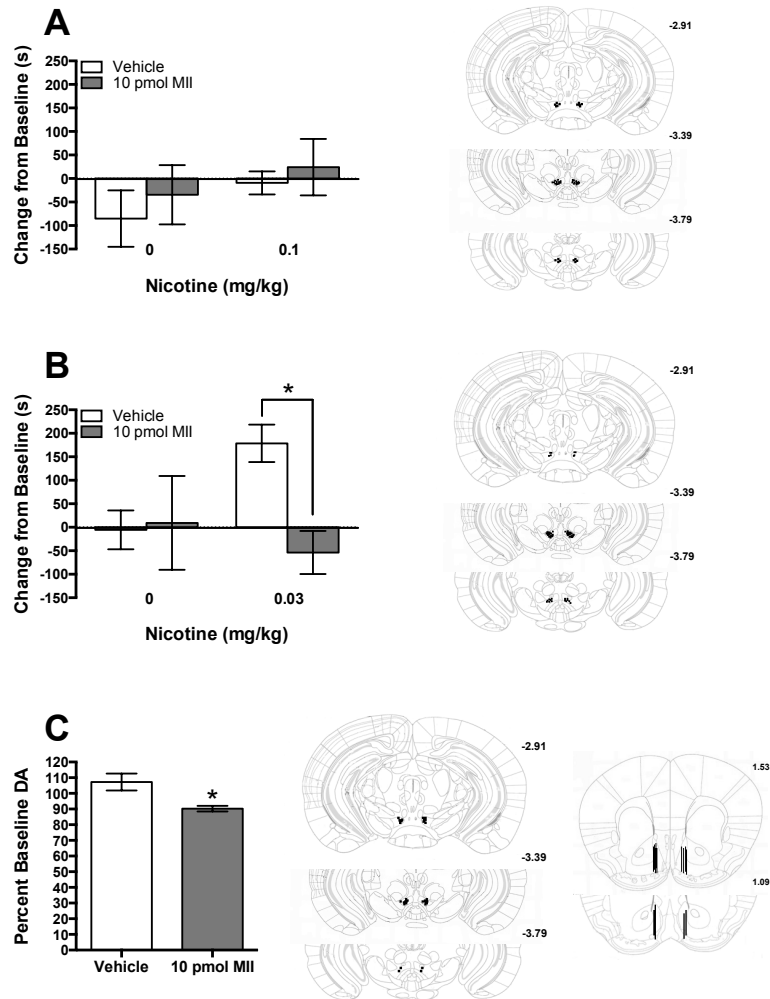


Figure 2.4 – Ventral tegmental area (VTA) α -Conotoxin MII (α -Ctx MII) blocks nicotine CPP and attenuates nucleus accumbens (NAc) dopamine (DA) levels in $\alpha 6L9^S$ mice. a) Left, Nicotine CPP with VTA saline or aCSF vehicle (VEH) or α -Ctx MII pretreatment expressed as change from baseline time spent in the nicotine-paired chamber in WT mice at 0 mg/kg (VEH: $n=10$; α -Ctx MII: $n=11$) or 0.1 mg/kg i.p. nicotine (VEH: $n=7$; α -Ctx MII: $n=7$). Right, schematic diagram of the VTA -2.91 to -3.79 from Bregma (adapted from Paxinos and Franklin). Black dots represent guide cannula placements within the VTA. **b)** Left, Nicotine CPP with VTA saline or aCSF vehicle (VEH) or α -Ctx MII pretreatment expressed as change from baseline time spent in the nicotine-paired chamber in $\alpha 6L9^S$ mice at 0 mg/kg (VEH: $n=7$; α -Ctx MII: $n=7$) or 0.03 mg/kg i.p. nicotine (VEH: $n=8$; α -Ctx MII: $n=8$). Right, schematic diagram of the VTA -2.91 to -3.79 from Bregma (adapted from Paxinos and Franklin). Black dots represent guide cannula placements within the VTA. **c)** NAc DA levels expressed as percent baseline DA following VTA infusion of aCSF vehicle ($n=4$) or 10 pmol α -Ctx MII ($n=5$). Right, schematic diagram of the VTA -2.91 to -3.79 from Bregma and the NAc 1.09 to 1.53 from Bregma (adapted from Paxinos and Franklin). Black dots represent guide cannula placements within the VTA and black lines on the schematic diagram represent 1 mm long microdialysis probe membrane placements within the NAc. * $p<0.05$ vs. vehicle. Data are expressed as mean \pm SEM.

DISCUSSION

Complementary to null mutant strategies, the development of $\alpha 6\beta 2^*$ nAChR gain-of-function ($\alpha 6L9^*S$) mice (Drenan *et al*, 2008) has provided a means of testing behavior under conditions where $\alpha 6\beta 2^*$ nAChR function is amplified and isolated. Our *in vivo* microdialysis studies support $\alpha 6L9^*S$ *in vitro* data demonstrating elevated ACh-elicited striatal DA release (Cohen *et al*, 2012; Wang *et al*, 2014b). $\alpha 6L9^*S$ mice showed higher basal NAc DA levels compared to WT littermate controls, suggesting hyper-excitability of $\alpha 6\beta 2^*$ nAChRs in response to endogenous ACh. It is unlikely that this effect resulted from altered DA transporter (DAT) function or DA turnover, as $\alpha 6L9^*S$ DAT levels are similar to or higher than in WT mice with DA turnover unchanged (Drenan *et al*, 2010; Wang *et al*, 2014). These *in vivo* studies provide further evidence that activation of $\alpha 6\beta 2^*$ nAChRs by ACh is sufficient to promote NAc DA release.

$\alpha 6L9^*S$ mice also showed leftward shifts in the dose response curve for nicotine CPP; $\alpha 6L9^*S$ mice showed nicotine CPP at doses sub-threshold for observation of this behavior in WT mice. This finding expands on previous data implicating the necessity of $\alpha 6\beta 2^*$ nAChRs for nicotine reward (Jackson *et al*, 2009; Sanjakdar *et al*, 2015), to suggest that $\alpha 6\beta 2^*$ nAChR activation is sufficient for this behavior. Similar findings have been reported in $\alpha 4\beta 2^*$ nAChR gain-of-function ($\alpha 4L9^*A$) mice (Tapper *et al*, 2004), raising the possibility that the high sensitivity $\alpha 4\alpha 6\beta 2^*$ nAChRs regulate nicotine reward (Liu *et al*, 2012), a hypothesis that warrants further study. Independent groups of $\alpha 6L9^*S$ mice showed augmented basal NAc DA levels, suggesting that changes in cholinergic signaling at $\alpha 6\beta 2^*$ nAChRs may enhance both NAc DA tone and nicotine reward (but see Laviolette and van der Kooy, 2003). While these data are only correlative, baseline striatal DA tone appears to be a risk factor for addiction (Volkow *et*

al, 2012). In PET studies, human smokers show lower baseline DA receptor availability, reflective of increases in DA release, in the striatum and putamen compared to non-smokers (Brown *et al*, 2012; Fehr *et al*, 2008). Thus, it is possible that $\alpha 6L9'S$ mice exhibit enhanced nicotine reward behavior due to the observed increase in basal DA levels.

$\alpha 6L9'S$ mice also showed an enhanced response to nicotine's locomotor stimulating effects, a behavior dependent on $\beta 2*nAChR$ -regulation of DA activity (King *et al*, 2004). Consistent with previous findings demonstrating that nicotine strongly activates locomotor activity in $\alpha 6L9'S$, but not WT mice (Berry *et al*, 2015; Cohen *et al*, 2012; Drenan *et al*, 2010; Drenan *et al*, 2008), these data support that $\alpha 6\beta 2*nAChRs$ regulate DA-mediated locomotor activating effects of nicotine. $\alpha 6L9'S$ mice are also hyperactive compared to WT mice under basal conditions with the lights off during the dark cycle (Cohen *et al*, 2012; Drenan *et al*, 2010; Drenan *et al*, 2008), an effect not seen in our studies where locomotor activity was measured with the lights on during the light cycle, conditions where mice are generally less active.

In vitro cyclic voltammetry studies show that tonic ACh released from cholinergic interneurons acts at $\beta 2*nAChRs$ on DA terminals in the NAc to control DA release independent of VTA DA neuron firing (Cachope *et al*, 2012; Threlfell *et al*, 2012). ACh released from cholinergic interneurons also acts on DA terminal $\beta 2*nAChRs$ to maintain a high probability of action potential-evoked DA release (Zhou *et al*, 2001). Moreover, α -Ctx MII or $\alpha 6$ genetic deletion reduces nicotine-stimulated NAc DA release (Champtiaux *et al*, 2003; Grady *et al*, 2002; Kulak *et al*, 1997; Salminen *et al*, 2004). Given the ability of $\alpha 6\beta 2*nAChRs$ on DA terminals to support NAc DA release (Champtiaux *et al*, 2003; Grady *et al*, 2002; Kulak *et al*, 1997; Salminen *et al*, 2004) and the putative role of NAc DA release in nicotine reinforcement

and reward (Corrigall *et al*, 1992; Sellings *et al*, 2008), we sought to determine if NAc $\alpha 6\beta 2^*$ nAChRs support nicotine reward.

Intra-NAc α -Ctx MII blocked nicotine CPP in previous mouse studies (Sanjakdar *et al*, 2015). The NAc is divided into two distinct regions, the NAc core and shell. These subdivisions differ anatomically, have unique neuronal connections, and are thought to play different roles in regard to abused drugs, including nicotine (Balfour, 2015). We report that $\alpha 6\beta 2^*$ nAChRs in the NAc shell subdivision are involved in nicotine reward. This finding in mice is consistent with our previous data showing that NAc shell $\alpha 6\beta 2^*$ nAChRs regulate nicotine reinforcement in rats (Brunzell *et al*, 2010).

Intra-NAc shell α -Ctx MII did not block nicotine CPP in $\alpha 6L9$ 'S mice, suggesting that $\alpha 6\beta 2^*$ nAChRs at another neuroanatomical locus contributed to elevated nicotine reward behavior. In fact, intra-VTA α -Ctx MII blocked nicotine CPP in $\alpha 6L9$ 'S mice, suggesting that the hyperactivity of $\alpha 6L9$ 'S VTA DA neurons may have overshadowed effects of NAc shell $\alpha 6\beta 2^*$ nAChRs on reward behavior in $\alpha 6L9$ 'S mice. A role for VTA $\alpha 6\beta 2^*$ nAChRs in nicotine reinforcement has been established (Gotti *et al*, 2010; Pons *et al*, 2008), but the present data are the first to our knowledge to show that VTA $\alpha 6\beta 2^*$ nAChRs modulate nicotine reward behavior in mice. These intra-VTA infusion experiments were also attempted in WT mice. However, WT VTA-infused mice failed to show nicotine CPP following vehicle infusions, precluding observation of intra-VTA α -Ctx MII effects on nicotine CPP. Normally, repeated micro-infusions are well tolerated in mice. However, some mice became lethargic or were unable to ambulate properly upon receiving VTA infusions, which could explain the failure to show nicotine CPP in these mice.

A preponderance of evidence suggests that VTA $\alpha6\beta2$ *nAChRs support DA release. ACh and nicotine fail to increase VTA DA neuron firing in $\alpha6$ null mutant mice or following intra-VTA α -Ctx MII (Champiaux *et al*, 2003; Liu *et al*, 2012; Zhao-Shea *et al*, 2011). Intra-VTA α -Ctx MII also decreases nicotine-stimulated and evoked phasic DA release (Gotti *et al*, 2010; Wickham *et al*, 2013). Studies show that $\alpha6\beta2$ *nAChR gain-of-function mutation results in enhancement of VTA DA neuron firing and NAc DA release *in vitro* (Cohen *et al*, 2012; Drenan *et al*, 2010; Drenan *et al*, 2008; Engle *et al*, 2013; Powers *et al*, 2013; Wang *et al*, 2014). We observed that intra-VTA infusion of α -Ctx MII reduced $\alpha6L9$ 'S NAc DA levels in awake, behaving mice; these findings suggest that elevations of cholinergic tone at VTA $\alpha6\beta2$ *nAChRs supports NAc DA release in these mice. Given the established role of DA in nicotine reward (Balfour, 2015; Sellings *et al*, 2008), our findings support a role for VTA $\alpha6\beta2$ *nAChR-driven DA neuron activity in nicotine reward behavior. Overall, our findings demonstrate that mesolimbic $\alpha6\beta2$ *nAChRs in the VTA and NAc shell independently support nicotine reward. Together with previous studies, these findings support a model of nicotine reward modulated by DA neuron activity-independent and activity-dependent DA release (Figure 5).

In summary, these *in vivo* studies demonstrate that elevated cholinergic tone at VTA $\alpha6\beta2$ *nAChRs is sufficient to enhance basal NAc DA tone and nicotine reward. Our findings also showed that NAc $\alpha6\beta2$ *nAChRs regulate nicotine reward behavior. These findings demonstrate a role for two independent pools of mesolimbic $\alpha6\beta2$ *nAChRs in nicotine reward, which may be regulated by activity-independent and activity-dependent NAc DA release, a hypothesis which needs to be directly tested in future experiments. Overall, this work provides information about the neural circuitry implicated in behaviors that can lead to nicotine addiction

and supports evidence to suggest that antagonism of $\alpha 6\beta 2$ *nAChRs may prove an effective therapeutic strategy for smoking cessation.

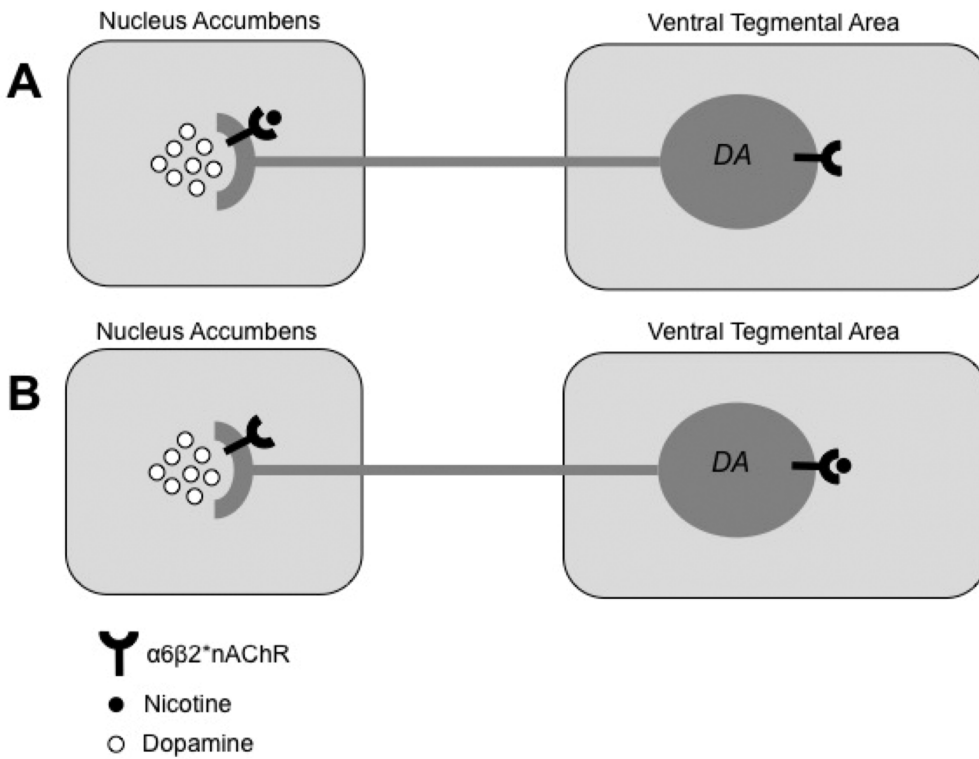


Figure 2.5 – A model for two potential mechanisms by which $\alpha6\beta2$ *nAChRs in the mesolimbic pathway may regulate nicotine reward. a) Nicotine activates $\alpha6\beta2$ *nAChRs on dopamine (DA) terminals in the nucleus accumbens to promote nicotine reward, which may be modulated by $\alpha6\beta2$ *nAChR-mediated DA release. b) Nicotine activates $\alpha6\beta2$ *nAChRs on ventral tegmental area neuron soma to drive DA release, which may promote nicotine reward.

Chapter 3 – Assessing the contributions of $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs to anxiety-like behavior

INTRODUCTION

Smokers report that they smoke to relieve anxiety, and stress can lead to escalation of smoking and relapse (Shiffman *et al*, 1997; Skara *et al*, 2001). Further, patients with anxiety disorders are at a greater risk for developing nicotine dependence (Kushner *et al*, 2012). Nicotinic acetylcholine receptors (nAChRs), the primary target of nicotine, are implicated in regulation of anxiety. Nicotine produces a bimodal effect on anxiety-like behavior in rodents, with low doses (0.01-0.1 mg/kg, i.p.) being anxiolytic and high doses (0.5-1.0 mg/kg, i.p.) being anxiogenic (Anderson *et al*, 2012, 2015; Cheeta *et al*, 2001a; Cheeta *et al*, 2001b; File *et al*, 2000; File *et al*, 1998; Irvine *et al*, 1999; McGranahan *et al*, 2011; Ouagazzal *et al*, 1999; Varani *et al*, 2012; Zarrindast *et al*, 2008). Nicotine appears to promote anxiolysis via desensitization of nAChRs, as nAChR antagonists produce anxiolytic-like effects (Anderson *et al*, 2012, 2015; Newman *et al*, 2002; Newman *et al*, 2001; Roni and Rahman, 2011). Further, recent studies from our lab show that a low dose of nicotine (0.05 mg/kg i.p.) blocks the anxiogenic-like effects of a high dose of nicotine (0.5 mg/kg i.p.) in the light-dark assay; this is presumably via nAChR desensitization (Anderson *et al*, 2015). Together, these studies suggest that activation of nAChRs promotes anxiety-like behavior.

Previous studies suggest that $\beta 2^*nAChRs$ regulate anxiety-like behavior. The $\beta 2^*nAChR$ -selective antagonist, dihydro-beta-erythroidine (DH β E) decreases anxiety-like behavior in a conditioned emotional response (CER) task, as well as in the EPM and marble burying tasks (Anderson *et al*, 2012). Moreover, low doses of the $\beta 2^*nAChR$ -selective agonist, 5I-A5830, decrease anxiety-like behavior, while high doses increase anxiety-like behavior (Anderson *et al*, 2015). DH β E (Grady *et al*, 2010; Papke *et al*, 2008) and 5I-A85830 (Mukhin *et al*, 2000) have similar potencies at $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*nAChRs$. The purpose of these experiments was to assess how $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*nAChRs$ independently contribute to anxiety-like behavior.

$\alpha 4\beta 2^*nAChRs$ are ubiquitously expressed throughout the brain, including in areas associated with anxiety-like behavior. The anxiolytic-like efficacy of a low dose of nicotine is reduced in mice with a selective genetic deletion of the $\alpha 4$ subunit from ventral tegmental area (VTA) dopamine (DA) neurons (McGranahan *et al*, 2011), suggesting that VTA $\alpha 4\beta 2^*nAChRs$ are important for nicotine's anxiolytic effects. Moreover, the $\alpha 4\beta 2^*nAChR$ gain-of-function L9'S mice ($\alpha 4L9'S$) exhibit greater levels of basal anxiety-like behavior compared to wild type (WT) mice (Labarca *et al*, 2001), suggesting that basal cholinergic activity at $\alpha 4\beta 2^*nAChRs$ promotes anxiogenic-like behavior. Interestingly, $\alpha 4$ null mutant ($\alpha 4KO$) mice also show increases in basal anxiety-like behavior (Ross *et al*, 2000). Unlike $\alpha 4\beta 2^*nAChRs$, $\alpha 6\beta 2^*nAChRs$ have a more selective expression pattern in brain, being enriched in catecholaminergic nuclei, as well as in the visual circuitry. However, $\alpha 6\beta 2^*nAChRs$ are not highly expressed in brain regions such as the amygdala, cingulate cortex, and lateral septum (Champtiaux *et al*, 2002; Klink *et al*, 2001; Le Novere *et al*, 1996), which are implicated in anxiety-like behavior. While $\alpha 6\beta 2^*nAChRs$ in the VTA and NAc have not been directly

implicated in anxiety-like behavior, these areas where $\alpha 6\beta 2^*$ nAChRs are enriched are involved in such behaviors. For example, mesocorticolimbic VTA DA projections to the prefrontal cortex (PFC) are involved in stress responses in animals and humans (Anstrom *et al*, 2009; Cha *et al*, 2014). In the NAc, the anxiolytic drug, buspirone reduces ACh levels (Kolasa *et al*, 1982), while local CRF administration increases anxiety-like behavior and local ACh levels (Chen *et al*, 2012). $\alpha 6\beta 2^*$ nAChRs are also expressed in the locus coeruleus, a norepinephrine (NE)-rich nucleus that has been implicated in anxiety-like behavior (Chmielarz *et al*, 2013; Itoi *et al*, 2011; Mazzone *et al*, 2016; McCall *et al*, 2015).

These studies assessed the independent contributions of $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs to anxiety-like behavior. First, we used complementary genetic approaches, assessing anxiety-like behavior in mice with a gain-of-function ($\alpha 6L9'S$) or loss-of-function ($\alpha 6KO$) to their $\alpha 6\beta 2^*$ nAChRs using the EPM, open field, and light-dark assays. $\alpha 6L9'S$ mice have a single point mutation in the M2 pore-forming region of the $\alpha 6$ subunit, rendering their $\alpha 6\beta 2^*$ nAChRs hypersensitive to nicotine and ACh as compared to WT littermates so that subthreshold concentrations of these agonists selectively activate $\alpha 6\beta 2^*$ nAChRs (Drenan *et al*, 2010; Drenan *et al*, 2008). Finally, we assessed anxiety-like behavior using the EPM, open field, and light-dark assays in $\alpha 4\beta 2^*$ nAChR gain-of-function ($\alpha 4L9'A$) mice with a single point mutation in the M2 pore-forming region of the $\alpha 4$ subunit, rendering their $\alpha 4\beta 2^*$ nAChRs hypersensitive to nicotine (Tapper *et al*, 2004), to determine whether activation of $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs may work together or in opposition in regards to regulation of anxiety-like behavior.

MATERIALS AND METHODS

Subjects

A total of 129 adult male mice participated in these studies: 33 α 6L9'S and 38 WT littermates; 15 α 6KO mice and 15 WT littermates; 13 α 4L9'A and 15 WT littermates. All mice were backcrossed at least 10 generations. A single allele for the α 6L9'S transgene produces the hypersensitive α 6 β 2*nAChR phenotype (Drenan et al, 2008) so that breedings to WT mice resulted in 50% α 6L9'S and 50% WT offspring. α 6KO mice were generated from heterozygous matings of mice backcrossed at least 10 generations on a C57BL/6J background. Mice were housed in polycarbonate cages with Teklad corn cob bedding (catalog number 7092) in a temperature- and humidity-controlled vivarium on a 12 h light/dark cycle (lights on at 6:00 a.m.). Mice had access to food (Teklad LM-485 Mouse/Rat Sterilizable Diet, catalog number 7012) and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. All animals were treated according to the Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health).

Behavioral procedures

Mice in these studies underwent anxiety testing in the following order: open field (fluorescent lighting), light-dark, locomotor (infrared lighting), EPM. Except for locomotor testing, which took place immediately following the light-dark assay, at least 24 h were allowed between tests.

Elevated plus maze assay (EPM)

A plus maze situated 68 cm above the floor had white plastic flooring on two open arms (5 cm x 30 cm) that were perpendicular to two closed arms (5 cm x 30 cm) that had black Plexiglas enclosures (15.25 cm H). Testing took place under fluorescent lighting. Mice ($n=12-15$ per genotype) were injected with i.p. SAL and returned to their home cage for 5 min before being placed on the center of the EPM facing a closed arm. Behavior was recorded using a ceiling-

mounted camera interfaced to a PC for collection of data using ANY-maze tracking software (Stoelting, Wood Dale, IL) for 10 min. Dependent measures included time spent in the open arms, entries made into the open arms, distance traveled in the open arms, and latency to explore the terminal zones of the open arms. Time spent in the open arms, entries made in the open arms, and distance traveled in the open arms data were separated into 5 min time bins.

Open field/locomotor assay

Open field and locomotor tests took place in a polycarbonate cage (30 cm L x 18 cm W) surrounded by a white plastic enclosure. Testing took place under fluorescent (open field) or infrared lighting conditions (500 mA emitting an 830 nm frequency) (locomotor). Mice ($n=6-8$ per genotype) were placed into the polycarbonate cage facing one of the corners. Data was recorded using a ceiling-mounted camera interfaced to a PC for collection of data using ANY-maze tracking software for 15 min. Dependent measures included total distance traveled, latency to enter the centre zone, center zone entries, and time spent in the corners.

Light-dark assay

The light-dark assay was conducted in modified mouse place conditioning chambers (Med Associates, St. Albans, VT) consisting of a small, enclosed dark chamber (16.8 cm L x 12.7 cm W x 12.7 cm H) adjacent to a larger, open brightly-lit chamber (26.5 cm L x 12.7 cm W x 26.2 cm H) illuminated by a 23W fluorescent light bulb. A retractable door was opened (5 cm W x 5.9 cm H) at the beginning of the test to provide mice with free access to explore both chambers. During testing, the experimental room was dark other than illumination required for the light dark apparatus. Mice ($n=13-14$ per genotype) received i.p. SAL immediately prior to evaluation in the light-dark assay. A separate cohort of mice ($n=8$ per genotype) did not receive i.p. SAL

injections prior to the light-dark test. Animals were placed in the dark chamber and had free access to the entire apparatus for 10 min. Data was collected using Med Associates software. Dependent measures included latency to enter the light chamber, % time spent in the light chamber, and movement counts.

Statistical Analysis

All statistical analyses were performed using SPSS. A one-way analysis of variance (ANOVA) comparing saline-injected and non-injected mice revealed no significant main effects of injection for any measure in the light-dark assay (F 's <1), so these groups were combined for subsequent analyses. Two-tailed t-tests were used to assess genotype effects in the light-dark and EPM tasks. A repeated measures 2x2 (time x genotype) ANOVA analyzed EPM data separated into 5 min time bins. Latency to enter the terminal zone of the open arms was analyzed using two-tailed t-tests. A two-way 2x2 (genotype x light condition) ANOVA was used to analyze the effects of genotype and light condition on locomotor activity and anxiety-like behavior in the open field test. Significant interactions were followed by two-tailed t-tests. The criterion for significance was set at $p<0.05$. Data points more than 2 standard deviations from the mean were considered outliers and excluded from analysis.

RESULTS

Assessment of $\alpha6\beta2$ *nAChR contributions to anxiety-like behavior

Elevated plus maze (EPM)

Repeated measures 2x2 ANOVAs revealed significant time bin by genotype interactions for time spent in the open arms ($F_{1,27}=6.114$, $p<0.05$) and distance traveled in the open arms ($F_{1,27}=7.729$, $p<0.05$). $\alpha6L9^S$ mice spent less time in the open arms ($t_{27}=2.341$, $p<0.05$; Figure 3.1a) and

traveled less distances on the open arms compared to WT littermates during the first 5 min ($t_{27}=2.745$, $p<0.05$; Figure 3.1b), but not the second 5 min ($t_{27}=1.424$, $p=0.166$; $t_{27}=0.899$, $p=0.377$). $\alpha 6L9'S$ mice also had longer latencies to enter the terminal zone of the open arms compared to WT controls ($t_{27}=2.812$, $p<0.05$; Figure 3.1d). Independent of genotype, mice made fewer entries into the open arms during the second 5 min ($F_{1,27}=13.04$, $p<0.01$; Figure 3.1c). These data demonstrate higher levels of anxiety-like behavior in $\alpha 6L9'S$ mice compared to WT controls in the EPM assay.

Repeated measures 2x2 ANOVAs revealed that mice spent more time (Figure 3.1e), traveled greater distances (Figure 3.1f), and made more entries in the open arms (Figure 3.1g) during the first 5 min independent of genotype ($F_{1,23}=27.53$; $F_{1,23}=18.32$; $F_{1,23}=16.93$, $p's<0.001$), but no significant effects were detected between WT and $\alpha 6KO$ mice for open arm time, open arm distance, or open arm entries ($F_{1,23}=1$, $p=0.423$; $F_{1,23}=1.052$, $p=0.316$; $F_{1,23}=1.087$, $p=0.308$). $\alpha 6KO$ mice did not show differences for latencies to enter the terminal zone of the open arms compared to WT mice ($t_{23}=0.240$, $p=0.814$; Figure 3.1h).

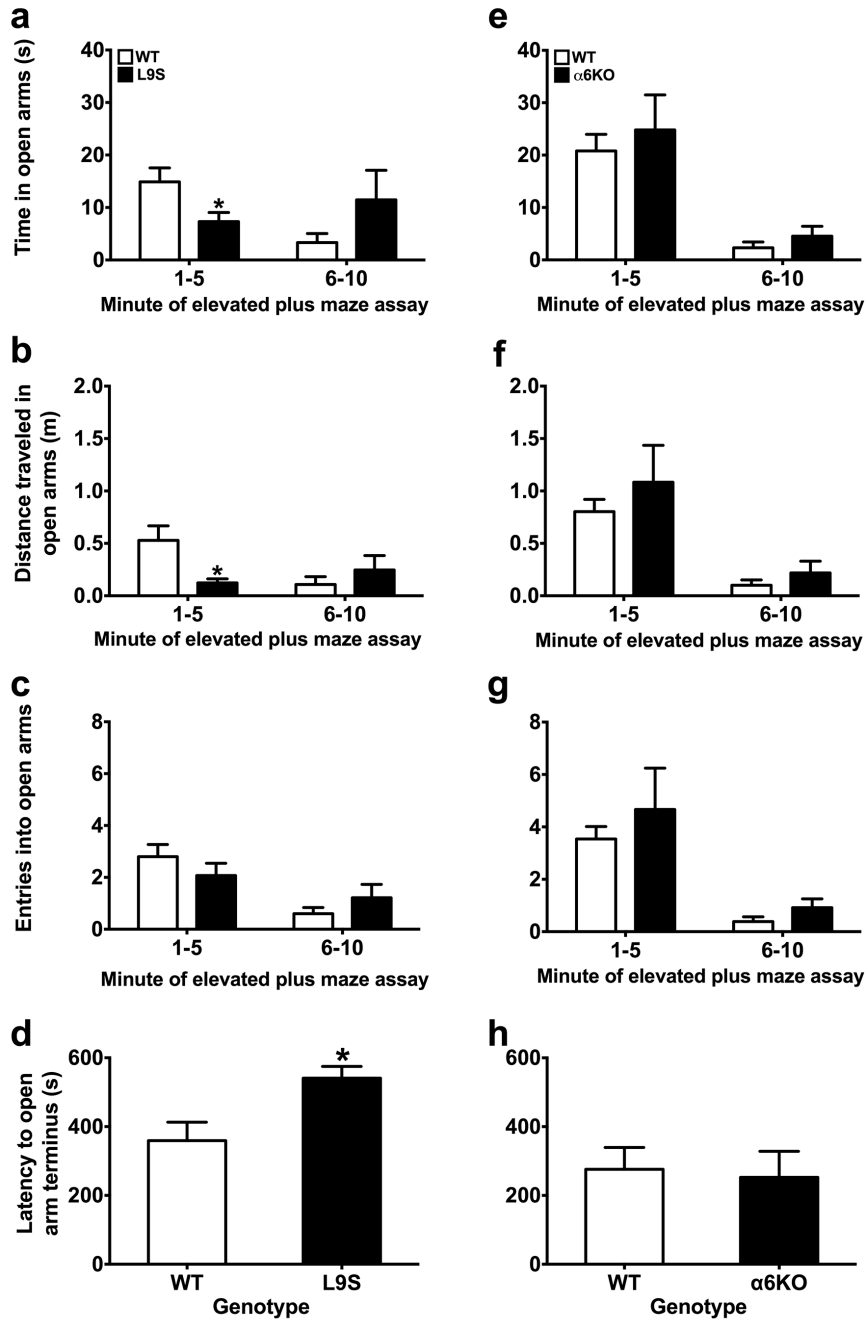


Figure 3.1 - $\alpha 6L9'S$ gain-of-function mice express increased anxiety-like behavior in the elevated plus maze (EPM) assay. a-d) Time in the open arms, distance traveled on the open arms, open arm entries, and time to explore the terminal 5 cm of the open arms on the EPM in wild type (WT) ($n=14$) versus $\alpha 6L9'S$ mice ($n=13$). **e-h)** Time in the open arms, distance traveled on the open arms, open arm entries, and time to explore the terminal 5 cm of the open arms on the EPM in WT ($n=13$) versus $\alpha 6$ null mutant ($\alpha 6KO$) mice ($n=12$). Data are represented as means \pm SEM. * $p < 0.05$ vs. WT.

Open field/locomotor assay

Two-way 2x2 ANOVAs revealed a genotype by light condition interaction for latency to enter the center zone ($F_{1,26}=10.625, p<0.01$) and total distance traveled ($F_{1,26}=4.356, p<0.05$). Under fluorescent lighting conditions, $\alpha 6L9'S$ mice had longer latencies to enter the center zone ($t_{12}=3.218, p<0.01$; Figure 3.2a) compared to WT mice, indicating higher levels of anxiety-like behavior in $\alpha 6L9'S$ mice. WT mice showed traveled greater distances compared to $\alpha 6L9'S$ mice under infrared, but not fluorescent lighting conditions ($t_{14}=2.296, p<0.05$; Figure 3.2d), suggesting that the longer latencies to enter the center zone in $\alpha 6L9'S$ mice under fluorescent lighting conditions was not due to an overall decrease in activity. Independent of lighting conditions, main effects of genotype revealed that $\alpha 6L9'S$ made fewer center entries ($F_{1,26}=9.969, p<0.01$, Figure 3.2b) and spent more time in the corners ($F_{1,26}=7.199, p<0.05$, Figure 3.2c) compared to WT controls, suggesting an anxiogenic-like phenotype in $\alpha 6L9'S$ mice. Independent of genotype, a main effect of lighting condition revealed that mice made fewer center entries ($F_{1,26}=10.350, p<0.01$, Figure 3.2b), indicating a more anxiogenic environment under fluorescent lighting.

