BETA 2 NICOTINIC ACETYLCHOLINE RECEPTOR CONTRIBUTIONS TO ANXIETY-LIKE BEHAVIOR

Shawn Anderson
Virginia Commonwealth University

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BETA 2 NICOTINIC ACETYLCHOLINE RECEPTOR CONTRIBUTIONS TO ANXIETY-LIKE BEHAVIOR

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

by

Rev. Shawn Matthew Anderson, O.S.B.
Bachelor of Science in Pharmacy, Duquesne University, 1992
Master of Divinity, Saint Vincent Seminary, 2007

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

Virginia Commonwealth University
Richmond, Virginia
November 21, 2013
Acknowledgements

Thomas Merton wrote a popular book titled, “No Man is an Island,” to indicate how necessary others are for us on this journey. Great things are never accomplished by one acting independently, but in constant communication with others. This work is no exception. I must begin by thanking God, who has supported me in countless ways, primarily in and through the people who have provided me with their guidance and assistance over these past 5-plus years. First among these are my family members, namely Mom, Mary, Derrek and my two wonderful nephews, Alex and Nicholas. I look forward to being able to spend more time with all of you in the coming years, now that I may have more liberty for visits! Maggie and Tom, two of my long-time friends over the years, thank you for the numerous phone conversations that you endured, patiently allowing me to vent my frustrations and anxieties concerning graduate school. Of course, no conversation would be complete without the literal army of monks who provided prayerful and personal support to me during these years of my exile from Saint Vincent.

Having mentioned those that I left behind, I must now address those I met during the days of my sojourn in Richmond. Kath, Litt, Fr. James, Nolte, Jeannette and Mario, just to name a few, thank you for taking this Yankee under your wings and providing such a warm welcome while I was here. I also need to thank the members of my advisory committee for providing fresh insights into my project over the years. Be assured that the experiments contained herein are truly a collaborative effort that emerged in no small part from my meetings with you. Jennifer, other than Dr. B, you’ve been the most constant member of the lab. It isn’t really possible to adequately thank you for the support and sense of community that you bring to the lab. Not to mention the mice!! Alex and Meredith, both of you helped to create an enjoyable work environment that is tremendously important, considering that we spend so much time together. Thank you. Dr. B, what can I say? Forming a future scientist must be a daunting task, especially when it is a student that had next to no lab experience and had not seriously studied science for over a decade. Thank you for taking a chance on the monk, and for patiently providing the necessary environment in which enthusiasm, dedication and scholarly pursuits are possible. The countless hours to hone a 10 minute talk, constantly challenging me to learn the scientific way, not the philosophical way, to express my data, all have given me tools that I will be able to pass on to my own students, very soon in fact. You also helped me to critically approach journal articles and instilled in me the importance of remaining current in the literature. I hope that I will be able to hand down some of what I have gleaned during my years in the Brunzell Lab. May God bless you.
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Clarification of Contributions

Other than the assistance noted below, all of the experiments performed in this dissertation, other than what is cited, is my own work.

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Dr. Petra Schulze of the Max Planck Institute of Molecular Biology in Vienna, Austria, generously performed the immunoprecipitation assays to quantify the expression of α4 nAChR subunit protein in the lateral septum, amygdala, hippocampus and anterior cingulate cortex of WT-Adult, WT-Aged, α4HET-Adult, α4HET-Aged, α4KO-Adult and α4KO-Aged mice.
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α3β4*nAChRs ...................... α3, and β4 containing nAChRs
α4α6β2*nAChRs .................... α4, α6 and β2 containing nAChRs
α4β2*nAChRs ...................... α4 and β2 containing nAChRs
α4HET ................................. α4 heterozygous mouse
α4KO .................................. α4 null mutant mouse
α6β2*nAChRs ...................... α6 and β2 containing nAChRs
α6KO .................................. α6 null mutant mouse
α7nAChRs .............................. α7 containing nAChRs
ACh ..................................... Acetylcholine
AChE ................................. Acetylcholinesterase
AD ..................................... Alzheimer’s Disease
α4HET-Adult ......................... α4HET mice 6-8 months of age
α4HET-Aged ......................... α4HET mice 22-24 months of age
α4KO-Adult .......................... α4KO mice 6-8 months of age
α4KO-Aged .......................... α4KO mice 22-24 months of age
AS ..................................... Anxiety sensitivity
ASI ................................. Anxiety Sensitivity Index
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<tr>
<td>β4*nAChRs</td>
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<tr>
<td>BLA</td>
<td>Basolateral amygdala</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Calcium modulated kinase II</td>
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<tr>
<td>CER</td>
<td>Conditioned emotional response</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>CPP</td>
<td>Conditioned place preference</td>
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<tr>
<td>CREB</td>
<td>Cyclic amp response element-binding protein</td>
</tr>
<tr>
<td>CRF</td>
<td>Corticotropin releasing factor</td>
</tr>
<tr>
<td>CRFR2</td>
<td>Corticotropin releasing factor subtype 2 receptors</td>
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<td>CS</td>
<td>Conditioned stimulus</td>
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<td>DHβE</td>
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<td>ERK</td>
<td>Extracellular regulated kinase</td>
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<td>FR</td>
<td>Fixed ratio</td>
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<td>HPA</td>
<td>Hypothalamic pituitary</td>
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<td>IP</td>
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<td>L9A</td>
<td>Mouse with single point L9'A mutation in α4 subunit</td>
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<td>LC</td>
<td>Locus coeruleus</td>
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LTP .......................... Long term potentiation
mA .......................... Milliamp
NAcc .......................... Nucleus accumbens
nAChR-KO .................... Nicotinic acetylcholine receptor knock-out mouse
nAChRs .......................... Nicotinic acetylcholine receptors
NE .............................. Norepinephrine
NIC ............................. Nicotine
0.01 NIC/0.5 NIC ............ Administration of a pre-injection of 0.01 mg/kg nicotine followed by an injection of 0.5 mg/kg nicotine
0.05 NIC/0.05 NIC ............ Administration of a pre-injection of 0.05 mg/kg nicotine followed by an injection of 0.05 mg/kg nicotine
0.05 NIC/0.5 NIC ............ Administration of a pre-injection of 0.05 mg/kg nicotine followed by an injection of 0.5 mg/kg nicotine
NO INJ .......................... Pre-injection not given
NO INJ/VEH .......................... Pre-injection not given before administration of saline vehicle
NON-CS .......................... Total lever presses in absence of CS
pCREB .......................... Phosphorylated CREB
pERK .......................... Phosphorylated ERK
PFC .............................. Prefrontal cortex
PKA .............................. Protein kinase A
PKC .............................. Protein kinase C
PRE DHβE .......................... Pre-injection of DHβE
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<tr>
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<td>PRE NIC</td>
<td>Pre-injection of nicotine</td>
</tr>
<tr>
<td>Pre-CS</td>
<td>60 second period immediately preceding presentation of CS</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus of the hypothalamus</td>
</tr>
<tr>
<td>Pyk-2</td>
<td>Proline-rich tyrosine kinase</td>
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<tr>
<td>SR</td>
<td>Suppression ratio</td>
</tr>
<tr>
<td>STEP</td>
<td>Striatal enriched protein phosphatase</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
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<td>TBS-T</td>
<td>Tris-buffered saline with Tween</td>
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<td>US</td>
<td>Unconditioned stimulus</td>
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<td>VCU</td>
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<td>Administration of a pre-injection of saline followed by an injection of 0.5 mg/kg nicotine</td>
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<td>VI</td>
<td>Variable interval schedule of reinforcement</td>
</tr>
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<td>VI-30s</td>
<td>Variable interval 30 sec schedule of reinforcement</td>
</tr>
<tr>
<td>VR</td>
<td>Variable ratio schedule of reinforcement</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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<td>WT-Adult</td>
<td>Wild type mice 6-8 months of age</td>
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WT-Aged ……………  Wild type mice 22-24 months of age
Abstract

BETA 2 NICOTINIC ACETYLCHOLINE RECEPTOR CONTRIBUTIONS TO ANXIETY-LIKE BEHAVIOR


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

Virginia Commonwealth University, 2013.

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

Nicotine is a major psychoactive ingredient in tobacco that is thought to promote smoking behavior via nicotinic acetylcholine receptors (nAChRs) in the brain. Given reports that people smoke to relieve anxiety and that anxiety precipitates relapse, the overarching goal of this dissertation research is to assess β2 subunit containing nAChR (β2*nAChR) contributions to anxiety-like behavior. Nicotine’s activity at β2*nAChRs is concentration-dependent, with high concentrations facilitating activation followed by rapid desensitization and low concentrations preferentially desensitizing β2*nAChRs; hence, activation or inhibition of β2*nAChRs may support smoking behavior. Rodent studies reveal that nicotine affects anxiety-like behavior dose-dependently: low doses promote anxiolysis- and high doses support anxiogenic-like behavior. These pharmacological and genetic studies in mice test the hypothesis that nicotine administration promotes anxiolysis via inactivation of β2*nAChRs and begin to identify which subunits, namely α4 and α6, work in concert with β2 to affect anxiety-like behavior. Low dose
nicotine and inhibition of β2*nAChRs supported anxiolysis-like behavior in a number of tasks with predictive validity for anxiolysis efficacy. These studies further suggest that activation of α6β2*nAChRs is sufficient to produce anxiogenic-like behavior and that inhibition of α4β2*nAChRs supports anxiolysis-like behavior. A secondary goal of these studies is to assess if β2*nAChRs affect anxiety-like behavior during aging. Dysregulation of cholinergic tone can increase anxiety in the elderly, but little is known regarding β2*nAChR contributions to anxiety in this population or where in the brain this may take place. These studies show that α4β2*nAChR expression differentially affects anxiety-like behavior in adult and aged mice. With a focus on the lateral septum, a GABA-ergic limbic nucleus thought to regulate anxiety-like responses to external stimuli, a third goal of these studies is to elucidate the neuroanatomical and intracellular underpinnings of anxiety-like behavior that are affected by β2*nAChR inhibition and expression. Previous studies demonstrate that exposure to stressors reduces phosphorylation of extracellular regulated kinase (ERK) in the lateral septum. In these studies, levels of pERK in the lateral septum were inversely associated with α4β2*nAChR expression as well as anxiogenic-like behavior. In sum, these preclinical studies suggest that inhibition α4β2*nAChRs may support cessation in those who smoke to relieve anxiety.
Chapter 1 – Introduction

Tobacco use remains the leading cause of preventable death in the US and is a growing global health concern (WHO, 2008). Some 443,000 deaths were attributed to tobacco use and another 49,000 were linked to secondhand smoke exposure in the United States in 2011 (CDC, 2011; NIH, 2011). Studies in human smokers reveal that multiple factors contribute to tobacco use; as well as the pleasure received from smoking, many report that they use tobacco to relieve anxiety (Berlin et al., 2003; Fidler & West, 2009; Livson & Leino, 1988; Perkins & Grobe, 1992; Shiffman et al., 1997; Shiffman & Waters, 2004; Skara, Sussman, & Dent, 2001). Studies further show that perceived stress contributes both to failed smoking cessation attempts and escalation of smoking behaviors (Byrne, Byrne, & Reinhart, 1995; Byrne & Mazanov, 2003; Finkelstein, Kubzansky, & Goodman, 2006; McCabe et al., 2004; Shiffman et al., 1997; Siqueira, Diab, Bodian, & Rolnitzky, 2000; Skara et al., 2001; Zvolensky, Marshall, et al., 2009; Zvolensky, Stewart, Vujanovic, Gavric, & Steeves, 2009). The exact mechanism underlying reported reductions in anxiety by smokers is not clearly understood. The preclinical studies in this dissertation aim to elucidate the mechanisms by which nicotine exerts its effects on anxiety-like behavior.

History of Nicotine

Nicotine is the principal alkaloid in tobacco, comprising some 1.5% of the weight and 95% of the cumulative alkaloid content of commercially available cigarette tobacco. The average
tobacco rod contains about 10-14 mg of nicotine, and approximately 1-1.5 mg of nicotine is absorbed during smoking (Benowitz & Jacob, 1984; Kozlowski et al., 1998). The tobacco plant is a member of the nightshade family. Other than its known use as a drug for recreational purposes, nicotine is also an effective insecticide, which is its endogenous role in the tobacco plant. Tobacco was used for centuries by the indigenous peoples of North and South America, chiefly in religious ceremonies and as a treatment for many ailments, including diarrhea, pain, wounds and burns. *Nicotiana tobacum*, the species of tobacco plant most commonly used in commercial tobacco products, is likely of South American origin, while another species, *Nicotiana rusticum*, is native to North America. Jean Nicot de Villemain, a French ambassador to Portugal, grew tobacco and observed the healing properties of extracts of the leaves. His enthusiasm for the healing properties of the plant grew and his sharing of tobacco was so liberal that the plant became known as the ambassador’s plant, or *nicotiane*, by which it is known today.

In 1807 Gaspare Cerioli and in 1809 Louis-Nicolas Vauquelin independently isolated nicotine from the tobacco plant. Later, in 1828, Wilhelm Heinrich Posselt and Karl Ludwig Reimann first isolated pure nicotine from the tobacco plant. Louise Melsens, a Belgian chemist and physicist, described nicotine's empirical formula in 1843, and Adolf Pinner and Richard Wolffenstein, described its structure in 1893. The first synthesis of nicotine occurred in 1904 by A. Pictet and P. Crepieux.

