alpha6 beta2 subunit containing nicotinic acetylcholine receptor contributions to abuse-related effects of nicotine and alcohol

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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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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 \( \alpha 6L9' \)S mice. Dr. J. Michael McIntosh provided the \( \alpha \)-conotoxin MII.

Chapter 3
Shawn M. Anderson performed the basal anxiety experiments in \( \alpha 6L9' \)S and \( \alpha 6KO \) mice and contributed to data analysis and interpretation. Jennifer Lee helped perform the basal anxiety studies in \( \alpha 4L9'A \) mice. Dr. Ryan M. Drenan provided the \( \alpha 6L9' \)S mice. Dr. Andrew Tapper provided the L9’A mice.

Chapter 4
Dr. Ryan M. Drenan provided the \( \alpha 6L9' \)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
α2KO .......................... alpha2 null mutant mouse
α3β2*nAChRs .................. alpha3 and beta2 containing nAChRs
α3β4*nAChRs .................. alpha3 and beta4 containing nAChRs
α4β2*nAChRs .................. alpha4 and beta2 containing nAChRs
α4α5β2*nAChRs .............. alpha4, alpha5 and beta2 containing nAChRs
α4α6β2*nAChRs .............. alpha4, alpha6 and beta2 containing nAChRs
α4KO .......................... alpha4 null mutant mouse
α4L9’A ......................... alpha4 gain-of-function mouse
α4L9’S .......................... alpha4 gain-of-function mouse
α5KO .......................... alpha5 null mutant mouse
α5*nAChRs .................... alpha5 nAChRs
(non-α4)α6β2*nAChRs ...... non-alpha4, alpha6, and beta2 subunit containing nAChRs
α6β2*nAChRs .................. alpha6 and beta2 subunit containing nAChRs
α6HET .......................... alpha6 heterozygous mouse
α6KO .......................... alpha6 null mutant mouse
α6L9’S .......................... alpha6 gain-of-function mouse
α6L9’S-α4KO ............... alpha6 gain-of-function mouse with the α4 null mutation

α7 nAChRs ............... alpha7 nAChRs

α7KO .................... 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

β2*nAChRs ..................... beta2 subunit containing nAChRs

β2KO ......................... beta2 null mutant mouse

β3KO ......................... beta3 null mutant mouse

β4KO ......................... beta4 null mutant mouse

β4*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

CHRNMB2 ..................... 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
DHβE ................................. Dihydro-beta-erythroidine
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.

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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 α6β2 subunit containing nicotinic acetylcholine receptor (α6β2*nAChR; *denotes possible assembly with other subunits) contributions to abuse-related effects of nicotine and alcohol. In the absence of α6β2*nAChR-selective agonists, α6β2*nAChR gain-of-function (α6L9’S) mice provide a tool for selective activation of α6β2*nAChRs. Using the α6L9’S mice together with nicotine doses sub-threshold for stimulation of native nAChRs, these studies tested the hypothesis that activation of α6β2*nAChRs is sufficient to promote neurochemical and behavioral effects relevant to nicotine addiction. Intracranial infusions of an α6β2*nAChR-selective antagonist further tested the neuroanatomical locus of α6β2*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 α6β2*nAChRs on ventral tegmental area (VTA) DA neurons, as well as on DA terminals in the nucleus accumbens (NAc) shell support nicotine reward. VTA α6β2*nAChR stimulation is required for elevated basal NAc DA levels in α6L9’S mice, who also show elevated nicotine CPP. These studies also showed elevated anxiety-like behavior in α6L9’S mice, but no change in α6 subunit null mutant (α6KO) mice to suggest that elevated cholinergic tone at α6β2*nAChRs promotes anxiety-like behavior. To better define the molecular make-up of α6β2*nAChRs supporting nicotine reward and anxiety-like behavior, these studies crossed α6L9’S to α4 subunit knockout mice to differentiate (non-α4)α6β2* and α4α6β2*nAChR contributions. (non-α4)α6β2*nAChRs appear to promote nicotine reward behavior, while the α6β2*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 α6 heterozygous (α6HET) and α6KO mice to characterize the role of α6β2*nAChRs in EtOH reinforcement. EtOH self-administration was similar to wild type mouse in α6KO mice, but not α6HET mice, suggesting that expression of α6β2*nAChRs modulates EtOH reinforcement. Together, these preclinical studies implicate α6β2*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 α6β2
subunit containing nAChR (α6β2*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 β2*nAChRs, α6β2*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, α6β2*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 α6β2*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<sub>A</sub> and glycine receptor function (Dildy-Mayfield et al, 1996; Mascia et al, 1996). In addition, n-alcohols can inhibit GABA<sub>C</sub> 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 acomprosate) 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 α7- and β2*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 cyste-loop ligand-gated ion channels that also includes γ-aminobutyric 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 αbungarotoxin, 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 α1, β1, 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 (α1)2β1δγ nAChr and the adult (α1)2β1δε 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 (α2-10) and three β subunits (β2-4) 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 α8, which has only been found in avian tissue. Homomeric nAChRs are composed of α7-α9 subunits. Heteromeric nAChRs are composed of a combination of α and β subunits, including α2-6 and β2-4. More recently, researchers have discovered that α9 and α10 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. α5 and β3 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 β3 is important for the formation of α6β2*nAChRs and the loss of β3 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 (1-3µM), 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 pleasurable (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 β2*nAChRs compared to other nAChR subtypes (Grady et al., 2010; Pauly et al., 1991; Whiting and Lindstrom, 1988; Xiao and Kellar, 2004). β2*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 β2 subunit primarily assembles with α4 and/or α6 to make up three subclasses of β2*nAChRs: α4β2*nAChRs, α4α6β2*nAChRs, and (non-α4)α6β2*nAChRs. The α4α6β2*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 α4β2*nAChRs are widely expressed in the brain, while α-Ctx MII-sensitive α6β2*nAChRs are more selectively expressed in catecholaminergic nuclei (Champtiaux et al, 2002; Klink et al, 2001; Le Nover et al, 1996). This dissertation aims to investigate the subunit make-up of β2*nAChRs, especially those expressed in the mesolimbic DA pathway, that modulate abuse-related effects of nicotine and alcohol.

*Studying the function of β2*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 α3-7, α9, β2-4 subunits have all been knocked out in mice, and all but the α3KO 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 β2KO mice (Picciotto et al, 1995) provide valuable insight into the function of β2*nAChRs but, these data should be carefully interpreted due to the limitations of using knockout mice discussed above. While β2KO mice are grossly normal, development of the visual system is altered (Bansal et al, 2000; Rossi et al, 2001). Other studies commonly use DHβE, a β2-selective antagonist, and A-85380, a β2-selective agonist, as pharmacological tools to elucidate the function of β2*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 DHβE and A-85380 are selective for β2*nAChRs, they are not necessarily specific, as they may have affinity for other nAChR subtypes. While these tools have provided great insight into β2*nAChR function, they are incapable of differentiating the various β2*nAChR subtypes.

The studies in this dissertation aim to investigate α6β2*nAChR contributions to abuse-related effects of nicotine and alcohol using genetic and pharmacological tools, including the α6β2*nAChR-selective antagonist, α-Ctx MII, as well as α6KO and α6β2*nAChR gain-of-function (α6L9’S) mice. α6KO mice (Champtiaux et al, 2002) are grossly normal, exhibiting no neurological or behavioral deficits. Specifically, there are no developmental alterations in α6KO mouse visual or dopaminergic systems, where α6β2*nAChRs are highly expressed. However, α6KO mice exhibit an increase in α4β2*nAChRs as shown by epibatidine binding studies (Champtiaux et al, 2003), indicating potential functional compensation that may confound
interpretations of results generated using these α6KO mice. The α6β2*nAChR-selective antagonist, α-Ctx MII (Cartier et al, 1996), is also available to study the effects of α6β2*nAChR loss-of-function and complement knockout studies. It should be noted that α-Ctx MII also binds to α3β2*nAChRs, which are expressed in some brains areas where α6β2*nAChRs are located. The binding affinity of α-Ctx MII for α3β2*nAChRs (Kᵢ=50 nM) is approximately 50-fold lower compared to α6β2*nAChRs (Kᵢ=1.1 nM), and α-Ctx MII is slightly more potent at α6β2*nAChRs (IC₅₀=0.39 nM) compared to α3β2*nAChRs (IC₅₀=0.5-2.2 nM) (Gotti et al, 2006). There are other conotoxin derivatives, such as α-Ctx PIA (Dowell et al, 2003), that are selective for α6β2* versus α3β2*nAChRs (IC₅₀=0.69 nM and 74.2 nM, respectively) (Gotti et al, 2006). More recently, another α6β2*nAChR-selective antagonist, r-bPiDI, was developed (Beckmann et al, 2015). Unlike α-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 α6KO mice and intracranial infusions of α-Ctx MII in combination with behavioral and neurochemical techniques to elucidate functions of α6β2*nAChRs.