For WT and $\alpha 6KO$ mice, there were no main effects of genotype for any measure in the locomotor assay ($F_{1,27}=1.645, p=0.211, F_{1,27}=1.698, p=0.204, F_{1,27}=1.645, p=0.862, F_{1,27}=1.292, p=0.266$; Figures 3.2e-h). Unexpectedly, there was a main effect of lighting condition, demonstrating that mice spent more time in the corners under infrared lighting conditions compared to fluorescent lighting conditions ($F_{1,27}=19.48, p<0.001$; Figure 3.2g). Mice also traveled greater distances ($F_{1,27}=5.817, p<0.05$; Figure 3.2h) under infrared lights compared to fluorescent lights independent of genotype.

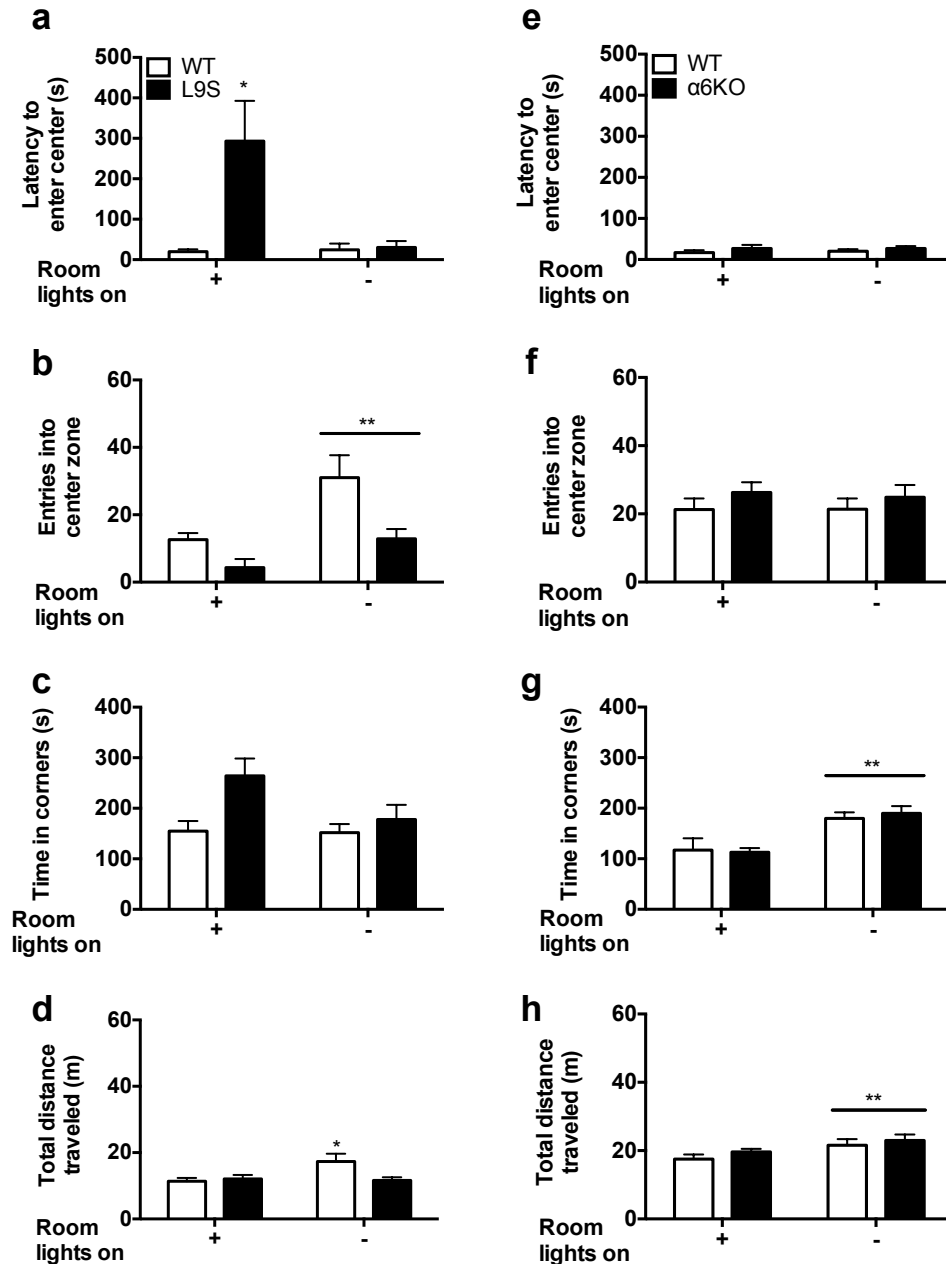


Figure 3.2 - $\alpha 6L9$ 'S gain-of-function mice showed higher levels of anxiety-like behavior during a locomotor activity test. a-d) Latencies to enter the center zone, center zone entries, time in the corners, and distance traveled under fluorescent or infrared lighting conditions in wild type (WT) (fluorescent lights: $n=8$; infrared lights: $n=8$) versus $\alpha 6L9$ 'S mice (fluorescent lights: $n=6$; infrared lights: $n=8$). **e-h)** Latencies to enter the center zone, center zone entries, time in the corners, and distance traveled under fluorescent or infrared lighting conditions in WT (fluorescent lights: $n=8$; infrared lights: $n=8$) versus $\alpha 6$ null mutant ($\alpha 6KO$) mice (fluorescent lights: $n=7$; infrared lights: $n=8$). Data are represented as means \pm SEM. * $p < 0.05$ vs. WT, ** $p < 0.05$ compared to room lights on +.

Light-dark assay

$\alpha 6L9$ 'S mice spent less time in the light chamber compared to WT littermates ($t_{41}=3.837$, $p<0.001$; Figure 3.3a), demonstrating an anxiogenic-like phenotype in $\alpha 6L9$ 'S mice. Despite similar trends, there was no significant effect of genotype for latency to enter the light chamber ($t_{41}=1.395$, $p=0.171$; Figure 3.3b). $\alpha 6L9$ 'S mice exhibited less movement counts compared to WT controls ($t_{41}=5.563$, $p<0.001$; Figure 3.3c), suggesting that decreases in activity may reflect less time spent in the light chamber. On the other hand, there were no significant differences between WT and $\alpha 6KO$ mice for any of these measures ($t_{25}=1.924, 1.206, 1.687$; p 's >0.05 ; Figure 3.3d-f).

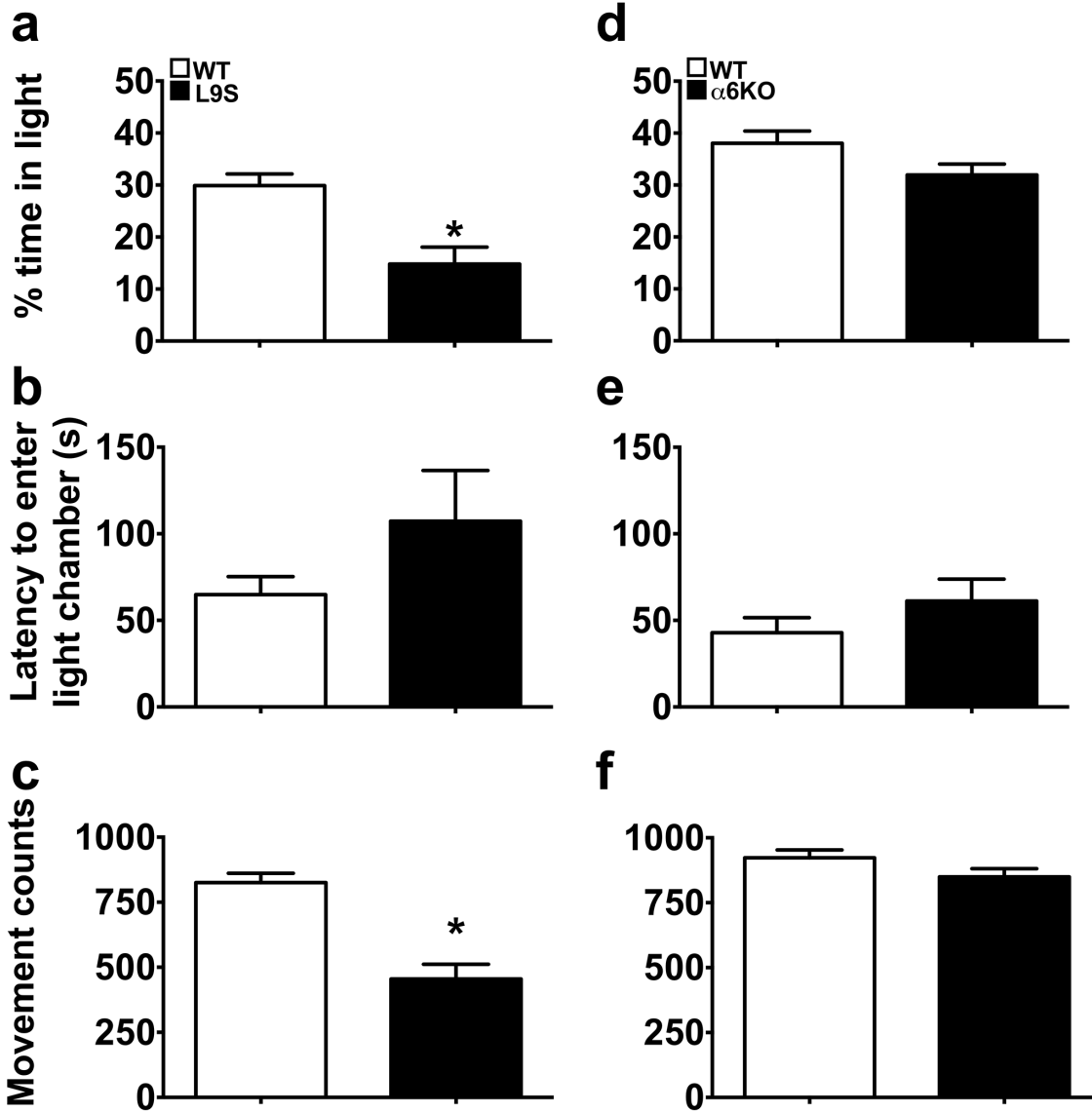


Figure 3.3 - $\alpha 6^{L9S}$ mice showed an elevated anxiety-like phenotype in the light-dark assay. a-c) Time in the light chamber, latency to enter the light chamber, and movement counts in wild type (WT) ($n=14$) versus $\alpha 6^{L9S}$ mice ($n=13$). **d-f)** Time in the light chamber, latency to enter the light chamber, and movement counts in WT ($n=8$) versus $\alpha 6$ null mutant ($\alpha 6^{KO}$) mice ($n=8$). Data are represented as means \pm SEM. * $p < 0.05$ vs. WT.

*Assessment of $\alpha 4\beta 2$ *nAChR contributions to anxiety-like behaviors*

Elevated plus maze

Repeated measures 2x2 ANOVAs detected significant time bin by genotype interactions for time spent in the open arms ($F_{1,26}=4.025$, $p<0.05$) and open arm entries ($F_{1,26}=5.28$, $p<0.05$). $\alpha 4L9$ 'A mice spent more time in the open arms ($t_{26}=-2.431$, $p<0.05$; Figure 3.4a) and made more entries onto the open arms of the EPM during the first 5 min ($t_{26}=-2.387$, $p<0.05$; Figure 3.4c), but not the second 5 min ($t_{26}=-0.215$, $p=0.832$; $t_{26}=-0.174$, $p=0.864$) compared to WT littermates, suggestive of an anxiolytic-like phenotype in $\alpha 4L9$ 'A mice. Independent of genotype, mice spent less time in the open arms ($F_{1,26}=4.361$, $p<0.05$) and made less entries into the opens arms of the EPM during the second 5 min ($F_{1,26}=9.969$, $p<0.01$). There was no significant time bin by genotype interaction for distance traveled in the open arms ($F_{1,26}=2.106$, $p=0.159$; Figure 3.4b), and no differences between WT and $\alpha 4L9$ 'A mice for latency to enter the open arm terminus ($t_{26}=0.689$, $p=0.517$; Figure 3.4d).

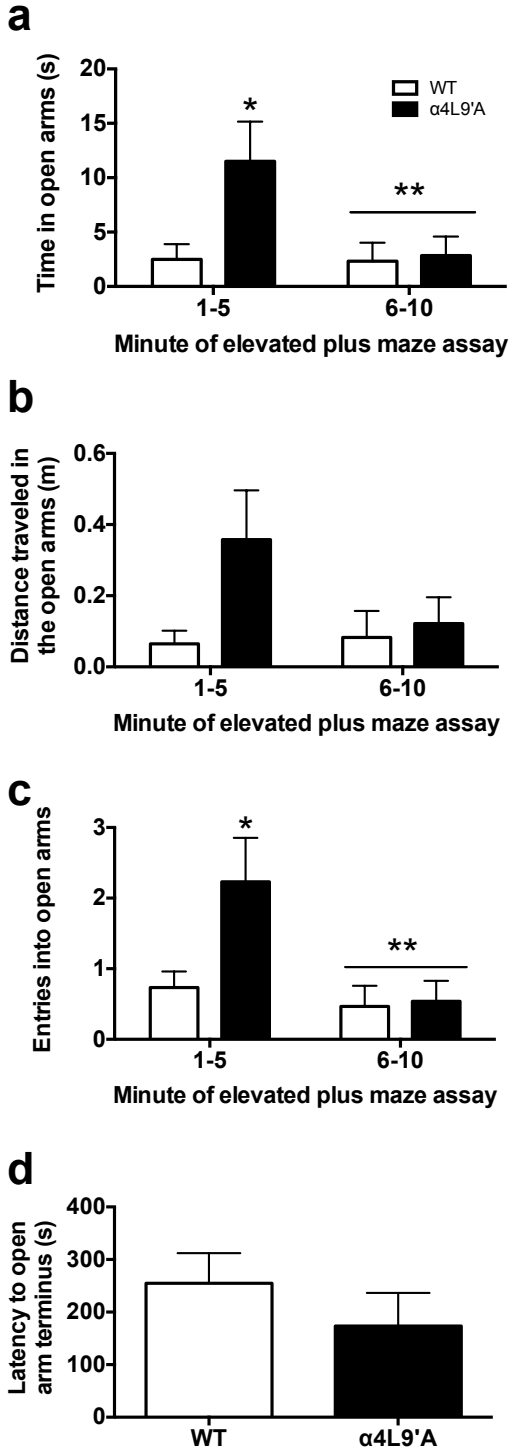


Figure 3.4 - $\alpha 4L9'A$ gain-of-function mice expressed decreased anxiety-like behavior in the elevated plus maze (EPM) assay. a-d) Time in the open arms, distance traveled on the open arms, open arm entries, and time to explore the terminal 5 cm of the open arms on the EPM in wild type (WT) ($n=15$) versus $\alpha 4L9'A$ mice ($n=13$). Data are represented as means \pm SEM. * $p < 0.05$ vs. WT, ** $p < 0.05$ compared to minute 1-5.

Open field/locomotor assay

There were no significant genotype by light condition interactions for latency to enter the center zone ($F_{1,52}=2.524$, $p=0.118$), entries into the center zone ($F_{1,52}=0.015$, $p=0.904$), time in the corners ($F_{1,52}=3.34$, $p=0.073$), or total distance traveled ($F_{1,52}=0.44$, $p=0.51$) during a locomotor test (Figure 3.5a-d). While genotype did not impact behavior in the open field (fluorescent lights) and locomotor (infrared lights) assays differently, main effects of genotype revealed that $\alpha 4L9^A$ mice made more center entries ($F_{1,52}=6.378$, $p<0.05$) and spent less time in the corners ($F_{1,52}=6.869$, $p<0.05$) compared to WT mice independent of lighting conditions, suggestive of an anxiolytic-like phenotype in $\alpha 4L9^A$ mice. However, $\alpha 4L9^A$ mice also traveled greater distances independent of lighting conditions ($F_{1,52}=19.751$, $p<0.001$), which could have influenced increases in center entries. Unexpectedly, mice had greater latencies to enter the center zone ($F_{1,52}=4.063$, $p<0.05$), made less entries into the center zone ($F_{1,52}=16.966$, $p<0.001$), and spent more time in the corners ($F_{1,52}=11.979$, $p=0.001$) under infrared lighting. Mice also traveled less distance overall ($F_{1,52}=17.652$, $p<0.001$) under infrared lighting independent of genotype, suggesting that reductions in center zone behavior under infrared lighting could have resulted from an overall reduction in behavior.

Light-dark assay

There were no significant differences for percent time spent in the light chamber ($t_{24}=-0.573$, $p=0.572$), latency to enter the light chamber ($t_{24}=0.045$, $p=0.964$), or total movement counts ($t_{24}=0.817$, $p=0.422$) during the light-dark test (Figure 3.6a-c), indicating no differences in anxiety-like behavior as measured by the light-dark test between WT and $\alpha 4L9^A$ mice.

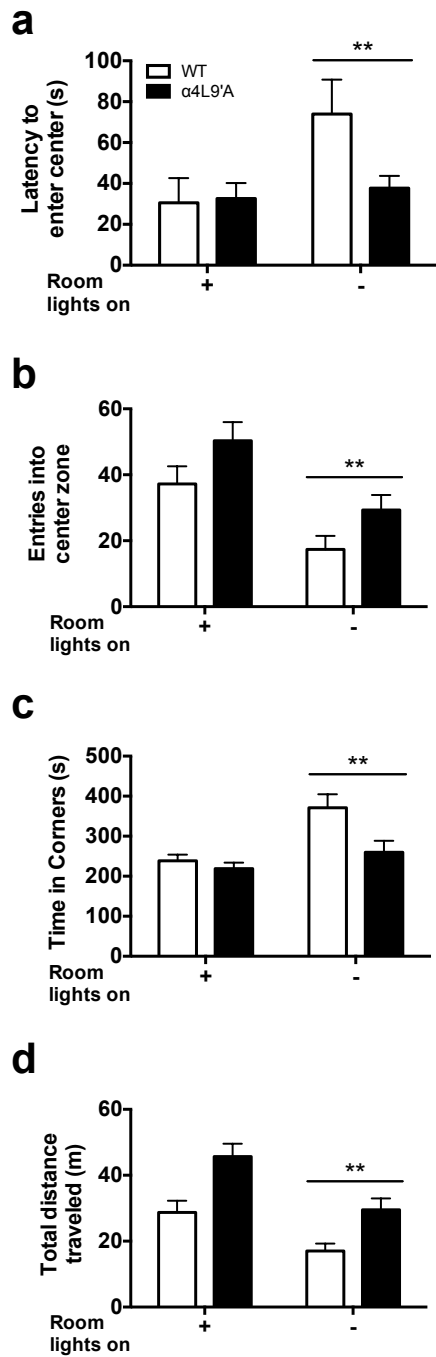


Figure 3.5 - $\alpha 4L9^A$ gain-of-function mice showed no significant differences in anxiety-like behavior compared to wild type (WT) mice in a locomotor activity test. a-d) Latencies to enter the center zone, center zone entries, time in the corners, and distance traveled under fluorescent or infrared lighting conditions in WT ($n=15$) versus $\alpha 4L9^A$ mice ($n=13$). Data are represented as means \pm SEM. $p < 0.05$ compared to room lights on +.**

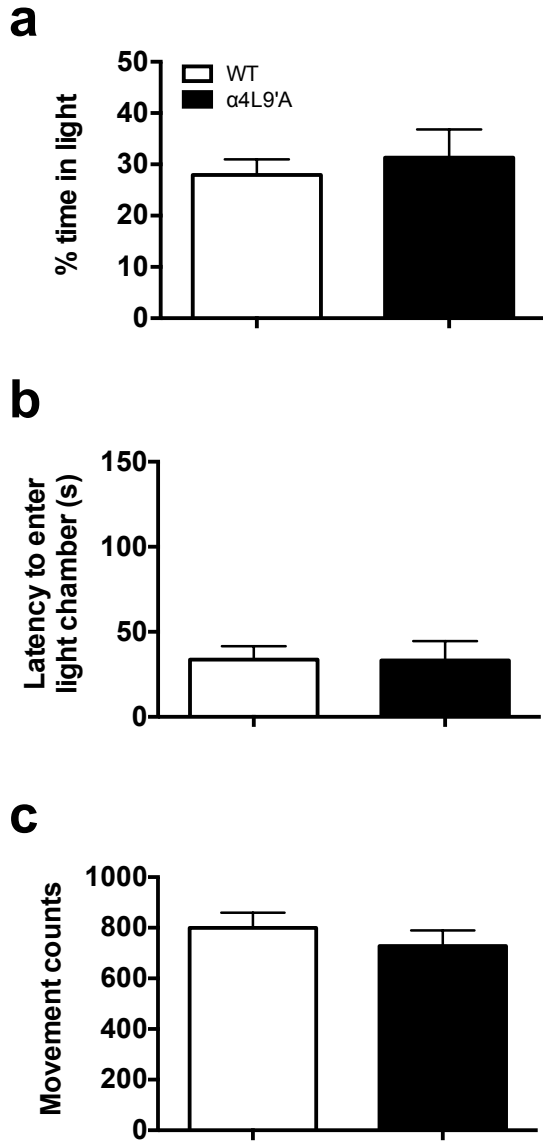


Figure 3.6 - $\alpha 4L9'A$ gain-of-function mice showed no significant differences in anxiety-like behavior compared to wild type (WT) mice in the light-dark test. a-c) percent time spent in the light chamber, latency to enter the light chamber, and total movement counts in WT ($n=15$) versus $\alpha 4L9'A$ mice ($n=11$). Data are represented as means \pm SEM.

DISCUSSION

In these studies, $\alpha 6L9'S$ gain-of-function mice showed elevated levels of anxiety-like behavior compared to WT controls in the EPM, open field, and light-dark assays, suggesting that activation of $\alpha 6\beta 2*nAChRs$ is sufficient to promote anxiogenic-like behavior. In contrast, WT and $\alpha 6KO$ mice did not differ for measures of anxiety-like behavior, suggesting that $\alpha 6\beta 2*nAChRs$ are not necessary for expression of anxiety-like behavior. Given the effects we observed in $\alpha 6L9'S$ mice, we subsequently tested anxiety-like behavior in $\alpha 4L9'A$ gain-of-function mice to determine whether activation of $\alpha 4\beta 2*$ and $\alpha 6\beta 2*nAChRs$ may work together or in opposition to regulate anxiety-like behavior. In contrast to $\alpha 6L9'S$ mice, $\alpha 4L9'SA$ mice showed decreased anxiety-like behavior compared to WT controls in the EPM and open field assays, suggesting that $\alpha 4\beta 2*nAChRs$ activation is sufficient to promote anxiolysis rather than anxiogenesis.

In support of the present data in $\alpha 4L9'A$ mice suggesting that activation of $\alpha 4\beta 2*nAChRs$ promotes anxiolysis, previous findings have demonstrated that genetically inactivating $\alpha 4\beta 2*nAChRs$ promotes anxiogenesis (Ross *et al*, 2000). These present findings are also consistent with studies in transgenic mice with their $\alpha 4$ subunit specifically knocked down in VTA DA neurons suggesting that $\alpha 4\beta 2*nAChRs$ in the mesolimbic DA pathway are important for the anxiolytic-like effects of nicotine (McGranahan *et al*, 2011). More specifically, DA neuron-selective $\alpha 4$ deletion resulted in an attenuation of the anxiolytic efficacy of 0.01 mg/kg i.p. nicotine in the EPM assay. It is interesting that, unlike $\alpha 4L9'A$ mice, $\alpha 4L9'S$ gain-of-function mice, whose $\alpha 4\beta 2*nAChRs$ are hypersensitive to nicotine like $\alpha 4L9'A$ mice, show increases rather than decreases in anxiety-like behavior (Labarca *et al*, 2001). These differences

between $\alpha 4L9'A$ and $\alpha 4L9'S$ mice may be due to differences in the M2 pore-forming region mutations, where $\alpha 4L9'A$ mice have a leucine to alanine mutation and the $\alpha 4L9'S$ mice have a leucine to serine mutation. In contrast to our results in $\alpha 4L9'A$ mice, selective activation of $\alpha 6\beta 2*nAChRs$ in $\alpha 6L9'S$ mice appears to promote, rather than attenuate anxiety-like behavior, acting in opposition of $\alpha 4\beta 2*nAChRs$.

An accumulation of evidence suggests that cholinergic hyperactivity promotes anxiety-like behavior in rodents (Hart *et al*, 1999; Kolasa *et al*, 1982; Lamprea *et al*, 2000; Luo *et al*, 2013; Mineur *et al*, 2013; Power and McGaugh, 2002; Revy *et al*, 2014). As our data suggests that activation of $\alpha 6\beta 2*$ and $\alpha 4\beta 2*nAChRs$ promotes opposite effects on anxiety-like behavior, it could be that hyperactive cholinergic tone has a greater effect at $\alpha 6\beta 2*nAChRs$ in regards to anxiety-like behavior to result in a net increase in this behavior. Brain areas where cholinergic activity regulates anxiety-like behavior include the amygdala, where cholinergic lesions decrease anxiety-like behavior (Power *et al*, 2002), the hippocampus, where acetylcholinesterase (AChE) inhibition increases cholinergic activation along with anxiety-like phenotypes (Lamprea *et al*, 2000; Luo *et al*, 2013; Mineur *et al*, 2013), and the prefrontal cortex (PFC), where a benzodiazepine partial inverse agonist, FG 7142 promotes anxiogenic-like behavior and reduces AChE levels (Hart *et al*, 1999). Unlike $\alpha 4\beta 2*nAChRs$, $\alpha 6\beta 2*nAChRs$ are not greatly enriched in these brain areas, suggesting that other brain regions contribute to $\alpha 6\beta 2*nAChRs$ regulation of anxiety-like behavior. $\alpha 6\beta 2*nAChRs$ are expressed on VTA DA neuron soma and DA terminals in the NAc, as well as on noradrenergic projection neurons in the locus coeruleus (Champtiaux *et al*, 2002; Klink *et al*, 2001; Le Novere *et al*, 1996). VTA DA projections to the PFC can promote stress (Anstrom *et al*, 2009; Cha *et al*, 2014). Further, a role for VTA $\alpha 4\beta 2*nAChRs$ has been established for nicotine-associated anxiolysis (McGranahan *et al*,

2011), but it is not clear if VTA $\alpha 4\alpha 6\beta 2^*$ nAChRs contribute to this phenotype. Buspirone, an anxiolytic drug, reduces ACh levels in the NAc (Kolasa *et al*, 1982) and local NAc shell infusion of CRF produces increases in anxiety-like behavior along with elevations in local NAc shell ACh levels (Chen *et al*, 2012), suggesting that cholinergic hyperactivity in the NAc may promote anxiety-like behavior. In addition, $\alpha 6\beta 2^*$ nAChRs are also found in the locus coeruleus, a brain area also implicated in anxiety-like behavior (Chmielarz *et al*, 2013; Itoi *et al*, 2011; Mazzone *et al*, 2016; McCall *et al*, 2015).

In contrast to the $\alpha 6L9'S$ gain-of-function mutation, genetic deletion of the $\alpha 6$ subunit had no effect on anxiety-like behavior in these studies. The anxiety tests we used may not have been stressful enough for this genetic manipulation to have an effect on behavior; exposing these mice to an external stressor, such as restraint stress, before anxiety testing may unmask an effect in $\alpha 6$ KO mice. While intracerebroventricular infusion of the $\alpha 6\beta 2^*$ nAChR-selective antagonist, α -Ctx MII [H9A;L15A], attenuated nicotine withdrawal-induced anxiety-like behavior in the EPM assay (Jackson *et al*, 2009), the lack of anxiolytic efficacy of α -Ctx MII [H9A;L15A] in naïve mice is consistent with our present findings demonstrating that anxiety-like behavior in $\alpha 6$ KO mice did not differ from WT mice. This further supports that our anxiety studies in $\alpha 6$ KO mice may not have been stressful enough to uncover an effect on basal anxiety-like behavior. Given the higher levels of anxiety-like behavior observed in $\alpha 6L9'S$ mice, the anxiolytic effect of α -Ctx MII [H9A;L15A] during spontaneous nicotine withdrawal suggests that activation of $\alpha 6\beta 2^*$ nAChRs by endogenous ACh during withdrawal may contribute to anxiety experienced by smokers in abstinence.

Taken together, these findings suggest that activation of $\alpha 6\beta 2^*$ nAChRs is sufficient to promote anxiogenic-like behavior, whereas $\alpha 6\beta 2^*$ nAChRs don't appear to be necessary for

expression of anxiety-like behavior under basal conditions. We also demonstrate that activation of $\alpha 4\beta 2^*$ nAChRs has the opposite effect of $\alpha 6\beta 2^*$ nAChRs, decreasing anxiety-like behavior. Thus, it appears that $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs act in opposition in regard to regulation of anxiety-like behavior. Overall, these studies provide insight into how cholinergic hyperactivity at subtypes of $\beta 2^*$ nAChRs might regulate anxiety-like behavior.

Chapter 4 – Differentiating the roles of (non- $\alpha 4$) $\alpha 6\beta 2$ and $\alpha 4\alpha 6\beta 2$ subunit containing nicotinic acetylcholine receptors to nicotine reward and anxiety-like behavior

INTRODUCTION

$\beta 2$ subunit containing nicotinic acetylcholine receptors ($\beta 2^*$ nAChRs, *denotes possible assembly with other subunits) are known to be responsible for many behavioral responses to nicotine. Studies show that activation of $\beta 2^*$ nAChRs promotes nicotine's rewarding and reinforcing properties (Besson *et al*, 2006; Corrigall *et al*, 1994; Maskos *et al*, 2005; Picciotto *et al*, 1998; Pons *et al*, 2008; Walters *et al*, 2006), as well as anxiety-like behavior (Anderson *et al*, 2012, 2015; Hussmann *et al*, 2014; Turner *et al*, 2010; Yohn *et al*, 2014). However, the exact molecular make-up of $\beta 2^*$ nAChRs involved with these addiction-like behaviors is not completely understood.

The $\beta 2$ subunit assembles with the $\alpha 4$ and/or $\alpha 6$ subunits to make subclasses of $\beta 2^*$ nAChRs, including $\alpha 4\beta 2^*$, $\alpha 6\beta 2^*$, and $\alpha 4\alpha 6\beta 2^*$ nAChRs. $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs are independently implicated in regulation of nicotine reward and reinforcement, and anxiety-like behavior. $\alpha 4$ null mutant mice ($\alpha 4$ KO) do not express nicotine CPP (McGranahan *et al*, 2011; Sanjakdar *et al*, 2015); but see Cahir *et al*, 2011), while $\alpha 4\beta 2^*$ nAChR gain-of-function ($\alpha 4$ L9'A) mice show enhanced CPP (Tapper *et al*, 2004). Moreover, selective $\alpha 4$ deletion in VTA DA neurons is sufficient to block nicotine CPP (McGranahan *et al*, 2011). $\alpha 4$ KO mice also fail to self-administer nicotine intravenously (i.v.) into the tail vein or intracranially into the VTA

(Exley *et al*, 2011; Pons *et al*, 2008). However, $\alpha 4$ KO mice are not different from WT mice in jugular i.v. nicotine self-administration (Cahir *et al*, 2011). Like CPP studies, $\alpha 4$ -S248F mice with enhanced sensitivity for nicotine (similar to $\alpha 4$ L9'A mice) show leftward shifts in the jugular i.v. nicotine self-administration dose response curve (Cahir *et al*, 2011). For anxiety-like behavior, nicotine loses its anxiolytic efficacy when the $\alpha 4$ subunit is selectively deleted in VTA DA neurons (McGranahan *et al*, 2011). $\alpha 4$ KO mice show increases in basal anxiety-like behavior (Ross *et al*, 2000). Interestingly, $\alpha 4\beta 2^*$ nAChR gain-of-function ($\alpha 4$ L9'S) mice also exhibit an increased basal anxiety phenotype compared to WT mice (Labarca *et al*, 2001), while $\alpha 4$ L9'A mice show decreases in basal anxiety-like behavior (see Chapter 3).

Similar findings have been reported in studies of mice with modifications to their $\alpha 6\beta 2^*$ nAChRs (see table 4.1 for a summary of previous findings regarding $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChR contributions). $\alpha 6$ null mutant mice ($\alpha 6$ KO) show rightward shifts in nicotine CPP (Sanjakdar *et al*, 2015), while $\alpha 6\beta 2^*$ nAChR gain-of-function ($\alpha 6$ L9'S) mice show enhanced nicotine CPP (see Chapter 2). Moreover, $\alpha 6$ KO mice do not self-administer nicotine i.v. or intracranially into the VTA (Exley *et al*, 2011; Pons *et al*, 2008). Further, local infusion of the $\alpha 6\beta 2^*$ nAChR-selective antagonist, α -Ctx MII, into the ventral tegmental area (VTA) or nucleus accumbens (NAc) shell reduces nicotine self-administration (Brunzell *et al*, 2010; Gotti *et al*, 2010), and intracerebroventricular (i.c.v.) or intra-NAc α -Ctx MII blocks nicotine CPP (Jackson *et al*, 2009; Sanjakdar *et al*, 2015). For anxiety-like behavior, we have shown that the $\alpha 6$ L9'S gain-of-function mutation produces basal anxiogenic effects (see Chapter 3). It is not clear from this body of literature whether $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs independently regulate

behaviors relevant to nicotine addiction or if $\alpha 4$ and $\alpha 6$ assemble together with $\beta 2$ in the $\alpha 4\alpha 6\beta 2^*nAChR$ confirmation to promote these behaviors.