**Nicotinic Acetylcholine Receptors (nAChRs)**

Nicotine binds to pentameric nicotinic acetylcholine receptors (nAChRs), which are members of the Cys-loop superfamily of ligand-gated ion channels (Karlin, 1993; Le Novere & Changeux,
The earliest studies that led to the discovery of nAChRs were performed on skeletal muscle by John Langley. In skeletal muscle, $\alpha_1$, $\beta_1$, $\delta$ and $\gamma$ or $\varepsilon$ subunits assemble to form a functional nAChR. He observed that nicotine and curare had opposing effects on skeletal muscle activity and that they continued to exert their effects in the absence of innervation (Langley, 1905). From this he theorized that nicotine and curare acted via a “receptive substance” other than the muscle that was able to affect the contractile activity of the muscle (Langley, 1907) and change its properties. Langley also discovered that the application of nicotine resulted in paralysis of peripheral ganglia (Langley & Dickinson, 1890). Although they did not realize it at the time, nicotine was exerting these effects via desensitization or inactivation of the nAChRs.

The activity of nicotine at nAChRs is dose-dependent; micromolar concentrations of nicotine activate nAChRs (Fenster, Rains, Noerager, Quick, & Lester, 1997; Grady, Wageman, Patzlaff, & Marks, 2012; Kuryatov, Berrettini, & Lindstrom, 2011; R. A. Lester & Dani, 1995; Lu, Marks, & Collins, 1999; Mansvelder, Keath, & McGehee, 2002; Pidoplichko, DeBiasi, Williams, & Dani, 1997), whereas nM concentrations of nicotine support preferential desensitization of most heteromeric nAChRs (Fenster et al., 1997; Grady et al., 2012; Kuryatov et al., 2011; Lu et al., 1999; Mansvelder et al., 2002; Pidoplichko et al., 1997). The binding of two agonist molecules to nAChRs generally promotes a conformational change in the nAChR protein, facilitating opening of the central pore, which allows the passage of the cations sodium and calcium into the cell. Following activation, nAChRs become desensitized, wherein they are resistant to subsequent activation by either endogenous or exogenous agonists (Fenster et al., 1997; R. A. Lester & Dani, 1995; Mansvelder et al., 2002; Pidoplichko et al., 1997). Consequently, nAChRs can exist in at least 3 distinct stoichiometries: Unbound and Resting, Bound and Activated (with
ligand bound to nAChR) and Bound and Desensitized (with ligand still bound) (Katz & Thesleff, 1957). nAChRs in the Bound and Desensitized state are in the thermodynamically preferred state (Changeux, Devillers-Thiery, & Chemouilli, 1984), with α4β2*nAChRs requiring upwards of 90 min washout time before returning to the Unbound and Resting state (Fenster et al., 1997).

In contrast to the effects of micromolar concentrations of nicotine, which activate and subsequently desensitize nAChRs, nanomolar concentrations of nicotine at least 62 fold lower than the EC$_{50}$ value of nicotine preferentially desensitize nAChRs without activation (Fenster et al., 1997; Grady et al., 2012; Kuryatov et al., 2011; Lu et al., 1999). However, since nicotine is an agonist, nAChRs are not statically in the Bound and Desensitized state, but are said to be in an intermediate “smoldering” state. Higher nicotine concentrations favor transition of nAChRs from Bound and Desensitized to the Bound and Activated state, whereas lower agonist concentrations are likely to result in nAChRs remaining Bound and Desensitized until nicotine is cleared from the receptor site (Kuryatov et al., 2011). Peak concentrations of nicotine in the brains of smokers reach micomolar levels, while they are likely maintained between 200-500 nM throughout the day (Rose et al., 2010). Consequently, fluctuating concentrations of nicotine in the brains of smokers have the potential to activate or desensitize nAChRs. Although the phenomenon of nAChR receptor desensitization was first observed following prolonged exposure of the endogenous ligand acetylcholine (ACh) at the neuromuscular junction, the activity of acetylcholinesterase (AChE), the degradative enzyme of ACh, normally prevents ACh-mediated nAChR desensitization (G. L. Brown, Dale, & Feldberg, 1936; Katz & Thesleff, 1957; Thesleff, 1955).
In the central nervous system, the affinity and activity of both ACh and exogenous agonists such as nicotine upon nAChRs further depend upon the subunit composition of the particular nAChR subclass that is the target of the ligand’s activity. α7nAChRs, the only homomeric nAChRs known to exist in the mammalian brain, have a high binding affinity for the antagonist α-bungarotoxin (Clarke, Schwartz, Paul, Pert, & Pert, 1985; Marks & Collins, 1982). Heteromeric nAChRs are comprised of a combination of alpha (α2-α6) and beta (β2-β4) subunits. β2 subunit-containing nAChRs (β2*nAChRs; * denotes other subunits may co-assemble to form a functional receptor) have the highest binding affinity and lowest EC₅₀ for ACh and nicotine, at least 15 fold lower than other subclasses of nAChRs (Grady et al., 2010; Pauly, Marks, Gross, & Collins, 1991; Whiting & Lindstrom, 1988; Y. Xiao & Kellar, 2004), and are widely distributed throughout the brain (Clarke et al., 1985; Court & Clementi, 1995; Fujita et al., 2000; Grady et al., 2010; Kimes et al., 2008; Marks et al., 2010; Marks et al., 1992; Pauly & Collins, 1993; Picciotto et al., 1995; Rubboli et al., 1994; Staley et al., 2005; Y. Xiao & Kellar, 2004), where they are enriched on the soma or terminals of neurons residing in limbic brain regions known to regulate appetitive and affective behaviors (Clarke et al., 1985; Court & Clementi, 1995; Fujita et al., 2000; Grady et al., 2010; Kimes et al., 2008; Marks et al., 2010; Marks et al., 1992; Pauly & Collins, 1993; Picciotto et al., 1995; Rubboli et al., 1994; Staley et al., 2005; Y. Xiao & Kellar, 2004), suggesting that modifying the activity of α4β2*nAChRs could affect both reward-like and anxiety-like behaviors. This is in contrast to a subclass of β2*nAChRs, which is sensitive to α-conotoxin MII, those that contain an α3 or α6 subunit. (α3β2*nAChRs and α6β2*nAChRs). Studies in α6 subunit knockout mice (α6KO) reveal that α6β2*nAChRs are more selectively expressed than other β2*nAChRs, being predominantly enriched on the soma and neuron terminals of catecholaminergic neurons, as well as in brain areas associated with the
visual circuit, including the superior colliculus (Champtiaux et al., 2002; Marks et al., 2010; Whiteaker, McIntosh, Luo, Collins, & Marks, 2000). α-conotoxin MII binding studies further show that α6β2*nAChRs are highly expressed on terminals of dopamine (DA) neurons projecting from the ventral tegmental area (VTA) to the dorsal striatum and nucleus accumbens (NAcc). This is in contrast to the α4β2*nAChRs, which in addition to their expression in the VTA and are also highly expressed in other DA projection regions including the amygdala, hippocampus, lateral septum and anterior cingulate cortex (Cartier et al., 1996; Champtiaux et al., 2002; Marks et al., 2010; Mineur et al., 2009; Whiteaker et al., 2000). In making a distinction between α4β2*nAChRs and α6β2*nAChRs, it should be noted that a subclass of receptors expresses both α4 and α6 subunits (α4α6β2*nAChRs). The α4α6β2*nAChRs are highly implicated in nicotine reinforcement and reward and compared to other heteroreceptors are resistant to desensitization in response to nM concentrations of nicotine that are achieved in the brains of smokers (Brunzell, Boschen, Hendrick, Beardsley, & McIntosh, 2010; Jackson, McIntosh, Brunzell, Sanjakdar, & Damaj, 2009; L. Liu, Zhao-Shea, McIntosh, Gardner, & Tapper, 2012; Pons et al., 2008; Tapper et al., 2004).

nAChR agonists bind at the juncture of the “front” of an α subunit and the “back” of the adjacent subunit. Heteromeric nAChRs only have two binding sites, at the junction of an α and β subunit, which is in contrast to the homomeric α7nAChRs which may have a ligand binding site at each of the five subunit junctures. In heteromeric nAChRs, the fifth or accessory subunit does not participate in binding but does contribute to the receptor binding and channel function (Bailey, De Biasi, Fletcher, & Lambe, 2010; Grady et al., 2012; Kuryatov, Onksen, & Lindstrom, 2008). The α5 and β3 subunits are thought to only function as accessory subunits, whereas α2-4, α6,
α7, β2 and β4 may act as binding or accessory subunits (Briggs et al., 2006; R. W. Brown, Collins, Lindstrom, & Whiteaker, 2007; Drenan, Nashmi, et al., 2008; Gerzanich, Wang, Kuryatov, & Lindstrom, 1998; Kuryatov, Luo, Cooper, & Lindstrom, 2005; Nelson, Kuryatov, Choi, Zhou, & Lindstrom, 2003; Tumkosit, Kuryatov, Luo, & Lindstrom, 2006). Additionally, studies in *xenopus* oocytes show that an increased expression ratio of α4:β2 subunits favors assembly of a lower sensitivity α4(3)β2(2) stoichiometry, whereas higher expression of β2:α4 favors the α4(2)β2(3) stoichiometry of α4β2*nAChRs with higher sensitivity for nAChR ligands (Anand, Conroy, Schoepfer, Whiting, & Lindstrom, 1991; Buisson & Bertrand, 2001; Cooper, Couturier, & Ballivet, 1991; Covernton & Connolly, 2000; Khiroug, Khiroug, & Yakel, 2004; Moroni, Zwart, Sher, Cassels, & Bermudez, 2006; Nelson et al., 2003; Zwart & Vijverberg, 1998). *In vitro* studies show that 86Rb+ efflux from striatal synaptosomes taken from mice with 50% reduction of the β2 subunit (β2HET), favoring the α4(3)β2(2) stoichiometry, is reduced following exposure to low ACh concentrations compared to synaptosomes harvested from mice with a 50% reduction of the α4 subunit (α4HET), which would more likely assemble in the higher sensitivity α4(2)β2(3) stoichiometry (Gotti et al., 2008). This suggests that assembly of α4β2*nAChRs with both high and low sensitivity modifies endogenous ACh tone in *in vivo* as well. The “front” of the binding pocket requires the Cys-Cys loop of the α subunit as well as a number of predominantly hydrophobic amino acids, including Tyr 93, Trp 149, Tyr 190 and Tyr 198 and the “back” of the binding site requires 3 amino acids (L112, M114, and Trp53) found on the adjacent subunit (Celie et al., 2004; Karlin et al., 1986; Sine & Engel, 2006).

Unlike other neurotransmitters which are largely expressed post-synaptically opposite the release site, nAChRs are predominantly expressed extrasynaptically, on pre-synaptic terminals, and on
cell bodies (Arroyo-Jiminez et al., 1999; Hill, Zoli, Bourgeois, & Changeux, 1993; Kawai, Lazar, & Metherate, 2007; McGehee, Heath, Gelber, Devay, & Role, 1995; Rice & Cragg, 2004; Vidal & Changeux, 1993; Wonnacott, 1997; H. Zhang & Sulzer, 2004). Rather, nAChRs act as neuromodulators. Positioned on neurons that release many different neurotransmitters, stimulation of nAChRs can facilitate the release of glutamate, GABA, DA, ACh, norepinephrine and serotonin, suggestive of pre-synaptic activity (McGehee et al., 1995; Wonnacott, 1997). Facilitation of neurotransmitter release via nAChRs is subclass specific and it varies between brain regions. For example, activation of α7nAChRs facilitates glutamate release and activation of α4β2 nAChRs facilitates GABA release in the VTA, whereas glutamate release is increased after β2*nAChR activation in thalamo-cortical neurons. (Mansvelder & McGehee, 2000; Parikh, Ji, Decker, & Sarter, 2010; Poorthuis, Bloem, Verhoog, & Mansvelder, 2013). Additionally, the release of ACh is enhanced following activation of α3β4*nAChRs on cholinergic neuron terminals (Grady et al., 2001). In support of ACh synaptic transmission, there is evidence demonstrating that nicotine and ACh can stimulate excitatory post-synaptic potentials (Alkondon, Pereira, & Albuquerque, 1998; Jones, Sudweeks, & Yakel, 1999; Lena & Changeux, 1999; Picciotto et al., 1995; Picciotto et al., 1998), but other studies suggest that ACh exerts its effects primarily through volume transmission (Arroyo, Bennett, Aziz, Brown, & Hestrin, 2012; Bell, Shim, Chen, & McQuiston, 2011; Yamasaki, Matsui, & Watanabe, 2010). The effect of nAChR activation on neuronal plasticity is also contingent upon the nAChR subclass involved and brain region investigated. For example, activation of α7nAChRs on glutamate terminals promotes long-term potentiation (LTP) via activation of DA neurons in the VTA (Mansvelder & McGehee, 2000). By contrast, activation of α4β2*nAChRs on GABA terminals reduces post-synaptic calcium levels (Couey et al., 2007). Furthermore, by activation of the protein
phosphatase calcineurin and subsequent inactivation of L-type calcium channels, activation of high affinity $\beta_2^*\text{nAChRs}$ can reduce calcium entry into post-synaptic neurons following the release of glutamate (Stevens, Krueger, Fitzsimonds, & Picciotto, 2003). These studies demonstrate that the disruption of cholinergic activity by nicotine and/or nicotinic antagonists or partial agonists has the potential to alter the valence of an external stimulus.