α6KO mice and α6β2*nAChR-selective antagonists used to reduce α6β2*nAChR function have been valuable in investigating the function of α6β2*nAChRs, but there are limited tools available to activate this nAChR subtype. To date, development of α6β2*nAChR-selective agonists has been unsuccessful due to poor function of α6β2*nAChRs in heterologous expression systems (Drenan et al, 2008). As result, Drenan et al developed an α6β2*nAChR gain-of-function mouse strain (Drenan et al, 2008), which can be used as an alternative strategy to selectively activate α6β2*nAChRs in the absence of a selective agonist. These BAC transgenic
mice (α6L9’S) possess a single leucine to serine point mutation at the 9’ position of the M2 pore-forming region of the α6 subunit. This mutation renders the α6β2*nAChRs hypersensitive to ACh and nicotine, so that sub-threshold concentrations of these agonists are able to selectively activate α6β2*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 α6L9’S synaptosome preparations and activate VTA DA neurons in α6L9’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 α6β2*nAChRs. Behaviorally, these gain-of-function α6L9’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). α6L9’S mice show normal expression of α6β2*nAChRs, with normal localization and intensity of [125I]-α-Ctx MII. To confirm this, [125I]-epibatidine binding coupled with inhibition by unlabeled α-Ctx MII revealed normal levels and localization of α6β2* and α4β2*nAChRs (Drenan et al, 2008). Further, α6L9’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 α6β2*nAChRs, the studies described in this dissertation also used α6L9’S gain-of-function mice as a tool to selectively activate α6β2*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 β2*nAChRs contribute to tobacco use and dependence. PET and SPECT imaging studies show that nicotine binds to about 88% of the β2*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 β2*nAChR occupancy (Esterlis et al., 2010). Postmortem studies show an up-regulation of α4β2*nAChR numbers in the brains of smokers (Benwell et al., 1988; Breese et al., 1997). Imaging studies have also shown α4β2*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 α4β2*nAChR expression in the brains of smokers has been associated with better smoking cessation outcomes (Brody et al., 2014). Further, the β2*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 β2 subunit (CHRNB2) in the risk for tobacco dependence. However, candidate gene studies have identified CHRNB2 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). β2 primarily assembles with the α4 and α6 subunits in brain areas associated with nicotine addiction. Not surprisingly, candidate gene studies have shown that variation in the genes that encode the α4 and α6 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 β2*nAChR binding and increased expression and function of α4β2*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-CHRNB3 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 CHRNB2 polymorphisms associated with nicotine dependence, they have identified single nucleotide polymorphisms (SNPs) within the CHRNA3-CHRNA5-CHRNB4 gene cluster, encoding the α3, α5, and β4 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; Munafo 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 CHRNA4 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 CHRNA4 gene cluster have been linked to heavy alcohol consumption (Hoft et al., 2009). Polymorphisms in the CHRNA3-CHRNA5-CHRN4 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-CHRN4 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 α7 nAChR activation, while β2*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 α4β2* and α6β2*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 α5 accessory subunit is also highly expressed in the VTA; about half of the α4β2*nAChRs expressed in the VTA assemble with the α5 subunit (Chatterjee et al, 2013). Other nAChRs expressed in the VTA include α7 and α3*nAChRs. In the NAc, there are four main populations of nAChRs expressed on DA terminals. These include the α4β2*, α4α5β2*, α6β2*, and α4α6β2*nAChRs. According to one study, there is very little α5 expression on these terminals (Salminen et al, 2004). α7 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).
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 β2*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). β2*nAChR availability is correlated with the urge to smoke to relieve withdrawal symptoms during early abstinence (Staley et al, 2006). In rodent studies, β2*nAChRs are critical for nicotine-stimulated mesolimbic DA neuron activity. Nicotine desensitizes GABAergic β2*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 β2 null mutant (β2KO) mouse slice preparations (Picciotto et al, 1998). Spontaneous VTA DA neuron firing is also absent in anesthetized β2KO mice and re-expression of β2 restores neuron firing (Maskos et al, 2005). Nicotine does not stimulate NAc DA release in β2KO 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 β2*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 β2*nAChR subtypes involved in regulation of mesolimbic DA activity. The α4 and α6 subunits, which assemble with β2, are important for mesolimbic DA activity. Inward currents of DA neurons in the VTA and nicotine-stimulated DA release is blunted in α4 null mutant (α4KO) 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 α4 subunit in the VTA restores nicotinic control of NAc DA release and nicotine-stimulated increases in VTA DA neuron firing (Exley et al, 2011). α4β2*nAChR gain-of-function mice with a single point mutation of the α4 subunit (α4L9’A) that renders their α4β2*nAChRs hypersensitive to nicotine
exhibit enhanced activation of DA neurons, suggesting that \( \alpha_4\beta_2^n \)AChR stimulation is sufficient for this effect (Liu et al., 2012; Tapper et al., 2004; Zhao-Shea et al., 2011).

In regards to \( \alpha_6\beta_2^n \)AChRs, 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 (\( \alpha_6KO \)) mice or upon administration of \( \alpha \)-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 \( \alpha_6KO \) mice or following local infusion of \( \alpha \)-Ctx MII (Champtiaux et al., 2003; Liu et al., 2012; Zhao-Shea et al., 2011). In \( \alpha_4L9'A \) mice, \( \alpha \)-Ctx MII infused into the VTA blocked the enhancement of VTA DA neuron activation in the response to physiologically relevant concentrations of nicotine, implicating \( \alpha_4\alpha_6\beta_2^n \)AChRs in the control of nicotine-stimulated DA neuron activity (Liu et al., 2012; Zhao-Shea et al., 2011). Infusion of \( \alpha \)-Ctx MII or \( \alpha \)-Ctx PIA in the VTA reduces nicotine-stimulated DA release in the NAc (Gotti et al., 2010). In addition, \( \alpha \)-Ctx MII robustly attenuates evoked phasic DA release in the NAc core of rats (Wickham et al., 2013). \( \alpha_6\beta_2^n \)AChR 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 \textit{in vitro} compared to wild type (WT) mice, suggesting that stimulation of \( \alpha_6\beta_2^n \)AChRs 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 \( \alpha_4\alpha_6\beta_2^n \)AChRs regulate DA neuron firing and DA release (Drenan et al., 2010; Engle et al., 2013).
α7 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 α7 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

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Manipulation</th>
<th>Behavioral Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2</td>
<td>KO</td>
<td>Evoked DA release blocked</td>
<td>Zhou et al. 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nicotine-elicited DA release blocked</td>
<td>Picciotto et al. 1998; Grady et al. 2002; Champtiaux et al. 2003; Salminen et al. 2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blocks ACh- and nicotine-stimulated DA neuron firing</td>
<td>Picciotto et al. 1998; Champtiaux et al. 2003; Maskos et al. 2005</td>
</tr>
<tr>
<td>DHβE</td>
<td></td>
<td>Evoked DA release blocked</td>
<td>Zhou et al. 2001; Rice and Cragg, 2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Evoked DA release blocked</td>
<td>Exley et al. 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blunted nicotine-stimulated DA release</td>
<td>Champtiaux et al. 2003; Marubio et al. 2003; McGranahan et al. 2011</td>
</tr>
<tr>
<td>α6</td>
<td>KO</td>
<td>Blocks ACh- and nicotine-stimulated DA neuron firing</td>
<td>Liu et al. 2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blunted nicotine-stimulated DA release</td>
<td>Champtiaux et al. 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blocks evoked DA release</td>
<td>Exley et al. 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hypersensitive DA release</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blunted nicotine-stimulated DA release</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blocks evoked DA release</td>
<td></td>
</tr>
<tr>
<td>α7</td>
<td>MLA</td>
<td>Blocks nicotine-elicited DA release</td>
<td>Schilstrom et al. 1998</td>
</tr>
</tbody>
</table>

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 β2*nAChRs in rodent models of reward and reinforcement. β2KO 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 β2*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. β2KO mice do not express nicotine CPP either (Walters et al, 2006), indicating that β2*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 β2KO mice as it does in WT mice (Brunzell et al, 2006). β2KO 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, β2*nAChRs expressed in the mesolimbic DA pathway are important for nicotine’s abuse-related effects. Local infusion of the β2-selective antagonist, dihydro-β-erythroidine (DHβE), 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 β2 subunit in the VTA of β2KO mice rescues nicotine self-administration (Pons et al, 2008). β2 transgenic mice expressing β2*nAChRs only in VTA neurons show a restoration of nicotine-stimulated
locomotor activity after seven days of nicotine administration compared to β2KO mice (Mineur et al., 2009).

Studies in this dissertation aim to investigate subtypes of β2*nAChRs that regulate nicotine reward. β2 primarily assembles with the α4 and α6 subunits, which are also involved in the rewarding and reinforcing effects of nicotine. α4KO 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 α4 subunit from DA neurons is sufficient to block nicotine reward (McGranahan et al., 2011). However, for jugular i.v. nicotine self-administration, α4KO mice do not differ from WT mice (Cahir et al., 2011). α4β2*nAChR gain-of-function (α4L9’A) mice exhibit leftward shifts in nicotine CPP (Tapper et al., 2004) and α4-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 α4KO mice, α6KO mice also show reductions in i.v. (tail vein) or VTA intracranial nicotine self-administration (Exley et al., 2011; Pons et al., 2008). α6KO mice also exhibit rightward shifts in the dose response curve for nicotine CPP (Sanjakdar et al., 2015). Local infusion of the α6β2*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 DHβE, which inhibits both α4β2* and α6β2*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.,
Moreover, basal and nicotine-stimulated locomotor activity is enhanced in α6L9’S mice, an effect blocked by D₁ (SCH 23390) and D₂ (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 α4 subunit, as knocking out the α4 subunit in α6L9’S mice reverses this locomotor effect (Drenan et al., 2010).

Initial studies indicated that α7 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 α7 nAChRs act in opposition to β2*nAChRs. That is, inhibiting α7 nAChRs enhances nicotine reward and reinforcement, while stimulating α7 nAChRs reduces nicotine’s rewarding and reinforcing effects (Brunzell and McIntosh, 2012; Harenza et al., 2014). Local infusion of an α7 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 α7 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 α6β2*nAChRs (Mogg et al., 2002). α7 null mutant (α7KO) mice show leftward shifts in nicotine CPP (Harenza et al., 2014). Consistently, α7-selective agonists inhibit nicotine reward and reinforcement, blocking nicotine CPP (Harenza et al., 2014) and reducing nicotine-self-administration (Brunzell et al., 2012). However, α7KO mice show reductions in oral nicotine self-administration in a two bottle choice paradigm after 40 days of exposure. These conflicting results suggest that α7 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. α2 null mutant (α2KO) mice exhibit increases in nicotine self-administration (Lotfipour et al, 2013). When the accessory α5 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 α5 is re-expressed in the medial habenula (Fowler et al, 2011). Overexpression of the β4 subunit produces reductions in nicotine intake, which is rescued by expression of the α5 variant, D398N (Frahm et al, 2011; Morel et al, 2014; Tammimaki et al, 2012). Thus, it appears that β4*nAChRs and α5*nAChRs may work against each other or that assembly of α3β4*nAChRs with the α5 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