Table 4.1 – $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*nAChR$ contributions to nicotine self-administration, nicotine CPP, and anxiety-like behavior

	$\alpha 4\beta 2^*nAChRs$		$\alpha 6\beta 2^*nAChRs$		
	$\alpha 4KO$	$\alpha 4$ gain-of-function	$\alpha 6KO$	$\alpha 6$ gain-of-function	α -Ctx MII
Nicotine self-administration	↓/–	↑	↓	?	↓
Nicotine CPP	↓/–	↑	↓	↑	↓
Basal anxiety-like behavior	↑	↑/↓	–	↑	?
Nicotine-associated anxiety-like behavior	↑	?	?	?	?

↓ indicates that the manipulation decreased the behavior, ↑ indicates that the manipulation increased the behavior, and – indicates that the manipulation had no effect on the behavior; ? indicates that the study has not been performed to date; Abbreviations: $\alpha 4$ null mutant mice ($\alpha 4KO$), $\alpha 6$ null mutant mice ($\alpha 6KO$), conditioned place preference (CPP); α -Conotoxin MII (α -Ctx MII)

Of all the nAChR subtypes, $\alpha 4\alpha 6\beta 2$ *nAChRs have the highest sensitivity to nicotine (Exley *et al*, 2008; Kuryatov *et al*, 2011; Liu *et al*, 2012; Salminen *et al*, 2007; Salminen *et al*, 2004), and are persistently activated in the VTA at physiologically relevant concentrations of nicotine (300 nM) that typically desensitize other nAChRs (Liu *et al*, 2012). These low concentrations of nicotine are similar to levels achieved in the brain during smoking, so it is likely that $\alpha 4\alpha 6\beta 2$ *nAChRs are being engaged to promote some of nicotine's effects. Not surprisingly, stimulation of $\alpha 4\alpha 6\beta 2$ *nAChRs appears to drive mesolimbic DA neuron activity and DA-related locomotor behavior (Drenan *et al*, 2010; Engle *et al*, 2013; Liu *et al*, 2012; Zhao-Shea *et al*, 2011). However, no known studies have investigated $\alpha 4\alpha 6\beta 2$ *nAChR contributions in nicotine reward or anxiety-like behavior. Thus, the purpose of these studies was to differentiate the role of (non- $\alpha 4$) $\alpha 6\beta 2$ *nAChRs and $\alpha 4\alpha 6\beta 2$ *nAChRs in nicotine reward and anxiety-like behavior by crossing $\alpha 6L9$ 'S gain-of-function mice with $\alpha 4$ KO mice. In support of a role for $\alpha 6\beta 2$ *nAChRs in nicotine reward and anxiety-like behavior, we have shown previously (see Chapters 2 and 3) that $\alpha 6L9$ 'S mice show elevated basal levels of anxiety and express nicotine CPP and locomotor activation at doses of nicotine that are subthreshold for activation of other nAChR subtypes. The $\alpha 6L9$ 'S x $\alpha 4$ KO cross will determine if elevated reward and anxiety-like behavior are regulated by hypersensitive $\alpha 4\alpha 6\beta 2$ *nAChRs or $\alpha 6\beta 2$ *nAChRs that do not contain an $\alpha 4$ subunit ((non- $\alpha 4$) $\alpha 6\beta 2$ *nAChRs). If (non- $\alpha 4$) $\alpha 6\beta 2$ *nAChRs are involved with nicotine reward and anxiety-like behavior, we expect that elevated $\alpha 6L9$ 'S nicotine CPP and anxiety-like behavior will be maintained in $\alpha 6L9$ 'S- $\alpha 4$ KO mice. On the other hand, if $\alpha 4$ and $\alpha 6$ assemble together in the $\alpha 4\alpha 6\beta 2$ *nAChR conformation to

regulate these behaviors, we expected that elevated $\alpha 6L9'S$ nicotine CPP and anxiety-like behavior will be reversed in $\alpha 6L9'S$ - $\alpha 4KO$ mice.

MATERIALS AND METHODS

Subjects

Adult male wild type C57BL/6J (WT) ($n=40$), $\alpha 4KO$ ($n=32$), $\alpha 6L9'S$ ($n=40$), and $\alpha 6L9'S$ - $\alpha 4KO$ mice ($n=31$) backcrossed > 10 generations on a C57BL/6J background were used in these studies. A single allele for the $\alpha 6L9'S$ transgene produces the hypersensitive $\alpha 6\beta 2^*nAChR$ phenotype (Drenan et al, 2008) so that breedings to WT mice resulted in 50% $\alpha 6L9'S$ and 50% WT offspring. Then, the $\alpha 6L9'S$ mice were crossed with $\alpha 4KO$ mice to generate $\alpha 4$ heterozygous mice ($\alpha 4HET$) with the $\alpha 6L9'S$ mutation ($\alpha 6L9'S$ - $\alpha 4HET$). Finally, the $\alpha 6L9'S$ - $\alpha 4HET$ mice were crossed with $\alpha 4HET$ mice to generate WT, $\alpha 6L9'S$, $\alpha 4KO$, and $\alpha 6L9'S$ - $\alpha 4KO$ mice (see Figure 4.1 for probable nAChR subtypes expressed as a result of these genetic manipulations). This cross also generated $\alpha 4HET$ and $\alpha 6L9'S$ - $\alpha 4HET$ mice, which were not used in these studies. Mice were housed in a temperature- and humidity-controlled vivarium in polycarbonate cages with Teklad corn cob bedding (catalog number 7092) on a 12 h light/dark cycle, with lights on at 6:00 a.m. All mice had access to food (Teklad LM-485 Mouse/Rat Sterilizable Diet, catalog number 7012) and water *ad libitum* and were gently handled at least three times before any experiments were performed. The experimental protocol was approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. All animals were treated according to the Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health).

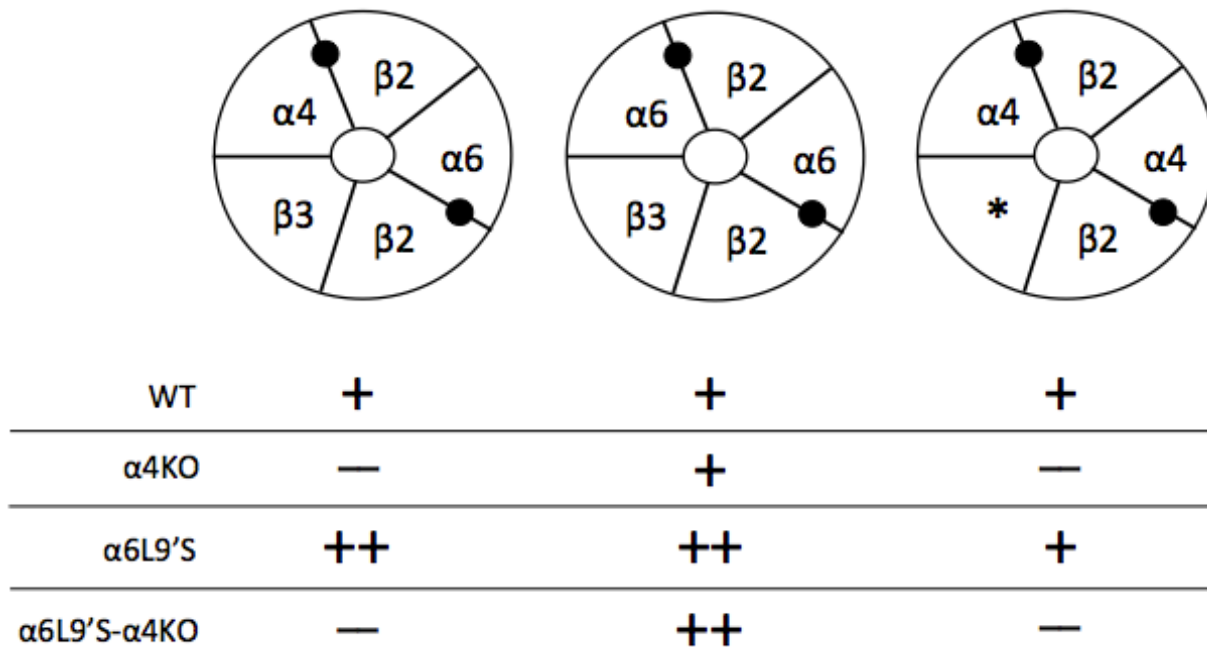


Figure 4.1 – Expression of $\beta 2^*$ nAChRs related to nicotine addiction-like behavior resulting from genetic manipulations in mice used in these studies. Wild type (WT) mice express $\alpha 4\beta 2^*$, $\alpha 4\alpha 6\beta 2\beta 3^*$, and (non- $\alpha 4$) $\alpha 6\beta 2\beta 3^*$ nAChRs, while $\alpha 4$ null mutant ($\alpha 4$ KO) mice only express (non- $\alpha 4$) $\alpha 6\beta 2\beta 3^*$ nAChRs. Like WT mice, $\alpha 6$ L9'S mice express all three subtypes as well, with the $\alpha 4\alpha 6\beta 2\beta 3^*$ and (non- $\alpha 4$) $\alpha 6\beta 2\beta 3^*$ nAChRs being hypersensitive. Like $\alpha 4$ KO mice, $\alpha 6$ L9'S- $\alpha 4$ KO mice only express (non- $\alpha 4$) $\alpha 6\beta 2\beta 3^*$ nAChRs, but in the hypersensitive state. + indicates the presence of the native receptor subtype, ++ indicates the presence of the subtype in the hypersensitive form, and – indicates the absence of the receptor subtype. * denotes presence of the $\alpha 4$, $\beta 2$, or $\alpha 5$ subunit. The $\alpha 5$ and $\beta 3$ subunits are accessory subunits that do not participate in ligand binding, but instead contribute to the receptor's channel permeability, binding affinity, desensitization, sensitivity to allosteric modulators, and sensitivity to upregulation (Kuryatov *et al*, 2008; Moroni *et al*, 2008; Moroni *et al*, 2006; Tapia *et al*, 2007). Further, evidence suggests that the $\beta 3$ subunit is important for the formation of $\alpha 6\beta 2^*$ nAChRs, as expression of $\alpha 6\beta 2^*$ nAChRs is drastically reduced in $\beta 3$ null mutant mice (Cui *et al*, 2003).

Drugs

Nicotine hydrogen tartrate salt (Sigma-Aldrich, St. Louis, MO) was dissolved in 0.9% sterile saline vehicle (SAL). Nicotine solutions were filter sterilized and titrated to a pH of 7.1-7.4. Mice received intraperitoneal (i.p.) injections of SAL or nicotine at a volume of 0.1 ml/30 g. Nicotine doses are expressed by free base weight.

Behavioral Procedures

Mice in these studies underwent testing in the following order: CPP, open field, light-dark, locomotor activity under dim lighting conditions, and contextual threat conditioning. With the exception of one cohort, mice originally tested in CPP were also used for subsequent anxiety and threat conditioning tests. Testing took place no less than 24 h apart.

Unbiased Nicotine Conditioned Place Preference (CPP)

Nicotine CPP was conducted in Med Associates mouse place conditioning chambers (Med Associates, St. Albans, VT). The CPP apparatus consisted of two unique but equally preferred conditioning chambers with distinct floors (parallel bars or grid) and walls (black or striped) separated by retractable doors and a small neutral (grey) chamber with Plexiglas floor. CPP training took place twice a day between the hours of 10:00 a.m. and 12:00 p.m. and 1:00 p.m. and 3:00 p.m., with baseline and testing taking place at an intermediate timepoint on the day prior to and following training, respectively. During baseline, mice ($n=7-12$ per group) received i.p. SAL prior to being placed in the neutral chamber with doors retracted to allow free exploration of the apparatus for 15 min. The following 3 days during the a.m. training session, mice received i.p. SAL prior to 30 min confinement in the saline-paired chamber. During the p.m. session, mice received i.p. nicotine (0, 0.03 or 0.1 mg/kg) prior to 30 min of confinement in

the opposite, nicotine-paired conditioning chamber. Control mice received i.p. SAL prior to exposure to both chambers. Assignment of conditioning chamber was counterbalanced with mice showing overall similar preferences across chambers. During test, mice received i.p. SAL and were placed in the chamber as during baseline and allowed to explore the entire apparatus for 15 min. Photobeams detected movement and data were recorded using Med-PC IV software (Med Associates, St. Albans, VT).

Contextual Threat Conditioning

To assess if genotypic differences in nicotine CPP were due to generalized changes in contextual learning, contextual threat conditioning was conducted in mouse operant conditioning chambers (Med Associates, St. Albans, VT). Training occurred during a 5.5 min session with WT ($n=14$), $\alpha 4\text{KO}$ ($n=11$), $\alpha 6\text{L9'S}$ ($n=13$), and $\alpha 6\text{L9'S-}\alpha 4\text{KO}$ mice ($n=10$) placed in the chamber for 2 min before presentation of a 30 s light plus tone cue that terminated with a 2 s, 0.5 mA footshock. This sequence was repeated followed by a 30 s post-shock period. The next day, mice were placed in the conditioning chamber for 5.5 min in the absence of the cue and footshock to test for context-specific freezing. Freezing (absence of voluntary movement except for respiration) was measured using Anymaze tracking software (Stoelting, Wood Dale, IL) and confirmed by a blind experimenter. Freezing during the first 2 minutes of the training session (before any footshock) was compared to freezing during the corresponding first 2 minutes of the test session to assess whether mice learned to associate the context with the previous footshocks received during training.

Open Field Assay

The open field assay was conducted under overhead fluorescent lighting in a polycarbonate cage (30 cm L x 18 cm W) inside a white plastic enclosure that surrounds the walls and floor. Mice ($n=7-14$ per group) received i.p. injections of SAL, 0.03, or 0.1 mg/kg nicotine (dose assigned based on dose received during CPP) and were immediately placed in the open field chamber for 15 min. Data was collected using AnyMaze tracking software. Dependent variables included total distance traveled (m) and time in the center (s).

Light-dark Assay

The light-dark assay was conducted in a rectangle Plexiglas box divided into a small, enclosed, dark chamber (25 cm H x 25.5 cm W x 18 cm L) adjacent to a large, open, brightly lit chamber (25 cm H x 25.5 cm W x 25.5 cm L) illuminated by a 23W fluorescent bulb. An opening in the wall shared by the two chambers allowed mice to move freely throughout the apparatus. Testing was conducted in a dark room except for the fluorescent light bulbs above each apparatus. Mice ($n=7-14$ per group) received i.p. injections of SAL, 0.03, or 0.1 mg/kg nicotine (dose assigned based on dose received during CPP). Mice were then immediately placed in the dark chamber of the light-dark apparatus and allowed to explore the apparatus for 10 min. Data was collected using AnyMaze tracking software. Dependent variables included time in the light (s) and light chamber entries.

Locomotor Activity-Dim Light Conditions

Locomotor activity was assessed in a polycarbonate cage (30 cm L x 18 cm W) under dim lighting with 500 mA intensity infrared light emitting an 830 nm frequency (Wisecomm, Cerritos, CA). On the first day, mice ($n=7-14$ per group) were habituated to the locomotor chamber for 15 min. The following day, mice received i.p. injections of SAL, 0.03, or 0.1 mg/kg

nicotine (dose assigned based on dose received during CPP) and were immediately placed in the locomotor chamber for 15 min. Distance traveled was measured using Anymaze tracking software.

Statistical Analysis

All statistical analyses were performed using SPSS. CPP, locomotor, and anxiety data were analyzed using a 3x4 (nicotine dose x genotype) analysis of variance (ANOVA). Significant nicotine dose x genotype interactions were further analyzed using one-way ANOVAs followed by Dunnett's post hoc tests for each genotype independently to assess nicotine effects within each genotype. Planned comparisons using two-tailed t-tests were used to compare basal anxiety-like behavior in WT versus $\alpha 4$ KO mice, WT versus $\alpha 6L9'S$ mice, and $\alpha 6L9'S$ versus $\alpha 6L9'S$ - $\alpha 4$ KO mice. For contextual threat conditioning, percent time freezing during the pre-shock period (first 2 min) was compared between the training and test sessions and was analyzed using a 2x4 (session x genotype) repeated measures ANOVA. The criterion for significance was set at $p < 0.05$. Data points more than 2 standard deviations from the mean were considered outliers and excluded from analysis.

RESULTS

Nicotine CPP

To differentiate (non- $\alpha 4$) $\alpha 6\beta 2^*$ and $\alpha 4\alpha 6\beta 2^*$ nAChR contributions to nicotine reward behavior, WT, $\alpha 4$ KO, $\alpha 6L9'S$, and $\alpha 6L9'S$ mice with the $\alpha 4$ null mutation ($\alpha 6L9'S$ - $\alpha 4$ KO) were tested in an unbiased nicotine CPP task (Figure 4.2a). A two-way 3x4 ANOVA revealed a main effect of nicotine dose ($F_{2,103}=13.311, p < 0.001$) and an interaction of nicotine dose by genotype ($F_{6,103}=2.875, p=0.013$). Consistent with previous data (Brunzell *et al*, 2009a; Mineur *et al*,

2009), WT mice expressed nicotine CPP at 0.1 mg/kg i.p. nicotine ($p < 0.001$). Similar to Chapter 2 findings, nicotine CPP was left-shifted in $\alpha 6L9'S$ mice, as 0.03 mg/kg ($p = 0.033$) and 0.1 mg/kg i.p. nicotine ($p = 0.003$) conditioned a place preference. The $\alpha 4$ subunit does not appear to be required for $\alpha 6L9'S$ pronounced nicotine reward behavior, as $\alpha 6L9'S$ - $\alpha 4KO$ mice did not differ from $\alpha 6L9'S$ mice; these mice also showed nicotine CPP at both 0.03 mg/kg ($p = 0.022$) and 0.1 mg/kg i.p. nicotine ($p = 0.050$). Like $\alpha 6L9'S$ and $\alpha 6L9'S$ - $\alpha 4KO$ mice, $\alpha 4KO$ showed increased preference for the nicotine-paired chamber following 0.03 mg/kg and 0.1 mg/kg i.p. nicotine, which could suggest that inhibiting $\alpha 4\beta 2$ *nAChRs enhances nicotine reward behavior; unusual positive changes from baseline in the saline control $\alpha 4KO$ mice precluded observation of statistically significant nicotine CPP however. In summary, the $\alpha 6L9'S$ gain-of-function mutation enhanced nicotine reward independent of $\alpha 4$ subunit expression, suggesting that stimulation of (non- $\alpha 4$) $\alpha 6\beta 2$ *nAChRs is sufficient to drive nicotine's rewarding effects. For contextual threat conditioning (Figure 4.2b), there was a main effect of session ($F_{3,44} = 65.631$, $p < 0.001$), where percent time freezing in a chamber under the same context where mice had previously received a footshock was greater during the first 2 minutes of the test compared to the first 2 minutes of the training, indicating that the mice learned to associate the context with the footshocks. It is unlikely that these genetic mutations alter reward behavior due to a change in general context-based learning, as there was no main effect of genotype ($F_{3,44} = 1.248$, $p = 0.304$) and no interaction of session by genotype ($F_{3,44} = 2.070$, $p = 0.118$) for percent time freezing during the contextual threat conditioning test.

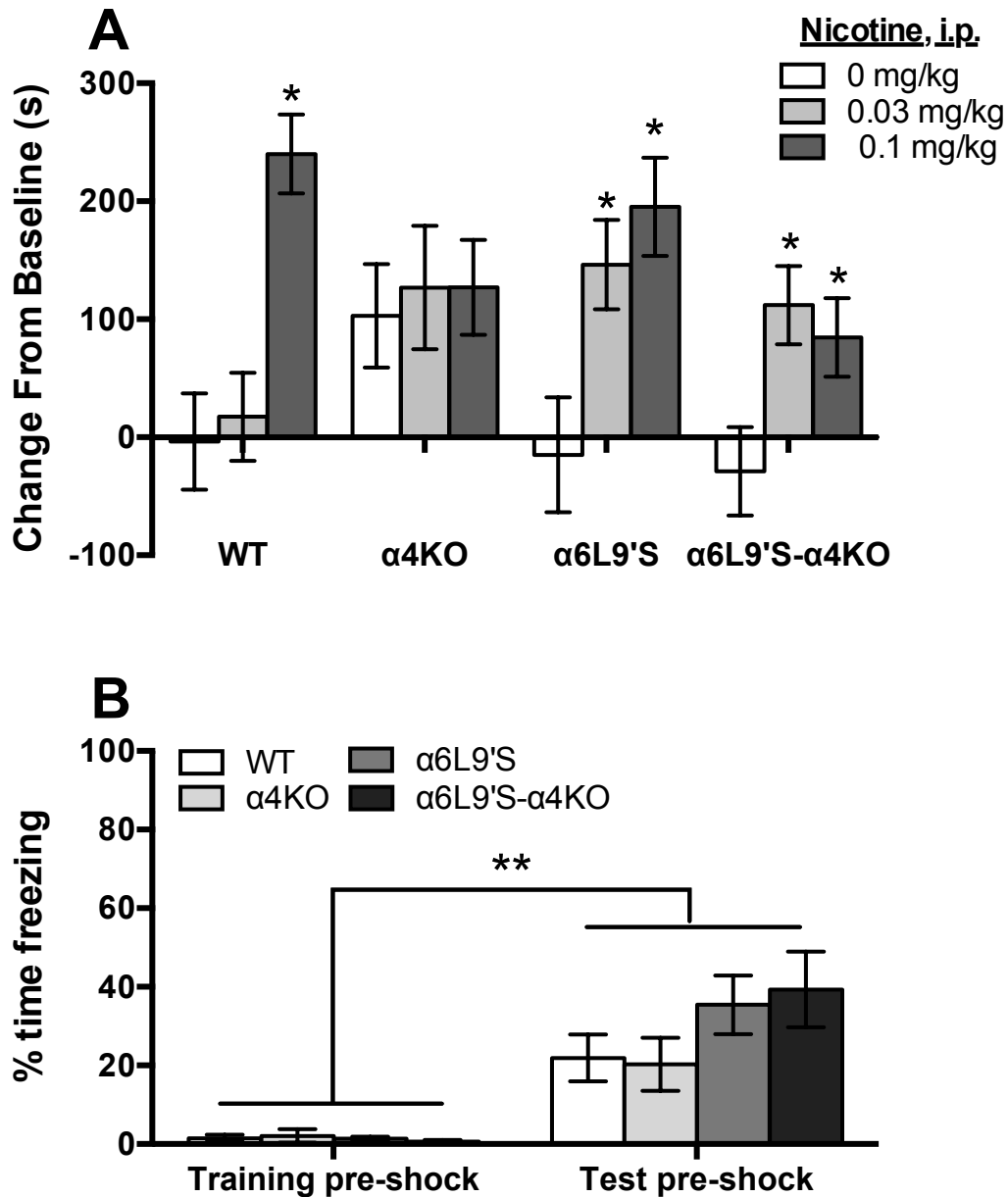


Figure 4.2 – α 6L9'S elevation of nicotine conditioned place preference (CPP) does not require the α 4 subunit. **a)** Nicotine CPP is expressed as change from baseline time spent in the nicotine-paired chamber in WT, α 4KO, α 6L9'S, and α 6L9'S- α 4KO mice receiving saline (WT: $n=10$; α 4KO: $n=8$; α 6L9'S: $n=11$; α 6L9'S- α 4KO: $n=8$), 0.03 mg/kg i.p. nicotine (WT: $n=11$; α 4KO: $n=8$; α 6L9'S: $n=9$; α 6L9'S- α 4KO: $n=7$) and 0.1 mg/kg i.p. nicotine (WT: $n=11$; α 4KO: $n=10$; α 6L9'S: $n=12$; α 6L9'S- α 4KO: $n=9$). **b)** Contextual threat conditioning expressed as percent time freezing during the training and test session for the 2 min time period corresponding to the time period before first footshock (pre-shock) during training in WT ($n=14$), α 4KO ($n=11$), α 6L9'S ($n=13$), and α 6L9'S- α 4KO mice ($n=10$). * p <0.05 vs. saline of same genotype; ** p <0.001 vs. training pre-shock.

Locomotor Activity-Dim Lighting Conditions

To assess (non- $\alpha 4$) $\alpha 6\beta 2^*$ and $\alpha 4\alpha 6\beta 2^*$ nAChR contributions to another DA-related behavior, basal and nicotine-associated locomotor activity was assessed under dim light conditions (Figure 4.3). A 3x4 ANOVA revealed main effects of nicotine dose ($F_{2,102}=27.274, p<0.001$) and genotype ($F_{3,102}=22.642, p<0.001$), as well as a significant interaction of nicotine dose by genotype ($F_{6,102}=7.573, p<0.001$) for distance traveled under dim lighting conditions. In response to i.p. saline, $\alpha 6L9'S$ - $\alpha 4KO$ mice showed a trend for greater distance traveled compared to $\alpha 6L9'S$ mice ($t_{21}=-1.986, p=0.060$). $\alpha 6L9'S$ mice, but not $\alpha 6L9'S$ - $\alpha 4KO$ mice, showed locomotor activation in response to the lower 0.03 mg/kg i.p. nicotine dose ($p=0.002$). Consistent with previous data (Drenan *et al*, 2010), this suggests that the $\alpha 4$ subunit assembles with $\alpha 6$ to support nicotine-stimulated locomotor activity. However, locomotor activity was increased in response to 0.1 mg/kg i.p. nicotine in both $\alpha 6L9'S$ ($p<0.001$) and $\alpha 6L9'S$ - $\alpha 4KO$ mice ($p<0.001$), suggesting a role for (non- $\alpha 4$) $\alpha 6\beta 2^*$ nAChR in locomotor effects of nicotine as well. There was a trend for 0.1 mg/kg i.p. nicotine to increase locomotor in $\alpha 4KO$ mice ($p=0.071$), that did not reach statistical significance.

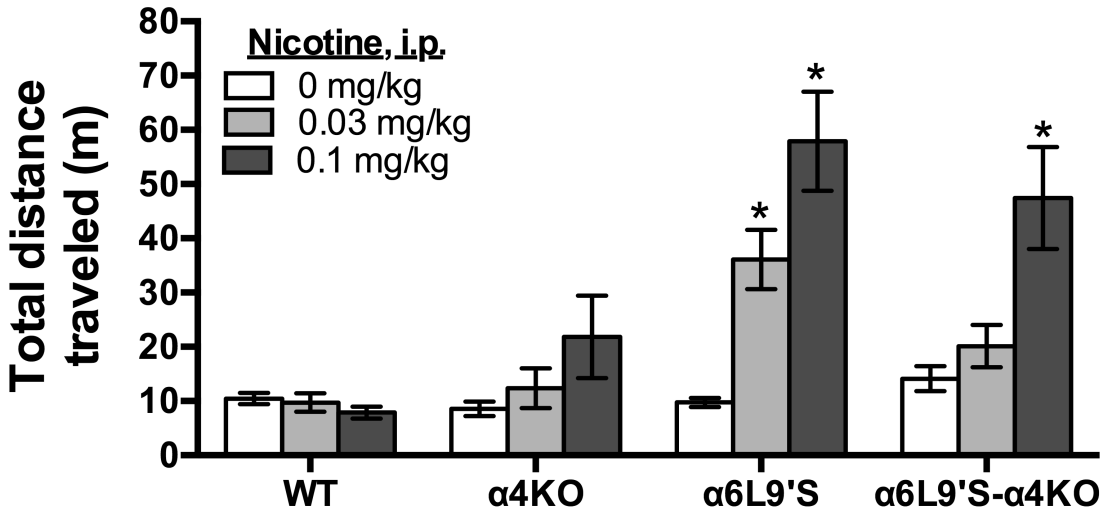


Figure 4.3 – (non- $\alpha 4$) $\alpha 6\beta 2^*$ and $\alpha 4\alpha 6\beta 2^*$ nAChRs play a role in nicotine-stimulated locomotor activity. a) Distance traveled under dim light conditions in WT, $\alpha 4$ KO, $\alpha 6L9'S$, and $\alpha 6L9'S$ - $\alpha 4$ KO mice at saline (WT: $n=13$; $\alpha 4$ KO: $n=10$; $\alpha 6L9'S$: $n=13$; $\alpha 6L9'S$ - $\alpha 4$ KO: $n=10$), 0.03 (WT: $n=11$; $\alpha 4$ KO: $n=7$; $\alpha 6L9'S$: $n=11$; $\alpha 6L9'S$ - $\alpha 4$ KO: $n=8$) and 0.1 mg/kg nicotine (WT: $n=8$; $\alpha 4$ KO: $n=7$; $\alpha 6L9'S$: $n=9$; $\alpha 6L9'S$ - $\alpha 4$ KO: $n=7$). * $p < 0.05$ vs. saline of same genotype.

Anxiety-like Behavior

Nicotine and endogenous ACh can impact anxiety-like behavior. In these studies WT, $\alpha 4$ KO, $\alpha 6$ L9'S, and $\alpha 6$ L9'S- $\alpha 4$ KO were utilized to assess (non- $\alpha 4$) $\alpha 6\beta 2$ *nAChR and $\alpha 4\alpha 6\beta 2$ *nAChR contributions anxiety-like behavior in an open field (Figure 4.4) and light-dark assay (Figure 4.5) in the presence or absence of nicotine.

Open Field

For time spent in the center zone of the open field (Figure 4.4a), a two-way 3x4 ANOVA revealed main effects of nicotine dose ($F_{2,105}=7.705$, $p=0.001$) and genotype ($F_{3,105}=16.726$, $p<0.001$), as well as a significant interaction of nicotine dose by genotype ($F_{6,105}=3.816$, $p=0.002$). Consistent with our previous data, $\alpha 6$ L9'S mice exhibited a heightened basal anxiety-like phenotype, showing decreased time spent in the center zone following i.p. saline compared to WT mice ($t_{25}=7.221$, $p<0.001$). The $\alpha 4$ subunit does appear to be necessary to promote $\alpha 6\beta 2$ *nAChR regulation of basal anxiety-like phenotype, as $\alpha 6$ L9'S- $\alpha 4$ KO mice did not differ from $\alpha 6$ L9'S mice ($t_{22}=-1.466$, $p=0.157$). This indicates that stimulation of (non- $\alpha 4$) $\alpha 6\beta 2$ *nAChRs promotes basal anxiogenic-like behavior as measured by the open field assay. $\alpha 4$ KO mice did not differ from WT mice ($t_{22}=1.201$, $p=0.243$), demonstrating that $\alpha 4\beta 2$ (non- $\alpha 6$)*nAChRs do not appear to regulate anxiety-like behavior in the open field.

Inconsistent with previous studies in our laboratory (Anderson *et al*, 2015), 0.03 mg/kg i.p. nicotine significantly increased anxiety-like behavior in WT mice, producing decreases in time spent in the center zone compared to saline controls ($p=0.013$). There was also a non-significant trend for 0.1 mg/kg i.p. nicotine to decrease time spent in the center zone in WT mice compared to saline controls ($p=0.1$). Similarly, both 0.03 mg/kg ($p=0.005$) and 0.1 mg/kg i.p.

nicotine ($p=0.005$) decreased time spent in the center zone in $\alpha4KO$ mice compared to saline controls, indicating that this anxiogenic effect of nicotine does not require $\alpha4\beta2^*nAChRs$. In $\alpha6L9'S$ and $\alpha6L9'S-\alpha4KO$ mice, neither 0.03 mg/kg or 0.1 mg/kg i.p. nicotine further altered time spent in the center zone compared to saline controls. ($p's>0.3$), suggesting the selective activation of (non- $\alpha4$) $\alpha6\beta2^*nAChRs$ blocks the anxiogenic effect of nicotine.

Total distance traveled in the open field was used as measure of locomotor activity (Figure 4.4b), and a two-way 3x4 ANOVA revealed main effects of nicotine dose ($F_{2,105}=37.262$, $p<0.001$) and genotype ($F_{3,105}=23.767$, $p<0.001$), as well as a significant interaction of nicotine dose by genotype ($F_{6,105}=9.347$, $p<0.001$). Unlike WT mice whose locomotor activity was unaffected by nicotine exposure ($p's>0.8$), 0.03 mg/kg and 0.1 mg/kg i.p. nicotine increased distance traveled in $\alpha6L9'S$ ($p's<0.001$) and $\alpha6L9'S-\alpha4KO$ ($p's<0.05$), with a similar trend for 0.1 mg/kg i.p. nicotine to increase locomotor activity in $\alpha4KO$ mice ($p=0.063$). However, nicotine-stimulated increases in locomotor activity did not impact center zone measures, as these groups did not show any increases in time spent in the center zone in response to nicotine.