Considering that activation of pre-synaptic $\beta_2^*\text{nAChRs}$ can facilitate neurotransmitter release and that activation of post-synaptic $\beta_2^*\text{nAChRs}$ can result in either elevated or reduced levels of intracellular calcium, preferential desensitization of $\beta_2^*\text{nAChRs}$ also has the potential to profoundly modify the central nervous system response to both ACh and nicotine. Currently approved pharmacotherapies for smoking cessation include the $\beta_2^*\text{nAChR}$ partial agonist varenicline and the nicotine patch, which has nicotine pharmacokinetics that would support desensitization of $\beta_2^*\text{nAChRs}$ (Benowitz, Jacob, Fong, & Gupta, 1994). Thus, it is possible that these therapies derive their efficacy by reducing the activity of $\beta_2^*\text{nAChRs}$ during the release of ACh. Varenicline is also a full agonist at $\alpha_7\text{nAChRs}$, and local infusions of the selective $\alpha_7\text{nAChR}$ agonist PNU 282987 into the NAcc shell reduce the motivation to self-administer nicotine in preclinical models, making it difficult to determine if the efficacy of varenicline is promoted primarily via $\beta_2^*\text{nAChRs}$ or $\alpha_7\text{nAChRs}$ (Brunzell & McIntosh, 2012).

Similar to nicotine’s dose-dependent effects at nAChRs, behavioral studies show that nicotine administration also has a bimodal effect on anxiety-like behavior; low doses of nicotine promote an anxiolytic-like phenotype (Cheeta, Irvine, Kenny, & File, 2001; File, Kenny, & Ouagazzal, 1998; Irvine, Cheeta, & File, 1999; McGranahan, Patzlaff, Grady, Heinemann, & Booker, 2011),
whereas high doses of nicotine result in an increase in anxiety-like behavior (Cheeta, Tucci, & File, 2001; File, Kenny, & Cheeta, 2000; File et al., 1998; Ouagazzal, Kenny, & File, 1999; Zarrindast, Solati, Oryan, & Parivar, 2008). Evidence suggests that inhibition of the β2*nAChRs with selective antagonists or partial agonists supports anxiolysis in animal models (Anderson & Brunzell, 2012; Dawson, Miles, & Damaj, 2013; Roni & Rahman, 2011; Turner, Castellano, & Blendy, 2010, 2011). This is in contrast to nicotine’s “reward-like” and reinforcing properties, which require activation of the high affinity β2*nAChRs (Brunzell et al., 2010; Corrigall, Coen, & Adamson, 1994; Gotti et al., 2010; Grabus, Martin, Brown, & Damaj, 2006; Jackson et al., 2009; Maskos et al., 2005; McGranahan et al., 2011; Mineur et al., 2009; Picciotto et al., 1998; Pons et al., 2008; Tapper et al., 2004; Walters, Brown, Changeux, Martin, & Damaj, 2006) (but see (Exley & Cragg, 2008). Nicotine self-administration and nicotine conditioned place preference are absent in α4, α6 and β2 null mutant mice (α4KO, α6KO, β2KO) (Jackson et al., 2009; McGranahan et al., 2011; Mineur et al., 2009; Orejarena et al., 2012; Picciotto et al., 1998; Pons et al., 2008; Walters et al., 2006). These rodent models of nicotine reinforcement and reward are also blocked by pre-injection of the β2*nAChR antagonist dihydro-beta-erythroidine (DHβE), which selectively antagonizes α4β2*nAChRs as well as α6β2*nAChRs, and by selective antagonists of α6β2*nAChRs (Brunzell et al., 2010; Corrigall et al., 1994; Gotti et al., 2010; Grabus et al., 2006; Jackson et al., 2009; Maskos et al., 2005; McGranahan et al., 2011; Mineur et al., 2009; Picciotto et al., 1998; Pons et al., 2008; Tapper et al., 2004; Walters et al., 2006) (but see (Exley & Cragg, 2008)). Studies utilizing gain-of-function mice with an L9*A single point mutation in the M2 pore-forming region of the α4 subunit (L9A) further show that activation of the α4 subunit is sufficient to support nicotine conditioned place preference (CPP) (Tapper et al., 2004). Furthermore, activation of α6β2*nAChRs on the soma of DA neurons
originating in the VTA is sufficient to stimulate release of DA in the NAcc (Drenan et al., 2010; Drenan, Grady, et al., 2008; Exley & Cragg, 2008; Pons et al., 2008; Zhao-Shea et al., 2011). Together these studies suggest that activation of $\alpha_4\alpha_6\beta_2^*nAChRs$ supports nicotine reward and nicotine self-administration. DA release in the dorsal striatum is thought to mediate nicotine-mediated locomotor activation (Cohen et al., 2012; Drenan et al., 2010; Drenan, Grady, et al., 2008; McGranahan et al., 2011; Mineur et al., 2009). However, in vivo microdialysis and cyclic voltammetry studies show that DA release is also increased in the NAcc following an aversive footshock and is predictive of execution of lever pressing behavior to avoid reception of a footshock (Kalivas & Duffy, 1995; Oleson, Gentry, Chioma, & Cheer, 2012; Takahashi, Takada, Nagai, Urano, & Takada, 1998). Thus, increased accumbal DA release is not ubiquitous with the rewarding properties of a reinforcer, but is also associated with determining the salience of aversive external stimuli.

**Neuroanatomy of nAChRs: pathways relevant to addiction phenotype**

Binding studies show that nAChRs are expressed in brain regions such as the NAcc and VTA that regulate the rewarding/reinforcing properties of drugs of abuse and natural reinforcers, such as food, as well as brain nuclei associated with emotive responses to environmental stimuli, such as the amygdala, hippocampus, prefrontal cortex (PFC) and lateral septum (Baddick & Marks, 2011; Clarke et al., 1985; Marks et al., 2010). Binding studies using the selective $\alpha_6/\alpha_3^*nAChR$ antagonist $\alpha$-conotoxin MII further demonstrate that $\alpha_7nAChRs$ and $\alpha_4\beta_2^*nAChRs$, but not $\alpha_6\beta_2^*nAChRs$ are expressed in the amygdala, hippocampus, PFC and lateral septum (Baddick & Marks, 2011; Champtiaux et al., 2002; Clarke et al., 1985; Marks et al., 2010; Mineur et al., 2009; Whiteaker et al., 2000). The activation of the amygdala promotes anxiety and fear, as
evidenced by reduced anxiety-like behaviors in rodents, non-human primates and humans following either excitotoxic lesions of the amygdala or amygdalar damage (Drevets, 2003; Kalin, Shelton, & Davidson, 2004; Masaoka, Hirasawa, Yamane, Hori, & Homma, 2003; Ventura-Silva et al., 2013). Additionally, human imaging studies show that amygdala activity is increased in individuals presenting with trait anxiety and anxiety related to dementia (Britton, Lissek, Grillon, Norcross, & Pine, 2011; Sehlmeyer et al., 2011). The selective removal of cholinergic input to the basolateral amygdala (BLA), where α4β2*nAChRs prevail, results in reduced anxiety-like behavior (Power & McGaugh, 2002), suggesting that reduced activity of α4β2*nAChRs in the amygdala would attenuate anxiety-like behavior.

The lateral septum is a GABA-ergic nucleus that receives a convergence of neuronal input from various brain regions known to regulate the motivational valence of external stimuli (e.g., DA from the VTA, the neuropeptide CRF indirectly from the amygdala via the bed nucleus of the stria terminalis, glutamate from the hippocampus and the PFC and ACh from the lateral dorsal tegmental nucleus (Risold & Swanson, 1997a, 1997b). Together with the hippocampus, the lateral and medial septal nuclei comprise the “septo-hippocampal” circuit that is thought to regulate anxiety-like behavior, with the hippocampus integrating information that is predictive of a real or perceived threat, and the lateral septum regulating the emotive anxiety response to an environmental stimulus (Gray, 1982; Risold & Swanson, 1997a, 1997b). Among primates, the septum achieves its maximal size in the human species, making it an appealing brain region to evaluate the anxiolytic-like properties of nicotine (Andy & Stephan, 1968). Excitotoxic lesions to the lateral septum result in the expression of an anxiogenic-like phenotype classified as the “septal rage syndrome” (Albert & Chew, 1980; Albert & Walsh, 1982; Brady & Nauta, 1953;
Olton & III, 1976). Activation of corticotropin releasing factor subtype 2 receptors (CRFR2) in the lateral septum, which increases anxiety-like behavior, also inhibits lateral septum activity, possibly via inhibition of excitatory glutamate release in the lateral septum from the PFC or hippocampus (Eberly, Dudley, & Moss, 1983; J. Liu et al., 2004). Pharmacological antagonism of both corticotropin releasing factor subtype 1 receptor (CRFR1) and CRFR2 in the lateral septum attenuates anxiogenic-like behavior following restraint stress (Radulovic, Ruhmann, Liepold, & Spiess, 1999).

A preponderance of the evidence suggests that stimulation of the lateral septum promotes anxiolysis-like behavior. Electrical stimulation of the lateral septum results in increased water consumption despite the reception of lick-contingent shocks and has been shown to reduce gastric ulceration following chronic stress (Yadin & Thomas, 1996; Yadin, Thomas, Grishkat, & Strickland, 1993). The lateral septum gives rise to GABA-ergic outputs to several brain areas thought to regulate anxiety and arousal and thus is positioned to inhibit these brain areas. The infusion of the benzodiazepine chlordiazepoxide results in activation of the lateral septum and reduced activity within the amygdala (Yadin & Thomas, 1998), suggesting that opposing activation of these two brain regions may be coordinated to regulate the emotive response to an aversive stimulus. Additionally, electrical stimulation of the lateral septum reduces activity of the paraventricular nucleus of the hypothalamus (PVN) by 50% and neurotoxic lesions in the lateral septum resulted in increased HPA-axis activation and expression of c-Fos in the PVN following forced swim stress than sham-lesioned controls, suggesting the activation of lateral septal nuclei results in an attenuation of the response of the PVN to stressors (Herman, Marciano, & Gash, 1986; Herman, Ostrander, Mueller, & Figueiredo, 2005; Pittman, Blume,
Taken together, these studies indicate that activation of the lateral septum reduces anxiety-like behaviors, and suggest that lateral septum inhibition may promote anxiogenic-like behavior (Fig 1.1).

**Nicotine effects on intracellular signaling: extracellular regulated kinase (ERK)**

ERK is a serine/threonine kinase critical for neuronal plasticity related to learning and memory and to the rewarding properties of drugs of abuse, including nicotine (Brunzell, Mineur, Neve, & Picciotto, 2009; Brunzell, Russell, & Picciotto, 2003; S. Davis, Vanhoute, Pages, Caboche, & Laroche, 2000; Schafe et al., 2000; Schafe, Swank, Rodrigues, Debiec, & Doyere, 2008; Sweatt, 2001; Valjent et al., 2006; Valjent, Pages, Herve, Girault, & Caboche, 2004). Nicotine-mediated activation of nAChRs facilitates the release of neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), which activate the Ras/Raf/MEK pathway via receptor tyrosine kinases (Belluardo, Mudo, Blum, et al., 1999; Belluardo, Mudo, Blum, & Fuxe, 2000; Belluardo, Mudo, Caniglia, et al., 1999; Ying et al., 2002). Activation of nAChRs also facilitates increased levels of intracellular calcium, leading to releases of intracellular stores of calcium or phosphorylation of protein kinase C (PKC), calcium modulated kinase II (CaMKII), or proline-rich tyrosine kinase (Pyk-2), which in turn may phosphorylate (activate) ERK (Chang & Berg, 2001; Dajas-Bailador, Soliakov, & Wonnacott, 2002; Fenster et al., 1999; Konu et al., 2001; Messing, Stevens, Kiyasu, & Sneade, 1989; Nakayama, Numakawa, Ikeuchi, & Hatanaka, 2001; Tahara et al., 2001; Tuominen et al., 1992).
The effect of external stressors on ERK phosphorylation is brain region specific. In rats exposed to restraint stress, pERK was significantly increased in the hippocampus, PFC and hypothalamus (Kwon et al., 2006; Meller et al., 2003; Sananbenesi, Fischer, Schrick, Spiess, & Radulovic, 2003; H. T. Wang, Han, Gao, & Shi, 2010), (however, see (ElBatsh, Assareh, Marsden, & Kendall, 2012)). An intracerebroventricular infusion of corticotropin releasing factor (CRF), associated with anxiogenic-like behavior, induced significant increases in pERK in the hippocampus and BLA respectively (Refojo et al., 2005). In contrast with the increased phosphorylation of ERK in these excitatory nuclei, levels of pERK were reduced in the lateral septum following restraint and chronic housing stress (Sheehan, Neve, Duman, & Russell, 2003; Singewald, Nguyen, Neumann, Singewald, & Reber, 2009). Additionally, overexpression of Pyk-2, which can activate Ras, leading to ERK phosphorylation, in the lateral septum led to a reduction in depression-like behavior (Sheehan et al., 2003). ERK, like c-Fos, can act as a biomarker for cellular activity. The studies above suggest that elevated levels of pERK, similarly to increased lateral septum neuronal activity, is associated with reductions in stress-like behavior whereas decreases are associated with increases in anxiety-like and depression-like behavior (Sheehan et al., 2003; Singewald et al., 2009; E. Thomas, Strickland, Yadin, & Burock, 2005; E. Thomas & Yadin, 1980; E. Thomas, Yadin, & Strickland, 1991; Yadin & Thomas, 1996, 1998; Yadin et al., 1993).

In addition to acting as a biomarker for anxiety-like behavior, lateral septum ERK may play a functional role in regulation of anxiety-like behavior. pERK can be translocated to the nucleus, where it activates the transcription factor CREB. The overexpression of CREB in the NAcc
Figure 1.1 - Promotion of increased neuronal activity in the lateral septum via elevations in pERK may result in reduced expression of anxiety-like behavior. Adapted from (Sheehan, Chambers, & Russell, 2004).
regulates the motivational salience of rewarding and aversive external stimuli (Barrot et al., 2002; Barrot et al., 2005). Like ERK, CREB activation is region specific as it associates to anxiety-like behavior. The expression of pCREB in the hippocampus and PFC is inversely associated with changes in anxiety-like behavior (Rubino et al., 2007; J. Zhang et al., 2010), whereas the opposite is found regarding pCREB expression in the periaqueductal gray (Adamec, Berton, & Abdul Razek, 2009). Increased expression of inducible cAMP early repressor is associated with increased anxiety-like behavior in the elevated plus maze (Green et al., 2006). Furthermore, increased pCREB in the hippocampus is associated with improved cognitive function in preclinical experiments (Trofimiuk, Holownia, & Braszko, 2010).