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Manipulation</th>
<th>Behavioral Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2</td>
<td>KO</td>
<td>CPP blocked</td>
<td>Walters et al. 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Self-administration blocked</td>
<td>Picciotto et al. 1998; Maskos et al. 2005; Besson et al. 2006; Pons et al. 2008; Brunzell et al. 2006; King et al. 2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Conditioned reinforcement blocked</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Locomotor activation blocked</td>
<td></td>
</tr>
<tr>
<td>DHβE</td>
<td>KO</td>
<td>CPP blocked</td>
<td>Walters et al. 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Self-administration blocked</td>
<td>Corrigall et al. 1994</td>
</tr>
<tr>
<td>α2</td>
<td>KO</td>
<td>Increased self-administration</td>
<td>Lotfipour et al. 2013</td>
</tr>
<tr>
<td>α4</td>
<td>KO</td>
<td>CPP blocked</td>
<td>McGranahan et al. 2011; Sanjakdar et al. 2015</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CPP unchanged</td>
<td>Cahir et al. 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Self-administration blocked</td>
<td>Pons et al. 2008; Exley et al. 2011</td>
</tr>
<tr>
<td></td>
<td>α4L97A</td>
<td>Enhanced nicotine CPP</td>
<td>Tapper et al. 2004</td>
</tr>
<tr>
<td></td>
<td>α4-S248F</td>
<td>Enhanced self-administration</td>
<td>Cahir et al. 2011</td>
</tr>
<tr>
<td>α5</td>
<td>KO</td>
<td>Increased self-administration</td>
<td>Fowler et al. 2011</td>
</tr>
<tr>
<td>α6</td>
<td>KO</td>
<td>CPP right-shifted</td>
<td>Sanjakdar et al. 2015</td>
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<td></td>
<td></td>
<td>Self-administration reduced</td>
<td>Pons et al. 2008; Exley et al. 2011</td>
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<tr>
<td>α-CTX MII</td>
<td></td>
<td>CPP blocked</td>
<td>Jackson et al. 2009; Sanjakdar et al. 2015</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Self-administration blocked</td>
<td>Brunzell et al. 2010; Gotti et al. 2010</td>
</tr>
<tr>
<td>α7</td>
<td>KO</td>
<td>CPP unaffected</td>
<td>Walters et al. 2006</td>
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<td></td>
<td></td>
<td>Leftward shift in CPP</td>
<td>Harenza et al. 2014</td>
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<tr>
<td></td>
<td></td>
<td>Self-administration unaffected</td>
<td>Pons et al. 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chronic oral nicotine intake decreased</td>
<td>Levin et al. 2009</td>
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<tr>
<td>MLA</td>
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<td>Self-administration unaffected</td>
<td>Grottick et al. 2000</td>
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<td></td>
<td></td>
<td>Self-administration blocked</td>
<td>Markou and Paterson 2001</td>
</tr>
<tr>
<td>α-CTX ArLB</td>
<td></td>
<td>Self-administration increased</td>
<td>Brunzell et al. 2012</td>
</tr>
<tr>
<td>PHA-543613</td>
<td></td>
<td>CPP blocked</td>
<td>Harenza et al. 2014</td>
</tr>
<tr>
<td>PNU-282987</td>
<td></td>
<td>Self-administration blocked</td>
<td>Brunzell et al. 2012</td>
</tr>
</tbody>
</table>

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 β2*nAChRs contributes to anxiety phenotypes (Picciotto et al., 2015). Chronic nicotine exposure produces an up-regulation of the α4β2*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; Slottkin 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 α6β2*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 α6β2*nAChRs in response to chronic nicotine depends on whether α4 is present, where α4α6β2*nAChRs are down-regulated and (non-
α4)α6β2*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 β2*nAChRs regulated anxiety-like behavior, preclinical rodent studies report that the β2*nAChR-selective antagonist, DHβE and β2*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 β2KO mice. High doses of the β2-selective agonist, 5I-A85380 also produce an anxiogenic-like phenotype (Anderson et al., 2015). These findings suggest that inactivation of β2*nAChRs, presumably via desensitization, promotes anxiolysis, while stimulation of β2*nAChRs promotes anxiogenesis.

The subtype of β2*nAChRs that contribute to anxiety-like behavior is a topic of question in this dissertation. α4β2*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 α4 subunit is genetically deleted in VTA DA neurons (McGranahan et al., 2011), suggesting that α4β2*nAChRs expressed in the VTA are required for nicotine’s anxiolytic properties. α4KO mice show elevated levels of basal anxiety-like behavior (Ross et al., 2000), suggesting that inhibition of α4β2*nAChRs promotes anxiety-like behavior. Interestingly, α4β2*nAChR gain-of-function (α4L9’S) mice also show increases in basal anxiety-like behavior in the EPM task.
(Labarca et al, 2001). Based on these data in α4KO and α4L9’S mice, it is not clear whether activation or inhibition of α4β2*nAChRs promotes anxiety-like behavior. For α6β2*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 α6β2*nAChRs regulate anxiety-like behavior in response to withdrawal from nicotine.

Other nAChR subtypes have also been implicated in anxiety-like behavior. β3 (β3KO) and β4 null mutant (β4KO) 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 β3 and β4 subunits promote anxiety-like behavior. α7KO 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 α7 nAChR-selective agonist, increases anxiety-like behavior (Pandya and Yakel, 2013). These data suggest that inhibition of α7 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

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Manipulation</th>
<th>Behavioral Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2</td>
<td>KO</td>
<td>Blunted nicotine-induced anxiogenesis (light-dark)</td>
<td>Anderson and Brunzell, 2015</td>
</tr>
<tr>
<td></td>
<td>DHβE</td>
<td>Anxiolytic (EPM; marble burying)</td>
<td>Anderson and Brunzell 2012</td>
</tr>
<tr>
<td></td>
<td>Varenicline</td>
<td>Anxiolytic (marble burying; hypophagia)</td>
<td>Turner et al. 2010; Hussman et al. 2014</td>
</tr>
<tr>
<td></td>
<td>ABT-089</td>
<td>Anxiogenic (hypophagia)</td>
<td>Yohn et al. 2014</td>
</tr>
<tr>
<td></td>
<td>51-A85380</td>
<td>Anxiogenic (light-dark; EPM)</td>
<td>Anderson and Brunzell, 2015</td>
</tr>
<tr>
<td>β3</td>
<td>KO</td>
<td>Decreased anxiety levels (EPM)</td>
<td>Booker et al. 2007</td>
</tr>
<tr>
<td>β4</td>
<td>KO</td>
<td>Decreased anxiety levels (EPM; light-dark)</td>
<td>Salas et al. 2003; Semenova et al. 2012</td>
</tr>
<tr>
<td>α4</td>
<td>KO</td>
<td>Nicotine-stimulated anxiolysis blocked (EPM)</td>
<td>McGranahan et al. 2011</td>
</tr>
<tr>
<td></td>
<td>α4L9'S</td>
<td>Anxiogenic (EPM; mirrored chamber)</td>
<td>Labarca et al. 2001</td>
</tr>
<tr>
<td></td>
<td>Sazetidine</td>
<td>Anxiolytic (hypophagia)</td>
<td>Hussman et al. 2014</td>
</tr>
<tr>
<td>α6</td>
<td>α-Ctx MII</td>
<td>Decreased nicotine withdrawal-induced anxiety-like behavior</td>
<td>Jackson et al. 2009</td>
</tr>
<tr>
<td>α7</td>
<td>KO</td>
<td>Anxiety-like behavior unaffected (EPM; light-dark; open field)</td>
<td>Salas et al. 2007; Jackson et al. 2008</td>
</tr>
<tr>
<td></td>
<td>MLA</td>
<td>Reversed nicotine-induced anxiogenesis</td>
<td>Tucci et al. 2003</td>
</tr>
<tr>
<td></td>
<td>PNU-282987</td>
<td>Increased anxiety levels</td>
<td>Pandya et al. 2013</td>
</tr>
</tbody>
</table>

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 β2*nAChRs in the behavioral and neurochemical effects of EtOH have generated conflicting results. DHβE or genetic knockdown of the β2 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 β2*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 β2*nAChR subtypes that modulate EtOH reinforcement. Previous data implicate that α4β2* and α6β2*nAChRs are involved in abuse-related effects of EtOH. α4KO mice consume less EtOH and show reduced preference for EtOH compared to WT mice (Hendrickson et al., 2010; Liu et al., 2013a), while α4L9’A mice exhibit increases in EtOH preference (Liu et al., 2013a). EtOH-induced VTA DA neuron activation is also reduced in α4KO mice and enhanced in α4L9’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 DHβE 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 α6β2*nAChRs, α6KO mice show no differences compared to WT mice for measures of EtOH consumption and preference (Guildford et al., 2016; Kamens et al., 2012). But, α6KO mice do show a loss of reward sensitivity at high doses of EtOH in CPP (Guildford et al., 2016). α6L9’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 α6 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, α7KO mice consumed less EtOH compared to WT mice (Kamens et al., 2010a). However, antagonizing α7 nAChRs has no effect on operant EtOH self-administration (Kuzmin et al., 2009) or EtOH-elicited DA release (Larsson et al., 2002). β3KO 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). α5 null mutant mice (α5KO) 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 β4KO 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

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Manipulation</th>
<th>Behavioral Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2 KO</td>
<td>No change in EtOH intake (two-bottle choice; intermittent access) or preference</td>
<td>Kamens et al. 2010a; Dawson et al. 2013; Lof et al. 2007</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No effect on EtOH-associated conditioned reinforcement</td>
<td>Dawson et al. 2013</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Decreased EtOH-induced sedation (LORR)</td>
<td>Tolu et al. 2017</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Decreased EtOH-induced VTA DA neuron firing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β2 DHβE</td>
<td>No effect on EtOH operant self-administration</td>
<td>Le et al. 2000; Kuzmin et al. 2009</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Decreased EtOH-induced hypnosis (LORR)</td>
<td>Dawson et al. 2013</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No effect on EtOH-elicited DA release</td>
<td>Larsson et al. 2002; Ericson et al. 2003</td>
<td></td>
</tr>
<tr>
<td>Varenicline</td>
<td>Reduced EtOH intake (two-bottle choice; DID)</td>
<td>Steensland et al. 2007; Hendrickson et al. 2010; Kamens et al. 2010a; Santos et al. 2013; Feduccia et al. 2014</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reduced EtOH operant self-administration</td>
<td>Steensland et al. 2007; Bito-Onon et al. 2011</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increased EtOH-induced sedation and ataxia (LORR; balance beam; dowel test)</td>
<td>Kamens et al. 2010b</td>
<td></td>
</tr>
</tbody>
</table>
|         | No effect on EtOH-elicited DA release | Ericson et al. 2009b;
| β3 KO  | No change in EtOH intake (two-bottle choice) or EtOH-induced sedation and ataxia (LORR; balance beam) | |
| β4 KO  | Increased EtOH intake (two-bottle choice) | Tolu et al. 2017 |
| α4 KO  | Reduced EtOH intake (DID) | Hendrickson et al. 2010 |
|         | EtOH CPP blocked | Liu et al. 2013a |
|         | Reduced EtOH-stimulation of DA neurons | Liu et al. 2013a |
| α4 L9A | Enhanced EtOH CPP | Liu et al. 2013a |
|         | Hypersensitive EtOH-stimulation of DA neurons | Liu et al. 2013a |
| α5 KO  | No change in EtOH intake (DID) | Santos et al. 2013 |
|         | Increased EtOH-induced sedation and ataxia (LORR; rotarod) | Santos et al. 2013 |
| α6 KO  | No change in EtOH intake or preference (two-bottle choice; DID) | Kamens et al. 2012; Guildford et al. 2016 |
|         | Increased EtOH-induced sedation | Kamens et al. 2012 |
|         | Reduced EtOH-stimulation of DA neurons | Liu et al. 2013b |
| α6L9’S | Increased EtOH intake (DID) and enhanced EtOH CPP | Powers et al. 2013 |
| α-CTX MII | Reduced EtOH operant self-administration | Kuzmin et al. 2009 |
|         | Reduced EtOH intake and preference (two-bottle choice) | Larsson et al. 2004 |
|         | EtOH-associated conditioned reinforcement | Lof et al. 2007 |
|         | Reduced EtOH-stimulation of DA neurons | Liu et al. 2013b |
|         | Reduced EtOH-induced DA release | Larsson et al. 2004 |
| α7 KO  | Reduced EtOH intake (two-bottle choice) | Kamens et al. 2010a |
| MLA    | No effect on EtOH operant self-administration | Kuzmin et al. 2009 |
|         | No effect on EtOH-elicited DA release | 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 α6L9’S mice. As with NAc DA release, we predict that α6L9’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 α6β2*nAChRs also promotes nicotine reward and locomotor activity.