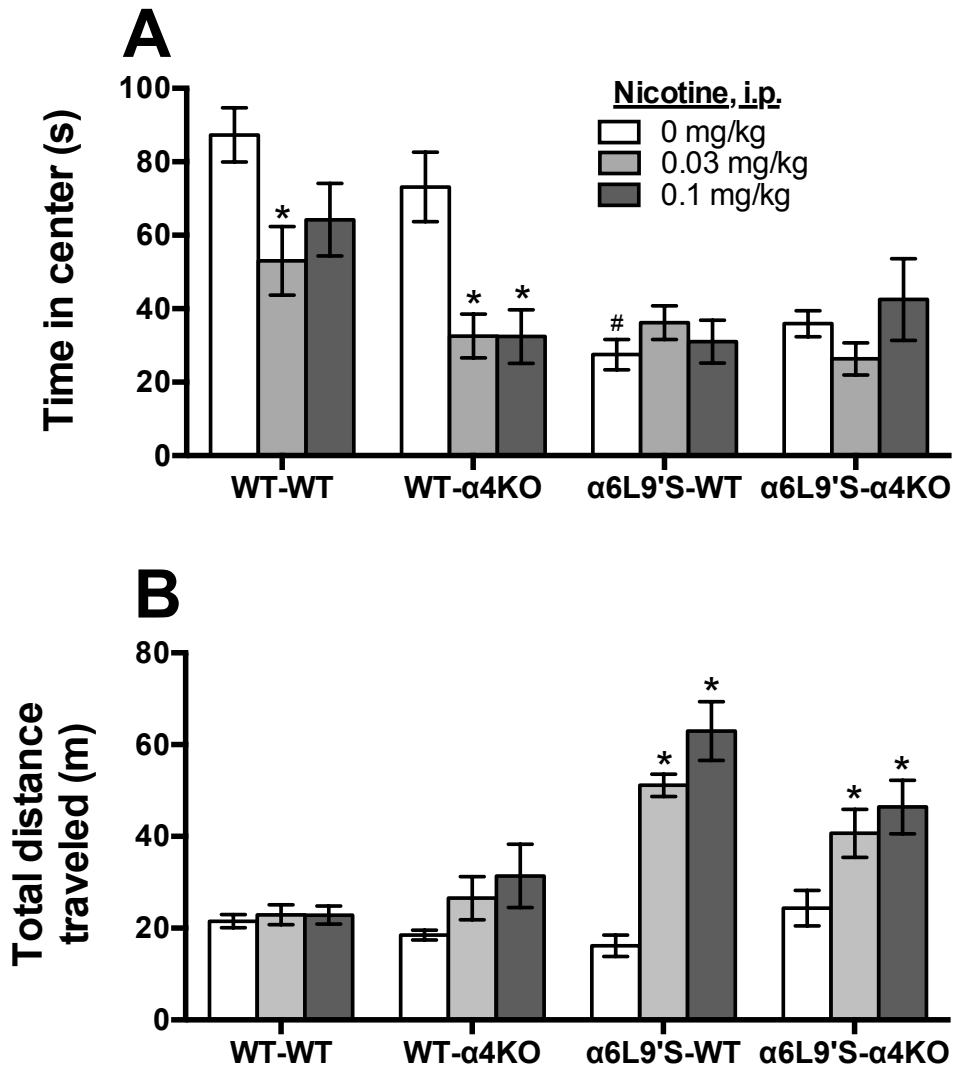


Figure 4.4 – (non- $\alpha 4$) $\alpha 6\beta 2$ *nAChRs modulate basal anxiogenic-like behavior and nicotine-induced anxiogenesis in the open field. a) Time spent in the center zone of the open field arena in WT, $\alpha 4$ KO, $\alpha 6L9'S$, and $\alpha 6L9'S$ - $\alpha 4$ KO mice in response to saline (WT: $n=13$; $\alpha 4$ KO: $n=11$; $\alpha 6L9'S$: $n=14$; $\alpha 6L9'S$ - $\alpha 4$ KO: $n=10$), 0.03 (WT: $n=11$; $\alpha 4$ KO: $n=7$; $\alpha 6L9'S$: $n=11$; $\alpha 6L9'S$ - $\alpha 4$ KO: $n=8$), and 0.1 mg/kg nicotine (WT: $n=9$; $\alpha 4$ KO: $n=7$; $\alpha 6L9'S$: $n=9$; $\alpha 6L9'S$ - $\alpha 4$ KO: $n=7$). **b)** Total distance traveled in the open field arena in WT, $\alpha 4$ KO, $\alpha 6L9'S$, and $\alpha 6L9'S$ - $\alpha 4$ KO mice in response to saline, 0.03, and 0.1 mg/kg i.p. nicotine. * $p < 0.05$ vs. saline of same genotype; # $p < 0.05$ vs. WT of same nicotine dose.

Light-Dark Assay

A two-way 3x4 ANOVA revealed a main effect of nicotine dose ($F_{2,105}=6.390, p=0.002$) and genotype ($F_{3,105}=16.328, p<0.001$), as well as a significant interaction of nicotine dose by genotype ($F_{6,105}=7.943, p<0.001$) for time spent in the light chamber. There were trends for $\alpha6L9'S$ saline-injected mice to spend less time in the light chamber than WT littermates ($t_{24}=1.352, p=0.095$), while $\alpha6L9'S-\alpha4KO$ mice spent significantly more time in the light chamber compared to $\alpha6L9'S$ mice ($t_{22}=-3.312, p=0.003$). $\alpha4KO$ mice showed no differences in time spent in the light chamber in response to i.p. saline compared to WT mice ($t_{22}=1.201, p=0.243$). Together, these data suggest that trends for increases in anxiety-like behavior require $\alpha4$ assembly with $\alpha6$ in the $\alpha4\alpha6\beta2*nAChR$ conformation.

Although nicotine did not increase time spent in the light chamber in WT mice ($p's>0.2$) in contrast to previous reports from this laboratory (Anderson *et al*, 2015), both 0.03 mg/kg ($p=0.002$) and 0.1 mg/kg i.p. nicotine ($p<0.001$) produced increases in time spent in the light chamber in $\alpha6L9'S$ mice. There was no such effect in $\alpha6L9'S-\alpha4KO$ ($p's>0.5$), suggesting that $\alpha4$ is required for $\alpha6L9'S$ reductions in nicotine-associated anxiety-like behavior. A significant interaction of nicotine dose x genotype ($F_{6,105}=7.260, p<0.001$) for light entries revealed that both $\alpha6L9'S$ and $\alpha6L9'S-\alpha4KO$ showed greater light entries following 0.3 ($p's=0.001$) and 0.1 mg/kg i.p. nicotine injection ($p's<0.01$) compared saline controls. This dissociation of light-dark and locomotor activity (as measured by light chamber entries) in $\alpha6L9'S-\alpha4KO$ mice suggests that this effect was not simply due to nicotine-associated changes in activity (Figure 4.5b). In addition, $\alpha6L9'S-\alpha4KO$ mice made more entries into the light chamber compared to $\alpha6L9'S$ mice ($t_{22}=-2.071, p=0.050$) in response to i.p. saline, so that increased time spent in the light chamber in these mice may be a reflection of a basal increase in behavior.

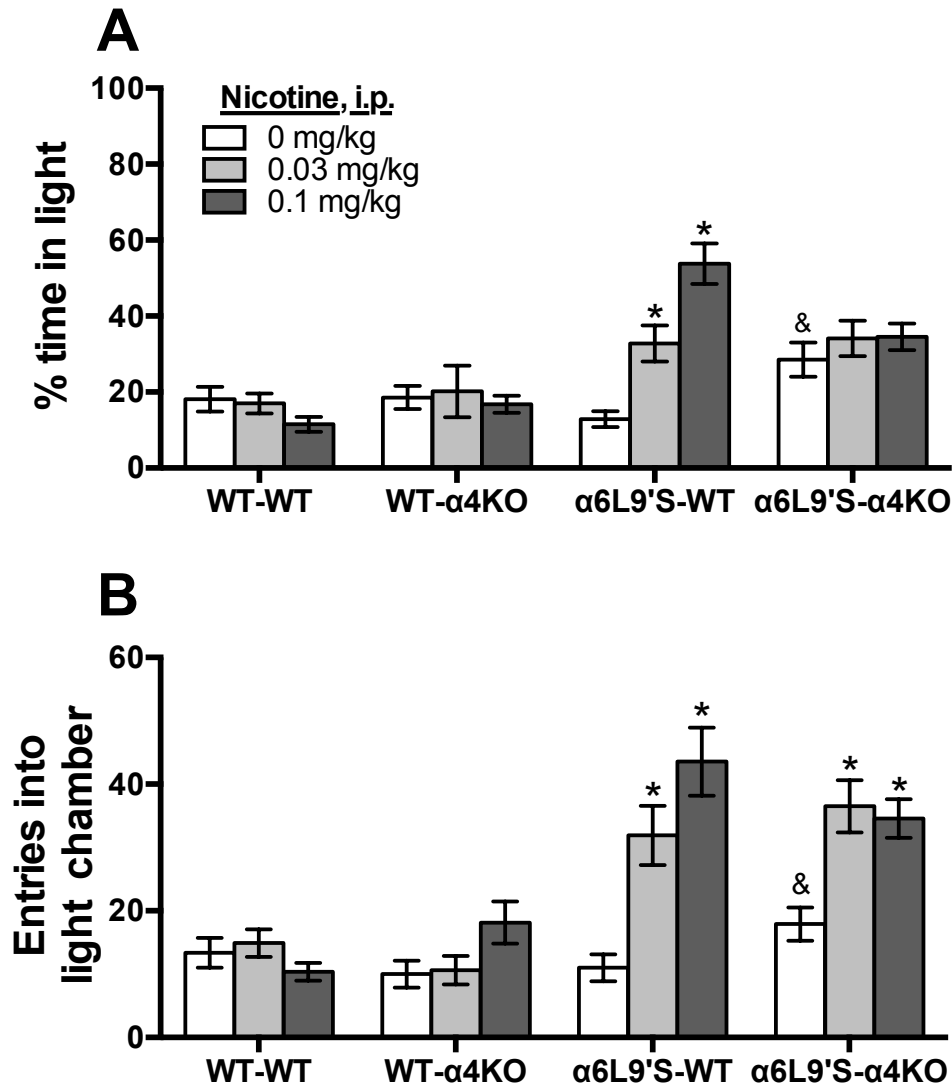


Figure 4.5 – $\alpha4\alpha6\beta2^*$ nAChRs modulate trending increases in basal anxiety-like behavior and are involved with nicotine-associated anxiolysis in the light-dark test. a) Percent time spent in the light chamber of the light-dark box in WT, $\alpha4$ KO, $\alpha6L9'S$, and $\alpha6L9'S$ - $\alpha4$ KO mice in response to saline (WT: $n=13$; $\alpha4$ KO: $n=11$; $\alpha6L9'S$: $n=13$; $\alpha6L9'S$ - $\alpha4$ KO: $n=11$), 0.03 (WT: $n=11$; $\alpha4$ KO: $n=8$; $\alpha6L9'S$: $n=11$; $\alpha6L9'S$ - $\alpha4$ KO: $n=8$), and 0.1 mg/kg nicotine (WT: $n=8$; $\alpha4$ KO: $n=7$; $\alpha6L9'S$: $n=9$; $\alpha6L9'S$ - $\alpha4$ KO: $n=7$). **b)** Entries into the light chamber of the light-dark box in WT, $\alpha4$ KO, $\alpha6L9'S$, and $\alpha6L9'S$ - $\alpha4$ KO mice in response to saline, 0.03, and 0.1 mg/kg i.p. nicotine. * $p<0.05$ vs. saline of same genotype; & $p<0.05$ vs. $\alpha6L9'S$ of same nicotine dose.

DISCUSSION

The present studies assessed contributions of $\alpha 4\alpha 6\beta 2^*$ and (non- $\alpha 4$) $\alpha 6\beta 2^*$ nAChRs to behaviors relevant to nicotine addiction in mice. Nicotine CPP was used as a model of nicotine reward, and the open field and light-dark tests were used as models of anxiety-like behavior. Overall, (non- $\alpha 4$) $\alpha 6\beta 2^*$ nAChRs appear to be sufficient for nicotine reward behavior, while the subtype that contributes to anxiety-like behavior depends on the model of anxiety, as well as whether anxiety-like behavior was measured basally or in response to nicotine.

Consistent with our previous findings (see Chapter 2), $\alpha 6L9'S$ mice showed nicotine CPP at doses subthreshold to support CPP in WT mice. Leftward shifts in these $\alpha 6\beta 2^*$ nAChR gain-of-function mice suggest that stimulation of $\alpha 6\beta 2^*$ nAChRs enhances nicotine reward behavior. This enhancement does not require the $\alpha 4$ subunit, as $\alpha 6L9'S$ - $\alpha 4KO$ mice don't differ from $\alpha 6L9'S$ mice, demonstrating that stimulation of (non- $\alpha 4$) $\beta \alpha 62^*$ nAChRs is sufficient to drive nicotine's rewarding properties. Of all nAChR subtypes, $\alpha 4\alpha 6\beta 2^*$ nAChRs are known to have the highest sensitivity to nicotine (Exley *et al*, 2008; Kuryatov *et al*, 2011; Liu *et al*, 2012; Salminen *et al*, 2007; Salminen *et al*, 2004). Moreover, previous electrophysiology, synaptosome, and behavioral studies demonstrate that $\alpha 4\alpha 6\beta 2^*$ nAChRs mediate augmentation of mesolimbic DA activity and associated locomotor activity in $\alpha 6L9'S$ mice, as enhancement of these phenotypes in $\alpha 6L9'S$ mice required the $\alpha 4$ subunit (Drenan *et al*, 2010; Engle *et al*, 2013). Further, α -Ctx MII blocks enhancement of VTA DA neuron firing produced by the $\alpha 4L9'A$ gain-of-function mutation (Liu *et al*, 2012; Zhao-Shea *et al*, 2011), suggesting that the $\alpha 4$ subunit gain-of-function acts primarily through $\alpha 4\alpha 6\beta 2^*$ nAChRs to support increased sensitivity to nicotine and elevated mesolimbic DA activity. As DA is associated with nicotine's rewarding properties, it was expected that $\alpha 4$ assembly with $\alpha 6$ in the $\alpha 4\alpha 6\beta 2^*$ nAChR

conformation would be required for expression of enhanced nicotine reward behavior in $\alpha 6L9'S$ mice. Given the divergent results regarding the $\alpha 6\beta 2*nAChR$ subtype that regulates mesolimbic DA activity and nicotine reward, it could be that DA is not a key regulator of enhanced nicotine reward behavior observed in $\alpha 6L9'S$ mice.

$\alpha 4KO$ mice showed similar levels of nicotine CPP at 0.03 mg/kg and 0.1 mg/kg i.p. nicotine compared to $\alpha 6L9'S$ and $\alpha 6L9'S-\alpha 4KO$ mice. Statistically speaking however, $\alpha 4KO$ mice did not show significant CPP, as $\alpha 4KO$ mice receiving nicotine did not differ from their saline controls. It should be noted that preference scores in the saline controls were unusually high, which could have precluded observation of nicotine CPP in these mice. Thus, it is not completely clear from these studies whether $\alpha 4KO$ mice express CPP or not. Further complicating matters, previous evidence is conflicting, with two studies showing that $\alpha 4KO$ mice don't express nicotine CPP (McGranahan *et al*, 2011; Sanjakdar *et al*, 2015), and one study showing that $\alpha 4KO$ mice express nicotine CPP similar to WT mice (Cahir *et al*, 2011). If in fact there is a leftward shift in the $\alpha 4KO$ mice similar to $\alpha 6L9'S$ and $\alpha 6L9'S-\alpha 4KO$ mice, this would suggest that $\alpha 4\beta 2*nAChRs$ act in opposition of other nAChR subtypes to reduce nicotine's rewarding properties, as genetically reducing $\alpha 4\beta 2*nAChR$ function would enhance nicotine reward behavior. Future studies repeating this CPP experiment is necessary to determine whether $\alpha 4KO$ mice show nicotine CPP or not in our studies.

Like nicotine reward, locomotor activity is also a DA-dependent behavior. Activity of the mesolimbic DA pathway is required for nicotine's locomotor activating effects, as studies show that 6-OHDA lesions of the NAc (Clarke *et al*, 1988) and VTA (Louis and Clarke, 1998), as well as DA receptor antagonists (King *et al*, 2004b) block the locomotor stimulant effect of nicotine. Further, nicotine-associated changes in locomotor activity are not observed in $\beta 2KO$ mice (King

et al, 2004b), suggesting that $\beta 2$ *nAChRs are important for locomotor activation driven by mesolimbic DA activity. Moreover, previous studies described in Chapter 2 of this dissertation demonstrate that activation of $\alpha 6\beta 2$ *nAChRs promote nicotine-stimulated locomotor activity, as $\alpha 6L9$ 'S mice showed an enhanced response to nicotine compared to WT mice. These present findings are consistent with previous studies showing that $\alpha 4\alpha 6\beta 2$ *nAChRs are involved in the locomotor stimulant effect of nicotine and related mesolimbic DA activity (Drenan *et al*, 2010; Engle *et al*, 2013).

Other than nicotine's rewarding properties, smokers also report that they smoke to relieve anxiety. Our previous data from Chapter 3 of this dissertation demonstrates that $\alpha 6L9$ 'S mice display enhanced basal levels of anxiogenic-like behavior, suggesting that stimulation of $\alpha 6\beta 2$ *nAChRs promote anxiogenesis. These effects are likely a result of an enhanced response of $\alpha 6\beta 2$ *nAChRs to endogenous acetylcholine (ACh). This interpretation is consistent with data from preclinical rodent studies suggesting that cholinergic hyperactivity promotes anxiety-like behavior (Hart *et al*, 1999; Kolasa *et al*, 1982; Lamprea *et al*, 2000; Luo *et al*, 2013; Mineur *et al*, 2009; Power *et al*, 2002; Revy *et al*, 2014). Consistent with our previous findings in $\alpha 6L9$ 'S mice (see Chapter 3), we observed that these $\alpha 6\beta 2$ *nAChR gain-of-function mice exhibited exaggerated basal anxiogenic-like behavior compared to WT mice in the open field, and showed trends for increases in basal anxiety-like behavior in the light-dark assay. In the open field, $\alpha 6L9$ 'S- $\alpha 4$ KO showed elevated levels of anxiety-like behavior like $\alpha 6L9$ 'S mice, indicating that endogenous cholinergic activity at (non- $\alpha 4$) $\alpha 6\beta 2$ *nAChRs is responsible for the elevated levels of anxiety in $\alpha 6L9$ 'S mice in this model of anxiety. In contrast to the open field, $\alpha 6L9$ 'S- $\alpha 4$ KO mice exhibited a decrease in anxiety-like behavior compared to $\alpha 6L9$ 'S mice in the light-dark

test, suggesting that ACh stimulation of $\alpha 4\alpha 6\beta 2$ *nAChRs modulates trending increases in anxiety-like behavior.

These studies also examined nicotine-associated anxiety-like behavior. In the open field test, 0.03 and 0.1 mg/kg i.p. nicotine produced decreases in center zone time in WT mice, reflecting an increase in anxiety-like behavior, while nicotine had no effect in WT mice in the light-dark test. This is inconsistent with previous studies showing that doses in this range or lower are anxiolytic (Anderson *et al*, 2012, 2015; File *et al*, 1998; McGranahan *et al*, 2011; Varani *et al*, 2012). While the mice used in these studies are backcrossed at least 10 generations on a C57BL/6J background, this discrepancy between WT mice in our studies and WT mice in previous studies may be explained by mouse strain differences. Different effects in WT mice may also be due to differences in basal levels of anxiety-like behavior produced by different experimenters and different environments.

In $\alpha 6L9$ 'S mice, selective activation of $\alpha 6\beta 2$ *nAChRs appeared to block nicotine-associated anxiogenesis observed in WT mice in the open field test. This did not require the $\alpha 4$ subunit, indicating that (non- $\alpha 4$) $\alpha 6\beta 2$ *nAChRs mediate this effect. It is interesting that stimulation of (non- $\alpha 4$) $\alpha 6\beta 2$ *nAChRs, presumably in response to ACh, promotes basal anxiety-like behavior, while stimulation of this same subtype decreased nicotine-induced anxiogenic-like behavior in the same open field test of anxiety-like behavior. As inhibition of $\beta 2$ *nAChRs reduces anxiety-like behavior, nicotine may have desensitized (non- $\alpha 4$) $\alpha 6\beta 2$ *nAChRs in $\alpha 6L9$ 'S mice to decrease this behavior, while ACh may have activated $\alpha 6L9$ 'S (non- $\alpha 4$) $\alpha 6\beta 2$ *nAChRs to increase anxiety-like behavior. This is supported by studies indicating that nAChR desensitization is prohibited by acetylcholinesterase (Brown *et al*, 1936; Katz *et al*, 1957; Thesleff, 1955), an enzyme that breaks down ACh, while nicotine desensitizes nAChRs

(Fenster *et al*, 1997; Grady *et al*, 2012; Kuryatov *et al*, 2011; Lester *et al*, 1995; Lu *et al*, 1999; Mansvelder *et al*, 2002; Pidoplichko *et al*, 1997).

In the light-dark test, nicotine decreased anxiety-like behavior in $\alpha 6\beta 2$ mice, suggesting that stimulation of $\alpha 6\beta 2$ nAChRs by nicotine reduces anxiety-like behavior. This is somewhat consistent with our open field data; that is, while selective stimulation of $\alpha 6\beta 2$ nAChRs did not decrease anxiety-like behavior in the open field, it did relieve the anxiogenic effect of nicotine. However, unlike in the open field where the $\alpha 4$ subunit was not required for this reversal, the $\alpha 4$ subunit was required to assemble with the $\alpha 6$ subunit in the $\alpha 4\alpha 6\beta 2$ nAChR conformation to promote this anxiolytic effect in the light-dark test. It is interesting that trending basal anxiogenic-like behavior measured by the light-dark test is mediated by enhanced cholinergic activity at $\alpha 4\alpha 6\beta 2$ nAChRs, while nicotine activates $\alpha 4\alpha 6\beta 2$ nAChRs to promote anxiolysis in this same anxiety model. As discussed above in regard to open field data, ACh may be stimulating these receptors to increase anxiety-like behavior, whereas nicotine may be desensitizing these receptors to promote anxiolysis.

We did expect for $\alpha 6\beta 2$ mice to show increases, instead of decreases in anxiety-like behavior in response to nicotine in our studies, given that activation of $\alpha 6\beta 2$ nAChRs promotes basal anxiety-like behavior and that $\alpha 6\beta 2$ nAChRs are persistently activated at concentrations of nicotine that typically desensitize other nAChRs (Liu *et al*, 2012). As discussed above, nicotine may in fact be desensitizing $\alpha 6\beta 2$ nAChRs in $\alpha 6\beta 2$ mice, but further studies are needed to characterize $\alpha 6\beta 2$ nAChR desensitization in these gain-of-function mice.

As with basal anxiety-like behavior, where (non- $\alpha 4$) $\alpha 6\beta 2$ nAChR promote anxiogenesis in the open field, but $\alpha 4\alpha 6\beta 2$ nAChRs promote trending anxiogenesis in the light-dark assay,

(non- $\alpha 4$) $\alpha 6\beta 2^*$ and $\alpha 4\alpha 6\beta 2^*$ nAChRs contribute to nicotine-associated anxiety-like behavior uniquely depending on the model of anxiety. Specifically, these studies suggest that selective stimulation of (non- $\alpha 4$) $\alpha 6\beta 2^*$ nAChRs blocks nicotine-induced anxiogenic-like behavior in the open field test, and while stimulation of $\alpha 4\alpha 6\beta 2^*$ nAChRs promotes anxiolysis in the light-dark test. Given that anxiety is a complex, multifaceted behavior, these tests of anxiety-like behavior could model different aspects of anxiety (Ramos and Mormede, 1998) that are controlled by unique mechanisms. This is supported by quantitative trait loci (QTL) studies showing that different models of anxiety have overlapping, but separate genetic underpinnings (Griebel *et al*, 2000; Henderson *et al*, 2004; Turri *et al*, 2001).

In conclusion, these experiments demonstrate distinct roles of (non- $\alpha 4$) $\alpha 6\beta 2^*$ nAChRs and $\alpha 4\alpha 6\beta 2^*$ nAChRs in behaviors relevant to nicotine addiction, namely reward and anxiety-like behavior. (non- $\alpha 4$) $\alpha 6\beta 2^*$ nAChRs appear to be sufficient to regulate nicotine's rewarding properties, while both (non- $\alpha 4$) $\alpha 6\beta 2^*$ nAChRs and $\alpha 4\alpha 6\beta 2^*$ nAChRs modulate different aspects of anxiety-like behavior. This data expands on the existing literature to further elucidate which nAChR subtypes regulate some of nicotine's addictive properties.

Chapter 5 – Oral operant ethanol self-administration in the absence of explicit cues, food restriction, water restriction and ethanol fading in C57BL/6J male mice

Published: Stafford AM, Anderson SM, Shelton KL, Brunzell DH (2015) *Psychopharmacology* 232(20):3783-95

INTRODUCTION

Alcohol abuse is a pervasive problem worldwide (WHO, 2011). Genetics play a major role in vulnerability to alcohol use disorder (AUD) (Gillespie *et al*, 2012; Prescott and Kendler, 1999; Schuckit and Smith, 1996), and understanding the molecular mechanisms that underlie ethanol (EtOH) use phenotypes may lead to novel treatment and prevention of AUD and alcoholism.

Alcohol consumption is motivated by environmental and psychosocial factors that are difficult to control in human experiments; hence, animal models are ideal for isolating biological and environmental factors which contribute to behaviors that promote EtOH use.

Mouse EtOH consumption models are commonly used to investigate the genetic and pharmacological mechanisms of EtOH endophenotypes (Rhodes and Crabbe 2003; Tabakoff and Hoffman, 2000). In mice, EtOH ingestion is typically measured using bottle choice paradigms or drinking-in-the-dark (DID) (Rhodes *et al*, 2005; Ryabinin *et al*, 2003). These non-operant self-administration models respectively assess EtOH preference compared to a vehicle solution and achieve high levels of EtOH intake but have been criticized as being less effective at assessing EtOH reinforcement (Tabakoff *et al*, 2000). Operant self-administration paradigms measure the

ability of positive reinforcers (e.g. EtOH) to increase the likelihood that a human or animal subject will exert effort to obtain the reinforcer.

Operant self-administration methods in rats (e.g. Augier *et al*, 2014; Cannady *et al*, 2013; Doyon *et al*, 2013a; van Erp and Miczek, 2007) and mice (e.g. Cunningham *et al*, 2000; Elmer *et al*, 1986; Ford, 2014; Middaugh *et al*, 1999a; Risinger *et al*, 1998; Samson, 1986) often utilize strategies such as food and water restriction to promote operant EtOH self-administration, which may introduce factors other than EtOH reinforcement (e.g. thirst, caloric intake). Gradual fading of sweetener (from high to low concentrations) and EtOH (from low to high concentrations) mimics the evolution of human patterns of alcoholic drink preference (Duncan *et al*, 2012) and has demonstrated success in promoting EtOH self-administration in mice (e.g. Elmer *et al*, 1986; Middaugh *et al*, 1999a; Risinger *et al*, 1998). Other models provide EtOH in the home cages of rodents to facilitate operant EtOH self-administration (Rodd *et al*, 2002). From the perspective of understanding the biology of the progression of EtOH use, however, it would be advantageous to employ a procedure that enables independent observation of how EtOH dose and length of exposure might impact EtOH reinforcement and physiological measures. A between-subject design using vehicle controls would also be advantageous for studies assessing the effects of EtOH self-administration on neuroplasticity. Another advantage of a between-subject design is that initial sensitivity to EtOH-associated sedation and reward (i.e., liking), which are predictive of heavy drinking and escalation of EtOH use in humans (King *et al*, 2011; Schuckit *et al*, 1996), may be assessed in mice during initial exposure to EtOH.

Environmental factors such as sweeteners and cues are physiologically relevant to promoting EtOH administration in humans (Dager *et al*, 2014; Dager *et al*, 2013; Garland *et al*, 2012; King *et al*, 2011; O'Connor and Colder, 2009; Petit *et al*, 2013; Schuckit *et al*, 1996;

Sjoerds *et al*, 2014) and hence are of interest to study in rodent models in a controlled fashion. Explicit cues and flavorants become secondary reinforcers when paired with drug (Browne *et al*, 2014; Brunzell *et al*, 2006) and may have reinforcing effectiveness on their own in rodents (Browne *et al*, 2014; Olsen and Winder, 2009; Regier *et al*, 2012). The development of an EtOH self-administration model in the absence of contingent sweeteners and cue presentation would facilitate isolation of biological factors which drive the primary reinforcing effects of EtOH in the absence of cues and sweeteners. The present studies controlled for sweetener that was paired with EtOH by providing EtOH in water or saccharin solution using a weekly overnight mouse model of oral operant EtOH self-administration that did not involve explicit cues, food restriction, water restriction, or the gradual fading of EtOH. The availability of a water bottle in the operant conditioning chamber further enabled comparison of water bottle and liquid dipper intake in order to assess the potential rewarding properties of EtOH under these conditions when compared against vehicle control subjects.

MATERIALS AND METHODS

Subjects

Fifty-four adult, male, C57BL/6J mice (Jackson Labs, Bar Harbor, ME) aged 14–17 weeks at the initiation of training were used for this study. Mice were group housed (4–5 per cage) in a temperature- and humidity-controlled vivarium. They were housed under a 12 h light/dark schedule (lights on at 0600 hours) and had *ad libitum* access to food and water. Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University and were in accordance with the Guidelines for the Care and Use of Laboratory Animals, as set forth by the National Institutes of Health.

Apparatus

Operant EtOH self-administration procedures were conducted in mouse operant conditioning chambers (21.6 cm × 17.8 cm × 12.7 cm; Med Associates, St. Albans, VT) housed inside sound-attenuating cabinets with a ventilation fan. Each chamber was equipped with two retractable levers placed 2.5 cm above the floor. One lever, designated active, resulted in the presentation of a liquid dipper that provided 0.01 ml of fluid; the other lever, designated inactive, had no consequence when depressed. The liquid dipper was located within a magazine equidistant between the two levers and equipped with a photobeam sensor to record head entries into the magazine during the presence or absence of the liquid dipper presentation. A 100 mA house light, located 11 cm above the floor on the opposite wall, was on during the session. A water bottle with sipper tube provided ad libitum access to water during EtOH self-administration sessions, and food pellets were placed on the floor. Med PC IV software and Med Associates interfacing controlled liquid dipper presentations and recorded active and inactive lever pressing, liquid dipper reinforcers earned, and magazine entries during the presence (correct entry) and absence (incorrect entry) of liquid dipper presentation. Data were collected in 15 min time bins.

Drugs

EtOH was diluted in tap water or 0.2% saccharin (SAC) in tap water and provided to mice for voluntary oral intake. Naltrexone was diluted in SAL and administered i.p. at a volume of 0.1 ml per 30 g mouse immediately prior to overnight EtOH self-administration sessions.

Magazine training

Mice received 80 liquid dipper presentations of 0.01 ml of SAC solution on a variable time 30 s schedule (13–47 s range). Mice were trained to a criterion of at least 20% magazine entry during

dipper presentations or for up to three training sessions. Magazine training took place during the light cycle between 1300 and 1500 hours.

Acquisition of lever pressing

Next, mice underwent acquisition of lever pressing in the absence of EtOH to assure that all groups of mice demonstrated reliable lever pressing and goal tracking behavior prior to their first exposure to EtOH. Mice were trained during 16 h overnight sessions to lever press for SAC dipper presentations. Sessions began between 1700 and 1800 hours. Active lever pressing for SAC was maintained on FR1 for the first 20 reinforcers, FR2 for the 20 subsequent reinforcers, and FR4 for the 10 subsequent reinforcers. For the remainder of the session, the FR4 schedule was shifted to a variable ratio (VR) 5 schedule (1–12 range). Training continued for two to five sessions until animals pressed the active lever at least 40 times and showed at least 100 s of head entry into magazine during fluid dipper presentation.