Intracellular changes in ERK phosphorylation may result from downregulation of ERK activation pathways (Raf/Ras/MEK) or upregulation of phosphatase activity, which would inactivate pERK. The phosphatase striatal enriched phosphatase (STEP) can dephosphorylate, or inactivate ERK (M. A. Johnson & Lombroso, 2012; Lombroso, Murdoch, & Lerner, 1991; Munoz, Tarrega, Blanco-Aparicio, & Pulido, 2003; Odagaki et al., 2001; Paul, Nairn, Wang, & Lombroso, 2003; Xu, Kurup, Bartos, et al., 2012). Outside of the striatum, STEP expression is the highest in the lateral septum, suggesting that it may be an important regulator of ERK phosphorylation in this brain region (Boulanger et al., 1995; Lombroso, Naegele, Sharma, & Lerner, 1993). STEP is increased in several neuropsychiatric disorders, including Alzheimer’s Disease (AD) and schizophrenia, disorders also known to involve dysregulation of nAChRs (Chin et al., 2005; Kurup et al., 2010; Xu, Kurup, Nairn, & Lombroso, 2012; Y. Zhang et al., 2010). Furthermore, the overexpression of a “substrate trapping” form of STEP that irreversibly binds to molecular targets such as ERK prevents fear conditioning when locally infused into the
amygdala (Paul et al., 2007); hence altering the activity of phosphatases that control pERK can regulate the motivational salience of external stimuli. Little is known about how α4β2*nAChRs may contribute to changes in lateral septum ERK that could support changes in anxiety behaviors.

**Nicotine and smoking behavior**

Human and animal studies demonstrate that nicotine exerts its psychoactive properties by binding to nAChRs in brain (Brunzell et al., 2010; Corrigall et al., 1994; DeNoble & Mele, 2006; Gotti et al., 2010; Pons et al., 2008). Like most drugs of abuse, nicotine activates DA projection neurons of the mesocorticlimbic reward pathway that originate in the VTA, resulting in the release of the neurotransmitter DA in brain areas known to be critical in determining the motivational salience of external stimuli, such as the NAcc, PFC and lateral septum (Brody, Mandelkern, Olmstead, et al., 2009; Brody et al., 2004; Domino et al., 2013; Domino, Tsukada, & Harada, 2009; Kleijn et al., 2011; Mansvelder et al., 2002; Mineur et al., 2009; Perez, Bordia, McIntosh, Grady, & Quik, 2008; Perez, Ly, McIntosh, & Quik, 2012; Perez, McIntosh, & Quik, 2013; Pidoplichko et al., 1997; Takahashi et al., 2008). Consequently, the majority of preclinical and clinical research has been directed at understanding the neuronal and molecular mechanisms regulating the pleasurable or “rewarding” effects exerted by nicotine.

The Fagerstrom Test for Nicotine Dependence (Table 1.1) is the most widely used instrument to determine the degree of nicotine dependence in smokers (Fagerstrom, 1978). Time elapsed before smoking the first cigarette of the day is the greatest predictor of nicotine dependence.
Table 1.1 – Fagerstrom Test for Nicotine Dependence

<table>
<thead>
<tr>
<th>Fagerstrom Test for Nicotine Dependence</th>
<th>Less than 5 min</th>
<th>6-30 min</th>
<th>30-60 min</th>
<th>1 pt</th>
<th>2 pt</th>
<th>3 pt</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. How soon after you wake up do you smoke your first cigarette?</td>
<td></td>
<td></td>
<td></td>
<td>1 pt</td>
<td>2 pt</td>
<td>3 pt</td>
</tr>
<tr>
<td>2. Do you find it difficult to refrain from smoking in places where it is prohibited?</td>
<td>Yes</td>
<td>1 pt</td>
<td>No</td>
<td>0 pt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Which cigarette would you hate to give up?</td>
<td>First one of the day</td>
<td>1 pt</td>
<td>Any other</td>
<td>0 pt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. How many cigarettes a day do you smoke?</td>
<td>10 or less</td>
<td>0 pt</td>
<td>11-20</td>
<td>1 pt</td>
<td>21-30</td>
<td>2 pt</td>
</tr>
<tr>
<td>5. Do you smoke more frequently in the morning?</td>
<td>Yes</td>
<td>1 pt</td>
<td>No</td>
<td>0 pt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Do you smoke even if you are sick in bed most of the day?</td>
<td>Yes</td>
<td>1 pt</td>
<td>No</td>
<td>0 pt</td>
<td></td>
<td></td>
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</tbody>
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<table>
<thead>
<tr>
<th>Total score</th>
<th>Score</th>
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<tbody>
<tr>
<td>1-2 = Low dependence</td>
<td>5-7 = Moderate dependence</td>
</tr>
<tr>
<td>3-4 = Low to moderate dependence</td>
<td>8+ = High dependence</td>
</tr>
</tbody>
</table>

(Adapted from (Fagerstrom, 1978)).
using this test, indicating that nicotine dependence is thought to be primarily driven by the reward-like experience of smoking the first cigarette of the day, which is reported by many smokers to be the most pleasurable. An emerging theory of drug dependence is that the positive reinforcing effects of drugs of abuse likely contribute to the onset of drug use, whereas avoidance of the negative effects of withdrawal underlie escalation of drug use, leading to dependency and addiction (Koob & Volkow, 2010).

While this may also hold true for tobacco use, there is a high concordance between tobacco use and affective disorders, including the range of anxiety-related disorders (Evatt & Kassel, 2010; Gilbert et al., 2008; Grillon, Avenevoli, Daurignac, & Merikangas, 2007; John, Meyer, Rumpf, & Hapke, 2004; J. L. Johnson, Eaton, Pederson, & Lowry, 2009; McCabe et al., 2004; Morissette, Brown, Kamholz, & Gulliver, 2006; Tsuda, Steptoe, West, Fieldman, & Kirschbaum, 1996; Vujanovic, Marshall, Gibson, & Zvolensky, 2010; Vujanovic, Marshall-Berenz, Beckham, Bernstein, & Zvolensky, 2010; Zvolensky, Marshall, et al., 2009; Zvolensky, Stewart, et al., 2009). Furthermore, stress and anxiety are cited as primary factors contributing to relapse during smoking cessation attempts and to the escalation of cigarette use (Byrne et al., 1995; Byrne & Mazanov, 2003; Finkelstein et al., 2006; McCabe et al., 2004; Shiffman et al., 1997; Siqueira et al., 2000; Skara et al., 2001; Zvolensky, Marshall, et al., 2009; Zvolensky, Stewart, et al., 2009). In light of this, clinical trials are evaluating subjects for trait anxiety scores using instruments such as the Anxiety Sensitivity Index (ASI) to help identify other factors contributing to the onset and maintenance of tobacco use. Various preclinical models of anxiety-like behavior are also being employed to determine the genetic and molecular factors that underlie initial sensitization to the interoceptive effects of acute nicotine that ultimately lead to escalation of
tobacco use and dependence. The studies described herein attempt to address the question of what subclass of nAChRs contributes to the anxiolytic-like effects of acute nicotine administration, to pharmacologically test if nicotine exerts anxiolytic-like effects via activation or inhibition of these nAChRs, and to identify the neurochemical substrates downstream of nAChRs that may contribute to these anxiolytic effects.

In smokers, daily concentrations of nicotine in the brains of average smokers are estimated to be as high as 1-3 µM, but thought to be maintained in the 200 - 450 nM range (Rose et al., 2010), which would favor desensitization of the high affinity β2*nAChRs. Nicotine absorption via the alveolar membrane is delayed due to sequestration of nicotine in the lung tissues of smokers (Rose et al., 2010). Nonetheless, the lag time between taking a puff of a cigarette to nicotine reaching crossing the blood-brain barrier is only 10-20 seconds (Rose, Behm, Westman, & Coleman, 1999). This relatively short time between cigarette smoking and nicotine delivery in the central nervous system affords the smoker the ability to titrate the desired dose, increasing the reinforcing properties of cigarette smoking and the addiction potential of cigarette use. In the morning, consumption of the first cigarette of the day, which many smokers report to be the most pleasurable, would result in a sharp increase in nicotine concentrations, facilitating activation of β2*nAChRs and nicotine-stimulated DA release, as is reported in human smokers during PET imaging studies (Benowitz, 1988; Benowitz & Jacob, 1984; Brody, Mandelkern, Olmstead, et al., 2009; Brody et al., 2004; Domino et al., 2013; Domino et al., 2009; Fagerstrom, 1978; Fagerstrom, Heatherton, & Kozlowski, 1990; Takahashi et al., 2008). When considered along with the fact that a single cigarette contains enough nicotine to occupy at least 75% of the high affinity β2*nAChRs for upwards of 4 hours, it is possible that many of these β2*nAChRs are in
a desensitized state for much of the day (Brody, Mandelkern, Costello, et al., 2009; Brody et al., 2006). Since the activation of β2*nAChRs regulates the release of DA in the NAcc, factors other than the reinforcing properties of nicotine may also contribute to the maintenance of smoking behavior. One possibility is that in susceptible populations, cigarette use may be employed as a means of self-medication to relieve anxiety.

**Anxiety and smoking**

Anxiety has been described as an emotional state arising from the anticipation of a future event and has been distinguished from fear, in that fear is often tied to a clearly identified threat, while anxiety lacks an objective threatening object or event (Estes, 1948; Grillon, 2002). In the human subject, anxiety can result from a seminal precipitating event, as occurs in post-traumatic stress disorder, or it can manifest itself as heightened worry and concern over every day events in general anxiety disorders or social phobias.

Although human imaging studies do not reveal a correspondence between the expression of β2*nAChRs and anxiety, there is a significant correlation between cigarette smoking and anxiety-related disorders such as panic attacks, specific phobias, social phobias and post-traumatic stress disorder (Evatt & Kassel, 2010; Gilbert et al., 2008; Grillon et al., 2007; John et al., 2004; J. L. Johnson et al., 2009; McCabe et al., 2004; Morissette et al., 2006; Tsuda et al., 1996; Vujanovic, Marshall, et al., 2010; Vujanovic, Marshall-Berenz, et al., 2010; Zvolensky, Marshall, et al., 2009; Zvolensky, Stewart, et al., 2009). Additionally, post-mortem [³H] nicotine binding and SPECT imaging studies show that cigarette smokers have a significantly higher number of high affinity β2*nAChRs than non-smokers (Benwell, Balfour, & Anderson, 1988;
Breese et al., 1997; Cosgrove et al., 2009; Gilbert et al., 2008; Staley et al., 2006; Staley et al., 2005; Tsuda et al., 1996). There is also a high concordance between cigarette smoking and other psychiatric disorders, such as depression (John et al., 2004; Kalman, Morissette, & George, 2005; Lasser et al., 2000; Leonard et al., 2000; Leonard et al., 1998).

Anxiety sensitivity (AS) is defined as the fear of behaviors or emotions related to anxiety. Individuals rating higher on the ASI assessment are more likely to experience improvement in positive affect following smoking, have lower smoking cessation success rates and are more likely to relapse in order to relieve negative affect than subjects who rate low on this scale (R. A. Brown, Kahler, Zvolensky, Lejuez, & Ramsey, 2001; Evatt & Kassel, 2010; Gonzalez, Zvolensky, Vujanovic, Leyro, & Marshall, 2008; K. A. Johnson, Farris, Schmidt, & Zvolensky, 2012; K. A. Johnson, Stewart, Rosenfield, Steeves, & Zvolensky, 2012; Wong et al., 2013). These data suggest that individuals exhibiting heightened trait anxiety may be more sensitive to the anxiety relief following tobacco use. Although prospective studies have not demonstrated causality between cigarette smoking and development of anxiety-related disorders, the high concordance between chronic tobacco use and anxiety disorders also has implications for longevity and well-being, as individuals with chronic anxiety have a higher risk of developing cardiovascular and cognitive degenerative diseases related to aging, as well as premature death (Benninghoven et al., 2006; Bjorngaard et al., 2013; Carroll et al., 2011; Eaker, Sullivan, Kelly-Hayes, D'Agostino, & Benjamin, 2005; Goodwin, Perkonigg, Hofler, & Wittchen, 2013; Kubzansky & Kawachi, 2000; C. Li et al., 2008; Shahab, Andrew, & West, 2013; Wong et al., 2013). Finally, since cigarette smoking is a risk factor for many chronic diseases, including cardiovascular and respiratory diseases, as well as cancer, it is not a desirable treatment for
anxiety. Understanding how nicotine relieves anxiety may not only help smokers to quit, but could identify a novel therapeutic target for anxiety disorders.