To expand on these initial studies, we seek to determine neuroanatomical loci where α6β2*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 α6β2*nAChR-selective antagonist, α-Ctx MII, prior to nicotine CPP training in a third group of WT and α6L9’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 α6L9’S mice. Consistent with our lab’s previous nicotine self-administration studies (Brunzell et al, 2010), this would suggest that α6β2*nAChRs located on terminals in the NAc shell are involved in nicotine’s rewarding properties.

Specific Aim 2 will assess α6β2*nAChR contributions to anxiety-like behavior. Chapter 3 will describe results from these studies where α6L9’S and α6KO 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 β2*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 α4α6β2*nAChRs by low concentrations of nicotine that typically desensitize other nAChRs (Liu et al, 2012), we hypothesize that activation of α6β2*nAChRs promotes anxiogenesis. Thus, we predict that α6L9’S mice will exhibit elevated anxiety-like behavior, while α6KO mice will show decreases in these measures.

The goal of Specific Aim 3 is to differentiate the contributions of α4α6β2*nAChRs and (non-α4)α6β2*nAChRs to nicotine reward and anxiety-like behavior in mice. Chapter 4 will describe the results from these experiments in which WT, α6L9’S, α4KO, and α6L9’S mice with an α4 null mutation (α6L9’S-α4KO) 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 α4α6β2*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 α6L9’S mice will be at least partially reversed in α6L9’S-α4KO mice, which would demonstrate that α6 assembles with α4 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 α4α6β2*nAChRs are solely responsible for α6β2*nAChR-mediated effects, or if α6L9’S-α4KO mice will show intermediate levels of nicotine CPP and anxiety-like
behavior between WT and α6L9’S mice to suggest that these effects are modulated by both (non-α4)α6β2*nAChRs and α4α6β2*nAChRs.

Finally, Specific Aim 4 focuses on developing a mouse model of oral, operant EtOH self-administration to utilize to test the hypothesis that α6β2*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 α6 subunit (α6HET), and α6KO 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 α6KO mice will fail to show EtOH reinforcement compared to WT mice to implicate α6β2*nAChRs in EtOH’s reinforcing properties. It is less clear whether α6HET mice will show EtOH reinforcement to indicate whether full expression of α6β2*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 α6β2*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). \( \alpha \)-Conotoxin MII (\( \alpha \)-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 α6β2*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 α6β2*nAChR-selective agonists that can demonstrate behaviors and physiological effects for which α6β2*nAChRs are sufficient (Drenan et al, 2008). The development of α6β2*nAChR gain-of-function (α6L9’S) mice has provided a complementary research approach to null mutant strategies to test the sufficiency of α6β2*nAChRs. α6L9’S mice possess a leucine to serine single point mutation in the M2 pore-forming region of the α6 subunit that renders their α6β2*nAChRs hypersensitive to ACh and nicotine (Drenan et al, 2008); nicotine doses that are sub-threshold for activation of native nAChRs can selectively activate α6β2*nAChRs, allowing for assessment of α6β2*nAChR function in isolation of other nAChRs. In vitro slice studies reveal that the α6β2*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 β2*nAChR-associated DA release (King et al, 2004b). These data suggest that activation of α6β2*nAChRs is sufficient for mesolimbic DA neuron activity, however, no studies to date have assessed this in vivo or determined if α6β2*nAChR activity is sufficient to support nicotine reward. Being that cholinergic tone from pedunculopontine 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 α6β2*nAChRs in α6L9’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 α6L9’S mice compared to WT littermates and demonstrate that doses sub-threshold to produce nicotine CPP in WT mice support nicotine reward behavior in α6L9’S mice. To identify neuroanatomical loci within the mesolimbic DA pathway where α6β2*nAChRs support nicotine reward behavior, we expanded on previous findings (Sanjakdar et al, 2015) to demonstrate that local antagonism of α6β2*nAChRs in the NAc shell subdivision blocked nicotine CPP in WT mice. In α6L9’S mice, however, which express global elevations in α6β2*nAChR sensitivity, local antagonism of VTA α6β2*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 α6β2*nAChR gain-of-function mice (α6L9’S) backcrossed > 10 generations on a C57BL/6J background were used in these studies. A single allele for the α6L9’S transgene produces the hypersensitive α6β2*nAChR phenotype (Drenan et al, 2008) so that breedings to WT mice resulted in 50% α6L9’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). Intracerebral infusions of α-Ctx MII were dissolved in SAL or artificial cerebrospinal fluid (aCSF) vehicle (VEH) and administered at 0.5 µl/min for a total volume of 0.25 µl per side.

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

WT (*n*=4) and α6L9’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 α6L9’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 α6β2* nAChR contributions to NAc DA release, a separate cohort of α6L9’S mice were additionally implanted with 26-gauge bilateral guide cannula that enabled infusion of the α6β2* 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)**

α6L9’S mice (n=13-20/dose) were compared to their WT littermates (n=14-19/dose) to determine if α6β2*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 α6β2*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, ±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 α6L9’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 µm 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 α6L9’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 α6L9’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 α6L9’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 α6L9S mice, indicating tissue sampling site sensitivity (Figure 1A, inset). These *in vivo* data provide further evidence that ACh activation of α6β2*nAChRs is sufficient to enhance basal NAc DA tone.

To determine if enhanced function of α6β2*nAChRs also supports nicotine reward behavior, α6L9’S mice were 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 α6L9’S mice spent in the nicotine-paired chamber; α6L9’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 suggest that $\alpha_6\beta_2*nAChR$ stimulation promotes nicotine reward, but it is possible that $\alpha_6L9'S$ 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 mintues 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 ± SEM.
**α6L9’S mice exhibit hyperactive nicotine-stimulated locomotor activity**

In addition to nicotine reward behavior, α6L9’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 α6L9’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 α6L9’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 ± SEM.
Nicotine CPP is blocked by intra-NAc shell α-Ctx MII in WT, but not α6L9’S mice

To investigate the neuroanatomical loci of α6β2*nAChR contributions to nicotine reward behavior, WT and α6L9’S mice received intra-NAc shell infusions of VEH or the α6β2*nAChR-selective antagonist, α-Ctx MII prior to systemic nicotine injection during CPP training. Given that WT and α6L9’S behaved differently in the above CPP task, we analyzed the effect of intra-NAc shell α-Ctx MII on WT and α6L9’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 α6β2*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 α6L9’S mice (Figure 2.3b), suggesting that α6β2*nAChRs elsewhere in the brain were driving nicotine reward behavior of α6L9’S mice.
Figure 2.3 – Nucleus accumbens (NAc) shell α-Conotoxin MII (α-Ctx MII) blocks nicotine CPP in WT, but not α6L9’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 α6L9’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 ± SEM.
Intra-VTA α-Ctx MII blocks enhanced nicotine reward behavior and attenuates elevated basal NAc DA release in α6L9’S mice

To determine if VTA α6β2*nAChRs promote enhanced nicotine reward, α6L9’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 α6L9’S mice ($t_{14}=3.228, p=0.006$; Figure 2.4a), suggesting that α6β2*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 α6β2*nAChRs also modulate enhanced α6L9’S basal NAc DA release, α6L9’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 α6β2*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 α6L9’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 α6L9’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 ± SEM.
DISCUSSION

Complementary to null mutant strategies, the development of α6β2*nAChR gain-of-function (α6L9’S) mice (Drenan et al., 2008) has provided a means of testing behavior under conditions where α6β2*nAChR function is amplified and isolated. Our in vivo microdialysis studies support α6L9’S in vitro data demonstrating elevated ACh-elicited striatal DA release (Cohen et al., 2012; Wang et al., 2014b). α6L9’S mice showed higher basal NAc DA levels compared to WT littermate controls, suggesting hyper-excitability of α6β2*nAChRs in response to endogenous ACh. It is unlikely that this effect resulted from altered DA transporter (DAT) function or DA turnover, as α6L9’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 α6β2*nAChRs by ACh is sufficient to promote NAc DA release.

α6L9’S mice also showed leftward shifts in the dose response curve for nicotine CPP; α6L9’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 α6β2*nAChRs for nicotine reward (Jackson et al., 2009; Sanjakdar et al., 2015), to suggest that α6β2*nAChR activation is sufficient for this behavior. Similar findings have been reported in α4β2*nAChR gain-of-function (α4L9’A) mice (Tapper et al., 2004), raising the possibility that the high sensitivity α4α6β2*nAChRs regulate nicotine reward (Liu et al., 2012), a hypothesis that warrants further study. Independent groups of α6L9’S mice showed augmented basal NAc DA levels, suggesting that changes in cholinergic signaling at α6β2*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
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 α6L9’S mice exhibit enhanced nicotine reward behavior due to the observed increase in basal DA levels.

α6L9’S mice also showed an enhanced response to nicotine’s locomotor stimulating effects, a behavior dependent on β2*nAChR-regulation of DA activity (King et al., 2004). Consistent with previous findings demonstrating that nicotine strongly activates locomotor activity in α6L9’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 α6β2*nAChRs regulate DA-mediated locomotor activating effects of nicotine. α6L9’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 β2*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 β2*nAChRs to maintain a high probability of action potential-evoked DA release (Zhou et al., 2001). Moreover, α-Ctx MII or α6 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 α6β2*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 α6β2*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 α6β2*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 α6β2*nAChRs regulate nicotine reinforcement in rats (Brunzell et al., 2010).