Weekly, overnight EtOH sessions

After meeting training criteria, mice underwent 16 h overnight operant self-administration sessions once every 7 days for 9 weeks. Individual mice lever pressed on a VR5 schedule of reinforcement maintained by 0, 3, 10, or 15% EtOH (v/v). Independent groups of mice were reinforced with EtOH in SAC vehicle ($n=4-5/\text{dose}$) or tap water vehicle ($n=5-9/\text{dose}$). Due to limited availability of operant conditioning chambers, each experiment was completed in three to four replicates. A subset of mice reinforced with EtOH in water was subsequently administered 1.25 mg/kg i.p. naltrexone or vehicle (weeks 10–11) in a counterbalanced order prior to EtOH self-administration. These tests were subsequently repeated with 0.3 mg/kg i.p. naltrexone or vehicle (weeks 12–13). These naltrexone doses have been previously shown to decrease rodent

operant self-administration of EtOH (Hay *et al*, 2013; Middaugh *et al*, 2000). Performance following the two saline sessions was averaged for analysis. Self-administration measures and EtOH intake were assessed during the first hour of the session, when peak naltrexone levels were most likely achieved (Wang *et al*, 2004). For all experiments, estimated drinking from the liquid dipper was calculated by multiplying the number of correct head entries (magazine head entry at the time of liquid dipper presentation) by 0.01 ml. Total EtOH dose consumed was estimated by multiplying intake volume by the EtOH concentration available for self-administration. The estimated total dose of EtOH self-administered was correlated with blood EtOH concentrations (BEC) on subsequent weeks upon completion of behavioral testing. Water bottle fluid intake was determined by measuring bottle weights immediately before and after each session. A separate dummy water bottle, located inside of the sound-attenuating cabinet, was weighed before and after each session to correct for water bottle drippage. Reinforcers earned and active lever presses provided measures of reinforcement. Response accuracy was determined by the percentage of active lever presses: active/ (active+inactive lever presses). Bouts of responding were evaluated during the overnight session using a skewness equation below, where x=each individual time bin value in the sample, \bar{x} =the average of the time bin values, n=sample size, and s=standard deviation of the sample. The percentage of fluid intake from the liquid dipper (Operant:*ad libitum* choice) was calculated by comparing estimates of liquid dipper intake to total fluid intake: liquid dipper fluid intake/ (water bottle+liquid dipper fluid intake).

$$\text{Skewness} = \frac{n}{(n-1)(n-2)} \sum \left(\frac{x_i - \bar{x}}{s} \right)^3$$

Blood ethanol concentration analysis

For validation of the g/kg EtOH estimate, trunk blood was collected after 30 min of self-administration from a separate group of mice reinforced with 15% EtOH in SAC ($n=4$). Additionally, blood samples were collected at 4 or 6 h into the session from a subset of mice reinforced with 0, 3, 10, or 15% EtOH in water ($n=5$ /dose) and 15% EtOH in SAC ($n=4$) by submandibular sampling using 5 mm Goldenrod Animal Lancets (MEDIpoinT, Mineola, NY). Samples were collected into BD Microtainer sampling tubes containing EDTA and 50 μ l whole blood aliquots were immediately pipetted into 20 ml headspace gas chromatography vials containing deionized water, 500 mg NaCl, and 1-propanol internal standard. Sample vials were tightly sealed and stored at -20°C until analysis. Blood samples were tested for EtOH concentration using an Agilent model 6890 gas chromatograph (GC) equipped with a flame ionization detector (FID), 0.53 mm ID Rtx BAC-1 capillary column (Restek, Bellefonte, PA) and CTC CombiPal headspace autosampler (Leap Technologies, Carrboro, NC). Samples were incubated and shaken for 10 min at 70°C prior to automated injection. The GC parameters were 1 ml headspace injection volume, 2/1 split ratio, 5 min sample run time, injector temperature 200°C , oven temperature isothermal 50°C , detector temperature 200°C , helium carrier gas flow rate 40 ml/min, nitrogen makeup gas flow rate 18 ml/min, hydrogen flame flow rate 25 ml/min, and FID air flow rate 300 ml/min. Data were collected and analyzed by Clarity GC software (Apex Data Systems, Prague, CZ) using a linear regression analysis with no weighting. EtOH concentrations were calculated by the internal standard method. A seven-point calibration curve preceded the analysis of blood EtOH concentrations. Quality control EtOH standards at concentrations similar to those found in the test samples were interspersed at regular intervals with blood samples.

Statistical analysis

SPSS software was used for all statistical analyses. Statistical analyses assessed reinforcers earned, active lever presses, percent active lever presses, EtOH consumed, skewness, and operant:*ad libitum* choice using 2×4×9 (water/SAC vehicle×EtOH concentration×session) repeated measures ANOVAs with vehicle and EtOH concentration as between-subject factors and weekly session as a repeated measure, within-subject factor. Separate 3×4 (naltrexone dose×EtOH concentration) repeated measures ANOVAs assessed reinforcers earned, active lever presses, and EtOH consumed following naltrexone administration with naltrexone dose as a within-subject factor and EtOH concentration as a between-subject factor. Significant interactions that included vehicle were followed by independent ANOVAs for SAC and water vehicle mice and pairwise comparisons across vehicle groups where relevant. Significant main effects were further assessed using Dunnett's post hoc tests; significant interactions were assessed using two-tailed t-tests. Planned comparisons compared session 1 to session 9 across EtOH concentrations. Estimates of EtOH intake were compared against BEC using a two-tailed Pearson correlation. The criterion for significance was set at $p<0.05$. Data points more than 2 standard deviations from the mean were considered outliers and excluded from analysis.

RESULTS

Operant self-administration of EtOH in SAC versus water vehicle

There was no significant difference between groups of mice on measures of reinforcers earned, active lever pressing, response accuracy, or head entries during dipper presentation for SAC prior to receiving any EtOH (Table 5.1).

Table 5.1 – Acquisition of lever pressing maintained by 0.2% saccharin (SAC) reinforcement

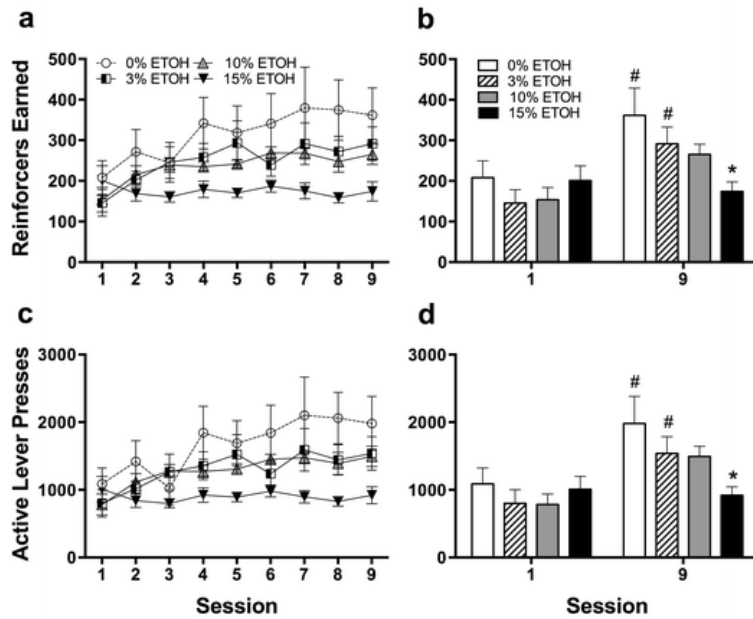
	EtOH in SAC vehicle				EtOH in water vehicle			
	0%	3%	10%	15%	0%	3%	10%	15%
Reinforcers Earned	219.0 (73.34)	199.4 (32.36)	211.6 (48.47)	139.8 (24.52)	157.0 (18.08)	172.7 (42.97)	163.6 (21.58)	161.4 (27.34)
Active Lever Presses	991.0 (387.90)	873.0 (172.19)	952.8 (245.32)	679.8 (162.36)	685.6 (94.26)	759.2 (211.86)	628.6 (180.00)	704.2 (131.36)
Response Accuracy	96.0 (1.63)	94.8 (0.26)	92.5 (4.85)	86.3 (3.22)	90.3 (2.05)	89.0 (2.88)	88.3 (1.33)	89.63 (2.56)
Correct Entries: Reinforcers Earned	0.93 (0.05)	0.93 (0.02)	0.82 (0.08)	0.85 (0.08)	0.89 (0.05)	0.88 (0.05)	0.79 (0.13)	0.90 (0.05)

Mean reinforcers earned, active lever presses, response accuracy (ratio of active lever presses:total lever presses), and ratio of correct head entries:reinforcers earned are depicted for all groups of mice at completion of training with 0.2% saccharin (SAC) reinforcement prior to initiation of ethanol (EtOH) in water or EtOH in SAC self-administration. SEM is shown in parentheses.

For EtOH self-administration, there was a significant interaction of vehicle by EtOH concentration by session for reinforcers earned ($F_{24,336}=4.635, p<0.001$) and active lever presses ($F_{24,336}=4.525, p<0.001$). Across EtOH sessions, mice that received EtOH in SAC vehicle showed concentration-dependent changes in EtOH reinforcement and intake as measured by a significant interaction of session by EtOH concentration for reinforcers earned ($F_{24,112}=1.701, p<0.05$) and active lever presses ($F_{24,112}=1.768, p<0.05$) (Figure 5.1). Animals receiving SAC vehicle and 3% EtOH in SAC, but not mice receiving higher concentrations of EtOH in SAC, showed significant increases in reinforcers earned and active lever presses across sessions ($p<0.05$). Of mice reinforced with EtOH in SAC, only mice reinforced with 15% EtOH differed on reinforcement measures from SAC vehicle controls. By the ninth week of EtOH exposure, mice receiving 15% EtOH in SAC earned significantly fewer reinforcers ($t_6= 2.632, p<0.05$) and provided fewer lever presses ($t_6=2.544, p<0.05$) than animals receiving SAC vehicle. In contrast, mice reinforced with EtOH in water vehicle showed a main effect of EtOH concentration on reinforcers earned ($F_{3,28}= 9.667, p<0.001$) and active lever presses ($F_{3,28}=9.045, p<0.001$), revealing that mice receiving 15% EtOH in water showed evidence of EtOH reinforcement as measured by significantly greater reinforcers earned and active lever presses compared to water vehicle control mice ($p<0.001$) (Figure 5.1). There was a significant interaction of session by EtOH concentration for reinforcers earned ($F_{24,224}=3.95, p<0.001$) and active lever presses ($F_{24,224}=3.79, p<0.001$) demonstrating that mice receiving 15% EtOH in water also showed an escalation of reinforcers earned ($t_8=-4.164, p<0.01$) and active lever presses ($t_8=-4.519, p<0.01$) across sessions. In the absence of SAC vehicle used during lever training, water and 3 and 10% EtOH mice showed significant decreases in reinforcers earned and lever pressing across sessions ($p<0.05$). SAC vehicle mice earned significantly more reinforcers and made significantly more

lever presses than water vehicle mice ($p < 0.001$) (note y-axis break for water vehicle mice), demonstrating that SAC was reinforcing and may have precluded observation of EtOH reinforcement in mice receiving SAC vehicle. Mice on average showed high levels of response accuracy, $>80\%$, but SAC vehicle mice achieved an overall higher level of response accuracy than water vehicle mice ($F_{1,32} = 22.456, p < 0.001$).

SAC Vehicle



Water Vehicle

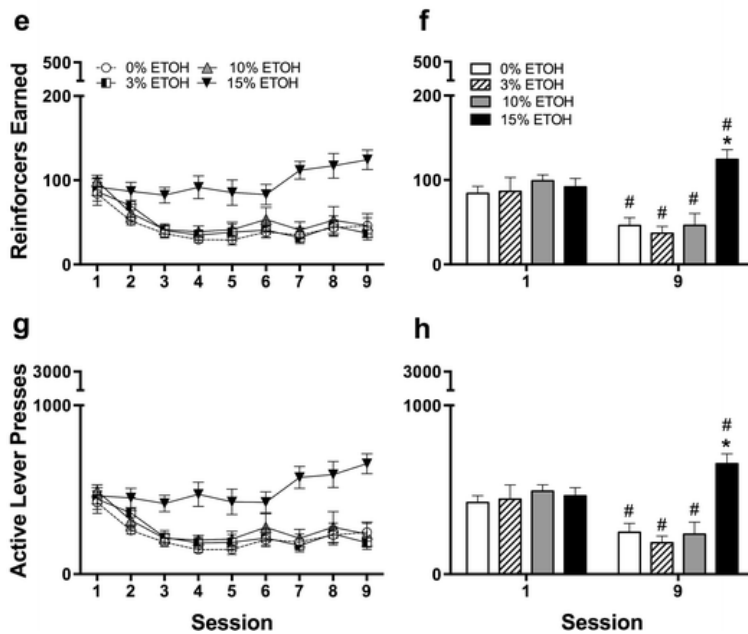


Figure 5.1 – 0.2% saccharin (SAC) vehicle masked ethanol (EtOH) reinforcement, while 15% EtOH in water was reinforcing in mice. Reinforcers earned and active lever presses are shown for mice reinforced with **a–d**) 0% ($n = 4$), 3% ($n = 5$), 10% ($n = 5$), and 15% EtOH ($n = 5$) in 0.2% saccharin (SAC) or **e–h**) 0% ($n = 9$), 3% ($n = 9$), 10% ($n = 5$), and 15% EtOH ($n = 9$) in water vehicle across weekly sessions, highlighting differences between sessions 1 and 9. Data are presented as means \pm SEM. * $p < 0.05$ compared to 0% EtOH controls during the same session; [#] $p < 0.05$ compared to the same concentration on session 1.

Intake of EtOH in SAC versus water vehicle

Estimates of g/kg EtOH intake were validated via positive correlation with BEC in a subgroup of mice reinforced with 15% EtOH in SAC ($r=0.959$; Figure 5.2).

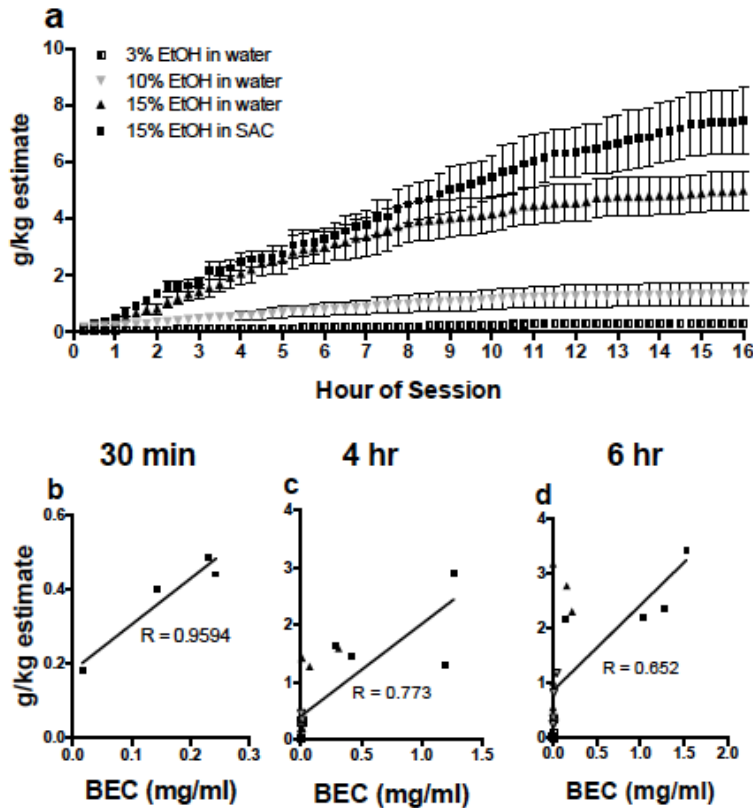


Figure 5.2 – Estimates of g/kg ethanol (EtOH) consumption is positively correlated with blood EtOH concentrations (BEC). **a)** Cumulative estimated g/kg EtOH consumption is depicted across a 16 hour oral operant EtOH self-administration session for a subset of mice reinforced with 3% EtOH in water ($n=5$), 10% EtOH in water ($n=5$), 15% EtOH in water ($n=5$), and 15% EtOH in 0.2% saccharin solution (SAC) ($n=4$). **b)** Estimates of g/kg EtOH consumption correlated with BEC in an independent cohort of mice that self-administered 15% EtOH in SAC during a 30 minute session. **c)** During overnight sessions, estimates of g/kg EtOH consumption correlated with BEC in mice that self-administered 3% EtOH in water, 10% EtOH in water, 15% EtOH in water, or 15% EtOH in SAC at a 4 hour and **d)** 6 hour time point. Mice reinforced with 15% EtOH in SAC achieved BEC that was similar to legally intoxicating doses in humans. Mice reinforced with EtOH in water showed much lower levels of EtOH intake; correlation analysis of mice that received EtOH in water only also resulted in a significant correlation of estimated g/kg EtOH intake with BEC at a 4 hour ($r=0.688$) and 6 hour ($r=0.626$) time point (p 's<0.05).

There was a significant main effect of EtOH concentration on g/kg EtOH consumed ($F_{2,31}=74.037, p<0.001$), indicating that higher concentrations of EtOH resulted in more g/kg EtOH consumed independent of session or vehicle. Reflective of more reinforcers earned and active lever presses, SAC vehicle mice ingested more g/kg EtOH than water vehicle mice ($F_{1,31}=113.575, p<0.001$), as measured by a main effect of vehicle on this measure (Figure 5.3). A significant interaction of vehicle by EtOH concentration by session for EtOH intake ($F_{16,248}=4.656, p<0.001$) showed that SAC and water vehicle also differentially impacted EtOH intake across sessions. Consistent with significant increases in reinforcers earned and active lever presses, mice reinforced with 15% EtOH in water also showed a significant increase in g/kg EtOH consumed ($t_8=-3.383, p<0.05$) across EtOH session. In contrast, mice reinforced with 3% EtOH in water ($t_8=3.078, p<0.05$) and 10% EtOH in water ($t_4=6.383, p<0.01$) showed decreases in EtOH consumed across sessions that paralleled declines in reinforcers earned and active lever presses in these groups. To the contrary, SAC vehicle appeared to promote escalation of low-dose EtOH intake as measured by increases in g/kg EtOH ingested by 3% EtOH mice on session 9 compared to session 1. No differences of total fluid consumed or body weights were detected between groups (Table 5.2).

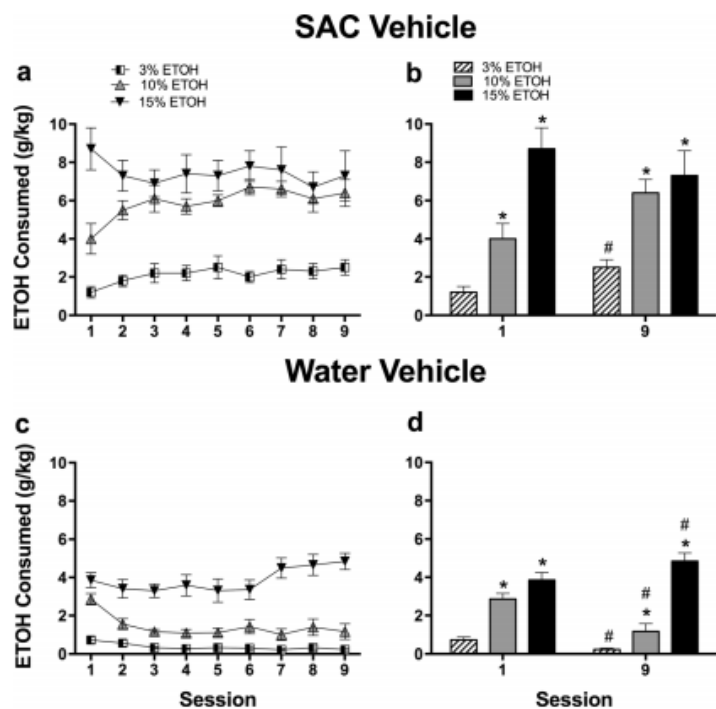


Figure 5.3 – Ethanol (EtOH) consumed (g/kg) in 0.2% saccharin (SAC) or water vehicle. EtOH consumed is shown for mice reinforced with 3, 10, and 15 % EtOH in **a–b)** 0.2 % saccharin (SAC, n=4–5/group) or **c–d)** water vehicle (n=5–9/group) across weekly sessions, highlighting differences between sessions 1 and 9. Data are presented as means±SEM. *p<0.05 compared to 3% EtOH during the same session; #p<0.05 compared to the same concentration on session 1.

Table 5.2 – Mean total fluid consumption and body weight

Vehicle	0 % EtOH		3 % EtOH		10 % EtOH		15 % EtOH	
	Total fluid	Weight	Total fluid	Weight	Total fluid	Weight	Total fluid	Weight
SAC	3.5 (0.59)	28.3 (1.13)	2.9 (0.45)	27.8 (1.59)	2.9 (0.21)	29.9 (0.76)	1.9 (0.24)	26.7 (1.07)
Water	2.5 (0.19)	27.9 (0.69)	2.2 (0.29)	28.0 (0.94)	2.1 (0.15)	26.8 (1.11)	1.9 (0.21)	28.3 (0.76)

Mean daily total fluid intake (ml) and average body weights (g) are depicted for groups of mice receiving EtOH in 0.2% SAC or EtOH in water. Total fluid volume was calculated as the sum of water bottle fluid consumed and liquid dipper fluid consumption estimates for mice. SEM is shown in parentheses.

Assessing the accuracy of estimated g/kg EtOH consumption in mice

EtOH consumed was estimated from magazine head entry occurring only during liquid dipper presentation. Mice showed concentration-associated increases in BEC (Table 5.3). There was a significant correlation between estimates of EtOH consumption and BEC at both the 4 h ($r=0.773$, $p<0.001$) and 6 h ($r=0.652$, $p=0.001$) time points, to support that mice were drinking EtOH during magazine entries (Figure 5.2).

Table 5.3 – BECs in mg/ml at 4 h and 6 h time points

EtOH concentration	4 h BEC	6 h BEC
3 % EtOH in water	0.005 (0.0013)	0.002 (0.0011)
10 % EtOH in water	0.006 (0.0017)	0.015 (0.0076)
15 % EtOH in water	0.081 (0.0590)	0.076 (0.4680)
15 % EtOH in SAC	0.793 (0.2527)	0.999 (0.3012)

Mean BEC are depicted for subsets of mice reinforced with EtOH in water or 0.2% SAC. SEM is shown in parentheses.

Bouts of responding as measured by skewness during overnight sessions

In order to assess patterns of responding, skewness of lever presses per 15 min time bin was calculated and averaged for each EtOH concentration group in SAC and water vehicle studies. As with reinforcers earned, active lever presses and g/kg EtOH intake, vehicle impacted skewness as indicated by a significant vehicle by EtOH concentration interaction ($F_{3,42}=9.547$, $p<0.001$) (Figure 5.4). Skewness measures within-subject variability in responding so that a low skewness value indicates a steady pattern of lever pressing and a high skewness value captures bouts of lever pressing via identification of a pattern of responding that includes more extreme peaks and troughs. There was a main effect of EtOH concentration ($F_{3,14}=4.627$, $p<0.05$) and a significant interaction of EtOH concentration by session ($F_{24,112}=1.607$, $p<0.05$) for skewness in mice receiving EtOH in SAC vehicle. Initial skewness scores reflected that mice showed similar patterns of lever pressing during session 1 but 15% EtOH in SAC mice showed significantly more bouts of responding than SAC vehicle mice in the final session as measured by elevated skewness ($t_6=-3.414$, $p<0.01$) (Figure 5.4). Mice receiving 3 and 10% EtOH did not differ from SAC controls.

In mice reinforced with EtOH in water, there was a main effect of EtOH concentration ($F_{3,28}=5.606$, $p<0.01$) for skewness (Fig. 5.4). Post hoc analysis revealed a significant difference in skewness between mice receiving 15% EtOH and water vehicle subjects ($p<0.05$). Unlike mice receiving 15% EtOH in SAC, mice receiving 15% EtOH in water exhibited a decrease in skewness or more stable responding than mice reinforced with water alone. The difference in pattern of responding between 15% EtOH in SAC and 15% EtOH in water mice may be due to the significantly greater g/kg EtOH consumed when 15% EtOH was delivered in SAC versus in water vehicle. There was an increase in skewness observed in water vehicle compared to SAC

vehicle patterns of self-administration. This could be an artifact of time spent drinking from the ad libitum water bottle, as water vehicle mice, but not SAC vehicle mice, achieved most of their fluid intake from this alternative source.

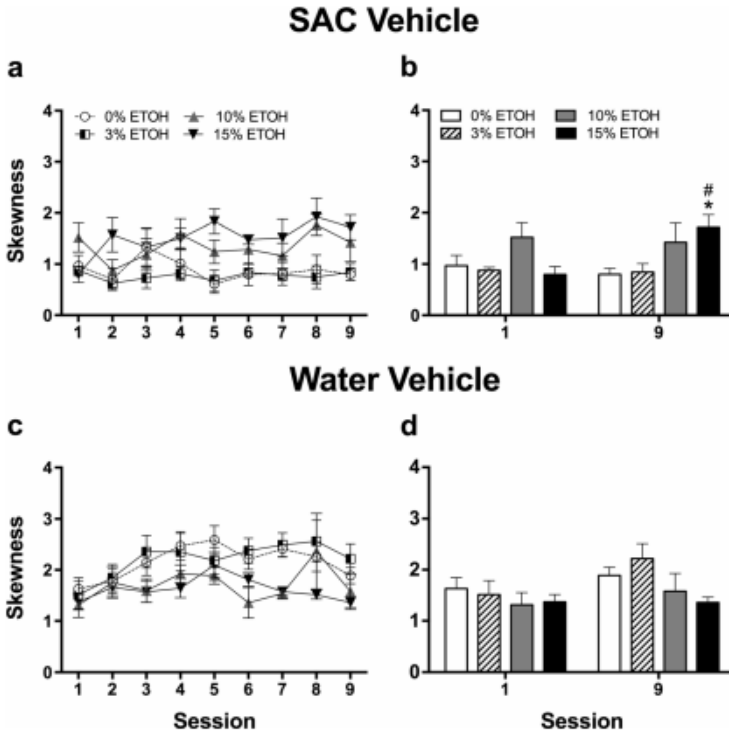


Figure 5.4 – Active lever pressing is more skewed in mice maintained on 15% ethanol (EtOH) in 0.2% saccharin (SAC). Skewness about the mean for active lever pressing are shown for mice receiving 0, 3, 10, and 15 % EtOH in **a–b**) 0.2 % saccharin (SAC, $n=4-5$ /group) or **c–d**) water vehicle ($n=5-9$ /group). Data are presented as means \pm SEM. * $p<0.05$ compared to 0% EtOH during the same session; # $p<0.05$ compared to the same concentration during session 1.

Operant: ad libitum fluid choice

Liquid dipper solution intake was compared to *ad libitum* water bottle fluid intake to assess the rewarding properties of the vehicle and EtOH reinforcers in these experiments. Operant:*ad libitum* choice was calculated as percentage of liquid dipper fluid intake compared to total fluid intake. There was a main effect of vehicle for this measure ($F_{1,42}=82.314, p<0.001$), revealing that despite having to work for liquid presentation, mice reinforced with fluids containing SAC vehicle consumed most of their total fluid intake from the liquid dipper, in contrast to mice reinforced with water vehicle, who ingested most of their fluid from the freely available water bottle (Figure 5.5). There was a significant interaction of vehicle by EtOH concentration by session for operant:*ad libitum* choice ($F_{24,336}=2.503, p<0.001$). In animals reinforced with EtOH in SAC ($F_{24,112}=1.88, p<0.05$) and water ($F_{24,224}=2.881, p<0.001$), there was an interaction of session by EtOH concentration on operant:*ad libitum* choice scores, revealing that mice receiving SAC vehicle ($t_3=-3.681, p<0.05$) and 3% EtOH in SAC ($t_4=-2.85, p<0.05$) showed significant increases in operant:*ad libitum* choice across sessions, whereas mice reinforced with 3% ($t_8=2.376, p<0.05$) and 10% EtOH in water ($t_4=7.909, p<0.01$) showed decreases in operant:*ad libitum* choice scores across sessions that were consistent with decreases in lever pressing following removal of the SAC reinforcer used during lever acquisition training. Independent of vehicle, mice reinforced with 15% EtOH showed a significantly greater percentage of liquid dipper fluid intake than vehicle mice ($p<0.05$), suggesting that this concentration of EtOH was rewarding. As early as session 1, mice reinforced with 15% EtOH in SAC showed a significantly greater percentage of their fluid intake from the liquid dipper compared to vehicle controls; 15% EtOH in water mice required extended training to reveal an increase in operant:*ad libitum* choice scores compared to vehicle control subjects

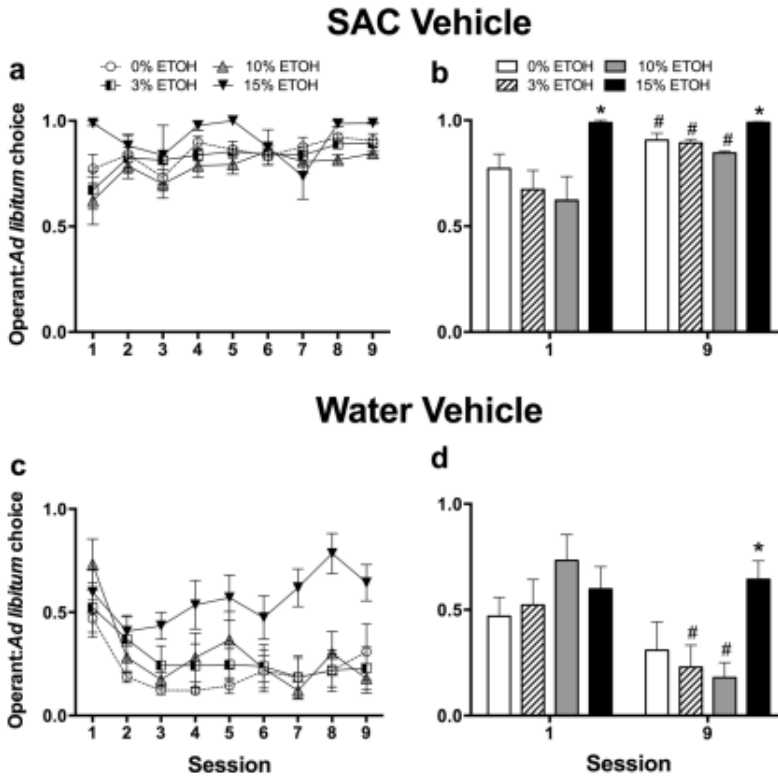


Figure 5.5 – Operant:*ad libitum* choice measures depend on vehicle and/or ethanol (EtOH) concentration. Operant:*ad libitum* choice measures are shown for mice receiving 0, 3, 10, and 15 % EtOH in a–b 0.2 % saccharin (SAC, n= 4–5/group) or c–d water vehicle (n=5–9/group). Data are presented as means±SEM. *p<0.05 compared to 0% EtOH during the same session; #p<0.05 compared to the same concentration during session 1.

Effect of naltrexone on operant responding maintained by EtOH in water

To assess the ability of our operant self-administration model to detect the effects of established treatment drugs with known effectiveness in reducing human alcohol intake, vehicle or 0.3 or 1.25 mg/kg i.p. naltrexone was administered immediately before operant EtOH self-administration with reinforcers earned, active lever pressing, and g/kg EtOH intake as dependent measures. During the first hour of the session, there was a significant interaction of naltrexone treatment (saline, 0.3, or 1.25 mg/kg naltrexone) by EtOH concentration on reinforcers earned ($F_{6,32}=3.788, p<0.01$) and EtOH consumed ($F_{4,24}=7.918, p<0.01$). Mice reinforced with 15% EtOH in water showed significant reductions in reinforcers earned following 0.3 mg/kg ($t_4=2.409, p<0.05$) and 1.25 mg/kg ($t_4=2.94, p<0.05$) i.p. naltrexone treatment compared to when mice received saline vehicle injection. At the highest concentration of EtOH, 1.25 mg/kg naltrexone also reduced g/kg EtOH consumed ($t_4=3.538, p<0.05$) (Figure 5.6). Similar trends for active lever pressing did not return a significant interaction. Planned t-tests showed that 1.25 mg/kg naltrexone significantly reduced active lever pressing of mice reinforced with 15% EtOH in water compared to when they were injected with saline ($t_4=-2.169, p<0.05$). Naltrexone had no effect on reinforcers earned, active lever presses, or EtOH consumed in water vehicle mice, demonstrating the specificity of naltrexone's effects on EtOH reinforcement. There was no significant effect of naltrexone on response accuracy during EtOH reinforcement. Consistent with reports indicating a limited 1 h bioavailability of naltrexone in mice (Wang *et al*, 2004), there was no effect of naltrexone detected by the end of the session (Figure 5.7).

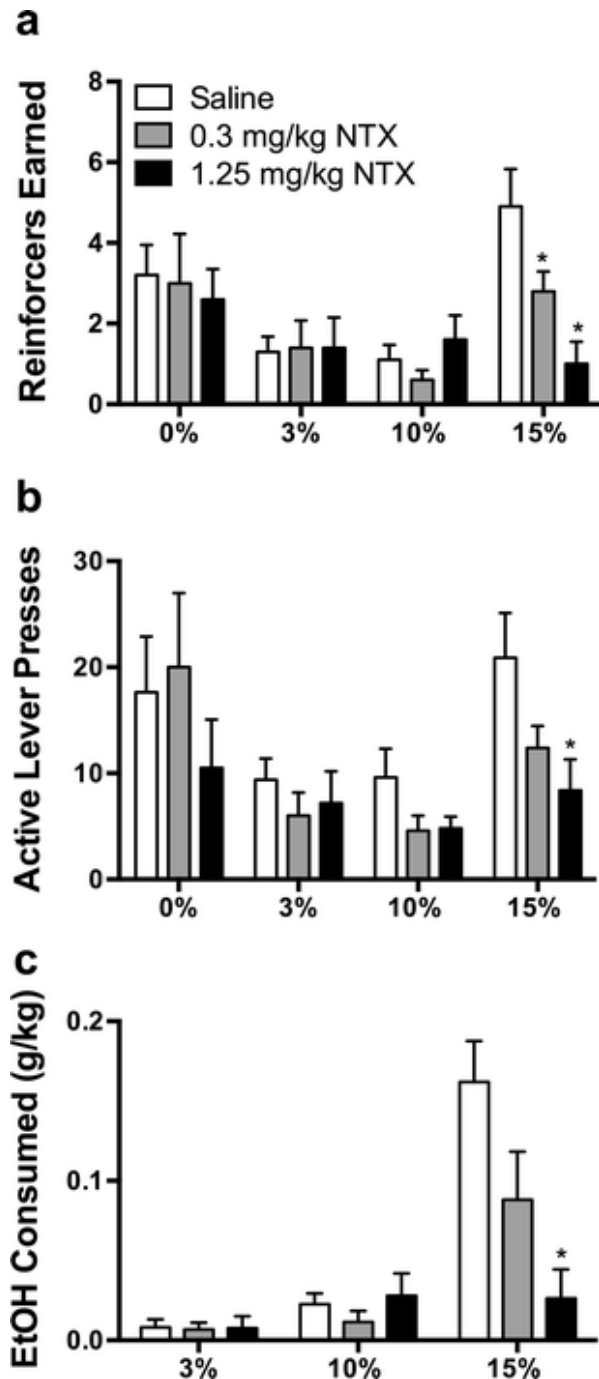


Figure 5.6 – Naltrexone decreases ethanol (EtOH) reinforcement and consumption in mice maintained on 15% EtOH in water. **a)** Reinforcers earned, **b)** active lever presses, and **c)** g/kg EtOH intake are shown for mice reinforced with 0 % (n=4), 3 % (n=5), 10 % (n=5), and 15 % (n=5) EtOH in water during the first hour of the session following pretreatment with 0.9 % saline vehicle or 0.3 or 1.25 mg/kg i.p. naltrexone. Data are presented as means±SEM; *p<0.05 compared to the same EtOH concentration following i.p. saline vehicle injection.