**Anxiety and Aging**

The preponderance of data suggests that although anxiety rates are lower in the geriatric population compared with the general population, they are increased in elderly subjects presenting with co-morbidities of AD or dementia (Baune et al., 2012; Beaudreau & O'Hara, 2009; Boralingaiah, Bettappa, & Kashyap, 2012; Bunce, Batterham, Mackinnon, & Christensen, 2012; Cairney, Corna, Veldhuizen, Herrmann, & Streiner, 2008; D'Onofrio et al., 2012; Flint, 1994; Goncalves, Pachana, & Byrne, 2011; Higgins et al., 2012; Hynnnin, Breitve, Rongve, Aarsland, & Nordhus, 2012; Kvaal, McDougall, Brayne, Matthews, & Dewey, 2008; Nilsson et al., 2012; O'Connor, 2006; Panza et al., 2012; Potvin, Forget, Grenier, Previle, & Hudon, 2011; Prina, Ferri, Guerra, Brayne, & Prince, 2011a, 2011b; Rozzini et al., 2009; Samuelsson, McCamish-Svensson, Hagberg, Sundstrom, & Dehlin, 2005; Seby, Chaudhury, & Chakraborty, 2011; Sinoff & Werner, 2003; Skoog, 2011; Smalbrugge, Jongenelis, Pot, Beekman, & Eefsting, 2005; Wolitzky-Taylor, Castriotta, Lenze, Stanley, & Craske, 2010; Yochim, Mueller, & Segal, 2013), (but see (Mehta et al., 2007; Prina et al., 2011a, 2011b; Wolitzky-Taylor et al., 2010)). Nevertheless, a diagnosis of general anxiety disorder is correlated with increased mortality and the onset of geriatric anxiety may be an early indicator of the development of cognitive neurodegenerative disorders such as AD or advanced dementia (Nilsson et al., 2012). Post-mortem epibatidine binding and SPECT imaging studies show that reductions in high affinity nAChRs in the human brain appear to coincide with normal aging, whereas pathologic reductions in this nAChR subclass are associated with cognitive declines and
the emergence of psychological co-morbidities, such as increased anxiety and depression (Brodaty et al., 2012; Mitsis et al., 2009; Sabbagh et al., 2006). FDA-approved pharmacotherapies for AD alleviate the symptoms of AD, including anxiety by increasing the endogenous ACh tone via inhibition of AChE (S. L. Rogers, Doody, Mohs, & Friedhoff, 1998). In addition to *in vitro* studies showing that β2*nAChRs serve a neuroprotective role, post-mortem epibatidine binding and western blot analyses, as well as imaging studies show that cognitive neurodegeneration is associated with reduced expression of β2*nAChRs (Gotti et al., 2006; Martin-Ruiz et al., 1999; Pimlott et al., 2004; Warpman & Nordberg, 1995; Wevers et al., 1999; Zanardi et al., 2007; Zoli, Picciotto, Ferrari, Cocchi, & Changeux, 1999). In summary, these data concerning anxiety-related behavior and the elderly population underscore the need to develop novel methods to improve treatment and early detection of anxiety in susceptible individuals.

**Experimental Aims**

In light of these data, Specific Aim 1 tested the hypothesis that nicotine supports anxiolysis-like behavior via inhibition of β2*nAChRs. These studies used a combination of genetic, pharmacological and behavioral manipulations to test this hypothesis. In Chapter 2, we evaluated the effects of a range of doses of nicotine (0, 0.0032, 0.01, 0.032, 0.1 mg/kg) and the selective β2*nAChR antagonist DHβE (0, 0.01 0.032, 0.1, 0.32 mg/kg) in multiple behavioral assays that have predictive and/or face validity for testing the anxiolytic efficacy of drugs, including the conditioned emotional response (CER), marble burying task and elevated plus maze assay. The anxiolytic efficacies were determined by the ability of nicotine and DHβE to increase behavior, as measured by increased exploration of the aversive open arms of an elevated maze.
plus maze, or increased lever pressing in the presence of an aversive conditioned stimulus or reduce behavior, as measured by less digging behavior in the marble burying task. We predict that the administration of both a low dose of nicotine and the selective $\beta_2*nAChR$ antagonist DH$\beta$E will similarly attenuate anxiety-like behavior in these behavioral assays without significantly affecting locomotor activity. In Chapter 3, we expanded upon these pharmacological studies in mice lacking $\beta_2*nAChRs$ (Picciotto et al., 1995). We tested if $\beta_2*nAChRs$ are necessary for the anxiolytic-like and anxiogenic-like efficacies of nicotine by evaluating low and high doses of nicotine (0, 0.01, 0.05, 0.1, 0.5 mg/kg) in WT and null mutant $\beta_2$KO mice in the light-dark assay. By expanding the range of doses administered in the light-dark assay, we predict that we will observe both an anxiolytic and anxiogenic-like dose of nicotine. In support of the hypothesis that $\beta_2*nAChRs$ are required for the anxiolytic and anxiogenic-like effects of acute nicotine, we predict that the effects of these doses of nicotine on anxiety-like behavior will be absent in $\beta_2$KO mice. The observation of an anxiolytic or anxiogenic-like effect of nicotine in $\beta_2$KO mice would suggest that some other subclass of nAChRs contributed to the observed effects. A third experiment assessed the effects of the selective $\beta_2*nAChR$ agonist 5I-A85380 (0, 0.001, 0.0032, 0.01, 0.032 mg/kg) in the light-dark and elevated plus maze assays. We predict that the administration of 5I-A85380 will also reduce anxiety-like behavior at low doses and promote anxiogenic-like behavior at high doses. Failure to observe either anxiolytic or anxiogenic efficacy of 5I-A85380 may result from not testing a wide enough range of doses. It may also suggest that other subclasses of nAChRs mediated the effects of nicotine observed in earlier experiments. The pharmacologic and genetic studies described thus far are correlative, and inform us whether the $\beta_2*nAChRs$ are required for the effects of nicotine, or whether nicotinic agonists and selective antagonism of $\beta_2*nAChRs$
similarly promote anxiolytic-like behavior. We therefore directly tested whether pre-injections of 2 mg/kg DHβE would block or accumulate with an anxiolytic low dose (0.05 mg/kg) or anxiogenic high dose (0.5 mg/kg) of nicotine in the light-dark assay. We also evaluated if 0.01 or 0.05 mg/kg doses of nicotine would accumulate with or also block the anxiogenic-like effects of 0.5 mg/kg during the light-dark and elevated-plus maze assays. In support of the hypothesis that low doses of nicotine promote anxiolysis via inhibition of β2*nAChRs, we predict that pre-injections of both DHβE and nicotine will significantly attenuate the anxiogenic-like effects of 0.5 mg/kg i.p. nicotine. If low doses of nicotine promote anxiolysis via activation of β2*nAChRs, these effects ought to be similarly blocked by DHβE and nicotine. In support of the hypothesis that the anxiolytic-like effects are promoted via inhibition of β2*nAChRs, we predict that neither DHβE nor nicotine pretreatment will significantly attenuate the anxiolytic-like effects of 0.05 mg/kg nicotine. If we do observe a significant attenuation of the anxiolytic-like effects of 0.05 mg/kg nicotine, this would suggest that reductions in anxiety-like behavior are in part mediated via activation of β2*nAChRs, and may necessitate the evaluation of 0.032 mg/kg nicotine in these assays.

The focus of Specific Aim 2 is to identify which subclass of β2*nAChRs is likely responsible for mediating the anxiolytic-like efficacy of acute nicotine. In contrast to *in vitro* and *in vivo* assays suggesting that low doses of nicotine may reduce anxiety-like behavior via activation of α4α6β2*nAChRs, we hypothesize that activation of α6β2*nAChRs will promote an anxiogenic phenotype (L. Liu et al., 2012; McGranahan et al., 2011). In order to test this hypothesis, in Chapter 4, we compared the anxiety-like behaviors of L9S mice with hypersensitive α6β2*nAChRs as well as null mutant α6KO mice with their WT controls in the ethological
light-dark, elevated plus maze and open field assays. To support the hypothesis that activation of \(\alpha_4\alpha_6\beta_2\text{nAChRs}\) promotes anxiety, we predict that anxiety-like behavior will be higher in L9S than WT mice in the behavioral tests used. We further predict that \(\alpha_6\text{KO}\) mice will show an anxiolytic-like phenotype compared to WT controls. Should L9S mice demonstrate reduced anxiety-like behavior, it may suggest that nicotine is promoting anxiolysis via activation of \(\alpha_4\alpha_6\beta_2\text{nAChRs}\). Failure of \(\alpha_6\text{KO}\) mice to show reduced anxiety-like behavior may suggest that the behavioral assays were not sufficiently stressful to render them sensitive to changes in anxiety-like behavior in this genotype. It may also reflect that other tasks, such as CER or the marble burying task, may be better assays to detect changes in basal anxiety-like behavior in these mice. It is also possible that neuroadaptations during development compensated for the deletion of \(\alpha_6\beta_2\text{nAChRs}\). In Chapter 5, using \(\alpha_4\text{HET}\) mice with a 50% reduction in \(\alpha_4\beta_2\text{nAChRs}\), we also evaluated whether reduced expression of \(\alpha_4\beta_2\text{nAChRs}\) would promote anxiolytic-like behavior in the light-dark, elevated plus and open field assays. In support of the hypothesis that nicotine promotes anxiolysis-like behavior via inactivation of \(\alpha_4\beta_2\text{nAChRs}\), we predict that \(\alpha_4\text{HET}\) mice will show reduced levels of anxiety-like behavior following a saline injection compared to WT controls.

The preponderance of evidence shows that anxiety rates are lower in the geriatric than the general population but higher in those with neurodegenerative diseases (Baune et al., 2012; Beaudreau & O'Hara, 2009; Boralingaiah et al., 2012; Bunce et al., 2012; Cairney et al., 2008; D'Onofrio et al., 2012; Flint, 1994; Goncalves et al., 2011; Higgins et al., 2012; Hynnenen et al., 2012; Kvaal et al., 2008; Ni Mhaolain et al., 2012; Nilsson et al., 2012; O'Connor, 2006; Panza et al., 2012; Potvin et al., 2011; Prina et al., 2011a, 2011b; Rozzini et al., 2009; Samuelsson et
al., 2005; Seby et al., 2011; Sinoff & Werner, 2003; Skoog, 2011; Smalbrugge et al., 2005; Wolitzky-Taylor et al., 2010; Yochim et al., 2013), and that α4β2*nAChRs may protect against age-dependent neurodegeneration (Gotti et al., 2006; Martin-Ruiz et al., 1999; Pimlott et al., 2004; Warpman & Nordberg, 1995; Wevers et al., 1999; Zanardi et al., 2007; Zoli et al., 1999).

In light of these data, Specific Aim 3 is to determine how α4β2*nAChRs may regulate age-related changes in anxiety-like behavior. Previous immunohistochemistry and [3H] nicotine binding studies show age-dependent reductions in α4β2*nAChRs in mice (Gahring, Persiyanov, & Rogers, 2005; S. W. Rogers, Gahring, Collins, & Marks, 1998). We further predict, using immunoprecipitation assays, that we will observe age-dependent reductions in levels of α4 subunit protein. Alternatively, expression of the α4 subunit may not decline, but may stay the same or increase in older animals. This may indicate increased sequestration of the α4 subunit in the endoplasmic reticulum, the assembly of α4β2*nAChRs in the lower sensitivity α4(3)β2(2) stoichiometry, or that the antibodies used in our studies are more selective for the α4 subunit than previous experiments (Anand et al., 1991; Buisson & Bertrand, 2001; Cooper et al., 1991; Covernton & Connolly, 2000; Gahring et al., 2005; Khiroug et al., 2004; H. A. Lester et al., 2009; Moroni et al., 2006; Nelson et al., 2003; S. W. Rogers et al., 1998; Wanamaker & Green, 2007; Zwart & Vijverberg, 1998). Finally, we desired to identify potential brain regions and cellular signaling molecules downstream of α4β2*nAChR activity related to age-dependent changes in anxiety-like behavior. Since ERK phosphorylation in the lateral septum is negatively correlated with exposure to both restraint and chronic housing stress (Sheehan et al., 2003; Singewald et al., 2009), we predict that western blot analyses of brain punches taken from the lateral septum will reveal that levels of ERK phosphorylation will be inversely associated with changes in anxiety-like behavior in Adult and Aged WT and α4HET mice. Failure to detect
significant changes in pERK are possible, since ERK phosphorylation is tightly regulated. We may need to repeat these studies at a different time point to detect relevant changes in pERK in the lateral septum.
Chapter 2 - Low dose nicotine and antagonism of β2 subunit containing nicotinic acetylcholine receptors have similar effects on affective behavior in mice

INTRODUCTION

Human and animal studies indicate that nicotine exerts its psychoactive effects by binding to nicotinic acetylcholine receptors (nAChRs) in the brain (DeNoble & Mele, 2006; Rose et al., 2003). nAChRs comprised of the β2 subunit (β2*nAChRs; *denotes assembly with other subunits) have high binding affinity for nicotine and the endogenous neurotransmitter, acetylcholine (ACh) (Grady et al., 2010; Pauly et al., 1991; Whiting & Lindstrom, 1988; Y. Xiao & Kellar, 2004). β2*nAChRs are enriched on neurons in limbic system brain areas that regulate both affect and reward (Clarke et al., 1985; Court & Clementi, 1995; Fujita et al., 2000; Grady et al., 2010; Kimes et al., 2008; Marks et al., 2010; Marks et al., 1992; Pauly & Collins, 1993; Picciotto et al., 1995; Rubboli et al., 1994; Staley et al., 2005; Y. Xiao & Kellar, 2004) suggesting that these nAChR subtypes may serve a dual role in supporting reward-like behavior and relieving negative affect. nAChRs are ion channels that can be activated as well as desensitized (inactivated) by nicotine (Fenster et al., 1997; R. A. Lester & Dani, 1995; Mansvelder et al., 2002; Pidoplichko et al., 1997). A preponderance of the evidence suggests that activation of β2*nAChRs supports nicotine conditioned place preference and nicotine self-administration, models of nicotine reward and reinforcement (Brunzell et al., 2010; Corrigall et
al., 1994; Gotti et al., 2010; Grabus et al., 2006; Jackson et al., 2009; Maskos et al., 2005; McGranahan et al., 2011; Mineur et al., 2009; Picciotto et al., 1998; Pons et al., 2008; Tapper et al., 2004; Walters et al., 2006) (but see (Exley & Cragg, 2008)). The studies herein used a conditioned emotional response (CER) assay, a marble burying task and an elevated plus maze experiment to test the hypothesis that inactivation of β2*nAChRs attenuates fear and anxiety-like behavior in mice.