Intra-NAc shell α-Ctx MII did not block nicotine CPP in α6L9’S mice, suggesting that α6β2*nAChRs at another neuroanatomical locus contributed to elevated nicotine reward behavior. In fact, intra-VTA α-Ctx MII blocked nicotine CPP in α6L9’S mice, suggesting that the hyperactivity of α6L9’S VTA DA neurons may have overshadowed effects of NAc shell α6β2*nAChRs on reward behavior in α6L9’S mice. A role for VTA α6β2*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 α6β2*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 α6β2*nAChRs support DA release. ACh and nicotine fail to increase VTA DA neuron firing in α6 null mutant mice or following intra-VTA α-Ctx MII (Champtiaux 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 α6β2*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 α6L9’S NAc DA levels in awake, behaving mice; these findings suggest that elevations of cholinergic tone at VTA α6β2*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 α6β2*nAChR-driven DA neuron activity in nicotine reward behavior. Overall, our findings demonstrate that mesolimbic α6β2*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 α6β2*nAChRs is sufficient to enhance basal NAc DA tone and nicotine reward. Our findings also showed that NAc α6β2*nAChRs regulate nicotine reward behavior. These findings demonstrate a role for two independent pools of mesolimbic α6β2*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 $\alpha_6\beta_2^*nAChRs$ in the mesolimbic pathway may regulate nicotine reward. a) Nicotine activates $\alpha_6\beta_2^*nAChRs$ on dopamine (DA) terminals in the nucleus accumbens to promote nicotine reward, which may be modulated by $\alpha_6\beta_2^*nAChR$-mediated DA release. b) Nicotine activates $\alpha_6\beta_2^*nAChRs$ on ventral tegmental area neuron soma to drive DA release, which may promote nicotine reward.
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 β2*nAChRs regulate anxiety-like behavior. The β2*nAChR-selective antagonist, dihydro-beta-erythroidine (DHβE) 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 β2*nAChR-selective agonist, 5I-A5830, decrease anxiety-like behavior, while high doses increase anxiety-like behavior (Anderson et al, 2015). DHβE (Grady et al, 2010; Papke et al, 2008) and 5I-A85830 (Mukhin et al, 2000) have similar potencies at α4β2* and α6β2*nAChRs. The purpose of these experiments was to assess how α4β2* and α6β2*nAChRs independently contribute to anxiety-like behavior.

α4β2*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 α4 subunit from ventral tegmental area (VTA) dopamine (DA) neurons (McGranahan et al, 2011), suggesting that VTA α4β2*nAChRs are important for nicotine’s anxiolytic effects. Moreover, the α4β2*nAChR gain-of-function L9’S mice (α4L9’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 α4β2*nAChRs promotes anxiogenic-like behavior. Interestingly, α4 null mutant (α4KO) mice also show increases in basal anxiety-like behavior (Ross et al, 2000). Unlike α4β2*nAChRs, α6β2*nAChRs have a more selective expression pattern in brain, being enriched in catecholaminergic nuclei, as well as in the visual circuitry. However, α6β2*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 α6β2*nAChRs in the VTA and NAc have not been directly
implicated in anxiety-like behavior, these areas where α6β2*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). α6β2*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 α4β2* and α6β2*nAChRs to anxiety-like behavior. First, we used complementary genetic approaches, assessing anxiety-like behavior in mice with a gain-of-function (α6L9’S) or loss-of-function (α6KO) to their α6β2*nAChRs using the EPM, open field, and light-dark assays. α6L9’S mice have a single point mutation in the M2 pore-forming region of the α6 subunit, rendering their α6β2*nAChRs hypersensitive to nicotine and ACh as compared to WT littermates so that subthreshold concentrations of these agonists selectively activate α6β2*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 α4β2*nAChR gain-of-function (α4L9’A) mice with a single point mutation in the M2 pore-forming region of the α4 subunit, rendering their α4β2*nAChRs hypersensitive to nicotine (Tapper et al., 2004), to determine whether activation of α4β2* and α6β2*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 α6L9’S and 38 WT littermates; 15 α6KO mice and 15 WT littermates; 13 α4L9’A and 15 WT littermates. All mice were backcrossed at least 10 generations. A single allele for the α6L9’S transgene produces the hypersensitive α6β2*nAChR phenotype (Drenan et al, 2008) so that breedings to WT mice resulted in 50% α6L9’S and 50% WT offspring. α6KO 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 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. 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 $\alpha_6\beta_2^{*nAChR}$ 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 ($F_{1,27}=7.729,$
$p<0.05$). $\alpha_6L9'S$ 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 - α6L9’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 α6L9’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 α6 null mutant (α6KO) mice \((n=12)\).

Data are represented as means ± 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, α6L9’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 α6L9’S mice. WT mice showed traveled greater distances compared to α6L9’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 α6L9’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 α6L9’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 α6L9’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 α6KO 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 - α6L9’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 α6L9’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 α6 null mutant (α6KO) mice (fluorescent lights: \(n=7\); infrared lights: \(n=8\)). Data are represented as means ± SEM. *\(p<0.05\) vs. WT, **\(p<0.05\) compared to room lights on +.
Light-dark assay

α6L9’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 α6L9’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). α6L9’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 α6KO 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 - α6L9’S 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 α6L9’S mice (n=13). d-f) Time in the light chamber, latency to enter the light chamber, and movement counts in WT (n=8) versus α6 null mutant (α6KO) mice (n=8). Data are represented as means ± 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 open 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 - α4L9’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 α4L9’A mice (n=13). Data are represented as means ± 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 α4L9’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 α4L9’A mice. However, α4L9’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 α4L9’A mice.
Figure 3.5 - α4L9’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 α4L9’A mice (n=13). Data are represented as means ± SEM. **p<0.05 compared to room lights on +.
Figure 3.6 - α4L9’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 α4L9’A mice (n=11). Data are represented as means ± SEM.
DISCUSSION

In these studies, α6L9’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 α6β2*nAChRs is sufficient to promote anxiogenic-like behavior. In contrast, WT and α6KO mice did not differ for measures of anxiety-like behavior, suggesting that α6β2*nAChRs are not necessary for expression of anxiety-like behavior. Given the effects we observed in α6L9’S mice, we subsequently tested anxiety-like behavior in α4L9’A gain-of-function mice to determine whether activation of α4β2* and α6β2*nAChRs may work together or in opposition to regulate anxiety-like behavior. In contrast to α6L9’S mice, α4L9’S A mice showed decreased anxiety-like behavior compared to WT controls in the EPM and open field assays, suggesting that α4β2*nAChRs activation is sufficient to promote anxiolysis rather than anxiogenesis.

In support of the present data in α4L9’A mice suggesting that activation of α4β2*nAChRs promotes anxiolysis, previous findings have demonstrated that genetically inactivating α4β2*nAChRs promotes anxiogenesis (Ross et al, 2000). These present findings are also consistent with studies in transgenic mice with their α4 subunit specifically knocked down in VTA DA neurons suggesting that α4β2*nAChRs in the mesolimbic DA pathway are important for the anxiolytic-like effects of nicotine (McGranahan et al, 2011). More specifically, DA neuron-selective α4 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 α4L9’A mice, α4L9’S gain-of-function mice, whose α4β2*nAChRs are hypersensitive to nicotine like α4L9’A mice, show increases rather than decreases in anxiety-like behavior (Labarca et al, 2001). These differences
between α4L9’A and α4L9’S mice may be due to differences in the M2 pore-forming region
mutations, where α4L9’A mice have a leucine to alanine mutation and the α4L9’S mice have a
leucine to serine mutation. In contrast to our results in α4L9’A mice, selective activation of
α6β2*nAChRs in α6L9’S mice appears to promote, rather than attenuate anxiety-like behavior,
acting in opposition of α4β2*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 α6β2* and α4β2*nAChRs promotes opposite effects on anxiety-like behavior,
it could be that hyperactive cholinergic tone has a greater effect at α6β2*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 α4β2*nAChRs, α6β2*nAChRs are not greatly enriched
in these brain areas, suggesting that other brain regions contribute to α6β2*nAChRs regulation
of anxiety-like behavior. α6β2*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
α4β2*nAChRs has been established for nicotine-associated anxiolysis (McGranahan et al,
2011), but it is not clear if VTA $\alpha_4\alpha_6\beta_2*n$AChRs 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, $\alpha_6\beta_2*n$AChRs 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 $\alpha_6$L9’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 $\alpha_6\beta_2*n$AChR-selective antagonist, $\alpha$-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 $\alpha$-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 $\alpha_6$L9’S mice, the anxiolytic effect of $\alpha$-Ctx MII [H9A;L15A] during spontaneous nicotine withdrawal suggests that activation of $\alpha_6\beta_2*n$AChRs by endogenous ACh during withdrawal may contribute to anxiety experienced by smokers in abstinence.