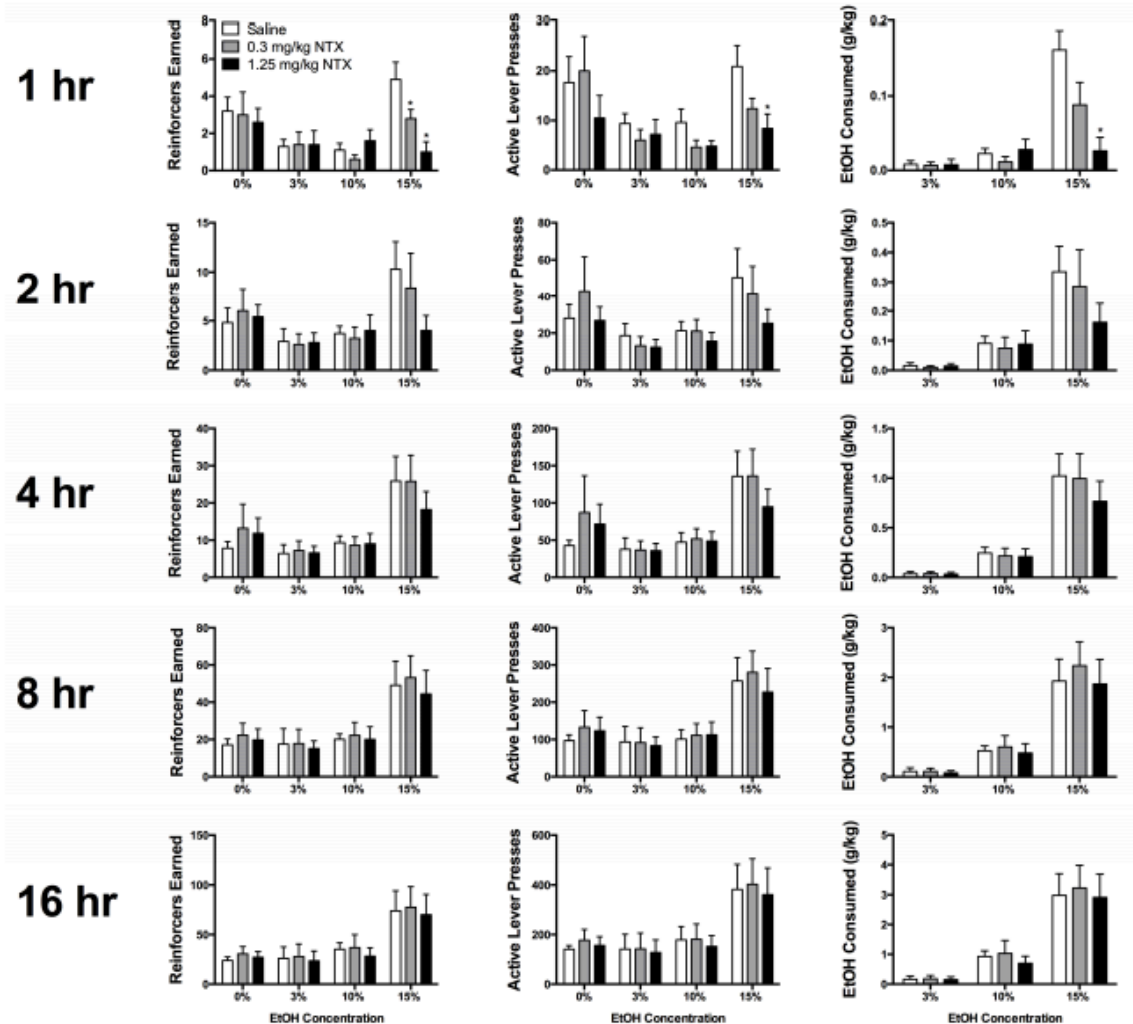


Figure 5.7 – Time course of naltrexone’s effects on responding and ethanol (EtOH) consumed. Reinforcers earned, active lever presses and g/kg EtOH consumed are shown for a subset of mice that received 0, 0.3 and 1.25 mg/kg i.p. naltrexone in a counterbalanced fashion immediately prior to their oral operant EtOH self-administration sessions. Naltrexone led to significant reductions in reinforcers earned, active lever pressing and g/kg EtOH consumed. This effect was limited to the first hour of self-administration, when naltrexone has been shown to peak in the plasma and brains of mice (Wang et al. 2004, “Basal signaling activity of μ opioid receptor in mouse brain: role of narcotic dependence”, *JPET* 308: 512-520). * $p < 0.05$ vs. 0 mg/kg naltrexone of same EtOH concentration.

DISCUSSION

Our results confirm previous reports that EtOH is reinforcing in C57BL/6J mice (Kelley and Middaugh, 1996; Middaugh *et al*, 1999a; Risinger *et al*, 1998). This observation was vehicle and concentration dependent. Mice ingested a range of concentrations of EtOH, but only mice receiving 15% EtOH in water vehicle showed evidence of EtOH reinforcement as measured by reinforcers earned and active lever presses compared to vehicle control mice. In the present study, EtOH reinforcement occurred in the absence of an added flavorant, explicit EtOH-paired cues, or food/water restriction, supporting the conclusion that lever pressing in these studies was motivated by the primary reinforcing effects of EtOH. These studies further showed an escalation of EtOH in water self-administration over weeks of exposure at this high concentration, suggesting that the overnight, weekly self-administration procedure used in these studies shows a progression of EtOH intake. Pretreatment with naltrexone attenuated EtOH reinforcers earned, lever pressing, and g/kg EtOH consumed in mice reinforced with 15% EtOH in water, suggesting that the present model in C57BL/6J mice may have potential predictive validity for therapeutic effectiveness.

Mice reinforced with EtOH in a SAC vehicle solution consumed nearly twice the EtOH compared to mice reinforced with the same concentrations of EtOH in water. C57BL/6J mice in these studies showed levels of EtOH consumption that are comparable to selectively bred high alcohol drinking rats (Bell *et al*, 2008). The SAC sweetener also supported escalation of lever pressing for 3% EtOH in SAC across weeks of exposure, an effect not observed in mice that received 3% EtOH in water vehicle. Despite this, mice reinforced with EtOH in SAC solution did not meet criteria of EtOH reinforcement as measured by significantly increased reinforcers earned or lever pressing compared to SAC vehicle mice. This appears to be due in part to a

ceiling effect resulting from the reinforcing effects of SAC. Previous research shows that saccharin has primary reinforcing properties in C57BL/6J mice on its own (e.g. Cason and Aston-Jones, 2013; Messier and White, 1984). It is therefore interesting that mice reinforced with 15% EtOH in SAC earned significantly fewer reinforcers and active lever presses than SAC vehicle controls, suggesting that this concentration of EtOH may reduce the reinforcing effects of SAC. Previous oral operant EtOH self-administration studies implementing the sucrose fading technique using a within-subject design in rats have similarly shown that increasing the concentration of EtOH produces concentration-dependent decreases in operant responding (Gonzales *et al*, 2004; Grant and Samson, 1985; Samson, 1986; Samson *et al*, 1988). Although it is possible that this high concentration of EtOH may have reduced the palatability of the SAC vehicle (Davison *et al*, 1976; Dudek, 1982), this conclusion is not supported by operant: ad libitum choice measures, which show that mice reinforced with 15% EtOH in SAC ingested a greater percentage of their fluid intake from the liquid dipper than mice reinforced with SAC vehicle alone. Concentration-dependent increases in g/kg EtOH in SAC consumed suggest that mice reinforced with 15% EtOH may have been titrating their dose of EtOH or that ingestion of nearly 8 g/kg of EtOH led to sedation in these mice (Blednov *et al*, 2014; Santos *et al*, 2013; Sharko and Hodge, 2008). This latter interpretation is supported by BEC of 0.8 mg/ml achieved in some of these mice as well as by skewness measures, which revealed that higher concentrations of EtOH in SAC, but not water, resulted in bouts of lever pressing followed by periods of quiescence in 15% EtOH mice compared to SAC vehicle subjects. This pattern of responding would not have been predicted based on the VR schedule of reinforcement used in these studies, which typically promotes a steady state of responding (Baum, 1993; Ferster and Skinner 1957). Bouts of responding in nonhuman primates predict the development of sustained

patterns of heavy drinking (Grant *et al*, 2008). It is of further interest that skewness increased across weeks of exposure for 15% EtOH in SAC mice while g/kg intake remained stable, suggesting that mice sensitized to this behavioral effect of EtOH intake.

Sweetener greatly increases the palatability of EtOH and, as such, encourages alcohol intake in humans (Kidorf *et al*, 1990). SAC sweetener appeared to facilitate responding for low-dose EtOH as demonstrated by a significant escalation of reinforcers earned, lever presses, and g/kg EtOH intake in mice reinforced with 3% EtOH in SAC but not in mice reinforced with 3% EtOH in water. When mixed with SAC, mice found all EtOH doses rewarding as measured by overall higher operant:*ad libitum* choice scores than those achieved with EtOH in water.

Although 15% EtOH in SAC mice did not earn significantly more reinforcers than SAC vehicle control mice, significantly higher operant:*ad libitum* choice scores reflected that they did appear to drink a greater percentage of liquid from the dipper compared to SAC controls, suggesting that the 15% EtOH concentration was rewarding. This observation was evident as early as session 1. Longitudinal studies in human drinkers indicate that alcohol subjective reward or liking is one of the best predictors for escalation of binge drinking (King and Byars, 2004a; King *et al*, 2016; King *et al*, 2002). It is therefore interesting that mice reinforced with 15% EtOH in SAC vehicle achieved BEC consistent with the legal definition of intoxication in humans. Despite this, mice reinforced with 15% EtOH in SAC did not demonstrate an escalation of responding as was evidenced in mice reinforced with 3% EtOH in SAC or with 15% EtOH in water. Although reinforcement was not confirmed compared to SAC vehicle controls, this model of EtOH in SAC self-administration could be used to promote high levels of voluntary operant EtOH drinking in the C57BL/6J mouse strain that is commonly used as a background for transgenic and null mutant genetic manipulations.

Using water vehicle unmasked EtOH reinforcement in mice receiving the highest concentration of EtOH. EtOH reinforcement is a precursor to AUD and alcohol dependence (Tabakoff and Hoffman, 2013). Consistent with subjective reports in humans, it is interesting that sub-intoxicating levels of EtOH intake led to reinforcement in this paradigm (King *et al*, 2011; King *et al*, 2002; McKee *et al*, 2009). Mice reinforced with 15% EtOH in water not only displayed a greater number of reinforcers earned and active lever presses compared to water vehicle controls, but they also showed a small but significant escalation of responding across exposure sessions. This weekly model of EtOH exposure mimics early patterns of alcohol intake observed with AUD in humans (Holdstock *et al*, 2000; King *et al*, 2004a; King *et al*, 2002). In rodents, intermittent EtOH exposure promotes an escalation of EtOH intake coined the alcohol deprivation effect (ADE) (Khisti *et al*, 2006; Rodd *et al*, 2003; Spanagel and Holter, 1999). Escalation of responding observed following abstinence from other drugs of abuse is referred to as an incubation effect to reflect changes in underlying brain processes that support the development of drug dependence (Grimm *et al*, 2001). It is not clear if chronic exposure, extended exposure sessions, protracted periods of abstinence, or all these factors are required for observation of ADE/incubation. Most studies in mice involve extended periods of EtOH exposure of at least 14 days (Bell *et al*, 2006; McBride and Li, 1998). In the present studies, where mice had weekly overnight access to EtOH, escalation of EtOH reinforcement and consumption first became evident during the seventh EtOH self-administration session for mice reinforced with 15% EtOH in water and as early as the third session in mice reinforced with 3% EtOH in SAC. During EtOH self-administration, it is not clear if residual responding maintained by SAC may have promoted a threshold level of EtOH intake necessary to engender reinforcing effects and later escalation of responding in mice reinforced with 15% EtOH in water and 3%

EtOH in SAC. Reductions in responding of mice reinforced with water vehicle and a significant difference between control mice and mice reinforced with 15% EtOH in water demonstrate that prior saccharin exposure alone was not sufficient to support reinforcement, however.

Reinforcing effects of EtOH in water were reduced in these studies by the EtOH treatment therapeutic, naltrexone (O'Malley *et al*, 2007; O'Malley *et al*, 2003). This opioid antagonist has previously been shown to decrease mouse operant responding maintained by EtOH (Middaugh *et al*, 1999b; Navarrete *et al*, 2014) and mouse EtOH intake during DID and two-bottle choice paradigms (Kamdar *et al*, 2007; Phillips *et al*, 1997). In the absence of any effect on water vehicle controls, naltrexone was effective at inhibiting 15% EtOH in water intake at sub-intoxicating doses consistent with its therapeutic profile in alcoholics.

In summary, these studies accomplished some, but not all, of the goals hoped to be achieved. Importantly, mice reinforced with 15% EtOH in water showed significantly greater responding than vehicle mice in the absence of food or water restriction; this finding suggests that EtOH has primary reinforcing properties that are not driven by thirst or caloric incentives. In the absence of EtOH fading, the between-subject design revealed an escalation of responding of mice reinforced with 15% EtOH in water and 3% EtOH in SAC that was not evident in other groups of mice. Together, the present data demonstrate that EtOH is reinforcing in the absence of contingent sweetener, but that contingent sweetener may facilitate responding of low-dose EtOH. Although the complete removal of all cues was not possible given the noise produced by the dipper mechanism and the scent of the EtOH, these studies were accomplished without the addition of more explicit tone and light cues that can serve as primary reinforcers and engage the dopamine system (Caggiula *et al*, 2001; Olsen *et al*, 2009; Olsen and Winder, 2012). Future studies may build upon this experimental design to test the regulation of cues on EtOH

reinforcement and reward. It was hoped that the between-subject design would lend itself to detection of initial sensitivity to EtOH concentration that might predict later behaviors (King *et al*, 2011; King *et al*, 2016; King *et al*, 2002; Schuckit *et al*, 1996). Unfortunately, prior training for SAC alone reinforcement may have overshadowed detection of differences in EtOH reinforcers earned and lever pressing between groups of mice during the first oral operant EtOH self-administration session. The addition of a water bottle in the operant conditioning chamber enabled operant: ad libitum choice measures, however, which revealed that mice reinforced with 15% EtOH in SAC ingested more of their fluid intake from the liquid dipper compared to SAC vehicle control mice on week 1. Interestingly, this group of mice achieved intoxicating levels of EtOH during later sessions as measured by estimates of EtOH intake and confirmed by BEC levels >1.0 mg/ml. The high levels of BEC achieved in mice reinforced with 15% EtOH in SAC may serve as a model for heavy EtOH use, even if these mice do not show an escalation of dosing across weeks of exposure or evidence of EtOH reinforcement as compared to SAC vehicle controls.

These studies establish a mouse model of oral operant EtOH self-administration that does not employ explicit cues, EtOH fading, food deprivation, or water deprivation to signal or promote ingestion of EtOH. Omission of these potentially confounding variables may be advantageous for studies designed to assess the genetic and biological mechanisms of EtOH use. As explicit cues and flavorants are important contributors to EtOH use in humans and animals alike, future studies can further manipulate these factors to explore the full biological complexities of behaviors that support EtOH use. Escalation of EtOH responding and consumption further provides a biological model to assess the neurochemical and molecular underpinnings that support elevations in EtOH reinforcement. The responsiveness of mice in

these studies to naltrexone further supports the predictive validity of this model for detection of drugs with therapeutic effectiveness for treatment of AUD as well as for understanding the genetic and neurobiological underpinnings of EtOH reinforcement.

Chapter 6 – Contribution of $\alpha 6\beta 2$ subunit containing nicotinic acetylcholine receptors to ethanol reinforcement in mice

INTRODUCTION

Alcohol abuse is a significant health concern across the globe. In 2014, WHO ranked alcohol abuse among the top five risk factors for disease, disability, and death (WHO, 2014). A better understanding of the mechanisms underlying alcohol's addictive effects is greatly needed, as treatment for alcohol use disorders (AUDs) has only proved modestly successful. Comorbid nicotine and alcohol abuse is highly prevalent; these two legal recreational drugs are the most common co-abused drugs (Sussman *et al*, 2011). As many as 96% of alcoholics also smoke tobacco (Ayers *et al*, 1976; De Leon *et al*, 2007; Falk *et al*, 2006; Marks *et al*, 1997; Miller *et al*, 1998) and smokers are more likely to binge drink, consume more alcohol, and are more likely to meet DSM-V criteria for an AUD compared to non-smokers (Britt *et al*, 2013; Carmody *et al*, 1985; DiFranza *et al*, 1990; McKee *et al*, 2013). The high rate of nicotine and alcohol co-abuse suggests that these two drugs share a common mechanism of action in the brain.

Both nicotine and ethanol (EtOH) independently activate the mesolimbic dopamine (DA) pathway through nicotinic acetylcholine receptor (nAChR)-dependent mechanisms. This interaction elicits stimulation of DA neurons in the ventral tegmental area (VTA), ultimately leading to an increase in nucleus accumbens (NAc) DA release, a process associated with the rewarding and reinforcing properties of most abused drugs (Hendrickson *et al*, 2013). It has been

well established that $\beta 2$ subunit containing nicotinic acetylcholine receptors ($\beta 2^*nAChRs$; * indicates possible assembly with other subunits) are involved in many abuse-related effects of nicotine (Brunzell *et al*, 2015). However, studies investigating the role of $\beta 2^*nAChRs$ in the behavioral and neurochemical effects of EtOH have generated mixed results. The $\beta 2$ -selective antagonist, DH β E or genetic knockdown of the $\beta 2$ subunit has no effect on EtOH intake and preference (Dawson *et al*, 2013; Kamens *et al*, 2010a; Kuzmin *et al*, 2009), EtOH self-administration (Kuzmin *et al*, 2009; Le *et al*, 2000), or associated EtOH-induced accumbal DA release (Ericson *et al*, 2003; Larsson *et al*, 2002) in rodent studies. However, varenicline, a partial agonist at $\beta 2^*nAChRs$, does reduce ethanol consumption (Feduccia *et al*, 2014; Hendrickson *et al*, 2010; Kamens *et al*, 2010b; Santos *et al*, 2013; Steensland *et al*, 2007) and operant self-administration in rodents (Bito-Onon *et al*, 2011; Steensland *et al*, 2007). Varenicline does not have an effect on EtOH-associated NAc DA release in rats however (Feduccia *et al*, 2014). These pharmacological findings are perhaps complicated by a lack of $\beta 2^*nAChR$ subtype selectivity.

$\beta 2$ primarily assembles with the $\alpha 4$ and $\alpha 6$ subunits to make functional $\alpha 4\beta 2^*$, $\alpha 6\beta 2^*$ and $\alpha 4\alpha 6\beta 2^*nAChRs$. $\alpha 6\beta 2^*nAChRs$ are of particular interest, as this subtype is selectively expressed in catecholaminergic nuclei, being enriched along the reward-related mesolimbic DA pathway (Champtiaux *et al*, 2002; Klink *et al*, 2001; Le Novere *et al*, 1996). Intra-VTA infusion of the $\alpha 6$ -selective antagonist, α -Conotoxin MII (α -Ctx MII) reduces EtOH intake and preference (Larsson *et al*, 2004), EtOH self-administration (Kuzmin *et al*, 2009), and associated EtOH-induced activation of the mesolimbic DA pathway (Larsson *et al*, 2004; Liu *et al*, 2013b). These effects of α -Ctx MII could be due to antagonism of $\alpha 3\beta 2^*nAChRs$ in the VTA, as α -Ctx MII binds to these nAChRs as well. In contrast, $\alpha 6$ null mutant mice ($\alpha 6KO$) show no

differences compared to WT mice for measures of EtOH intake and preference (Guildford *et al*, 2016; Kamens *et al*, 2012). But, $\alpha 6$ KO mice do show a loss of high dose EtOH reward sensitivity in CPP (Guildford *et al*, 2016) and associated reductions in EtOH-induced stimulation of VTA DA neurons (Liu *et al*, 2013b). Complementary to null mutant strategies, $\alpha 6\beta 2^*$ nAChR gain-of-function ($\alpha 6L9'S$) mice show increases in EtOH intake and conditioned place preference (CPP) compared to WT mice (Powers *et al*, 2013). However, no known studies have investigated how genetic manipulation of the $\alpha 6$ subunit affects EtOH's reinforcing effects. Thus, these studies sought to further characterize the role of $\alpha 6\beta 2^*$ nAChRs in EtOH reinforcement using a mouse model of oral, operant EtOH self-administration in WT, $\alpha 6$ HET, and $\alpha 6$ KO mice.

MATERIALS AND METHODS

Subjects

Adult male wild type (WT) ($n=11$), $\alpha 6$ heterozygous ($\alpha 6$ HET) ($n=13$), and $\alpha 6$ subunit null mutant mice ($\alpha 6$ KO) ($n=12$) were used in these studies. $\alpha 6$ KO mice were backcrossed to C57BL/6J WT mice for at least 10 generations. WT, $\alpha 6$ HET, and $\alpha 6$ KO mice were produced by heterozygous breeder pairs. Mice were housed in a temperature- and humidity-controlled vivarium in polycarbonate cages with Teklad corn cob bedding (catalog number 7092). They were housed under a 12 h light/dark schedule (lights on at 6:00 a.m.) and had *ad libitum* access to food (Teklad LM-485 Mouse/Rat Sterilizable Diet, catalog number 7012) and water. Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University and were in accordance with the Guidelines for the Care and Use of Laboratory Animals, as set forth by the National Institutes of Health.

Drugs

EtOH was diluted in tap water and provided to mice for voluntary oral intake. For rotarod and locomotor experiments, EtOH was diluted in double deionized water and administered intraperitoneal (i.p.) at a volume of 0.3 ml/30 g.

Operant ethanol self-administration

Apparatus

Operant EtOH self-administration procedures were conducted in mouse operant conditioning chambers (21.6 cm × 17.8 cm × 12.7 cm; Med Associates, St. Albans, VT) housed inside sound-attenuating cabinets with a ventilation fan. Each chamber was equipped with two retractable levers placed 2.5 cm above the floor. One lever, designated active, resulted in the presentation of a liquid dipper that provided 0.01 ml of fluid; the other lever, designated inactive, had no consequence when depressed. The liquid dipper was located within a magazine equidistant between the two levers and equipped with a photobeam sensor to record head entries into the magazine during the presence or absence of the liquid dipper presentation. A 100 mA house light, located 11 cm above the floor on the opposite wall, was on during the session. A water bottle with sipper tube provided ad libitum access to water during EtOH self-administration sessions, and food pellets were placed on the floor. Med PC IV software and Med Associates interfacing controlled liquid dipper presentations and recorded active and inactive lever pressing, liquid dipper reinforcers earned, and magazine entries during the presence (correct entry) and absence (incorrect entry) of liquid dipper presentation. Data were collected in 15 min time bins.

Magazine training

Mice received 80 liquid dipper presentations of 0.01 ml of SAC solution on a variable time 30 s schedule (13–47 s range). Mice were trained to a criterion of at least 20% magazine entry during

dipper presentations or for up to three training sessions. Magazine training took place during the light cycle between 1300 and 1500 hours.

Acquisition of lever pressing

Next, mice underwent acquisition of lever pressing in the absence of EtOH to assure that all groups of mice demonstrated reliable lever pressing and goal tracking behavior prior to their first exposure to EtOH. Mice were trained during 16 h overnight sessions to lever press for SAC dipper presentations. Sessions began between 1700 and 1800 hours. Active lever pressing for SAC was maintained on FR1 for the first 20 reinforcers, FR2 for the 20 subsequent reinforcers, and FR4 for the 10 subsequent reinforcers. For the remainder of the session, the FR4 schedule was shifted to a variable ratio (VR) 5 schedule (1–12 range). Training continued for two to five sessions until animals pressed the active lever at least 40 times and showed at least 100 s of head entry into magazine during fluid dipper presentation.

Weekly, overnight EtOH sessions

After meeting training criteria, mice ($n=5-7$ per group) underwent 16 h overnight operant self-administration sessions once every 7 days for 10 weeks. Individual mice lever pressed on a VR5 schedule of reinforcement maintained by 0, 3, 10, or 15% EtOH (v/v). Due to limited availability of operant conditioning chambers, each experiment was completed in two replicates. Reinforcers earned and active lever presses provided measures of reinforcement. Response accuracy was determined by the percentage of active lever presses: $\text{active}/\text{total}(\text{active}+\text{inactive lever presses})$. For all experiments, estimated drinking from the liquid dipper was calculated by multiplying the number of correct head entries (magazine head entry at the time of liquid dipper presentation) by 0.01 ml as has been shown previously to correlate well with BEC (Stafford *et al*, 2015). Total

EtOH dose consumed was estimated by multiplying intake volume by the EtOH concentration available for self-administration, only when mice had their head in the magazine during EtOH delivery.

Rotorod

Mice were habituated to an accelerating rotorod (5-45 rpm in 300 s) to reliably achieve at least 60 s without falling. The next day, mice first received i.p. injections of saline (SAL) and immediately tested for latency to fall. Mice then received 2 g/kg i.p. EtOH injections and were tested for latency to fall at 0, 15, 30, 45, and 60 min post-injection.

Locomotor assay

The locomotor assay was conducted in a polycarbonate cage (33 cm x 21 cm) under dim lighting. On the first day, mice were habituated to the locomotor chamber for 15 min. The following day, mice received i.p. injections of SAL or 2 g/kg EtOH and were immediately placed in the locomotor chamber for 15 min. Distance traveled was measured using Anymaze tracking software.

Statistical analysis

All data were analyzed using SPSS. Active lever presses, reinforcers earned, and lever pressing accuracy data were analyzed using a 2x3x6 (EtOH concentration x genotype x session) repeated measures analysis of variance (ANOVA). EtOH consumed was analyzed using a 3x6 (genotype x session) repeated measures ANOVA. Significant EtOH concentration x genotype x session interactions were further assessed using one-way ANOVAs to examine differences in active lever presses and reinforcers earned during session 7 for each genotype separately, and paired t-tests using a Bonferroni correction examined differences between sessions 2 and 7 for each

genotype. Session 2 was used in analysis of the data, instead of session 1, as there appeared to be residual responding for 0.2% saccharin that was used during training. This residual responding disappeared by session 2. A 3x5 (genotype x timepoint) repeated measures ANOVA was used to analyze rotorod performance. A 2x3 (EtOH dose x genotype) ANOVA was used to analyze locomotor activity. The criterion for significance was set at $p < 0.05$ for all analyses. Data points more than 2 standard deviations from the mean were considered outliers and excluded from analysis.

RESULTS

Active Lever Presses

A 2x3x6 repeated measures ANOVA revealed a main effect of session ($F_{5,140}=3.086, p=0.011$) and EtOH concentration ($F_{1,28}=13.734, p=0.001$), as well as interactions of EtOH concentration by session ($F_{5,140}=6.379, p < 0.001$), genotype by session ($F_{10,140}=2.328, p=0.014$), and EtOH concentration by genotype by session ($F_{10,140}=1.91, p=0.048$) for active lever presses (Figure 6.1a-b). WT ($p=0.009$) and $\alpha 6$ KO mice ($p=0.029$) maintained on 15% EtOH showed significantly elevated levels of active lever presses compared to water control mice, suggesting that these mice found EtOH reinforcing by the last session (session 7). However, $\alpha 6$ HET mice maintained on 15% EtOH did not show significant elevations of active lever presses by session 7 compared to vehicle controls ($p=0.472$), demonstrating that $\alpha 6$ HET mice did not find EtOH reinforcing. WT mice reinforced with 15% EtOH also showed a significant elevation in responding across sessions ($t_4=-7.615, p=0.002$) that was not observed in $\alpha 6$ HET or $\alpha 6$ KO mice maintained on 15% EtOH (p 's > 0.05). A 2x3x6 repeated measures ANOVA revealed no main effects or interactions for lever pressing accuracy, revealing that genotype and EtOH

concentration had no effect on accuracy and that accuracy remained stable over sessions (Figure 6.1e-f).

Reinforcers Earned

A 2x3x6 repeated measures ANOVA revealed a main effects of session ($F_{5,140}=2.738, p=0.022$) and EtOH concentration ($F_{1,28}=14.096, p=0.001$), and interactions of EtOH concentration by session ($F_{5,140}=6.696, p<0.001$), genotype by session ($F_{10,140}=2.306, p=0.015$), and EtOH concentration by genotype by session ($F_{10,140}=1.911, p=0.048$) for reinforcers earned (Figure 6.1c-d). WT ($p=0.008$) and $\alpha 6$ KO mice ($p=0.029$) maintained on 15% EtOH showed significantly greater levels of reinforcers earned compared to water control mice, suggesting that these mice found EtOH reinforcing by the last session. However, $\alpha 6$ HET mice maintained on 15% EtOH did not differ from water control mice, indicating that $\alpha 6$ HET mice did not find EtOH reinforcing ($p=0.472$). Only WT mice maintained on 15% EtOH showed an increase in reinforcers earned across weeks of training ($t_4=-6.848, p=0.002$).

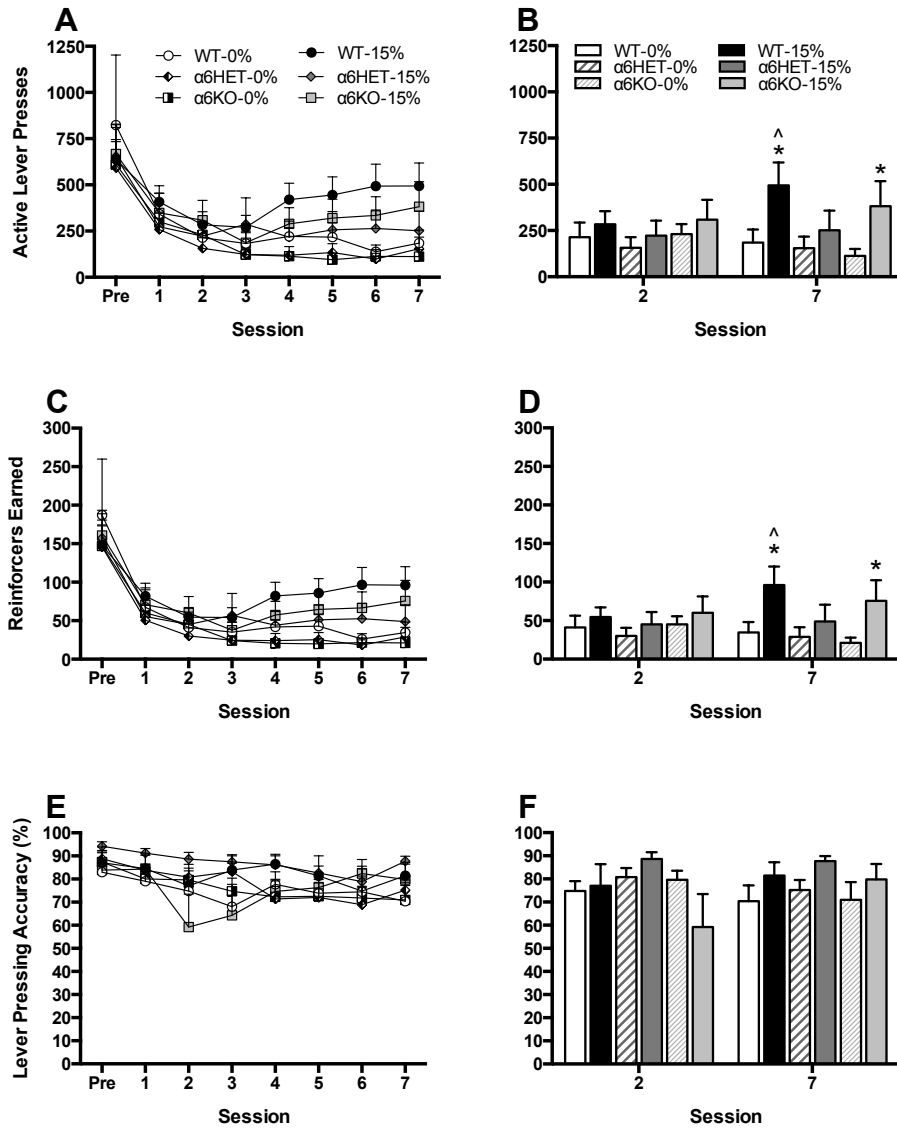


Figure 6.1 $-\alpha 6\beta 2^*nAChR$ expression modulates ethanol (EtOH) reinforcement. **a)** Active lever presses over weekly, overnight EtOH self-administration sessions for WT (0%: $n=5$; 15%: $n=5$), $\alpha 6HET$ (0%: $n=6$; 15%: $n=7$), and $\alpha 6KO$ mice (0%: $n=6$; 15%: $n=5$). **b)** Active lever presses for sessions 2 and 7. **c)** Reinforcers earned across weekly, overnight EtOH self-administration sessions. **d)** Reinforcers earned for sessions 2 and 7. **e)** Lever pressing accuracy across weekly, overnight EtOH self-administration sessions. **f)** Lever pressing accuracy for sessions 2 and 7. * $p < 0.05$ vs. 0% EtOH of same genotype; ^ $p < 0.05$ vs. week 2 of same group. Data are expressed as mean \pm SEM.