CER is an appetitive, operant task in which lever pressing maintained by a positive reinforcer (saccharin solution) is interrupted by presentation of a conditioned stimulus (CS, light and tone) that co-terminates with an aversive unconditioned stimulus (US, 0.1 or 0.3 mA, 0.5 s mild footshock). This study tested if nicotine and a selective antagonist of β2*nAChRs, dihydro-beta-erythroidine (DHβE) would attenuate conditioned suppression of responding in the presence of an aversive CS in the absence of footshock. During CER, subjects serve as their own controls within sessions to return a suppression ratio (SR) score = A/(A+B) where A is lever pressing during the 60 s CS period and B is lever pressing during the 60 s prior to CS presentation. An SR ≈ 0 is indicative of conditioned suppression whereas SR ≈ 0.5 indicates that rodent responding is unaffected by presentation of the CS. The CER assay has good face validity for tobacco users who experience stressors during goal-oriented behavior on a daily basis. Separate groups of mice were tested using an ethological marble burying task where increased digging, rather than suppression of activity, is thought to be interpretable of negative affective behavior, and in an elevated plus maze assay where increased activity in the open arms, relative to the enclosed arms of the maze, is thought to reflect anxiolysis-like behavior. As a follow-up to experiments that evaluated affective-like phenotype, a locomotor activity assay using a beam-
break apparatus confirmed that doses of nicotine and DHβE in these experiments did not affect locomotor activity.

**MATERIALS AND METHODS**

**Subjects**

Twenty-nine C57BL/6J, adult, male mice from Jackson Labs (Bar Harbor, ME) or derived in a Virginia Commonwealth University (VCU) breeding colony were used in these studies. Mice had *ad libitum* access to both food and water in their home cages. Animals were group-housed (2-5 per cage) with 1/8 inch corn cob bedding in a vivarium with a 12h light/dark cycle (lights on 0600). Mice were habituated to the test room and experimenter handling for 3 days prior to any training or testing.

**Ethics Statement**

Efforts were made to minimize mouse discomfort in these experiments. Mild footshock without analgesia and experimenter injections were necessary to perform these studies that model affective-like behavior in mice. Experiments were approved by the VCU Institutional Animal Care and Use Committee (Protocol Number: AM-10163) and were in compliance with the *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 2010).

**Apparatus**

CER experiments were conducted in mouse operant chambers (21.6 cm x 17.8 cm x 12.7 cm; Med Associates, St. Albans, VT). An LED cue light with an opaque cover was positioned 5.5
cm above the operant lever with a liquid dipper receptacle centered on the same wall. A speaker and a 2.24 watt incandescent house-light were positioned 9.5 cm from the floor on the opposite wall of the operant chamber. The floor consisted of steel rods (0.32 cm in diameter placed 0.79 cm apart) connected to a Med Associates shock generator/scrambler. All data were collected via Med PC software. Marble burying took place in a polycarbonate cage (33 cm × 21 cm × 9 cm high) filled with 5 cm of loose wood chip bedding (Harlan Sani-Chip, Indianapolis, IN). The elevated plus maze was constructed of wood with white laminated flooring on two (5 x 30 cm) open arms that were perpendicular to two equivalent, white, laminated, enclosed arms with 15.25 cm black Plexiglas wall enclosures. The entire apparatus was elevated 68 cm above the floor. Experimentation took place under fluorescent light illumination. A ceiling-mounted camera was interfaced to a PC for collection of data using ANY-maze tracking software by Stoelting (Wood Dale, IL). Locomotor testing was conducted in two adjoining chambers (measuring 26.5 cm x 12.7 cm x 26.2 cm and 16.8 cm x 12.7 cm x 12.7 cm). A locomotor unit was defined as the breaking of two adjacent light beams (3 cm apart). Illumination was provided by a single 23 watt fluorescent light bulb. Data was collected using Med Associates software. All experimental chambers were cleaned between animals with 2% Nolvosan (Pfizer Animal Health, Madison, NJ).

Drugs
Nicotine hydrogen tartrate (Sigma Aldrich, St. Louis, MO) was dissolved in 0.9 % sterile saline with pH adjusted to 7.1-7.3. DHβE (Sigma Aldrich, St. Louis, MO) was dissolved in 0.9 % sterile saline. Nicotine doses are expressed as freebase and DHβE doses are expressed as hydrogen bromide salt. As with previous nicotine place conditioning studies (Brunzell et al.,
2009; Mineur et al., 2009), injections were delivered i.p. in volumes of 0.1 ml/30 g. Nicotine was administered immediately prior to CER, marble burying and locomotor tests. After DHβE injection, animals were returned to their home cages for 15 minutes before CER, elevated plus maze and locomotor testing and for 30 minutes prior to the marble burying task. Weights were measured approximately 1 h prior to behavioral assays.

**Behavioral Procedures**

*Conditioned Emotional Response (CER)*

The CER paradigm consisted of several phases of operant and Pavlovian training followed by drug testing sessions where operant responding was tested in the presence of a footshock-paired CS but in the absence of the US footshock (Figure 2.1).

*Magazine Training*

Subjects received eighty presentations of 10 mMol saccharin solution according to a variable interval 30 second (VI-30s) schedule. Mice met criteria when they entered the magazine for 20% of the dipper presentations. Animals failing to meet this criteria were given a second, and if necessary, a third exposure to magazine training before moving on to acquisition of operant responding during overnight sessions.

*CER Acquisition of Lever-Pressing Behavior*

Mice were trained to lever press for 0.01 ml of 10 mMol saccharin solution via a liquid dipper in a single 16 hour overnight session (adapted from (Morris, Dawson, Reynolds, Atack, & Stephens, 2006)). Responding for saccharin reinforcement was maintained on increasing fixed
Figure 2.1 - CER training and drug testing schedule. To introduce mice to the location of saccharin delivery, magazine training (Phase I) occurred over days 1-3. CER acquisition of lever pressing maintained by 10 mMol saccharin solution took place during overnight sessions with increasingly demanding schedules of reinforcement (Phase II; days 4-9) until mice reached criteria of 70 reinforcers and 100 s of correct magazine entries in a session. This was followed by daily 30 minute sessions (Phase III) where mice lever pressing was maintained by saccharin under a variable schedule of reinforcement. Mice moved onto the next Phase of training when they reached criteria of 40 lever presses and 10 reinforcers during a single 30 minute session (days 10-13). During CER training mice continued operant training but also received 2 explicit pairings of a light and tone conditioned stimulus (CS) which co-terminated with a 0.1 or 0.3 mA footshock unconditioned stimulus (US) (Phase IV; days 14-43). Phase IV continued until all mice showed a stable level of CS and NON-CS responding over 3 days. Drug testing (Phase V) consisted of lever pressing maintained by saccharin in the presence of the CS but in the absence of the US. For these studies that used a within-subject, Latin square design, there were at least 2 CER training days in between drug testing sessions to assure that mice returned to baseline prior to the next injection of drug.
ratio (FR) schedules, FR 1 up to 10 reinforcers, FR 2 up to the subsequent 10 reinforcers and FR 4 for the subsequent 20 reinforcers. This was followed by a variable ratio (VR) 5 or variable interval (VI) 15 second schedule of reinforcement until the end of the session. Mice were next trained to lever press during 30 minute daily sessions (1400-1800 h) for a saccharin reinforcer at the assigned variable schedule of reinforcement for which they were trained. Mice meeting a criterion of 10 reinforcers and 40 lever presses in a single session advanced to CER training.

The house light was on during all acquisition and CER procedures in the absence of the CS.

**CER Training (Operant responding with Pavlovian CS + US footshock conditioning)**

During 30 minute CER sessions, saccharin continued to be available according to the variable schedule of reinforcement presented during acquisition. Pseudo-random presentation of two 60 s, compound CSs (house light off + cue light on + 70 dB, 2000 Hz tone) co-terminated with a 0.5 s, 0.3 mA footshock US. A second cohort of animals received all the same conditions but was administered a 0.1 mA footshock US shown previously to not significantly affect suppression ratios (Dixon, Rosahl, & Stephens, 2008). The first CS presentation occurred between min 3 and 12 and the second CS between min 18 and 27. The number of lever presses were recorded both for the 60 s immediately preceding the onset of the CS (Pre-CS) as well as during the CS. The suppression ratio was calculated using the equation \( A/(A+B) \), where \( A \) is the number of responses during the CS and \( B \) equals lever presses in the Pre-CS period (Annau & Kamin, 1961). A suppression ratio of 0.5 indicates no suppression of responding during the CS and a suppression ratio of 0 reflects total suppression of responding during the CS. All active lever-pressing in the absence of the CS was also evaluated (NON-CS). Increases in suppression ratio following drug treatment were interpreted as anxiolytic-like behavior. Once stable baseline
responding and suppression ratios (≤ 0.1 for the 0.3 mA condition) were established for 3 consecutive days, mice proceeded to drug testing.

**CER Drug Testing**

Drug testing took place in the absence of footshock US using a within-subject Latin square design. Animals received 0, 0.0032, 0.01, 0.032 or 0.1 mg/kg i.p. nicotine or 0, 0.1, 0.3, 1 or 3 mg/kg i.p. DHβE before CER. At least 2 days of CER training were administered between doses to allow for wash-out of drug. These intermediate training sessions further assured that responding returned to baseline between doses of drug.

**Marble Burying**

Using a within-subject, Latin Square design, separate groups of mice received 0, 0.01 and 0.032 mg/kg i.p. nicotine or 0 and 3 mg/kg i.p. DHβE. Marble burying sessions were separated by at least 5 days as has been demonstrated to provide a steady level of digging behavior in the absence of treatment (Broekkamp, Rijk, Joly-Gelouin, & Lloyd, 1986; A. Thomas et al., 2009). Prior to each test, 20 green, glass marbles (10 mm diameter) were evenly arranged in a 4 x 5 grid on loose sawdust bedding. Individual mice were placed into the side of the experimental cage so as to not disturb any of the marbles. At the conclusion of the 15 minute test, mice were returned to the home cage; marbles at least 50% covered by the bedding were counted as buried.

**Elevated Plus Maze**

Using a between-subject design, mice receiving 0, 0.03 or 3 mg/kg i.p. DHβE were returned to their home cage for a 15 min wait period and subsequently placed on the center of an elevated
plus maze apparatus \( n = 9 - 11 \) per dose. Behavior was evaluated for a period of 10 minutes. Subjects were scored for open arm entries, time spent in the open arms and latency to explore the terminal zones (the extreme 5 cm) of the open arms.

**Locomotor Test**

Using a between-subject design, animals received nicotine (0, 0.01 or 0.032 mg/kg i.p. by weight of freebase) immediately prior to placement into the small chamber of the Med Associates apparatus. The door separating the two chambers was opened, allowing animals free mobility throughout the apparatus. Breaking of two adjacent beams (3 cm equidistant apart) constituted a locomotor activity count. Behavior was assessed for ten minutes. Mice that received DHβE (0, 0.3 or 3 mg/kg i.p.) were placed in their home cages for 15 min following injections with the other locomotor procedures as described for nicotine subjects.

**Statistical Analysis**

For CER experiments, repeated measures ANOVA assessed the effect of nicotine and DHβE on suppression ratio, lever presses/minute during the CS period and lever presses/min during the NON-CS period. Paired t-tests were used as post-hoc tests where appropriate. Student’s t-test was used to assess the effect of footshock intensity on suppression ratios. Repeated measures ANOVA and paired t-tests were used to evaluate the effect of nicotine and DHβE on number of marbles buried. ANOVA tests assessed elevated plus maze activity as measured by open arm entries, time on the open arms and latency to reach the end terminal 5cm of the open arms. Post-hoc t-tests and planned comparisons were performed between vehicle and drug-injected subjects.
One-way ANOVA tested the effects of drug doses on locomotor activity. Confidence intervals of $P < 0.05$ were reported as significant.