Taken together, these findings suggest that activation of $\alpha_6\beta_2*n$AChRs is sufficient to promote anxiogenic-like behavior, whereas $\alpha_6\beta_2*n$AChRs 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). $\alpha$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, α4KO mice are not different from WT mice in jugular i.v. nicotine self-administration (Cahir et al, 2011). Like CPP studies, α4-S248F mice with enhanced sensitivity for nicotine (similar to α4L9’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 α4 subunit is selectively deleted in VTA DA neurons (McGranahan et al, 2011). α4KO mice show increases in basal anxiety-like behavior (Ross et al, 2000). Interestingly, α4β2*nAChR gain-of-function (α4L9’S) mice also exhibit an increased basal anxiety phenotype compared to WT mice (Labarca et al, 2001), while α4L9’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 α6β2*nAChRs (see table 4.1 for a summary of previous findings regarding α4β2* and α6β2*nAChR contributions). α6 null mutant mice (α6KO) show rightward shifts in nicotine CPP (Sanjakdar et al, 2015), while α6β2*nAChR gain-of-function (α6L9’S) mice show enhanced nicotine CPP (see Chapter 2). Moreover, α6KO 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 α6β2*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 α6L9’S gain-of-function mutation produces basal anxiogenic effects (see Chapter 3). It is not clear from this body of literature whether α4β2* and α6β2*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

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\( \downarrow \) indicates that the manipulation decreased the behavior, \( \uparrow \) 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); \( \alpha \)-Conotoxin MII (\( \alpha \)-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 $\alpha_4KO$ 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 $\alpha_6L9’S \times \alpha_4KO$ 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 ($\text{(non-}\alpha_4)\alpha_6\beta_2*nAChRs$). 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 $\alpha_6L9’S-\alpha_4KO$ 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 $\alpha_{6\beta2*nAChR}$ 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 corncob 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 ($n=14$), α4KO ($n=11$), α6L9’S ($n=13$), and α6L9’S-α4KO 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 α4KO mice, WT versus α6L9’S mice, and α6L9’S versus α6L9’S-α4KO 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-α4)α6β2* and α4α6β2*nAChR contributions to nicotine reward behavior, WT, α4KO, α6L9’S, and α6L9’S mice with the α4 null mutation (α6L9’S-α4KO) 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 mintues 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 – α6L9’S elevation of nicotine conditioned place preference (CPP) does not require the α4 subunit. a) Nicotine CPP is expressed as change from baseline time spent in the nicotine-paired chamber in WT, α4KO, α6L9’S, and α6L9’S-α4KO mice receiving saline (WT: n=10; α4KO: n=8; α6L9’S: n=11; α6L9’S-α4KO: n=8), 0.03 mg/kg i.p. nicotine (WT: n=11; α4KO: n=8; α6L9’S: n=9; α6L9’S-α4KO: n=7) and 0.1 mg/kg i.p. nicotine (WT: n=11; α4KO: n=10; α6L9’S: n=12; α6L9’S-α4KO: 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), α4KO (n=11), α6L9’S (n=13), and α6L9’S-α4KO 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-α4)α6β2* and α4α6β2*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, α6L9’S-α4KO mice showed a trend for greater distance traveled compared to α6L9’S mice ($t_{21}=-1.986$, $p=0.060$). α6L9’S mice, but not α6L9’S-α4KO 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 α4 subunit assembles with α6 to support nicotine-stimulated locomotor activity. However, locomotor activity was increased in response to 0.1 mg/kg i.p. nicotine in both α6L9’S ($p<0.001$) and α6L9’S-α4KO mice ($p<0.001$), suggesting a role for (non-α4)α6β2*nAChR in locomotor effects of nicotine as well. There was a trend for 0.1 mg/kg i.p. nicotine to increase locomotor in α4KO mice ($p=0.071$), that did not reach statistical significance.
Figure 4.3 – (non-α4)α6β2* and α4α6β2*nAChRs play a role in nicotine-stimulated locomotor activity. a) Distance traveled under dim light conditions in WT, α4KO, α6L9’S, and α6L9’S-α4KO mice at saline (WT: n=13; α4KO: n=10; α6L9’S: n=13; α6L9’S-α4KO: n=10), 0.03 (WT: n=11; α4KO: n=7; α6L9’S: n=11; α6L9’S-α4KO: n=8) and 0.1 mg/kg nicotine (WT: n=8; α4KO: n=7; α6L9’S: n=9; α6L9’S-α4KO: 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, α4KO, α6L9’S, and α6L9’S-α4KO where utilized to assess (non-α4)α6β2*nAChR and α4α6β2*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, α6L9’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 α4 subunit does appear to be necessary to promote α6β2*nAChR regulation of basal anxiety-like phenotype, as α6L9’S-α4KO mice did not differ from α6L9’S mice ($t_{22}=-1.466, p=0.157$). This indicates that stimulation of (non-α4)α6β2*nAChRs promotes basal anxiogenic-like behavior as measured by the open field assay. α4KO mice did not differ from WT mice ($t_{22}=1.201, p=0.243$), demonstrating that α4β2(nonne-α6)*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 $\alpha$4KO mice compared to saline controls, indicating that this anxiogenic effect of nicotine does not require $\alpha$4$\beta$2*nAChRs. In $\alpha$6L9’S and $\alpha$6L9’S- $\alpha$4KO mice, neither 0.03 mg/kg or 0.1 mg/kg i.p. nicotine further altered time spent in the center zone compared to saline controls. ($p$'s $>0.3$), suggesting the selective activation of (non-$\alpha$4)$\alpha$6$\beta$2*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 $\alpha$6L9’S ($p$’s $<0.001$) and $\alpha$6L9’S- $\alpha$4KO ($p$’s $<0.05$), with a similar trend for 0.1 mg/kg i.p. nicotine to increase locomotor activity in $\alpha$4KO mice ($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-α4)α6β2*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, α4KO, α6L9’S, and α6L9’S-α4KO mice in response to saline (WT: n=13; α4KO: n=11; α6L9’S: n=14; α6L9’S-α4KO: n=10), 0.03 (WT: n=11; α4KO: n=7; α6L9’S: n=11; α6L9’S-α4KO: n=8), and 0.1 mg/kg nicotine (WT: n=9; α4KO: n=7; α6L9’S: n=9; α6L9’S-α4KO: n=7). b) Total distance traveled in the open field arena in WT, α4KO, α6L9’S, and α6L9’S-α4KO 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 α6L9’S saline-injected mice to spend less time in the light chamber than WT littermates ($t_{24}=1.352$, $p=0.095$), while α6L9’S-α4KO mice spent significantly more time in the light chamber compared to α6L9’S mice ($t_{22}=-3.312$, $p=0.003$). α4KO 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 α4 assembly with α6 in the α4α6β2*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 α6L9’S mice. There was no such effect in α6L9’S-α4KO ($p’s>0.5$), suggesting that α4 is required for α6L9’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 α6L9’S and α6L9’S-α4KO 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 α6L9’S-α4KO mice suggests that this effect was not simply due to nicotine-associated changes in activity (Figure 4.5b). In addition, α6L9’S-α4KO mice made more entries into the light chamber compared to α6L9’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 – α4α6β2*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, α4KO, α6L9’S, and α6L9’S-α4KO mice in response to saline (WT: n=13; α4KO: n=11; α6L9’S: n=13; α6L9’S-α4KO: n=11), 0.03 (WT: n=11; α4KO: n=8; α6L9’S: n=11; α6L9’S-α4KO: n=8), and 0.1 mg/kg nicotine (WT: n=8; α4KO: n=7; α6L9’S: n=9; α6L9’S-α4KO: n=7). b) Entries into the light chamber of the light-dark box in WT, α4KO, α6L9’S, and α6L9’S-α4KO 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. α6L9’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 $\alpha_6L9'S$-$\alpha_4$KO mice don’t differ from $\alpha_6L9'S$ mice, demonstrating that stimulation of (non-$\alpha_4)\beta\alpha_6^2*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, $\alpha$-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 α6L9’S mice. Given the divergent results regarding the α6β2*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 α6L9’S mice.

α4KO mice showed similar levels of nicotine CPP at 0.03 mg/kg and 0.1 mg/kg i.p. nicotine compared to α6L9’S and α6L9’S-α4KO mice. Statistically speaking however, α4KO mice did not show significant CPP, as α4KO 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 α4KO mice express CPP or not. Further complicating matters, previous evidence is conflicting, with two studies showing that α4KO mice don’t express nicotine CPP (McGranahan et al., 2011; Sanjakdar et al., 2015), and one study showing that α4KO mice express nicotine CPP similar to WT mice (Cahir et al., 2011). If in fact there is a leftward shift in the α4KO mice similar to α6L9’S and α6L9’S-α4KO mice, this would suggest that α4β2*nAChRs act in opposition of other nAChR subtypes to reduce nicotine’s rewarding properties, as genetically reducing α4β2*nAChR function would enhance nicotine reward behavior. Future studies repeating this CPP experiment is necessary to determine whether α4KO 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 β2KO mice (King
et al, 2004b), suggesting that β2*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 α6β2*nAChRs promote nicotine-stimulated locomotor activity, as α6L9’S mice showed an enhanced response to nicotine compared to WT mice. These present findings are consistent with previous studies showing that α4α6β2*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 α6L9’S mice display enhanced basal levels of anxiogenic-like behavior, suggesting that stimulation of α6β2*nAChRs promote anxiogenesis. These effects are likely a result of an enhanced response of α6β2*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 α6L9’S mice (see Chapter 3), we observed that these α6β2*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, α6L9’S-α4KO showed elevated levels of anxiety-like behavior like α6L9’S mice, indicating that endogenous cholinergic activity at (non-α4)α6β2*nAChRs is responsible for the elevated levels of anxiety in α6L9’S mice in this model of anxiety. In contrast to the open field, α6L9’S-α4KO mice exhibited a decrease in anxiety-like behavior compared to α6L9’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 \( \alpha_6 \text{L9}^* \)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 \( \alpha_6 \text{L9}^* \)S mice to decrease this behavior, while ACh may have activated \( \alpha_6 \text{L9}^* \)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 α6L9’S mice, suggesting that stimulation of α6β2*nAChRs by nicotine reduces anxiety-like behavior. This is somewhat consistent with our open field data; that is, while selective stimulation of α6β2*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 α4 subunit was not required for this reversal, the α4 subunit was required to assemble with the α6 subunit in the α4α6β2*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 α4α6β2*nAChRs, while nicotine activates α4α6β2*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 α6L9’S mice to show increases, instead of decreases in anxiety-like behavior in response to nicotine in our studies, given that activation of α6β2*nAChRs promotes basal anxiety-like behavior and that α6β2*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 α6β2*nAChRs in α6L9’S mice, but further studies are needed to characterize α6β2*nAChR desensitization in these gain-of-function mice.

As with basal anxiety-like behavior, where (non-α4)α6β2*nAChR promote anxiogenesis in the open field, but α4α6β2*nAChRs promote trending anxiogenesis in the light-dark assay,
(non-α4)α6β2* and α4α6β2*nAChRs contribute to nicotine-associated anxiety-like behavior uniquely depending on the model of anxiety. Specifically, these studies suggest that selective stimulation of (non-α4)α6β2*nAChRs blocks nicotine-induced anxiogenic-like behavior in the open field test, and while stimulation of α4α6β2*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-α4)α6β2*nAChRs and α4α6β2*nAChRs in behaviors relevant to nicotine addiction, namely reward and anxiety-like behavior. (non-α4)α6β2*nAChRs appear to be sufficient to regulate nicotine’s rewarding properties, while both (non-α4)α6β2*nAChRs and α4α6β2*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


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/dose) or tap water vehicle (n=5–9/ 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, $x_i=$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).