EtOH consumed

A 3x6 repeated measures ANOVA revealed a main effect of session ($F_{5,70}=3.738, p=0.005$) for EtOH consumed (Figure 6.2a-b), revealing that EtOH consumption increased over sessions independent of genotype. However, there was no main effect of genotype ($F_{2,14}=1.242, p=0.319$) and no significant interaction of genotype by session ($F_{10,70}=1.742, p=0.088$), demonstrating that EtOH consumed did not differ between genotypes. There were also no main effects or interactions for % correct head entries (Figure 6.2 c-d) for which EtOH consumption is based.

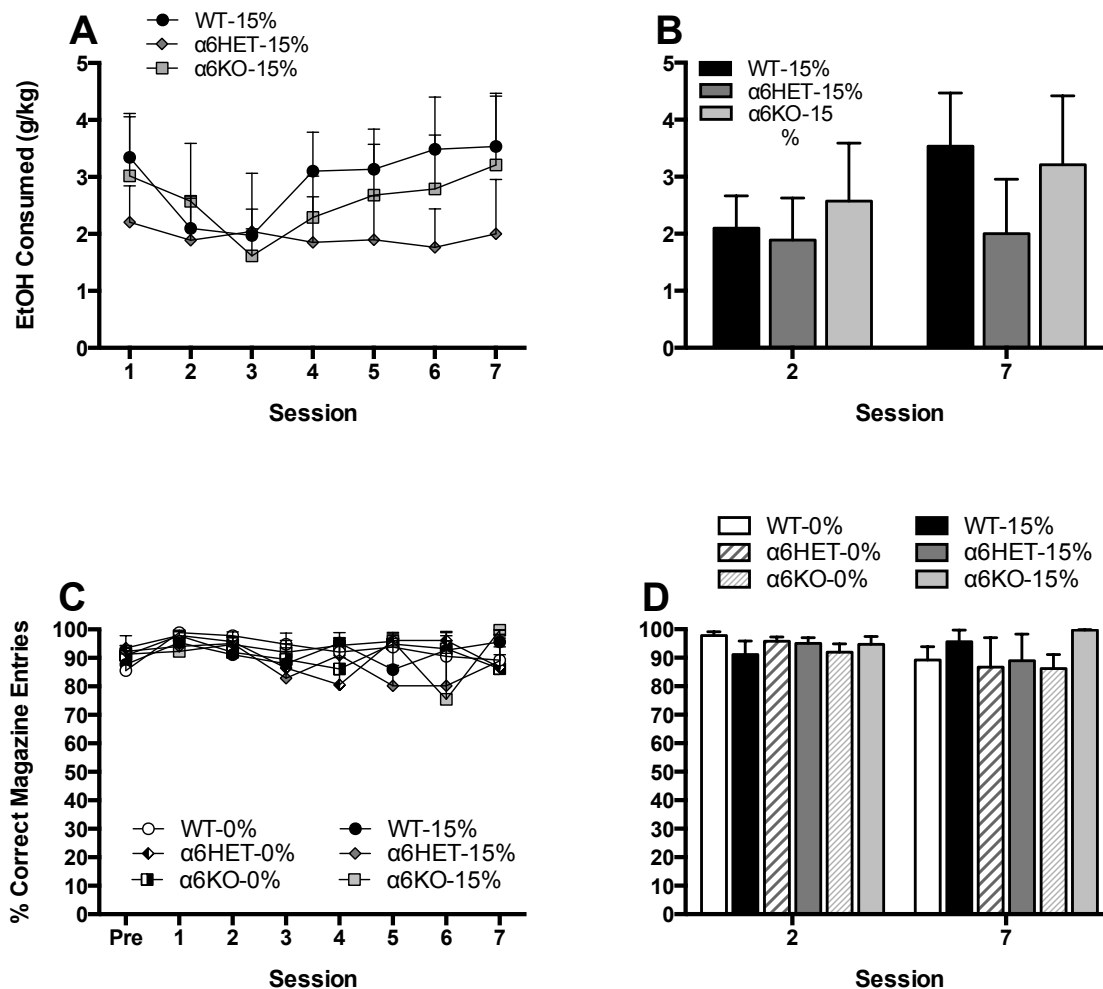


Figure 6.2 $-\alpha 6\beta 2^*nAChR$ expression does not significantly affect ethanol (EtOH) intake. **a)** EtOH consumed (g/kg) over weekly, overnight EtOH self-administration sessions for WT ($n=5$), $\alpha 6HET$ ($n=5$), and $\alpha 6KO$ mice ($n=5$). **b)** EtOH consumed (g/kg) for sessions 2 and 7. **c)** Percent correct magazine entries over weekly, overnight EtOH self-administration sessions. **d)** Percent correct magazine entries for sessions 2 and 7. Data are expressed as mean \pm SEM.

Rotorod

A 3x5 repeated measures ANOVA revealed a main effect of time ($F_{4,132}=22.322, p<0.001$), demonstrating that 2 g/kg i.p. EtOH administration impaired rotorod performance as expected. However, there was no main effect of genotype ($F_{2,33}=0.085, p=0.919$) and no interaction of genotype by time ($F_{8,132}=0.543, p=0.822$), suggesting that rotorod performance was not impacted by genotype (Figure 6.3a).

Locomotor activity

A 2x3 ANOVA revealed a main effect of EtOH dose ($F_{1,30}=14.846, p=0.001$), showing that 2 g/kg i.p. EtOH increased locomotor activity independent of genotype. However, there was no main effect of genotype ($F_{2,30}=0.091, p=0.913$) and no interaction of genotype by EtOH dose ($F_{2,30}=0.7261, p=0.492$), showing that locomotor activity did not differ between the genotypes (Figure 6.3b).

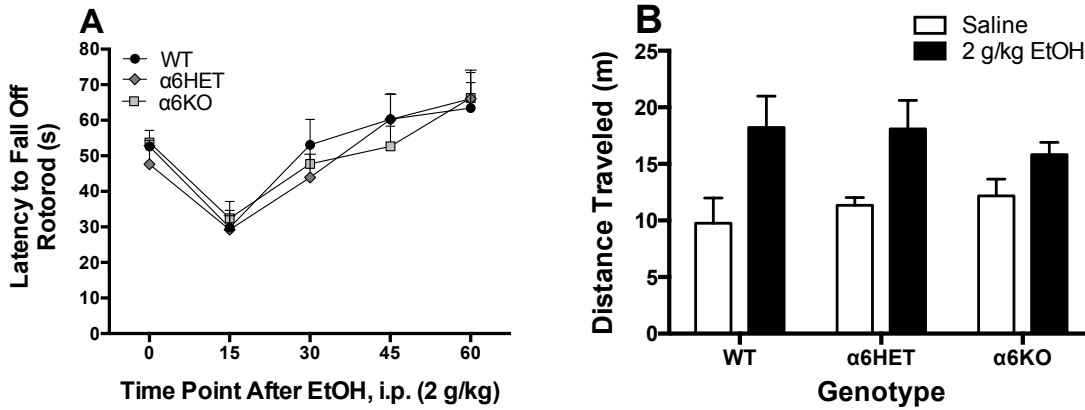


Figure 6.3 $-\alpha6\beta2^*$ nAChR expression does not appear to be involved in the ataxic or locomotor effects of ethanol (EtOH). **a)** Latency to fall off the rotarod for WT ($n=11$), $\alpha6$ HET ($n=13$), and $\alpha6$ KO mice ($n=12$) in response to 2 g/kg i.p. EtOH. **b)** Distance traveled in WT (saline: $n=5$; EtOH: $n=6$), $\alpha6$ HET (saline: $n=6$; EtOH: $n=7$), and $\alpha6$ KO mice (saline: $n=6$; EtOH: $n=6$) in response to saline or 2 g/kg i.p. EtOH. Data are expressed as mean \pm SEM.

DISCUSSION

The present study investigated the contribution of $\alpha 6\beta 2$ *nAChRs to EtOH reinforcement using genetic reduction ($\alpha 6$ HET) or deletion ($\alpha 6$ KO) of $\alpha 6\beta 2$ *nAChR expression in mice. A number of studies have used the $\alpha 6$ KO mice to elucidate the role of $\alpha 6\beta 2$ *nAChRs in EtOH intake, preference, and reward (Guildford *et al*, 2016; Kamens *et al*, 2012). To our knowledge however, this is the first study where EtOH reinforcement has been investigated in $\alpha 6$ HET and $\alpha 6$ KO mice using operant self-administration. Using our model of oral, operant EtOH self-administration (Stafford *et al*, 2015), we showed that WT mice showed EtOH reinforcement as measured by greater active lever presses and reinforcers earned compared to water control mice, while $\alpha 6$ HET mice did not show EtOH reinforcement, as no difference between $\alpha 6$ HET mice reinforced with 0% and 15% EtOH was observed for active lever presses and reinforcers earned. These results indicate that $\alpha 6\beta 2$ *nAChR expression modulates EtOH reinforcement. Curiously, we demonstrated that EtOH has similar reinforcing efficacy in $\alpha 6$ KO mice compared to WT mice, where WT and $\alpha 6$ KO mice maintained on 15% EtOH showed similar levels of lever pressing during operant EtOH self-administration. This is consistent with previous studies reporting similar EtOH preference and intake using two-bottle choice or drinking-in-the-dark (DID) in WT and $\alpha 6$ KO mice (Guildford *et al*, 2016; Kamens *et al*, 2012). However, it was unexpected that reducing $\alpha 6\beta 2$ *nAChR expression by approximately half in the $\alpha 6$ HET mice would prevent EtOH reinforcement while knocking out $\alpha 6\beta 2$ *nAChRs in $\alpha 6$ KO mice would have no effect. $\alpha 6$ KO mice do exhibit a decrease in EtOH reward sensitivity, where 2.0 and 3.0 g/kg EtOH conditioned a place preference in WT mice, but $\alpha 6$ KO mice only expressed CPP at 2.0 g/kg EtOH (Guildford *et al*, 2016). It is possible that there is a similar shift in the dose response curve for operant EtOH self-administration. It is also possible that there is a

compensatory mechanism responsible for the lack of effect in $\alpha 6$ KO mice, where another nAChR subtype may be compensating for the absence of $\alpha 6\beta 2^*$ nAChRs to promote EtOH reinforcement in $\alpha 6$ KO mice. Although the preponderance of evidence from binding studies suggests that there is no compensation of nAChRs in the knockout mice, one study reports that [3 H]epibatidine binding, which has affinity for $\alpha 7$, $\alpha 3\beta 2^*$, $\alpha 3\beta 4^*$, $\alpha 4\beta 2^*$, $\alpha 6\beta 2^*$ nAChRs (Badio and Daly, 1994; Champtiaux *et al*, 2002), is unchanged in $\alpha 6$ KO mice, suggesting that there may be compensation of another nAChR subtype in these mice (Champtiaux *et al*, 2003).

It is interesting that estimated EtOH consumed was not significantly different between the three genotypes as was seen with active lever presses and reinforcers earned, although there are non-significant trends for greater EtOH intake in WT and $\alpha 6$ KO mice compared to $\alpha 6$ HET mice similar to the other measures. Non-significant trends could indicate that this study is underpowered and warrants addition of additional subjects. This estimation of g/kg EtOH intake is based on the assumption that mice drink the reinforcer when they make a correct magazine entry (entry during liquid dipper presentation). Alternatively, this dichotomy between lever presses and reinforcers earned, and EtOH intake could be a result of mice making not making correct head entries every time they earn a liquid dipper presentation of EtOH. However, there were no significant differences between genotypes for measures of correct head entries.

In contrast to our present results in $\alpha 6$ KO mice, previous studies using intra-VTA infusion of the $\alpha 6$ -selective antagonist, α -Ctx MII, report reductions in EtOH intake and preference in rodents (Larsson *et al*, 2004), as well as in EtOH self-administration in rats (Kuzmin *et al*, 2009). Intra-VTA α -Ctx MII also reduces EtOH-induced activation of VTA DA neurons (Liu *et al*, 2013b) and EtOH-induced DA release in mice (Larsson *et al*, 2004), effects that are associated with EtOH's rewarding and reinforcing properties. These paradoxical findings

could be attributed the fact that intra-VTA α -Ctx MII inactivates $\alpha 6\beta 2^*nAChRs$ in one brain region, while $\alpha 6KO$ mice lack $\alpha 6\beta 2^*nAChRs$ throughout the whole brain. This could suggest that $\alpha 6\beta 2^*nAChRs$ in discrete brain regions oppose each other to regulate EtOH reinforcement. Measuring these behaviors following intracerebroventricular (i.c.v.) infusion of α -Ctx MII can provide information about brain systemic inactivation that more closely resembles global $\alpha 6$ knockout. If results from i.c.v. α -Ctx MII studies remain different from studies in $\alpha 6KO$ mice, this may further suggest a compensatory mechanism in these genetic mutants, as discussed above. It should be noted that α -Ctx MII also binds to $\alpha 3\beta 2^*nAChRs$, which are expressed in the VTA as well; it is therefore possible that effects of α -Ctx MII may be mediated by $\alpha 3\beta 2^*nAChRs$. Conotoxin derivatives more selective for $\alpha 6\beta 2^*nAChRs$, such as α -Ctx PIA (Dowell *et al*, 2003) could test whether α -Ctx MII effects are mediated by $\alpha 6\beta 2^*$ versus $\alpha 3\beta 2^*nAChRs$.

Our operant EtOH self-administration paradigm used in these studies provided mice with intermittent access to EtOH once per week. Intermittent EtOH exposure promotes escalation of EtOH intake, which is referred to as the “alcohol deprivation effect” (ADE) (Khisti *et al*, 2006; Rodd *et al*, 2003; Spanagel *et al*, 1999). This effect reflects neuroadaptations thought to support the transition to dependence (Ron and Barak, 2016). The ADE is considered a model of alcohol seeking, loss of control, or relapse. Similarities exist between ADE in rodents and humans, giving it face validity as a model for these behaviors associated with dependence (Martin-Fardon and Weiss, 2013). As we have previously reported in mice on the C57BL/6J background strain (Stafford *et al*, 2015), there was a small but significant elevation of responding in WT mice across weeks of exposure. While $\alpha 6KO$ mice did not differ from WT littermates in measures of EtOH self-administration, neither $\alpha 6HET$ nor $\alpha 6KO$ mice exhibited escalation of responding

across weekly self-administration sessions. This suggests that $\alpha 6\beta 2$ *nAChRs are involved in the escalation of operant EtOH intake.

The interesting genotypic effects on EtOH self-administration observed in these studies did not appear to result from different sensitivities to EtOH's ataxic and locomotor effects, as there was no difference between the three genotypes in rotorod performance or locomotor activity in response to 2 g/kg i.p. EtOH. This interpretation is supported by data showing that EtOH metabolism is not different between WT, $\alpha 6$ HET, and $\alpha 6$ KO mice (Kamens *et al*, 2012). $\alpha 6$ KO mice have been shown to be more sensitive to the sedative effects of EtOH, taking longer to recover their righting reflex in response to 4.1 g/kg i.p. EtOH (Kamens *et al*, 2012). However, this bolus dose of EtOH is much higher than the mice would have achieved during overnight oral operant self-administration in our experiments.

In conclusion, these data suggest that $\alpha 6\beta 2$ *nAChR expression modulates EtOH reinforcement. Moreover, our data demonstrate a role for $\alpha 6\beta 2$ *nAChRs in the escalation of EtOH intake seen following intermittent exposure. The present studies expand on previous data to further implicate $\alpha 6\beta 2$ *nAChRs in behaviors relevant to alcohol abuse and dependence.

Chapter 7 – Concluding Discussion: Implications and Future Directions

An accumulation of evidence suggests that activation of mesolimbic $\alpha6\beta2$ *nAChRs promotes nicotine-stimulated mesolimbic DA activity and associated behaviors relevant to nicotine addiction, namely nicotine self-administration and place conditioning (Brunzell *et al*, 2010; Champtiaux *et al*, 2003; Gotti *et al*, 2010; Grady *et al*, 2002; Kulak *et al*, 1997; Liu *et al*, 2012; Pons *et al*, 2008; Salminen *et al*, 2007; Salminen *et al*, 2004; Sanjakdar *et al*, 2015; Wickham *et al*, 2013; Zhao-Shea *et al*, 2011). These previous studies utilizing pharmacological and genetic techniques to inactivate $\alpha6\beta2$ *nAChRs demonstrate the necessity of this nAChR subtype. However, little progress has been made in the development of $\alpha6\beta2$ *nAChR-selective agonists that can selectively activate these receptors to demonstrate their sufficiency (Drenan *et al*, 2008). Thus, *in vivo* studies in this dissertation first aimed to use $\alpha6L9$ 'S gain-of-function mice as a way to selectively activate $\alpha6\beta2$ *nAChRs to determine if activation of $\alpha6\beta2$ *nAChRs is sufficient to promote NAc DA release and nicotine CPP, a model of nicotine reward.

Our *in vivo* microdialysis studies in awake, behaving mice showed that $\alpha6L9$ 'S mice exhibited elevated levels of basal NAc DA compared to WT mice, suggesting that activation of $\alpha6\beta2$ *nAChRs is sufficient to promote NAc DA release. Consistent with previous *in vitro* reports (Cohen *et al*, 2012), this effect is presumably a result of $\alpha6\beta2$ *nAChR hyperexcitability in response to endogenous ACh. In support of this hypothesis, it appears that endogenous activity at $\alpha6\beta2$ *nAChRs in the VTA promotes basal NAc DA release, as local VTA infusion of α -Ctx

MII blocked basal DA levels in the NAc of $\alpha 6L9$ 'S mice. This endogenous cholinergic tone is presumably from inputs projecting from either the pedunclopontine or laterodorsal tegmental nucleus, as studies show that these cholinergic inputs to the VTA control NAc DA release (Blaha *et al*, 1996; Lanca *et al*, 2000; Lester *et al*, 2008; Xiao *et al*, 2016). To confirm this, future studies using local infusion of a viral vector to selectively express channel rhodopsin YFP in mice with a CHAT CRE promotor could be used to optogenetically stimulate VTA cholinergic inputs from the peduncopontine and laterodorsal tegmental nuclei, which we predict would increase NAc DA levels. Based on our data, it is expected that antagonizing $\alpha 6\beta 2$ *nAChRs in the VTA with local infusions of α -Ctx MII would block this effect. For consistency across our present studies, we used α -Ctx MII, a putative selective antagonist of $\alpha 6\beta 2$ *nAChRs. But, this antagonist also blocks the activity of $\alpha 3\beta 2$ *nAChRs (Cartier *et al*, 1996), which are also expressed in the VTA, albeit at lower levels compared to $\alpha 6\beta 2$ *nAChRs. There are conotoxin derivatives that are more selective for $\alpha 6\beta 2$ *nAChRs compared to $\alpha 3\beta 2$ *nAChRs, like α -Ctx PIA (Dowell *et al*, 2003), that we could use in future studies to more selectively antagonize $\alpha 6\beta 2$ *nAChRs in the VTA.

It has also been reported that optogenetic stimulation of striatal cholinergic interneurons elicits DA release from NAc DA terminals, which is blocked by the $\beta 2$ -selective antagonist, DH β E (Cachope *et al*, 2012; Threlfell *et al*, 2012). This indicates that ACh released from these interneurons acts at $\beta 2$ *nAChRs to promote DA release independent of DA neuron firing. DH β E binds to both $\alpha 4\beta 2$ * and $\alpha 6\beta 2$ *nAChRs with similar potency (Grady *et al*, 2010; Papke *et al*, 2008). Together with these previous data, our present microdialysis results in $\alpha 6L9$ 'S mice suggest that activity of NAc cholinergic interneurons could stimulate $\alpha 6\beta 2$ *nAChRs on DA terminals to promote DA release. To test this hypothesis, local NAc infusions of α -Ctx MII

could be used in combination with optogenetic studies to assess if $\alpha 6\beta 2$ *nAChR antagonism blocks NAc DA release stimulated by NAc cholinergic activity. Compared to the high expression of $\alpha 6\beta 2$ *nAChRs, there is not a prevalence of $\alpha 3\beta 2$ *nAChRs in the NAc, so effects of α -Ctx MII in this brain area are likely due to antagonism of $\alpha 6\beta 2$ *nAChRs. Further supporting this, genetically deleting the $\alpha 3$ subunit in mice does not affect α -Ctx MII binding in the NAc (Whiteaker *et al*, 2002), while α -Ctx MII binding completely disappears in this brain area of $\alpha 6$ KO mice (Champtiaux *et al*, 2002). Effects of endogenous cholinergic signaling are relevant to nicotine addiction, as evidence has implicated these processes in abuse-related effects of nicotine (e.g. Avena *et al*, 2012; Hoebel *et al*, 2007; Lanca *et al*, 2000; Rada *et al*, 2001; Xiao *et al*, 2016). Moreover, human imaging studies have shown that basal DA tone is also related to smoking, as it is enhanced in the striatum of smokers compared to non-smokers (Brown *et al*, 2012; Fehr *et al*, 2008).

Similar to observations of elevated $\alpha 6$ L9'S basal NAc DA levels, we expected that $\alpha 6$ L9'S mice would show an enhanced response to nicotine as has been previously reported in $\alpha 6$ L9'S synaptosome preparation studies (Cohen *et al*, 2012; Drenan *et al*, 2010; Drenan *et al*, 2008), to suggest that stimulation of $\alpha 6\beta 2$ *nAChRs by nicotine also promotes NAc DA release *in vivo*. However, nicotine had no effect on NAc DA levels in WT or $\alpha 6$ L9'S mice (Appendix A). There are several possibilities that may explain the failure to replicate the *in vitro* studies showing elevated DA release following nicotine exposure (Cohen *et al*, 2012; Drenan *et al*, 2010; Drenan *et al*, 2008). In an effort to use behaviorally relevant doses of nicotine, we employed an escalating nicotine dose regimen, where mice first received a lower, rewarding dose of nicotine (0.1 mg/kg i.p.), followed 45 minutes later by a higher, anxiogenic dose of nicotine (0.5 mg/kg i.p.). This time course should be sufficient to support desensitization of nAChRs after

the first, lower nicotine dose (Grady *et al*, 2012). Desensitization by the lower dose of nicotine could have precluded any observable increase in DA levels in response to the higher nicotine dose, which has been shown to increase NAc DA levels (Champtiaux *et al*, 2003; Jerlhag and Engel, 2011; Picciotto *et al*, 1998). This interpretation is supported by previous studies demonstrating the nicotine desensitizes nAChRs, blocking effects of subsequent nicotine administration (Anderson *et al*, 2015; Buccafusco *et al*, 2007; Hulihan-Giblin *et al*, 1990; Sharp and Beyer, 1986). However, desensitization may have recovered before the second, higher dose of nicotine was administered in our studies (Grady *et al*, 2012).

It is also possible that our technique was not sensitive enough to detect changes in DA levels in response to nicotine. Even with drugs of abuse such as cocaine that result in robust DA release in the NAc, it is typical that doses that are higher than those required to observe behavior are required to observe changes in DA. Moreover, nicotine's effect on DA release is subtle, so use of a more time-sensitive technique, like fast scan cyclic voltammetry (FSCV) may be warranted in this case. In our experiments, we quantified DA levels from dialysis samples collected every 15 minutes. FSCV has much greater temporal resolution compared to microdialysis, with the ability to detect changes in DA levels on a sub-second time scale (Chefer *et al*, 2009). Future studies using *in vivo* FSCV in WT and $\alpha 6L9'S$ mice have the potential to detect subtle nicotine-associated changes in DA release in real time to further evaluate if stimulation of $\alpha 6\beta 2$ *nAChRs supports nicotine-stimulated DA release.

While we were not able to detect any effect of nicotine on NAc DA levels, behaviorally, we did observe that $\alpha 6L9'S$ mice were hyperactive in response to nicotine compared to WT littermates during nicotine CPP training. The mesolimbic DA pathway is known to play a role in drug-induced locomotor activation, providing a behavioral model that can assess the activity of

this pathway (Phillips and Shen, 1996; Wise and Bozarth, 1987), and antagonism of DA receptors with pimoziide (King *et al*, 2004b), as well as 6-OHDA lesions of the NAc (Clarke *et al*, 1988) and VTA (Louis *et al*, 1998) can block the locomotor stimulating effects of nicotine. Our findings are consistent with previous findings which showed hyperactivity in $\alpha 6L9$ 'S mice in response to nicotine (Berry *et al*, 2015; Cohen *et al*, 2012; Drenan *et al*, 2010; Drenan *et al*, 2008), further suggesting that stimulation of $\alpha 6\beta 2$ *nAChRs promotes the locomotor activating effects of nicotine. This data provides a clue that $\alpha 6L9$ 'S mice may show an elevated DA response to nicotine *in vivo* that our current techniques were not capable of detecting as discussed above.

It is well known that most drugs of abuse, including nicotine, activate the mesolimbic DA pathway, ultimately resulting in an increase in NAc DA release (Di Chiara *et al*, 1988), a process known to be involved in nicotine's rewarding and reinforcing properties (Corrigall *et al*, 1992; Sellings *et al*, 2008). We observed a leftward shift in the dose response curve for $\alpha 6L9$ 'S nicotine CPP, suggesting that stimulation of $\alpha 6\beta 2$ *nAChRs is sufficient for nicotine's rewarding properties. As $\alpha 6L9$ 'S mice showed both enhanced basal NAc DA levels and left-shifted nicotine CPP, it would appear that augmented reward behavior in $\alpha 6L9$ 'S mice results from enhanced NAc DA release. Future nicotine CPP experiments should test this directly using a DA receptor antagonist. Unfortunately, our pilot studies using doses of SCH 23390, a DA₁ receptor antagonist, reported in the literature (and lower) were unable to find a dose that did not promote aversion on its own. DA signaling could be manipulated in other ways including the use of optogenetic or chemogenetic inhibition of DA neurons, or 6-OHDA lesions. It would also be informative to quantify NAc DA levels during nicotine CPP to test whether nicotine exposure alters DA levels during the training phase of CPP, and further whether changes in DA

corresponds with expression of CPP during the test, where mice are in a drug-free state. We attempted this but ultimately abandoned the experiment when it was clear that the sensitivity of the microdialysis preparation was not detecting nicotine-stimulated changes in NAc DA levels. It would also be informative to record *in vivo* VTA DA neuron firing using electrophysiology at baseline and in response to nicotine, as *in vitro* ACh- and nicotine-stimulation of VTA DA neuron activity is enhanced in $\alpha 6L9'S$ midbrain slices (Drenan *et al*, 2008; Engle *et al*, 2013; Powers *et al*, 2013).

Expanding on previous findings demonstrating the necessity of $\alpha 6\beta 2$ *nAChRs for nicotine CPP (Jackson *et al*, 2009; Sanjakdar *et al*, 2015), our findings indicate that this nAChR subtype is sufficient for the rewarding properties of nicotine. However, there are two subclasses of $\alpha 6\beta 2$ *nAChRs and their individual contributions to nicotine reward have yet to be elucidated. Therefore, subsequent studies in this dissertation used an $\alpha 6L9'S$ x $\alpha 4KO$ cross to differentiate the contributions of $\alpha 4\alpha 6\beta 2$ *nAChRs and (non- $\alpha 4$) $\alpha 6\beta 2$ *nAChRs to nicotine reward behavior. As with $\alpha 6L9'S$ mice, nicotine doses sub-threshold for nicotine CPP in WT mice were capable of supporting nicotine CPP in $L9'S$ - $\alpha 4KO$ mice, suggesting that $\alpha 4$ is not required to assemble with $\alpha 6$ to promote nicotine CPP, but rather that (non- $\alpha 4$) $\alpha 6\beta 2$ *nAChRs promote this abuse-related behavior. This result was not expected, as both $\alpha 6L9'S$ and $\alpha 4L9'A$ (Tapper *et al*, 2004) gain-of-function mice show enhanced nicotine CPP. Moreover, $\alpha 4\alpha 6\beta 2$ *nAChRs, which have the highest known sensitivity to nicotine, are the only receptors in brain that ought to be activated by physiologically relevant levels of nicotine (Exley *et al*, 2008; Kuryatov *et al*, 2011; Liu *et al*, 2012; Salminen *et al*, 2007; Salminen *et al*, 2004). Indeed, the $\alpha 4\alpha 6\beta 2$ *nAChRs are persistently activated in VTA slices at concentrations of nicotine (300 nM) achieved in smokers that typically desensitize other nAChRs (Liu *et al*, 2012). *In vitro* slice studies have reported that

enhanced $\alpha 6L9$ 'S DA neuron firing and DA release requires the $\alpha 4$ subunit (Drenan *et al*, 2010; Engle *et al*, 2013). DA-associated behavior also requires the $\alpha 4$ subunit, as enhanced nicotine-stimulated locomotor activity observed in $\alpha 6L9$ 'S mice was reversed when the $\alpha 4$ subunit was deleted (Drenan *et al*, 2010). Future microdialysis studies comparing $\alpha 6L9$ 'S mice to $\alpha 6L9$ 'S- $\alpha 4$ KO mice are planned to test which $\alpha 6\beta 2$ *nAChR subclass ($\alpha 4\alpha 6\beta 2$ * or (non- $\alpha 4$) $\alpha 6\beta 2$ *nAChRs) supports *in vivo* elevations of NAc DA release observed in $\alpha 6L9$ 'S mice, as measurements of DA in $\alpha 6L9$ 'S- $\alpha 4$ KO mice have only been performed *in vitro*. If these future studies corroborate the *in vitro* data to demonstrate that $\alpha 4\alpha 6\beta 2$ *nAChRs are required for *in vivo* NAc DA release, this would suggest that different $\alpha 6\beta 2$ *nAChR subtypes are involved in DA release and nicotine reward, as our CPP studies implicate (non- $\alpha 4$) $\alpha 6\beta 2$ *nAChRs in nicotine reward behavior. It would thus appear that nicotine reward behavior may not be regulated by $\alpha 6\beta 2$ *nAChR modulation of NAc DA release; future studies could investigate other mechanisms by which $\alpha 6\beta 2$ *nAChRs control nicotine reward behavior. The noradrenergic system has also been implicated in drug addiction (Ouzir and Errami, 2016; Weinschenker and Schroeder, 2007). Being that $\alpha 6\beta 2$ *nAChRs are also located on NE projection neurons in the locus coeruleus (Le Novere *et al*, 1996; Lena *et al*, 1999), it would be appropriate to determine whether $\alpha 6\beta 2$ *nAChRs in the locus coeruleus are involved in nicotine reward using local infusions of $\alpha 6\beta 2$ *nAChR-selective antagonists. If so, microdialysis studies could further be used to quantify NE release in brain regions receiving NE projections from the locus coeruleus to elucidate whether NE activity is involved in nicotine reward.

To expand on our initial CPP study, we were interested in determining the neuroanatomical locus where $\alpha 6\beta 2$ *nAChRs modulate nicotine reward behavior. $\alpha 6\beta 2$ *nAChRs

are enriched along the mesolimbic pathway, on VTA DA neuron soma and on DA projection terminals in the NAc (Champtiaux *et al*, 2002; Klink *et al*, 2001; Le Novere *et al*, 1996), brain regions associated with drug reward. Previous studies have shown that antagonizing $\alpha 6\beta 2$ *nAChRs specifically in the NAc blocks nicotine CPP in mice (Sanjakdar *et al*, 2015). Our studies expanded on this to show that activation of $\alpha 6\beta 2$ *nAChRs in the NAc shell subdivision, where acute nicotine elicits a preferential initial increase in DA (Pontieri *et al*, 1996), are involved in nicotine reward behavior in WT mice. This is consistent with previous data from our lab demonstrating that NAc shell $\alpha 6\beta 2$ *nAChRs are important for nicotine self-administration in rats (Brunzell *et al*, 2010).

Interestingly, intra-NAc shell α -Ctx MII had no effect on nicotine reward behavior in $\alpha 6L9$ 'S mice. Initially, we thought that a higher dose of α -Ctx MII was needed to decrease $\alpha 6L9$ 'S $\alpha 6\beta 2$ *nAChR function given their hypersensitivity to agonists (Drenan *et al*, 2008). However, increasing the dose of α -Ctx MII from 10pmol to 20 pmol was not effective in blocking nicotine CPP in $\alpha 6L9$ 'S mice. Based on this, it does not appear that the α -Ctx MII dose response curve is shifted in $\alpha 6L9$ 'S mice to explain the lack of effect in these gain-of-function mice. However, we need to do a more complete dose response curve with additional doses of α -Ctx MII in future studies to definitively conclude that there is no shift in the $\alpha 6L9$ 'S mice. Another explanation for the lack of effect of α -Ctx MII on nicotine CPP in $\alpha 6L9$ 'S mice lies in the fact that $\alpha 6L9$ 'S mice are heterozygous, so that only some of the $\alpha 6\beta 2$ *nAChRs are hypersensitive. If the hypersensitive $\alpha 6\beta 2$ *nAChRs are not antagonized by α -Ctx MII the same as WT $\alpha 6\beta 2$ *nAChRs (i.e. different affinity or potency of α -Ctx MII in $\alpha 6L9$ 'S mice), the hypersensitive $\alpha 6\beta 2$ *nAChRs may be masking the effect of α -Ctx MII at the normal

$\alpha 6\beta 2$ *nAChRs. Previous studies have shown that α -Ctx MII blocks enhanced DA neuron firing and DA release observed in $\alpha 6L9$ 'S mice (Drenan *et al*, 2008), but no known studies have been done to assess binding affinity and potency of α -Ctx MII in $\alpha 6L9$ 'S mice, which should be investigated in future studies. Given that increasing the dose of α -Ctx MII had no effect on nicotine CPP in $\alpha 6L9$ 'S mice, we hypothesized that hyperactivity of $\alpha 6\beta 2$ *nAChRs on VTA DA projection neurons may have been overcoming any effect of blocking these receptors on DA terminals the NAc shell. This hypothesis was supported by data suggesting for the first time that VTA $\alpha 6\beta 2$ *nAChRs are also important for nicotine reward behavior in mice.