**RESULTS**

On the 3 days of CER training prior to CER testing, NON-CS responding was stable ($F$’s < 1) and suppression ratios were consistently lower than 0.1 for mice trained to a 0.3 mA footshock. The suppression ratios for mice receiving 0.1 mA footshock US were significantly higher than mice exposed to 0.3 mA footshock US following saline injection (0.1 mA = 0.69 ± 0.15; 0.3 mA = 0.03 ± 0.02) ($t_{14} = 5.691, P < 0.001$). In contrast to mice that received a 0.3 mA footshock, mice trained with a 0.1 mA footshock did not show a suppression of responding during the CS, indicating that this suppression of behavior in 0.3 mA-trained mice was a conditioned response to an aversive CS and not due to a more generalized orienting response to the compound stimulus CS used in these experiments. There was no effect of footshock intensity on overall NON-CS or Pre-CS lever pressing ($F$’s < 1). Supplemental Figure 2.1 shows average lever presses per minute during the entire 30 minute sessions. There was no significant effect of schedule of reinforcement on suppression ratios in 0.3 mA mice receiving 0.01 ml saccharin solution according to VI-15 sec or VR5 schedules of operant reinforcement following nicotine ($F_{1,8} = 0.060, P = 0.812$) or DHβE ($F_{1,5} = 0.290, P = 0.613$), so these groups were combined during subsequent analyses. Administration of nicotine resulted in a dose-dependent increase in suppression ratio ($F_{4,9} = 3.101, P < 0.05$) for mice exposed to the 0.3 mA US. Post hoc t-tests revealed that low doses of nicotine (0.01 and 0.032 mg/kg) significantly reversed conditioned suppression of responding in comparison to when animals received saline ($t_{9} = 2.663$ and $2.331$, respectively).
Despite consistent trends for elevated responding at doses of nicotine that reversed conditioned suppression, raw scores for CS lever pressing failed to reach significance following nicotine injection ($F_{4,9} = 0.867, P > 0.05$; Table 2.1). Unlike suppression ratios, there was no effect of drug treatment on Pre-CS responding ($F_{4,9} = 1.771, P > 0.05$). There was an effect of nicotine treatment observed for NON-CS responding, however ($F_{4,9} = 9.832, P < 0.001$). Post hoc tests showed that NON-CS lever pressing was elevated in mice following 0.0032 mg/kg ($t_9 = 3.820, P < 0.01$), 0.032 mg/kg ($t_9 = 4.941, P < 0.001$) and 0.1 mg/kg ($t_9 = 2.483, P < 0.05$) compared to treatment with saline (Table 2.2). It is possible that nicotine was enhancing the reinforcing efficacy of the saccharin stimulus as has been observed for a visual cue (Caggiula et al., 2001; Donny et al., 2000). Further analysis comparing mice against their responding prior to any injections indicated that this may have been due in part to anxiolytic effects of nicotine as well. Compared to days when they had received no injection, there was a significant decrease in NON-CS responding of mice following saline injection ($t_9 = 4.683, P < 0.001$), suggesting that the stress of injection led to an overall reduction in lever pressing activity (Table 2.2). Nicotine injection appeared to reverse this effect; low doses of nicotine (0.01 and 0.032 mg/kg i.p.) that elevated suppression ratio responding resulted in NON-CS lever pressing that did not differ from pre-injection responding. A rewarding-like dose of nicotine (0.1 mg/kg i.p.) (Brunzell et al., 2009; McGranahan et al., 2011; Mineur et al., 2009) resulted in a similar elevation of responding compared to saline injection, however, there were also significantly fewer lever presses during the NON-CS compared to when no injection was given, suggesting that this dose was not effective at reversing suppression of overall responding that was stimulated by the stress of injection.
Figure 2.2 - Low dose nicotine and the β2*nAChR antagonist DHβE reverse conditioned suppression. In mice conditioned to a 0.3 mA unconditioned stimulus footshock during CER training, a) administration of nicotine resulted in a dose-dependent reversal of conditioned suppression as measured by increased suppression ratios ($F_{4,9} = 3.101, P < 0.05; n = 10$). b) The β2*nAChR antagonist DHβE also resulted in a significant increase in suppression ratios in these mice ($F_{4,6} = 2.934, P < 0.05; n = 7$), suggesting that inhibition of β2*nAChRs supports anxiolytic-like behavior in the CER assay. c, d) Neither nicotine ($F_{4,5} = 1.991, P > 0.05; n = 6$) nor DHβE ($F_{4,5} = 1.263, P > 0.05; n = 6$) resulted in significant changes in suppression ratio responding in mice exposed to 0.1 mA US footshock during CER training. Data are presented as means ± SEM. *$P < 0.05$ compared to when mice received saline control injections.
Table 2.1 - Lever pressing activity during the CS and Pre-CS period.

<table>
<thead>
<tr>
<th>Nicotine (mg/kg)</th>
<th>Pre-CS 0.1 mA</th>
<th>CS 0.1 mA</th>
<th>Pre-CS 0.3 mA</th>
<th>CS 0.3 mA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Drug</td>
<td>2.83±1.17</td>
<td>1.50±0.26</td>
<td>1.60±0.40</td>
<td>0.30±0.17</td>
</tr>
<tr>
<td>0</td>
<td>1.58±0.97</td>
<td>1.50±0.47</td>
<td>0.80±0.40</td>
<td>0.10±0.07</td>
</tr>
<tr>
<td>0.0032</td>
<td>1.00±0.51</td>
<td>1.50±0.47</td>
<td>2.07±0.11</td>
<td>0.21±0.12</td>
</tr>
<tr>
<td>0.01</td>
<td>2.83±1.38</td>
<td>1.58±0.52</td>
<td>1.90±0.49</td>
<td>0.45±0.16</td>
</tr>
<tr>
<td>0.032</td>
<td>4.00±1.48</td>
<td>1.58±0.97</td>
<td>3.86±1.79</td>
<td>0.36±0.14</td>
</tr>
<tr>
<td>0.1</td>
<td>1.25±0.69</td>
<td>1.17±0.54</td>
<td>2.25±0.68</td>
<td>0.20±0.20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DHβE (mg/kg)</th>
<th>Pre-CS</th>
<th>CS</th>
<th>Pre-CS</th>
<th>CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Drug</td>
<td>2.75±1.33</td>
<td>3.92±1.48</td>
<td>1.86±0.66</td>
<td>0.14±0.09</td>
</tr>
<tr>
<td>0</td>
<td>1.58±0.97</td>
<td>1.50±0.47</td>
<td>1.07±0.55</td>
<td>0.14±0.09</td>
</tr>
<tr>
<td>0.1</td>
<td>3.50±1.48</td>
<td>2.25±0.68</td>
<td>1.25±0.36</td>
<td>0.25±0.14</td>
</tr>
<tr>
<td>0.3</td>
<td>0.75±0.34</td>
<td>1.83±0.38</td>
<td>1.93±0.63</td>
<td>0.14±0.09</td>
</tr>
<tr>
<td>1</td>
<td>2.80±0.88</td>
<td>0.80±0.21</td>
<td>2.36±0.70</td>
<td>0.50±0.29</td>
</tr>
<tr>
<td>3</td>
<td>1.75±0.48</td>
<td>1.67±0.69</td>
<td>2.86±1.29</td>
<td>1.71±0.79</td>
</tr>
</tbody>
</table>

Lever presses per minute are depicted for mice during presentation of the 60 s conditioned stimulus (CS) and during the 60 s period prior to CS presentation (Pre-CS). Pre-Drug levels of responding are depicted for the last day of training prior to drug testing sessions (Pre-Drug) and during test sessions following each of five i.p. doses of nicotine or DHβE. Data are represented as means ± SEM.
Table 2.2 - Lever pressing activity in the absence of the CS.

<table>
<thead>
<tr>
<th>Nicotine (mg/kg)</th>
<th>0.1 mA</th>
<th>0.3 mA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-CS</td>
<td>NON-CS</td>
<td>Non-CS</td>
</tr>
<tr>
<td>Pre Drug</td>
<td>2.68±0.95</td>
<td>1.92±0.28</td>
</tr>
<tr>
<td>0</td>
<td>1.89±0.64</td>
<td>0.94±0.22*</td>
</tr>
<tr>
<td>0.0032</td>
<td>1.31±0.48</td>
<td>1.78±0.30*</td>
</tr>
<tr>
<td>0.01</td>
<td>1.58±0.57</td>
<td>1.40±0.15</td>
</tr>
<tr>
<td>0.032</td>
<td>1.90±0.67</td>
<td>2.09±0.27*</td>
</tr>
<tr>
<td>0.1</td>
<td>1.10±0.42</td>
<td>1.30±0.18**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DHβE (mg/kg)</th>
<th>Non-CS</th>
<th>Non-CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre Drug</td>
<td>3.33±0.98</td>
<td>2.87±0.49</td>
</tr>
<tr>
<td>0</td>
<td>1.89±0.64*</td>
<td>1.21±0.24*</td>
</tr>
<tr>
<td>0.1</td>
<td>1.98±0.69</td>
<td>2.30±0.41</td>
</tr>
<tr>
<td>0.3</td>
<td>2.21±0.87</td>
<td>1.83±0.30</td>
</tr>
<tr>
<td>1</td>
<td>2.43±0.72</td>
<td>2.17±0.26</td>
</tr>
<tr>
<td>3</td>
<td>1.99±0.50</td>
<td>2.41±0.51</td>
</tr>
</tbody>
</table>

Lever presses per minute are depicted for mice in the absence of the conditioned stimulus (NON-CS) on the day prior to drug testing sessions (Pre-Drug) and during test sessions following each of five i.p. doses of nicotine or DHβE. Data are represented as means ± SEM; *Significantly different from Pre-drug training (P < 0.05); **Significantly different from test sessions following saline injection (0 mg/kg; P < 0.05).
Administration of nicotine did not significantly affect suppression ratios ($F_{4,5} = 1.991, P = 0.135$; Fig 2.2c), CS responding ($F_{4,5} = 0.103, P > 0.05$), Pre-CS responding ($F_{4,5} = 2.245, P > 0.05$; Table 2.1) or NON-CS responding ($F_{4,5} = 1.46, P > 0.05$; Table 2.2) in mice exposed to the 0.1 mA footshock US. A dose-dependent reversal of conditioned suppression was also observed following treatment with the selective β2*nAChR antagonist DHβE ($F_{4,6} = 2.934, P < 0.05$). Compared to when they received saline, mice showed a significant increase in suppression ratios following injection of 3 mg/kg i.p. DHβE ($t_6 = 2.614, P < 0.05$; Fig 2.2b) suggesting that antagonism of the β2*nAChRs, like low dose nicotine, reverses conditioned inhibition of behavior in this task. Pre-treatment with DHβE resulted in a dose-dependent increase in total lever pressing in the presence of the CS ($F_{4,6} = 3.338, P < 0.05$) reflecting a trend for elevated responding during the CS after administration of 3 mg/kg i.p. DHβE compared to saline ($t_9 = 2.049, P = 0.086$; Table 2.1). As observed during the nicotine treatment regimen above, Pre-CS responding was not significantly affected by DHβE exposure ($F_{4,6} = 1.382, P > 0.05$; Table 2.1), but total NON-CS lever pressing was reduced in mice following saline injection compared to the training session that immediately preceding the drug testing phase for DHβE ($t_6 = 3.113, P < 0.05$; Table 2.2). Unlike nicotine, DHβE did not significantly affect responding in the absence of the CS (Table 2.2). Similarly to nicotine, there were no effects of i.p. DHβE on suppression ratios ($F_{4,5} = 1.263, P = 0.317$; Fig 2.2d), CS lever pressing ($F_{4,5} = 1.334, P > 0.05$), Pre-CS responding ($F_{4,5} = 2.274, P > 0.05$; Table 2.1) or NON-CS responding ($F_{4,5} = 1.112, P > 0.05$; Table 2.2) in mice trained with 0.1 mA footshock US. As observed with 0.3 mA-trained mice, NON-CS responding was lower in mice following saline injection compared to responding during training sessions immediately prior to DHβE drug testing ($t_5 = 3.451, P < 0.05$; Table
suggesting that the stress of injection may have led to a suppression of overall lever-pressing activity.

A separate group of mice were tested in a marble burying task, an ethological measure of digging behavior that is thought to reflect changes in rodent affect (Broekkamp et al., 1986; Bruins Slot, Bardin, Auclair, Depoortere, & Newman-Tancredi, 2008; Deacon, 2006; Egashira et al., 2008; Ichimaru, Egawa, & Sawa, 1995; Kinsey, O'Neal, Long, Cravatt, & Lichtman, 2011; Nicolas, Kolb, & Prinssen, 2006; Njung'e & Handley, 1991; Pelleymounter, Joppa, Ling, & Foster, 2002; Turner et al., 2010). Doses of i.p. nicotine (0.01 and 0.032 mg/kg) and i.p. DHβE (3 mg/kg) that were capable of increasing lever pressing maintained by saccharin during presentation of an aversive CS also led to a significant decrease of marble burying in an open, exposed environment. Repeated measures ANOVA revealed a significant effect of nicotine exposure on digging behavior as measured by marbles buried ($F_{2,13} = 4.022, P < 0.05$). Consistent with results from CER, post hoc t-tests revealed that mice buried fewer marbles after 0.01 and 0.032 mg/kg i.p. nicotine than when they received saline ($t_{13} = 2.747, P < 0.05$ and $t_{13} = 2.376, P < 0.05$, respectively; Fig 2.3a). DHβE-injected mice also buried significantly fewer marbles than after saline vehicle in the marble burying task ($t_{14} = 1.781, P < 0.05$; Fig 2.3b).

Antagonism of β2*nAChRs also affected the behavior of mice in the elevated plus maze.

ANOVA analysis revealed a significant effect of DHβE treatment on latency to reach the terminus of the open arms ($F_{2,30} = 4.449, P < 0.05$). Post-hoc tests revealed that mice receiving 0.3 mg/kg i.p. DHβE required significantly less time to explore the terminal ends of the open arms compared to mice receiving saline ($t_{17.341} = 2.769, P < 0.05$; Fig 2.4a). Despite similar
Figure 2.3 - Nicotine and DHβE resulted in fewer marbles buried.  

a) The 0.01 and 0.032 mg/kg i.p. nicotine that promoted anxiolytic-like behavior in the CER task also resulted in a significant reduction in digging behavior as measured by fewer marbles buried compared to when mice were administered saline (n = 14). 

b) Similarly, mice treated with 3 mg/kg i.p. DHβE also buried less marbles compared to when they received saline treatment (n = 15). Data are presented as means ± SEM; *P < 0.05 compared to when mice received saline control injections.
Figure 2.4. Antagonism of β2*nAChRs promoted anxiolysis-like behavior in the elevated plus maze. a) Mice receiving 0.3 mg/kg i.p. DHβE required less time to explore the end terminus of the open arms of an elevated plus maze, b) to enter the open arms of the maze and c) spent more time in the open arms than saline-injected mice. Data are presented as means ± SEM; *$P < 0.05$, $#P = 0.067$ compared to saline controls.
trends for DHβE-associated increases in open arm entries and total time spent in the open arms, ANOVA tests failed to return a significant effect of treatment for these respective measures ($F_{2,30} = 2.258, P > 0.05$; $F_{2,30} = 2.219, P > 0.05$; Fig 2.4b), but planned comparisons revealed that mice receiving 0.3 mg/kg i.p. DHβE had significantly greater number of open arm entries than saline-injected mice ($t_{12.682} = 2.610, P < 0.05$; Fig 2.4b) and spent significantly more time in the open arms of the maze compared to saline controls ($t_{13.490} = 2.753, P < 0.05$; Fig 2.4c). Although there was a trend for mice receiving 3 mg/kg DHβE to spend more time in the open arms ($t_{10.992} = 2.034, P = 0.068$), behavioral measures for this dose of DHβE failed to reach significance for any behavioral measure in the elevated plus maze.