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

<table>
<thead>
<tr>
<th></th>
<th>EtOH in SAC vehicle</th>
<th>EtOH in water vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0%</td>
<td>3%</td>
</tr>
<tr>
<td>Reinforcers Earned</td>
<td>219.0 (73.34)</td>
<td>199.4 (32.38)</td>
</tr>
<tr>
<td>Active Lever Presses</td>
<td>991.0 (387.90)</td>
<td>873.0 (172.19)</td>
</tr>
<tr>
<td>Response Accuracy</td>
<td>96.0 (1.63)</td>
<td>94.8 (0.26)</td>
</tr>
<tr>
<td>Correct Entries: Reinforcers Earned</td>
<td>0.93 (0.05)</td>
<td>0.93 (0.02)</td>
</tr>
</tbody>
</table>

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 \((F_{1,32}=22.456, p<0.001)\).
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±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

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>0 % EtOH</th>
<th>3 % EtOH</th>
<th>10 % EtOH</th>
<th>15 % EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total fluid (ml)</td>
<td>Weight (g)</td>
<td>Total fluid (ml)</td>
<td>Weight (g)</td>
</tr>
<tr>
<td>SAC</td>
<td>3.5 (0.59)</td>
<td>28.3 (1.13)</td>
<td>2.9 (0.45)</td>
<td>27.8 (1.59)</td>
</tr>
<tr>
<td>Water</td>
<td>2.5 (0.19)</td>
<td>27.9 (0.69)</td>
<td>2.2 (0.25)</td>
<td>28.0 (0.94)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>EtOH concentration</th>
<th>4 h BEC</th>
<th>6 h BEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 % EtOH in water</td>
<td>0.005 (0.0013)</td>
<td>0.002 (0.0011)</td>
</tr>
<tr>
<td>10 % EtOH in water</td>
<td>0.006 (0.0017)</td>
<td>0.015 (0.0076)</td>
</tr>
<tr>
<td>15 % EtOH in water</td>
<td>0.081 (0.0590)</td>
<td>0.076 (0.4680)</td>
</tr>
<tr>
<td>15 % EtOH in SAC</td>
<td>0.793 (0.2527)</td>
<td>0.999 (0.3012)</td>
</tr>
</tbody>
</table>

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± 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% ($t_5=2.376, p<0.05$) 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.
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; Mc Kee 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 β2 subunit containing nicotinic acetylcholine receptors (β2*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 β2*nAChRs in the behavioral and neurochemical effects of EtOH have generated mixed results. The β2-selective antagonist, DHβE or genetic knockdown of the β2 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 β2*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 β2*nAChR subtype selectivity.

β2 primarily assembles with the α4 and α6 subunits to make functional α4β2*, α6β2* and α4α6β2*nAChRs. α6β2*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 α6-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 α3β2*nAChRs in the VTA, as α-Ctx MII binds to these nAChRs as well. In contrast, α6 null mutant mice (α6KO) show no
differences compared to WT mice for measures of EtOH intake and preference (Guildford et al, 2016; Kamens et al, 2012). But, α6KO 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, α6β2*nAChR gain-of-function (α6L9’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 α6 subunit affects EtOH’s reinforcing effects. Thus, these studies sought to further characterize the role of α6β2*nAChRs in EtOH reinforcement using a mouse model of oral, operant EtOH self-administration in WT, α6HET, and α6KO mice.

MATERIALS AND METHODS

Subjects

Adult male wild type (WT) (n=11), α6 heterozygous (α6HET) (n=13), and α6 subunit null mutant mice (α6KO) (n=12) were used in these studies. α6KO mice were backcrossed to C57BL/6J WT mice for at least 10 generations. WT, α6HET, and α6KO mice were produced by heterozygous breeder pairs. Mice were housed in a temperature- and humidity-controlled vivarium in polycarbonate cages with Teklad corncob 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: active/total(active+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$6KO mice ($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$6HET mice maintained on 15% EtOH did not show significant elevations of active lever presses by session 7 compared to vehicle controls ($p=0.472$), demonstrating that $\alpha$6HET mice did not find EtOH reinforcing. WT mice reinforced with 15% EtOH also showed a significant elevation in responding across sessions ($t_{4}=-7.615, p=0.002$) that was not observed in $\alpha$6HET or $\alpha$6KO mice maintained on 15% EtOH ($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$6KO mice ($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$6HET mice maintained on 15% EtOH did not differ from water control mice, indicating that $\alpha$6HET mice did not find EtOH reinforcing ($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 –α6β2*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), α6HET (0%: n=6; 15%: n=7), and α6KO 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 ± 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 –α6β2*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), α6HET (n=5), and α6KO 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 ± 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 –α6β2* nAChR expression does not appear to be involved in the ataxic or locomotor effects of ethanol (EtOH). a) Latency to fall off the rotorod for WT (n=11), α6HET (n=13), and α6KO mice (n=12) in response to 2 g/kg i.p. EtOH. b) Distance traveled in WT (saline: n=5; EtOH: n=6), α6HET (saline: n=6; EtOH: n=7), and α6KO mice (saline: n=6; EtOH: n=6) in response to saline or 2 g/kg i.p. EtOH. Data are expressed as mean ± SEM.
DISCUSSION

The present study investigated the contribution of α6β2*nAChRs to EtOH reinforcement using genetic reduction (α6HET) or deletion (α6KO) of α6β2*nAChR expression in mice. A number of studies have used the α6KO mice to elucidate the role of α6β2*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 α6HET and α6KO 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 α6HET mice did not show EtOH reinforcement, as no difference between α6HET mice reinforced with 0% and 15% EtOH was observed for active lever presses and reinforcers earned. These results indicate that α6β2*nAChR expression modulates EtOH reinforcement.

Curiously, we demonstrated that EtOH has similar reinforcing efficacy in α6KO mice compared to WT mice, where WT and α6KO 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 α6KO mice (Guildford et al., 2016; Kamens et al., 2012). However, it was unexpected that reducing α6β2*nAChR expression by approximately half in the α6HET mice would prevent EtOH reinforcement while knocking out α6β2*nAChRs in α6KO mice would have no effect. α6KO 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 α6KO 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 α6KO mice, where another nAChR subtype may be compensating for the absence of α6β2*nAChRs to promote EtOH reinforcement in α6KO 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 [³H]epibatidine binding, which has affinity for α7, α3β2*, α3β4*, α4β2*, α6β2*nAChRs (Badio and Daly, 1994; Champtiaux et al, 2002), is unchanged in α6KO 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 α6KO mice compared to α6HET 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 α6KO mice, previous studies using intra-VTA infusion of the α6-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 α6β2*nAChRs in one brain region, while α6KO mice lack α6β2*nAChRs throughout the whole brain. This could suggest that α6β2*nAChRs is 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 α6 knockout. If results from i.c.v. α-Ctx MII studies remain different from studies in α6KO 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 α3β2*nAChRs, which are expressed in the VTA as well; it is therefore possible that effects of α-Ctx MII may be mediated by α3β2*nAChRs. Conotoxin derivatives more selective for α6β2*nAChRs, such as α-Ctx PIA (Dowell et al., 2003) could test whether α-Ctx MII effects are mediated by α6β2* versus α3β2*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 α6KO mice did not differ from WT littermates in measures of EtOH self-administration, neither α6HET nor α6KO 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 α6β2*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 α6β2*nAChRs demonstrate the necessity of this nAChR subtype. However, little progress has been made in the development of α6β2*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 α6L9’S gain-of-function mice as a way to selectively activate α6β2*nAChRs to determine if activation of α6β2*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 α6L9’S mice exhibited elevated levels of basal NAc DA compared to WT mice, suggesting that activation of α6β2*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 α6β2*nAChR hyperexcitability in response to endogenous ACh. In support of this hypothesis, it appears that endogenous activity at α6β2*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 α6L9’S mice. This endogenous cholinergic tone is presumably from inputs projecting from either the pedunculopontine 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 pedunculopontine and laterodorsal tegmental nuclei, which we predict would increase NAc DA levels. Based on our data, it is expected that antagonizing α6β2*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 α6β2*nAChRs. But, this antagonist also blocks the activity of α3β2*nAChRs (Cartier et al, 1996), which are also expressed in the VTA, albeit at lower levels compared to α6β2*nAChRs. There are conotoxin derivatives that are more selective for α6β2*nAChRs compared to α3β2*nAChRs, like α-Ctx PIA (Dowell et al, 2003), that we could use in future studies to more selectively antagonize α6β2*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 β2-selective antagonist, DHβE (Cachope et al, 2012; Threlfell et al, 2012). This indicates that ACh released from these interneurons acts at β2*nAChRs to promote DA release independent of DA neuron firing. DHβE binds to both α4β2* and α6β2*nAChRs with similar potency (Grady et al, 2010; Papke et al, 2008). Together with these previous data, our present microdialysis results in α6L9’S mice suggest that activity of NAc cholinergic interneurons could stimulate α6β2*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 $\alpha$-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 $\alpha$-Ctx MII binding in the NAc (Whiteaker et al, 2002), while $\alpha$-Ctx MII binding completely disappears in this brain area of $\alpha_6KO$ 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 $\alpha_6L9'S$ basal NAc DA levels, we expected that $\alpha_6L9'S$ mice would show an enhanced response to nicotine as has been previously reported in $\alpha_6L9'S$ 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 $\alpha_6L9'S$ 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 α6L9’S mice have the potential to detect subtle nicotine-associated changes in DA release in real time to further evaluate if stimulation of α6β2*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 α6L9’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 pimozide (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 α6L9’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 α6β2*nAChRs promotes the locomotor activating effects of nicotine. This data provides a clue that α6L9’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 α6L9’S nicotine CPP, suggesting that stimulation of α6β2*nAChRs is sufficient for nicotine’s rewarding properties. As α6L9’S mice showed both enhanced basal NAc DA levels and left-shifted nicotine CPP, it would appear that augmented reward behavior in α6L9’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 α6L9’S midbrain slices (Drenan et al, 2008; Engle et al, 2013; Powers et al, 2013).