In addition to reward behavior, we observed that intra-VTA α -Ctx MII attenuates NAc DA release in $\alpha 6L9$ 'S mice, indicating that $\alpha 6\beta 2$ *nAChR regulation of DA neuron firing, ultimately resulting in DA release from terminals in the NAc, may drive the enhanced reward phenotype seen in $\alpha 6L9$ 'S mice. As discussed above, further investigation into a causative role between DA activity and reward would confirm this hypothesis. Future studies should also utilize a more selective $\alpha 6\beta 2$ *nAChR antagonist, as discussed previously, to rule out effects of $\alpha 3\beta 2$ *nAChR in supporting nicotine CPP.

Together with previous findings, these preclinical data demonstrate that reduction of $\alpha 6\beta 2$ *nAChR function may be an effective therapeutic strategy for treatment of nicotine addiction, as activating $\alpha 6\beta 2$ *nAChRs promotes behaviors and associated neurochemistry related to nicotine addiction, while inhibiting $\alpha 6\beta 2$ *nAChRs reduces these effects. Current approved smoking cessation aids such as nicotine replacement therapy and varenicline are only modestly successful, indicating a need for novel therapeutic targets. Varenicline, which is a full agonist at $\alpha 7$ nAChRs and a partial agonist at $\beta 2$ *nAChRs, is more efficacious compared to

NRT (Wu *et al*, 2006), indicating that the selectivity of these compounds predicts their effectiveness. Therefore, a more selective target such as $\alpha 6\beta 2$ *nAChRs may prove to be more effective for smoking cessation. While α -Ctx MII and its derivatives don't cross the blood brain barrier, a potent and selective $\alpha 6\beta 2$ *nAChR antagonist (r-bPiDI) that crosses the blood brain barrier and reduces nicotine-elicited DA release and nicotine reinforcement has been recently established as a potential compound for treatment of smoking cessation (Beckmann *et al*, 2015).

It is promising that $\alpha 6\beta 2$ *nAChRs have a highly restricted expression pattern in catecholaminergic nuclei in the brain (Champtiaux *et al*, 2002; Klink *et al*, 2001; Le Novere *et al*, 1996), being enriched in areas associated with addiction (e.g. VTA, NAc). A molecular target with more selective expression could reduce adverse side effects associated with varenicline that may arise from activity at nAChRs that may more generally control processes such as cognition, mood, and motivation (Brunzell and Picciotto, 2009b; Levin *et al*, 2006). However, it should be noted that $\alpha 6\beta 2$ *nAChRs are also expressed in the visual system on retinal ganglion cells (Champtiaux *et al*, 2002; Clarke and Pert, 1985; Marks *et al*, 2010; Whiteaker *et al*, 2000). It is possible that targeting $\alpha 6\beta 2$ *nAChRs may have unwanted effects related to the visual system, though it is encouraging that genetic manipulation of the $\alpha 6$ subunit does not appear to have an effect on this system in rodents (Champtiaux *et al*, 2002).

It is also encouraging that $\alpha 6\beta 2$ *nAChRs have been associated with nicotine use and dependence in humans. Candidate gene studies demonstrate that polymorphisms in the gene cluster encoding the $\alpha 6$ and $\beta 3$ subunits are associated with measures of nicotine dependence, such as cigarettes per day, FTND scores, positive subjective response to nicotine, and smoking initiation (Culverhouse *et al*, 2014; Hoft *et al*, 2009; Saccone *et al*, 2009; Saccone *et al*, 2010; Saccone *et al*, 2007; Stevens *et al*, 2008; Thorgeirsson *et al*, 2010; Wang *et al*, 2014a; Zeiger *et*

al, 2008). These findings further indicate that targeting $\alpha 6\beta 2$ *nAChRs may prove successful for treatment of tobacco dependence.

Of note, these studies are limited, only focusing on behavioral and neurochemical effects of acute nicotine exposure. These acute effects are important to study, as the initial rewarding effects of drugs are thought to be important in the initial development of addiction (Wise and Koob, 2014), and initial responses to abused drugs can predict risk for later development of drug dependence (de Wit *et al*, 2012). In fact, mood and subjective states induced by a drug is used by the FDA as the primary indicator of its abuse liability (Balster and Bigelow, 2003). Further studies examining how $\alpha 6\beta 2$ *nAChRs are involved in the chronic effects of nicotine, such as maintenance of self-administration and withdrawal behavior, have the potential to provide a more comprehensive understanding of the contributions of $\alpha 6\beta 2$ *nAChRs to nicotine dependence. Previous studies using genetic and pharmacological inhibition strategies have shown that $\alpha 6\beta 2$ *nAChRs regulate nicotine self-administration maintenance, where rodents are exposed to nicotine chronically (Brunzell *et al*, 2010; Exley *et al*, 2011; Gotti *et al*, 2010), and nicotine withdrawal behaviors (Jackson *et al*, 2009), which arise after abstinence from chronic exposure to nicotine. Future studies examining nicotine self-administration and withdrawal in $\alpha 6L9$ 'S mice would nicely complement this existing literature to elucidate how activation of $\alpha 6\beta 2$ *nAChRs effects these behaviors associated with nicotine dependence. Rodent behavioral models where animals will increase drug self-administration with extended access to the drugs or upon initiation of withdrawal are also of interest, as these models are thought to reflect on the transition to dependence (Koob and Le Moal, 2008). Future studies could test the effect of $\alpha 6\beta 2$ *nAChR antagonism and $\alpha 6$ null or gain-of-function mutations in these models to assess whether $\alpha 6\beta 2$ *nAChRs are involved in this transition.

In addition to the pleasurable effects of nicotine, nAChRs also regulate anxiety-like behavior. Using the $\alpha 6L9'S$ mice, our anxiety studies assessed the contribution of $\alpha 6\beta 2^*$ nAChR activation to basal anxiety-like phenotypes. While these studies were done in the absence of nicotine, this is still relevant to nicotine addiction, as studies have shown that smokers experience anxiety more intensely than non-smokers (Fidler *et al*, 2009; Parrott, 1999; Perkins *et al*, 1992) and stress can lead to escalation of smoking and relapse (Shiffman *et al*, 1997; Skara *et al*, 2001). These behaviors reflect on emotional states that occur in the absence of nicotine that may promote tobacco use. Evidence suggests that elevations in cholinergic tone could support anxiety (Hart *et al*, 1999; Kolasa *et al*, 1982; Lamprea *et al*, 2000; Luo *et al*, 2013; Mineur *et al*, 2013; Power *et al*, 2002; Revy *et al*, 2014) and blockade of $\beta 2^*$ nAChR supports relief from basal anxiety-like phenotypes (Anderson *et al*, 2012). Our studies show that activation of $\alpha 6\beta 2^*$ nAChRs is sufficient for expression of basal anxiety-like behavior, while stimulation of $\alpha 4\beta 2^*$ nAChRs reduces anxiety-like behavior, possibly working in opposition to $\alpha 6\beta 2^*$ nAChRs.

Subsequent studies were performed to differentiate the contributions of (non- $\alpha 4$) $\alpha 6\beta 2^*$ and $\alpha 4\alpha 6\beta 2^*$ nAChRs to basal anxiety-like behavior. We originally hypothesized that activation of $\alpha 4\alpha 6\beta 2^*$ nAChRs promotes anxiogenesis, as this nAChR is the most sensitive to nicotine (Exley *et al*, 2008; Kuryatov *et al*, 2011; Salminen *et al*, 2007; Salminen *et al*, 2004) and is persistently activated by low concentrations of nicotine that desensitize most other nAChRs (Liu *et al*, 2012). Given that the $\alpha 6L9'S$ and $\alpha 4L9'A$ gain-of-function mutations produced opposite effects on anxiety-like behavior in our studies, demonstrating that $\alpha 6\beta 2^*$ and $\alpha 4\beta 2^*$ nAChRs appear to act in opposition in the EPM and open field assays, however, we expected that (non- $\alpha 4$) $\alpha 6\beta 2^*$ nAChRs would be responsible for $\alpha 6\beta 2^*$ nAChR regulation of basal anxiogenic-like behavior. Consistent with this hypothesis, $\alpha 6L9'S$ - $\alpha 4$ KO mice did not differ from $\alpha 6L9'S$ mice,

suggesting that the $\alpha 4$ subunit is not required for assembly in $\alpha 6\beta 2$ *nAChRs to support anxiogenic-like behavior in the open field assay. However, our light-dark results were inconsistent, as activation of $\alpha 4\alpha 6\beta 2$ *nAChRs appeared to promote trending increases in anxiety-like behavior in the light-dark test. As discussed, it was unexpected that $\alpha 4\alpha 6\beta 2$ *nAChRs would promote anxiogenic-like behavior considering the opposite effects of the $\alpha 6L9'S$ and $\alpha 4L9'A$ gain-of-function mutations in the EPM and open field tests (see Chapter 3). However, these opposing effects were absent in the light-dark test, as $\alpha 6L9'S$ mice showed increases in anxiety-like behavior, but there was no change in $\alpha 4L9'A$ mice. This could explain why $\alpha 4$ and $\alpha 6$ appear to work together in the $\alpha 4\alpha 6\beta 2$ *nAChR conformation to regulate anxiogenesis in the light-dark test, but not the open field or EPM assays.

The effect of $\alpha 6\beta 2$ *nAChR hyperactivity may be stronger than that of $\alpha 4\beta 2$ *nAChRs, as cholinergic hyperactivity in the amygdala (Power *et al*, 2002) and hippocampus (Luo *et al*, 2013; Mineur *et al*, 2013) promotes anxiety-like behaviors in rodent studies. While $\alpha 6\beta 2$ *nAChRs are not expressed in these brain areas at substantial levels, hyperactive cholinergic tone in other brains areas, such as the NAc, may similarly promote anxiety-like behavior. One study found that the anxiolytic drug buspirone reduced ACh levels in the NAc (Kolasa *et al*, 1982), suggesting that cholinergic hyperactivity in this region may also promote anxiety-like behavior. Future optogenetic studies stimulating NAc cholinergic interneurons can test this hypothesis. It would also be interesting to use optogenetics to stimulate noradrenergic neurons in the locus coeruleus as well. $\alpha 6\beta 2$ *nAChRs are enriched in the locus coeruleus (Le Novere *et al*, 1996; Lena *et al*, 1999), which has been implicated in anxiety-like behavior (Chmielarz *et al*, 2013; Itoi *et al*, 2011; Mazzone *et al*, 2016; McCall *et al*, 2015). If we find that cholinergic tone in the NAc or noradrenergic activity in the locus coeruleus regulates anxiety-like phenotypes in our future

studies, subsequent studies could assess a potential role for $\alpha 6\beta 2$ *nAChRs in these brain areas using local brain infusions of $\alpha 6\beta 2$ *nAChR-selective antagonists.

While $\alpha 6L9$ 'S mice exhibited an anxiogenic-like phenotype compared to WT littermates, genetic deletion of the $\alpha 6$ subunit had no effect on anxiety-like behavior, suggesting that $\alpha 6\beta 2$ *nAChRs are not necessary for expression of this behavior. This is somewhat unexpected as i.c.v. infusion of α -Ctx MII reduces nicotine withdrawal-induced anxiety-like behavior in mice (Jackson *et al*, 2009). It is possible that our anxiety assays were not stressful enough to reveal an effect of reduced $\alpha 6\beta 2$ *nAChR function. Future studies providing an external stressor (e.g. restraint stress) may uncover an effect in these anxiety tasks in $\alpha 6$ KO mice. Given the results from studies assessing nicotine withdrawal-associated anxiety-like behavior in response to α -Ctx MII (Jackson *et al*, 2009), it would be interesting to assess the effect of nicotine withdrawal on anxiety-like behavior in $\alpha 6L9$ 'S mice to determine whether activation of $\alpha 6\beta 2$ *nAChRs exacerbates this behavior. Studies assessing $\alpha 6\beta 2$ *nAChR function in relation to withdrawal-induced anxiety-like behavior are important, as nicotine withdrawal is known to contribute to relapse (Le Foll and Goldberg, 2009). It will also be important to assess anxiety-like behavior in response to genetic deletion of $\alpha 4$ as we did in $\alpha 6$ KO mice. We would expect to see a decrease in anxiety-like behavior in $\alpha 4$ KO mice, as previous studies have shown that selectively deleting the $\alpha 4$ subunit in VTA DA neurons blocks the anxiolytic efficacy of low dose nicotine (McGranahan *et al*, 2011). A decrease in $\alpha 4$ KO mice without an effect in $\alpha 6$ KO mice would suggest that DH β E produces its anxiolytic effects primarily through interaction with $\alpha 4\beta 2$ *nAChRs.

In our studies assessing basal and nicotine-associated anxiety-like behavior, we observed divergent findings, where different $\alpha 6\beta 2^*$ nAChR subtypes contribute to this behavior based on the model of anxiety. The seemingly paradoxical findings generated from the open field and light-dark experiments are perhaps explained by previous QTL studies reporting that different anxiety models have overlapping, but separate genetic influences (Griebel *et al*, 2000; Henderson *et al*, 2004; Turri *et al*, 2001), suggesting that these models measure different aspects of anxiety that have unique underlying mechanisms. It would be informative to perform future studies assessing (non- $\alpha 4$) $\alpha 6\beta 2^*$ and $\alpha 4\alpha 6\beta 2^*$ nAChR contributions to anxiety-like behavior using other behavioral models (e.g. elevated plus maze, marble burying, novelty-induced hypophagia, the social interaction test) to more comprehensively understand how $\alpha 6\beta 2^*$ nAChRs are involved in anxiety, which is a complex, multidimensional behavior.

These studies investigating the role of $\alpha 6\beta 2^*$ nAChRs in regulation of anxiety-like behavior indicate that targeting this nAChR subtype for smoking cessation to reduce nicotine's rewarding effects (as discussed above) may also help with anxiety symptoms that can trigger relapse and escalation of tobacco use (Shiffman *et al*, 1997; Skara *et al*, 2001), as well as those which arise from withdrawal (Le Foll *et al*, 2009). Our studies show that genetically reducing $\alpha 6\beta 2^*$ nAChR function has no effect on basal anxiety-like behavior, so inhibition of $\alpha 6\beta 2^*$ nAChRs ought not provide adverse emotive effects from a smoking cessation therapeutic standpoint. As discussed, these assays may not have been stressful enough to reveal an effect of the $\alpha 6$ null mutation as discussed; future studies could provide a stressor prior to testing anxiety-like behavior in $\alpha 6$ KO mice. We can also give brain infusions of selective $\alpha 6\beta 2^*$ nAChR antagonists to complement the genetic studies and determine neuroanatomical loci where $\alpha 6\beta 2^*$ nAChRs regulate anxiety-like behavior. Candidate brain regions for $\alpha 6\beta 2^*$ nAChR

regulated anxiety-like behavior include the mesolimbic pathway and the locus coeruleus, two areas involved in stress- and anxiety-related phenotypes where $\alpha 6\beta 2$ *nAChRs are selectively expressed. It is important to further characterize the effect of $\alpha 6\beta 2$ *nAChR inhibition in order to determine whether reducing $\alpha 6\beta 2$ *nAChR function may be an effective therapeutic strategy for smoking cessation, as it would be a concern if inhibiting $\alpha 6\beta 2$ *nAChRs increases anxiety-like behavior. It is promising that one study reports that i.c.v. infusion of α -Ctx MII reduced withdrawal-associated anxiety-like behavior (Jackson *et al*, 2009).

Finally, these studies sought to determine whether $\alpha 6\beta 2$ *nAChRs are important for EtOH reinforcement in mice, as alcohol is another abused legal drug that is commonly co-abused with nicotine. First, we developed a mouse model of oral, operant EtOH self-administration. Consistent with previous reports (Kelley *et al*, 1996; Middaugh *et al*, 1999a; Risinger *et al*, 1998), EtOH was reinforcing in C57BL/6J WT mice. This model expanded on previous models to specifically allow for assessment of EtOH's primary reinforcing effects, as EtOH self-administration took place in the absence of added sweetener, explicit EtOH-paired cues, and food or water restriction, factors used in previous models. We compared EtOH reinforcement at multiple concentrations of EtOH between-subject, comparing them to water controls, instead of using an EtOH fading procedure, which is commonly used in other models, where EtOH concentrations are gradually increased to promote self-administration. In the development of this model, we also detected subtle, but significant escalation of EtOH self-administration in WT mice, similar to what has been observed in previous studies following intermittent exposure to EtOH, termed the alcohol deprivation effect (ADE) (Khisti *et al*, 2006; Rodd *et al*, 2003; Spanagel *et al*, 1999). Further, our model appears to have predictive validity for therapeutic

effectiveness, as naltrexone decreased operant responding and EtOH intake in WT mice maintained on 15% EtOH.

Admittedly, this model of oral, operant EtOH self-administration is not without limitations. We chose not to pair explicit cues with presentation of EtOH reinforcers, but it is possible that the sound of the liquid dipper mechanism serves as a cue. While we were able to estimate EtOH consumption in g/kg using correct magazine entry measurements, which was positively correlated with BECs at various time points (30 m, 4 h, 6 h), this model does not allow for exact determination of EtOH consumption. Mice can put their head in the magazine without ingesting the EtOH reinforcer. The use of technology such as a lickometer would be useful for more precise measurements of EtOH intake. Another limitation of this model involves the use of a sweetener during acquisition of lever pressing behavior. While sweetener was not available with EtOH, 0.2% saccharin was used to train mice to lever press for the liquid dipper reinforcer. It is possible that there was some residual responding for saccharin, even after it was taken away and replaced with an EtOH solution, which could confound interpretation of reinforcement. However, control mice maintained on water showed reductions in responding by the second EtOH self-administration session, while 15% EtOH maintained significantly higher levels of responding and even promoted subtle increases across weekly self-administration sessions. Therefore, it is unlikely that prior exposure to saccharin supported reinforcement on its own. Future studies could attempt to use water instead of saccharin during lever pressing acquisition in order to eliminate any confounds produced by exposure to saccharin, as it is possible that residual responding for saccharin after its removal could promote initial consumption of EtOH, leading to maintenance of self-administration.

Antagonizing $\alpha 6\beta 2$ *nAChRs locally into the VTA produces reductions in operant EtOH self-administration in rats (Kuzmin *et al*, 2009). However, no known studies have examined the effect of $\alpha 6$ genetic deletion on operant EtOH self-administration. In these mouse genetic studies complementing the pharmacological studies, we used our model of oral operant EtOH self-administration in WT, $\alpha 6$ HET, and $\alpha 6$ KO mice to further assess $\alpha 6\beta 2$ *nAChR contributions to EtOH reinforcement. Consistent with studies showing that genetically deleting the $\alpha 6$ subunit has no effect on EtOH intake or preference in mice (Guildford *et al*, 2016; Kamens *et al*, 2012), $\alpha 6$ KO mice showed similar levels of 15% EtOH self-administration compared to WT littermates. Based on the lack of effect in $\alpha 6$ KO mice, it was unexpected that EtOH reinforcement was not observed in $\alpha 6$ HET mice. A lack of EtOH reinforcement in $\alpha 6$ HET mice suggests that expression of $\alpha 6\beta 2$ *nAChRs does modulate the reinforcing effects of EtOH. $\alpha 6$ KO mice may show shifts in the dose response curve as has been seen previously in EtOH CPP studies (Guildford *et al*, 2016). Future studies are planned to establish a full dose response curve for EtOH self-administration in these mice to assess whether $\alpha 6$ KO mice show a similar shift to explain the lack of effect in $\alpha 6$ KO, but not $\alpha 6$ HET mice at 15% EtOH. Alternatively, these interesting divergent effects in $\alpha 6$ HET versus $\alpha 6$ KO mice may be a result of compensation by other nAChR subtypes for the complete lack of $\alpha 6$ subunits in $\alpha 6$ KO mice. Future autoradiography binding studies are planned to assess potential compensation in these mice.

Further, future studies are planned to assess oral operant EtOH self-administration in $\alpha 6$ L9'S mice to complement our studies in $\alpha 6$ loss-of-function mice and assess whether cholinergic hyperactivity at $\alpha 6\beta 2$ *nAChRs promotes EtOH reinforcement. As reduced $\alpha 6\beta 2$ *nAChR function appears to decrease EtOH reinforcement, we predict that selective

activation of $\alpha 6\beta 2^*$ nAChRs in $\alpha 6L9'S$ mice will enhance EtOH reinforcement. This would support previous data reporting enhanced EtOH intake and place conditioning in these gain-of-function mice (Powers *et al*, 2013). In addition to EtOH's behavioral effects, $\alpha 6\beta 2^*$ nAChRs have also been implicated in the neurochemical effects of EtOH. Intra-VTA infusion of α -Ctx MII reduces EtOH-induced activation of the mesolimbic DA pathway (Larsson *et al*, 2004; Liu *et al*, 2013b) and $\alpha 6KO$ mice show reductions in EtOH-induced stimulation of VTA DA neurons (Liu *et al*, 2013b). Future studies could also assess EtOH-associated VTA DA neuron activation and NAc DA release using *in vivo* electrophysiology and microdialysis or cyclic voltammetry in $\alpha 6L9'S$ mice to determine if activation of $\alpha 6\beta 2^*$ nAChRs supports these neurochemical effects related to EtOH abuse. If these studies in $\alpha 6L9'S$ mice demonstrate that activation of $\alpha 6\beta 2^*$ nAChRs promotes EtOH reinforcement and associated neurochemistry, we would expand these studies to differentiate the role of (non- $\alpha 4$) $\alpha 6\beta 2^*$ and $\alpha 4\alpha 6\beta 2^*$ nAChRs using the $\alpha 6L9'S$ - $\alpha 4KO$ mice.

These studies have established a role for $\alpha 6\beta 2^*$ nAChRs in abuse-related effects of nicotine and alcohol separately. In addition to their independent use, these legal drugs are the most widely co-abuse drugs (Sussman *et al*, 2011). Future studies are planned to assess the effect of systemic nicotine administration on EtOH reinforcement using our model of oral operant EtOH self-administration. Studies show that nicotine enhances EtOH reinforcement in humans (McKee *et al*, 2013) and in rodent models of self-administration (Doyon *et al*, 2013b). If nicotine increases EtOH self-administration as expected, subsequent experiments will assess the role of $\alpha 6\beta 2^*$ nAChRs on this effect using $\alpha 6L9'S$, $\alpha 6HET$ and $\alpha 6KO$ mice, as well as intracranial infusions of $\alpha 6\beta 2^*$ nAChR-selective antagonists. Given that our studies and others have independently implicated $\alpha 6\beta 2^*$ nAChRs in abuse-related effects of nicotine and alcohol, we

predict that genetically and pharmacologically reducing $\alpha 6\beta 2$ *nAChR function would abolish nicotine-elicited increases in EtOH self-administration, while genetically enhancing $\alpha 6\beta 2$ *nAChR function would exaggerate the effect of nicotine on EtOH reinforcement. While cigarette use has declined, the rate of nicotine and alcohol co-dependency remains high, likely due to the emergence of e-cigarettes. Thus, there is a great need to identify novel treatment options for this new generation of co-abusers (Tarren and Bartlett, 2017). It is important to begin to better understand the mechanisms underlying the comorbidity of nicotine and alcohol use in order to identify potential therapeutic targets that may be efficacious in treating the co-abuse of these drugs.

Overall, the studies in this dissertation provide evidence that activation of $\alpha 6\beta 2$ *nAChRs promotes neurochemical and associated behaviors that are related to nicotine addiction. Given the recent popularity of e-cigarettes, especially among adolescents who are particularly vulnerable to developing dependence issues (Lamb *et al*, 2016), it is important to identify novel therapeutic targets for nicotine dependence; our preclinical data, together with previous studies, indicate that targeting $\alpha 6\beta 2$ *nAChRs may prove successful. Additionally, $\alpha 6\beta 2$ *nAChRs appear to regulate effects of EtOH that are related to alcohol dependence, providing evidence that targeting these receptors may also be effective for treatment for AUDs. Further, the fact $\alpha 6\beta 2$ *nAChRs similarly effect nicotine and alcohol abuse-related phenotypes suggests that targeting this class of nAChRs may be effective in treating the common co-abuse of these drugs. It is promising that human genetic studies implicate $\alpha 6\beta 2$ *nAChRs in multiple measures of nicotine and alcohol dependence independently, supporting our preclinical rodent data. The development of techniques that are able to image $\alpha 6\beta 2$ *nAChR binding and expression in the human brain and how this is related to addiction phenotypes would be a tremendous

advancement in the field to better understand if these receptors are directly involved in the effects of nicotine and alcohol, independently and together, that promote dependence in humans. It is hopeful that these preclinical studies will advance our understanding of mechanisms underlying independent abuse of alcohol and nicotine so that more effective treatments may be developed in the future.

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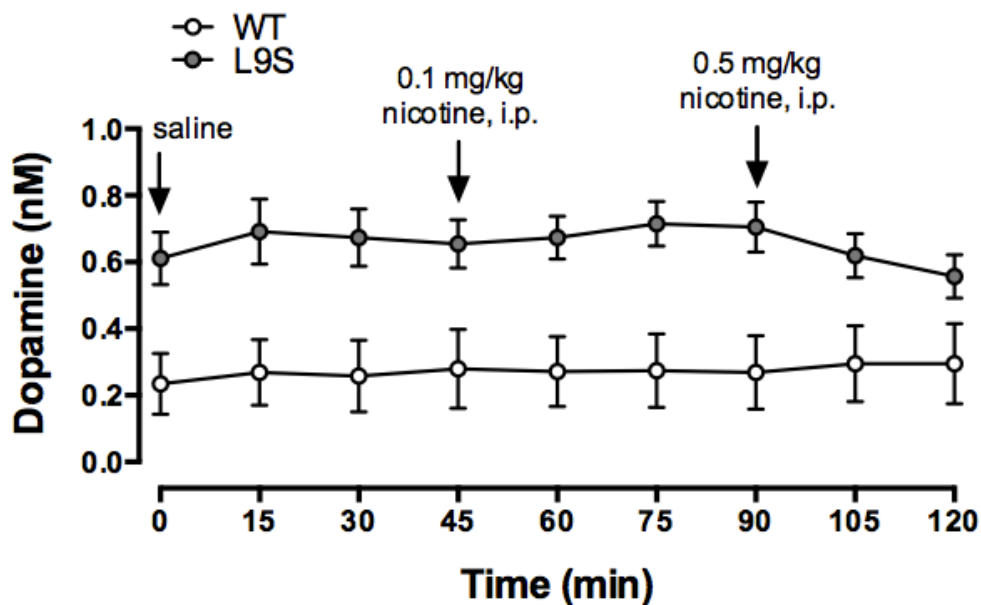
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Appendix A

Nicotine had no significant effect on nucleus accumbens (NAc) dopamine (DA) levels in wild type (WT) or $\alpha6\beta2^$ nAChR gain-of-function ($\alpha6L9'S$) mice*



WT and $\alpha6L9'S$ mouse NAc DA levels were measured using *in vivo* microdialysis following intraperitoneal (i.p.) injections of saline, 0.1 mg/kg, and 0.5 mg/kg nicotine. NAc DA levels remained elevated in $\alpha6L9'S$ mice compared to WT mice as with basal measurements. However, saline and both doses of nicotine had no effect on NAc DA levels in WT or $\alpha6L9'S$ mice.

Vita

Alexandra McIver Stafford was born on July 14, 1990 in Greenville, South Carolina, and is an American citizen. She graduated cum laude with a Bachelor of Science in Genetics with a minor in Philosophy from Clemson University in Clemson, South Carolina in May, 2012.

RESEARCH EXPERIENCE

Virginia Commonwealth University, School of Medicine, Richmond, VA

Ph.D. Dissertation Research with Dr. Darlene Brunzell, Nov 2012-May 2017

Established a mouse model of oral, operant ethanol self-administration in the absence of explicit cues, food restriction, water restriction, or ethanol fading. Utilized this model together with genetic and pharmacological manipulations to examine the contributions of alpha6 and alpha7 subunit containing nicotinic acetylcholine receptors to ethanol reinforcement in male and female mice. Investigated the role of alpha6 subunit containing nicotinic acetylcholine receptors in nicotine reward, anxiety-like behavior, and nucleus accumbens dopamine release.

Laboratory skills

- Drug dosing via intraperitoneal injections
- Local brain infusions
- Stereotaxic surgery
- Brain tissue sectioning
- Immunohistochemistry
- Conditioned place preference
- Operant ethanol self-administration
- Pavlovian fear conditioning
- Anxiety assays (open field, light-dark, elevated plus maze, marble burying)
- *in vivo* microdialysis in awake mice

Laboratory Rotation with Drs. Andrew Davies and Jill Bettinger, Aug-Nov 2012

Investigated the molecular targets of alcohol in *C. elegans*.

Laboratory skills

- Maintaining *C. elegans* colonies
- RNA interference in *C. elegans*
- Observing *C. elegans* behavior such as locomotion and body contraction in response to ethanol

Clemson University, Clemson, SC

Undergraduate Research Assistant with Dr. Haiying Liang, Aug 2010-Jan 2011

Assisted with research projects exploring the molecular underpinning of the vegetative/reproductive transition in *Metasequoia glyptostroboides*, genetic engineering for Septoria disease resistance in hybrid poplar, developing yellow-popular as a new tree model research system for comparative genomics of secondary cell wall formation, and modification of lignin by protein-crosslinking to facilitate production of biofuels from poplar.

Laboratory skills

- PCR
- DNA and RNA isolation
- Bacterial transformation of plant tissue

PUBLICATIONS

Stafford AM, Anderson SM, Shelton KL, Brunzell DH. Oral operant ethanol self-administration in the absence of explicit cues, food restriction, water restriction and ethanol fading in C57BL/6J mice. *Psychopharmacology*. 2015 Oct;232(20):3783-95

Brunzell DH, **Stafford AM**, Dixon CI. Nicotinic receptor contributions to smoking: insights from human studies and animal models. *Current Addiction Reports*. 2015 Mar;2(1):33-46

CONFERENCE POSTER ABSTRACTS

Stafford AM, Drenan RM, Banks ML, Brunzell DH. alpha6beta2 subunit containing nicotine acetylcholine receptors regulate nicotine reward and dopamine release in the nucleus accumbens. Central Virginia Chapter Society for Neuroscience, University of Virginia, Charlottesville, VA, March 2016.

Stafford AM and Brunzell DH. Assessment of alpha7 nicotinic acetylcholine receptor expression on mouse oral operant ethanol self-administration. Virginia Youth Tobacco Project, Richmond, VA, April 2015.

Stafford AM and Brunzell DH. alpha7 nicotinic acetylcholine receptor expression does not appear to modulate oral operant ethanol self-administration in female mice. Central Virginia

Chapter Society for Neuroscience, Virginia Commonwealth University, Richmond, VA, March 2015.

Stafford AM and Brunzell DH. alpha7 nicotinic acetylcholine receptors do not appear to regulate oral operant ethanol self-administration in female mice.” Society for Research on Nicotine and Tobacco, Philadelphia, PA, February 2015.

Stafford AM, Anderson SM, Shelton KL, Brunzell DH. Oral operant ethanol self-administration in the absence of explicit cues, food restriction, water restriction, or sweetener fading in C57BL/6J mice. Society for Neuroscience, Washington, DC, November 2014.

Stafford AM and Brunzell DH. Sex-related differences in oral operant ethanol self-administration in C57BL/6J mice. College on Problems of Drug Dependence, San Juan, PR, June 2014.

Stafford AM, Anderson SM, Shelton KL, Brunzell DH. Oral operant ethanol self-administration in the absence of explicit cues, food restriction, water restriction, or sweetener fading in C57BL/6J mice. Central Virginia Chapter Society for Neuroscience, Virginia Commonwealth University, Richmond, VA, March 2014.

Stafford AM, Engle SE, Lester HA, McIntosh JM, Drenan RM, Brunzell DH. Activation of alpha6beta2*nAChRs promotes nicotine reward-like behavior in mice. Central Virginia Chapter Society for Neuroscience, Virginia Tech Carillion Research Institute, Roanoke, VA, February 2013.

CONFERENCE ORAL PRESENTATIONS

Stafford AM, Drenan RM, Banks ML, Brunzell DH. Activation of alpha6beta2 subunit containing nicotinic acetylcholine receptors in the nucleus accumbens shell promotes nicotine reward. Society for Research on Nicotine and Tobacco, Chicago, IL, March 2016.

Stafford AM and Brunzell DH. alpha6beta2* and alpha7 nAChR contributions to oral operant ethanol self-administration in male and female mice. Virginia Youth Tobacco Project, Richmond, VA, March 2016.

Stafford AM and Brunzell DH. Activation of alpha6*nAChRs is sufficient for nicotine reward in mice: putative involvement of the nucleus accumbens shell. Virginia Academy of Sciences, James Madison University, Harrisonburg, VA, May 2015.

ACADEMIC AND PROFESSIONAL HONORS

- Virginia Foundation for Healthy Youth Poster Presentation Award Winner, April 2015
- B.S. awarded with high honors, Clemson University, 2012
- Dean’s List, Clemson University, 2008-2012

- Sistine Scholarship, 2008-2012
- Palmetto Fellows Scholarship, 2008-2012

PROFESSIONAL MEMBERSHIPS

- Society for Neuroscience, Member, 2014
- Central Virginia Chapter for the Society for Neuroscience, 2013-2016