Expanding on previous findings demonstrating the necessity of α6β2*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 α6β2*nAChRs and their individual contributions to nicotine reward have yet to be elucidated. Therefore, subsequent studies in this dissertation used an α6L9’S x α4KO cross to differentiate the contributions of α4α6β2*nAChRs and (non-α4)α6β2*nAChRs to nicotine reward behavior. As with α6L9’S mice, nicotine doses sub-threshold for nicotine CPP in WT mice were capable of supporting nicotine CPP in L9’S-α4KO mice, suggesting that α4 is not required to assemble with α6 to promote nicotine CPP, but rather that (non-α4)α6β2*nAChRs promote this abuse-related behavior. This result was not expected, as both α6L9’S and α4L9’A (Tapper et al, 2004) gain-of-function mice show enhanced nicotine CPP. Moreover, α4α6β2*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 α4α6β2*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 α6L9’S DA neuron firing and DA release requires the α4 subunit (Drenan et al, 2010; Engle et al, 2013). DA-associated behavior also requires the α4 subunit, as enhanced nicotine-stimulated locomotor activity observed in α6L9’S mice was reversed when the α4 subunit was deleted (Drenan et al, 2010). Future microdialysis studies comparing α6L9’S mice to α6L9’S-α4KO mice are planned to test which α6β2*nAChR subclass (α4α6β2* or (non-α4)α6β2*nAChRs) supports in vivo elevations of NAc DA release observed in α6L9’S mice, as measurements of DA in α6L9’S-α4KO mice have only been performed in vitro. If these future studies corroborate the in vitro data to demonstrate that α4α6β2*nAChRs are required for in vivo NAc DA release, this would suggest that different α6β2*nAChR subtypes are involved in DA release and nicotine reward, as our CPP studies implicate (non-α4)α6β2*nAChRs in nicotine reward behavior. It would thus appear that nicotine reward behavior may not be regulated by α6β2*nAChR modulation of NAc DA release; future studies could investigate other mechanisms by which α6β2*nAChRs control nicotine reward behavior. The noradrenergic system has also been implicated in drug addiction (Ouzir and Errami, 2016; Weinshenker and Schroeder, 2007). Being that α6β2*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 α6β2*nAChRs in the locus coeruleus are involved in nicotine reward using local infusions of α6β2*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 α6β2*nAChRs modulate nicotine reward behavior. α6β2*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 α6β2*nAChRs specifically in the NAc blocks nicotine CPP in mice (Sanjakdar et al, 2015). Our studies expanded on this to show that activation of α6β2*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 α6β2*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 α6L9’S mice. Initially, we thought that a higher dose of α-Ctx MII was needed to decrease α6L9’S α6β2*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 α6L9’S mice. Based on this, it does not appear that the α-Ctx MII dose response curve is shifted in α6L9’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 α6L9’S mice.

Another explanation for the lack of effect of α-Ctx MII on nicotine CPP in α6L9’S mice lies in the fact that α6L9’S mice are heterozygous, so that only some of the α6β2*nAChRs are hypersensitive. If the hypersensitive α6β2*nAChRs are not antagonized by α-Ctx MII the same as WT α6β2*nAChRs (i.e. different affinity or potency of α-Ctx MII in α6L9’S mice), the hypersensitive α6β2*nAChRs may be masking the effect of α-Ctx MII at the normal
α6β2*nAChRs. Previous studies have shown that α-Ctx MII blocks enhanced DA neuron firing and DA release observed in α6L9’S mice (Drenan et al, 2008), but no known studies have been done to assess binding affinity and potency of α-Ctx MII in α6L9’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 α6L9’S mice, we hypothesized that hyperactivity of α6β2*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 α6β2*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 α6L9’S mice, indicating that α6β2*nAChR regulation of DA neuron firing, ultimately resulting in DA release from terminals in the NAc, may drive the enhanced reward phenotype seen in α6L9’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 α6β2*nAChR antagonist, as discussed previously, to rule out effects of α3β2*nAChR in supporting nicotine CPP.

Together with previous findings, these preclinical data demonstrate that reduction of α6β2*nAChR function may be an effective therapeutic strategy for treatment of nicotine addiction, as activating α6β2*nAChRs promotes behaviors and associated neurochemistry related to nicotine addiction, while inhibiting α6β2*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 α7 nAChRs and a partial agonist at β2*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 \( \alpha 6\beta 2^*\text{nAChRs} \) may prove to be more effective for smoking cessation. While \( \alpha \)-Ctx MII and its derivatives don’t cross the blood brain barrier, a potent and selective \( \alpha 6\beta 2^*\text{nAChR} \) 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 \( \alpha 6\beta 2^*\text{nAChRs} \) 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 \( \alpha 6\beta 2^*\text{nAChRs} \) 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 \( \alpha 6\beta 2^*\text{nAChRs} \) 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 \( \alpha 6\beta 2^*\text{nAChRs} \) 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
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 α6L9’S mice, our anxiety studies assessed the contribution of α6β2*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 β2*nAChR supports relief from basal anxiety-like phenotypes (Anderson et al, 2012). Our studies show that activation of α6β2*nAChRs is sufficient for expression of basal anxiety-like behavior, while stimulation of α4β2*nAChRs reduces anxiety-like behavior, possibly working in opposition to α6β2*nAChRs.

Subsequent studies were performed to differentiate the contributions of (non-α4)α6β2* and α4α6β2*nAChRs to basal anxiety-like behavior. We originally hypothesized that activation of α4α6β2*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 α6L9’S and α4L9’A gain-of-function mutations produced opposite effects on anxiety-like behavior in our studies, demonstrating that α6β2* and α4β2*nAChRs appear to act in opposition in the EPM and open field assays, however, we expected that (non-α4)α6β2*nAChRs would be responsible for α6β2*nAChR regulation of basal anxiogenic-like behavior. Consistent with this hypothesis, α6L9’S-α4KO mice did not differ from α6L9’S mice,
suggesting that the α4 subunit is not required for assembly in α6β2*nAChRs to support anxiogenic-like behavior in the open field assay. However, our light-dark results were inconsistent, as activation of α4α6β2*nAChRs appeared to promote trending increases in anxiety-like behavior in the light-dark test. As discussed, it was unexpected that α4α6β2*nAChRs would promote anxiogenic-like behavior considering the opposite effects of the α6L9’S and α4L9’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 α6L9’S mice showed increases in anxiety-like behavior, but there was no change in α4L9’A mice. This could explain why α4 and α6 appear to work together in the α4α6β2*nAChR conformation to regulate anxiogenesis in the light-dark test, but not the open field or EPM assays.

The effect of α6β2*nAChR hyperactivity may be stronger than that of α4β2*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 α6β2*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. α6β2*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 $\alpha$-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 $\alpha_6KO$ mice. Given the results from studies assessing nicotine withdrawal-associated anxiety-like behavior in response to $\alpha$-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 $\alpha_6KO$ mice. We would expect to see a decrease in anxiety-like behavior in $\alpha_4KO$ 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 $\alpha_4KO$ mice without an effect in $\alpha_6KO$ mice would suggest that DH$\beta$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 α6β2*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-α4)α6β2* and α4α6β2*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 α6β2*nAChRs are involved in anxiety, which is a complex, multidimensional behavior.

These studies investigating the role of α6β2*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 α6β2*nAChR function has no effect on basal anxiety-like behavior, so inhibition of α6β2*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 α6 null mutation as discussed; future studies could provide a stressor prior to testing anxiety-like behavior in α6KO mice. We can also give brain infusions of selective α6β2*nAChR antagonists to complement the genetic studies and determine neuroanatomical loci where α6β2*nAChRs regulate anxiety-like behavior. Candidate brain regions for α6β2*nAChR
regulated anxiety-like behavior include the mesolimbic pathway and the locus coeruleus, two areas involved in stress- and anxiety-related phenotypes where $\alpha 6\beta 2^*n$AChRs are selectively expressed. It is important to further characterize the effect of $\alpha 6\beta 2^*n$AChR inhibition in order to determine whether reducing $\alpha 6\beta 2^*n$AChR function may be an effective therapeutic strategy for smoking cessation, as it would be a concern if inhibiting $\alpha 6\beta 2^*n$AChRs increases anxiety-like behavior. It is promising that one study reports that i.c.v. infusion of $\alpha$-Ctx MII reduced withdrawal-associated anxiety-like behavior (Jackson et al, 2009).

Finally, these studies sought to determine whether $\alpha 6\beta 2^*n$AChRs 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 \( \alpha_6 L9^* \) 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 \( \alpha \)-Ctx MII reduces EtOH-induced activation of the mesolimbic DA pathway (Larsson et al, 2004; Liu et al, 2013b) and \( \alpha_6 \)KO 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 \( \alpha_6 L9^* \) mice to determine if activation of \( \alpha_6 \beta_2^* \) nAChRs supports these neurochemical effects related to EtOH abuse. If these studies in \( \alpha_6 L9^* \) 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 \( \alpha_6 L9^* \)-\( \alpha_4 \)KO 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 \( \alpha_6 L9^* \), \( \alpha_6 \)HET and \( \alpha_6 \)KO 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.
Literature Cited


Berry JN, Engle SE, McIntosh JM, Drenan RM (2015). alpha6-Containing nicotinic acetylcholine receptors in midbrain dopamine neurons are poised to govern dopamine-mediated behaviors and synaptic plasticity. Neuroscience 304: 161-175.


Brunzell DH, McIntosh JM (2012). Alpha7 nicotinic acetylcholine receptors modulate motivation to self-administer nicotine: implications for smoking and schizophrenia. *Neuropsychopharmacology* 37(5): 1134-1143.


Ebbert JO (2013). Varenicline and combination nicotine replacement therapy are the most effective pharmacotherapies for treating tobacco use. Evid Based Med 18(6): 212-213.


Engle SE, Shih PY, McIntosh JM, Drenan RM (2013). alpha4alpha6beta2* nicotinic acetylcholine receptor activation on ventral tegmental area dopamine neurons is sufficient to stimulate a depolarizing conductance and enhance surface AMPA receptor function. Mol Pharmacol 84(3): 393-406.


Kahler CW, Spillane NS, Metrik J (2010). Alcohol use and initial smoking lapses among heavy drinkers in smoking cessation treatment. *Nicotine Tob Res* **12**(7): 781-785.


NIDA (2017). Cigarettes and Other Tobacco Products.


Pandya AA, Yakel JL (2013). Activation of the alpha7 nicotinic ACh receptor induces anxiogenic effects in rats which is blocked by a 5-HT(1)a receptor antagonist. *Neuropharmacology* **70**: 35-42.


Nicotine had no significant effect on nucleus accumbens (NAc) dopamine (DA) levels in wild type (WT) or α6β2* nAChR gain-of-function (α6L9’S) mice. WT and α6L9’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 α6L9’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 α6L9’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-poplar 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**


**CONFERENCE POSTER ABSTRACTS**

**Stafford AM**, Drenan RM, Banks ML, Brunzell DH. alpha6beta2 subunit containing nicotinic 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** 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
• Sirrine 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