To further determine if the observed behavioral effects of nicotine and DHβE were due in part to non-specific changes in locomotion, mice were tested in a locomotor activity beam-break apparatus following administration of saline and doses of nicotine (0.01 and 0.032 mg/kg) and DHβE (0.3 and 3 mg/kg) that reversed conditioned suppression in the CER assay, decreased digging in the marble burying task, or that increased open arm activity during the elevated plus maze test. In comparison to saline-injected animals, there were no observable effects of i.p. nicotine ($F_{2,14} = 0.072, P > 0.05$) or i.p. DHβE ($F_{2,13} = 1.451, P > 0.05$) on locomotor activity (Fig 2.5).

**DISCUSSION**

In the present experiments, low dose nicotine and a selective antagonist of β2*nAChRs decreased fear- and anxiety-like behavior in three separate animal models of affect. There was a
Figure 2.5 - Nicotine and DHβE did not affect locomotor activity. a) Doses of nicotine (n = 5 - 6) and b) DHβE (n = 4 - 5) that promoted anxiolytic-like behavior in the CER and marble burying tasks did not affect locomotor activity as measured by beam breaks ($F’$s = 0.072, 1.451, $P’$s > 0.05). Data are presented as means ± SEM.
significant reversal of conditioned suppression of lever pressing in mice treated with 0.01 and 0.032 mg/kg i.p. nicotine but a 0.1 mg/kg i.p. dose of nicotine that has been shown to be rewarding during conditioned place preference (Brunzell et al., 2009; McGranahan et al., 2011; Mineur et al., 2009) had no effect. Mice treated with these low doses of nicotine also buried fewer marbles compared to when they were treated with saline in an ethological marble burying task, and previous studies show that similarly low doses of nicotine decrease anxiety-like behavior as measured by increases in open arm activity in an elevated plus maze (McGranahan et al., 2011; Varani, Moutinho, Bettler, & Balero, 2012). The present findings expand on previous data to show that inactivation of the high affinity β2*nAChRs has similar effects of low dose nicotine on these affective tasks. DHBβE dose dependently increased responding for a saccharin reinforcer during the presentation of an aversive CS, significantly decreased marble burying in an open, exposed environment and significantly increased exploration of the open arms of an elevated plus maze. These divergent behavior-stimulating and behavior-inhibiting measures indicate that these observations were not due to non-selective effects of DHBβE or nicotine on activity. Neither effective doses of nicotine nor DHBβE showed any change in beam break activity during a locomotor task. Together these findings suggest that low dose nicotine and DHBβE attenuate negative affective and anxiety-like behavior.

Together with previous findings, these studies identify a dual role for β2*nAChRs in regulating nicotine reinforcement and relieving negative affective behavior. Whereas nicotine reinforcement and reward-like behavior requires activation of β2*nAChRs (Brunzell et al., 2010; Corrigall et al., 1994; Gotti et al., 2010; Grabus et al., 2006; Jackson et al., 2009; X. Liu et al., 2003; Maskos et al., 2005; McGranahan et al., 2011; Picciotto et al., 1998; Pons et al., 2008;
Tapper et al., 2004; Walters et al., 2006), the studies described herein suggest that inactivation of β2*nAChRs decreases fear-like and anxiety-like behavior as measured by increased suppression ratios during CER, decreased digging behavior in a marble burying task, and increased exploratory behavior in the open arms of an elevated plus maze. Rats will self-administer A-85830, a selective agonist of β2*nAChRs (X. Liu et al., 2003). Administration of selective β2*nAChR antagonists blocks nicotine conditioned place preference and greatly attenuates nicotine self-administration (Brunzell et al., 2010; Corrigall et al., 1994; Gotti et al., 2010; Grabus et al., 2006; Jackson et al., 2009). This is in contrast to the current studies which show that systemic administration of DHβE promotes lever pressing maintained by saccharin during presentation of a stressful cue. The current studies also showed that subthreshold doses for nicotine conditioned place preference, but not a reward-like dose, were capable of increasing suppression ratios during the CER operant task. The non-selective nAChR antagonist mecamylamine has been shown to have anxiolytic efficacy in the elevated plus maze, social interaction and marble burying tasks (Hall, Pearson, & Buccafusco, 2010; Newman, Manresa, Sanberg, & Shytle, 2002; Newman, Nazian, Sanberg, Diamond, & Shytle, 2001; Roni & Rahman, 2011). The present findings expand on this work to show that inhibition of β2*nAChRs is sufficient to decrease fear-like behavior and increase anxiolytic-like behavior in both operant and ethological tasks.

Low dose nicotine had similar effects as DHβE to decrease negative affective behavior. Although the mechanism of how DHβE and nicotine act at nAChRs has not been clearly elucidated in an awake, behaving animal, in vitro and ex vivo studies show that nicotine promotes both activation and desensitization of nAChRs (Fenster et al., 1997; Grady et al., 2012;
Kuryatov et al., 2011; R. A. Lester & Dani, 1995; Lu et al., 1999; Mansvelder et al., 2002; Pidoplichko et al., 1997); hence nicotine-associated desensitization could result in a behavioral phenotype that is similar to nAChR antagonism. Micromolar concentrations of nicotine activate β2*nAChRs, facilitating neurotransmitter release (Fenster et al., 1997; R. A. Lester & Dani, 1995; Mansvelder et al., 2002; Pidoplichko et al., 1997). This is followed by rapid desensitization of the β2*nAChRs (Fenster et al., 1997; R. A. Lester & Dani, 1995; Mansvelder et al., 2002; Pidoplichko et al., 1997). In vitro studies further show that nanomolar concentrations of nicotine can result in preferential desensitization of β2*nAChRs (Fenster et al., 1997; Grady et al., 2012; Kuryatov et al., 2011; Lu et al., 1999). Low levels of nicotine increase the likelihood that β2*nAChR stoichiometry will favor the desensitized state. Consistent with these observations, reductions in marble burying are also observed following administration of partial agonists of β2*nAChRs, including varenicline and sasetidine-a (Levin et al., 2010; Newman et al., 2002; Turner et al., 2010; Y. Xiao et al., 2006). The present data suggest that behavioral effects of partial agonists in the marble burying and CER tasks are likely due to inhibition rather than activation of the β2*nAChRs.

It is not clear from these studies which nAChR subunits in combination with β2 might require inactivation to promote the anxiolytic-like effects of nicotine. DHβE has high affinity for α6β2*nAChRs and α4β2*nAChRs (Grady et al., 2010; Papke et al., 2008) although a large part of the sensitivity appears to be driven by the α4 subunit (Papke et al., 2008). Recent work using the elevated plus maze as a measure of anxiety reported that α4 knockout mice fail to show nicotine-associated anxiolysis (McGranahan et al., 2011). There was no genotypic effect of the α4 null mutation in the absence of drug, so it is not clear if activation or inhibition of
α4β2*nAChRs or perhaps some other α4*nAChR is regulating open arm activity in this task (Bencan & Levin, 2008; Decker et al., 1995; McGranahan et al., 2011). Selective deletion of the α4 subunit in ventral tegmental area (VTA) DA neurons attenuated the effects of nicotine on open arm entries in the elevated plus maze, suggesting a possible role for the mesolimbic DA pathway in support of anxiolysis-like behavior (McGranahan et al., 2011). Given recent data to suggest that VTA GABA neurons promote conditioned aversion and counter appetitive behavior via inhibition of DA neuron signaling (Tan et al., 2012; van Zessen, Phillips, Budygin, & Stuber, 2012), it is possible that blockade of α4β2*nAChR activity on GABA terminals could promote anxiolysis-like behavior via disinhibition of DA neurons (Mansvelder et al., 2002; Mineur et al., 2009; Pidoplichko et al., 1997). It is not clear if α6β2*nAChRs, which are enriched in catecholaminergic nuclei (Champtiaux et al., 2002; Marks et al., 2010; Whiteaker et al., 2000) (but see (Yang et al., 2011)) might contribute to anxiety-like behavior. Slice electrophysiology studies show that α4α6β2*nAChRs on DA neurons in the posterior VTA are highly sensitive to even nM concentrations of nicotine and are resistant to desensitization (L. Liu et al., 2012) suggesting their activity, rather than their inhibition, may promote nicotine-associated anxiolysis in response to low doses of nicotine. As further support that desensitization of α4β2*nAChRs promotes anxiolysis-like behavior, the α4β2*nAChRs, but not α6β2*nAChRs, are localized in the basolateral amygdala where selective removal of ACh inputs decreases anxiety-like behavior (Bahi, Mineur, & Picciotto, 2009; Champtiaux et al., 2002; Clarke et al., 1985; Marks et al., 2010; Power & McGaugh, 2002; Whiteaker et al., 2000). A lack of compounds with selectivity for α6β2*nAChRs that cross the blood brain barrier make it difficult to assess the stoichiometry of the β2*nAChRs that support the systemic effects of nicotine; future studies using selective peptide infusions in brain e.g. (Brunzell et al., 2010; Gotti et al., 2010; Jackson et al., 2009) will
help parse the subunit configurations in combination with β2 that promote anxiolysis via inhibition of β2*nAChRs.

Behaviorally, these nicotine findings are consistent with previous data. A preponderance of the evidence suggests that low doses of nicotine promote anxiolysis-like behavior (Cheeta, Irvine, et al., 2001; File et al., 1998; Irvine et al., 1999; McGranahan et al., 2011), moderate doses of nicotine support reward-like behavior (Brunzell et al., 2009; McGranahan et al., 2011; Mineur et al., 2009), and high doses of nicotine increase anxiety-like behaviors (Cheeta, Tucci, et al., 2001; File et al., 2000; File et al., 1998; Ouagazzal et al., 1999). Similarly to low dose nicotine and DHβE, anxiolytic drugs such as benzodiazepines increase lever pressing during a presentation of an aversive CS compared to when saline is administered (Annau & Kamin, 1961; M. Davis, 1990; Dixon et al., 2008; Martin, Ballard, & Higgins, 2002; Mathiasen, Rodgers, & Mirza, 2007; Morris et al., 2006; Scobie & Garske, 1970; Stanhope & Dourish, 1996), decrease digging in the marble burying task (Broekkamp et al., 1986; Deacon, 2006; Kinsey et al., 2011; Nicolas et al., 2006; Turner et al., 2010) and increase open arm activity in an elevated plus maze. Studies in humans show that trait anxiety leads to elevated cued fear conditioning of aversive stimuli and imaging studies show this behavioral tendency is positively correlated with an exaggerated activation of the amygdala and anterior cingulate cortex, brain areas shown to regulate rodent behavior during fear conditioning tasks (Britton et al., 2011; Craske et al., 2008; Delgado, Olsson, & Phelps, 2006; Lang & McTeague, 2009; Sehlmeyer et al., 2011; Waters, Henry, & Neumann, 2009). CER, marble burying and the elevated plus maze have good predictive validity for anxiolytic drug efficacy (Annau & Kamin, 1961; Broekkamp et al., 1986; M. Davis, 1990; Deacon, 2006; Dixon et al., 2008; Garrett, Nekrasz, Haque, Parker, & Seale, 1998;
Together with previous data, the present studies suggest that inactivation of nAChRs may promote anxiolysis-like behavior and may have mechanistic implications for why individuals smoke to relieve anxiety.

These studies utilized CER, marble-burying and an elevated plus maze task to show that nicotine and DHβE could both stimulate and suppress behavior in a way that is consistent with currently available anxiolytic drugs (Broekkamp et al., 1986; Garrett et al., 1998; Griebel et al., 2000; N. J. Johnson & Rodgers, 1996; Kinsey et al., 2011; Nicolas et al., 2006; Njung'e & Handley, 1991; Pellemounter et al., 2002; Pellow et al., 1985; Turner et al., 2010; Wiley et al., 1995). Marble burying, however is also sensitive to antidepressant drugs and antipsychotics (Bruins Slot et al., 2008; Egashira et al., 2008; Ichimaru et al., 1995; Nicolas et al., 2006) suggesting that digging behavior in rodents may be driven by an underlying system that is common to the effects of these diverse drug classes. Individuals diagnosed with anxiety disorder, depression or schizophrenia all have a significantly elevated risk for tobacco dependence (Leonard et al., 2001; Ziedonis et al., 2008). In addition to the high concordance with tobacco use, there is a high comorbidity for diagnosis of anxiety with depression and schizophrenia, suggesting that there is a common underlying etiology for these disorders (Buckley, Miller, Lehrer, & Castle, 2009). Some suggest that the “non-purposeful” digging behavior in the marble burying task may model obsessive compulsive disorder (Bruins Slot et al., 2008; Egashira et al., 2008; Ichimaru et al., 1995).
Drugs such as clozapine, aripiprazole and risperidone that are used to augment the effects of mood stabilizers also reduce marble burying activity (Bruins Slot et al., 2008; Egashira et al., 2008). β2*nAChRs are ubiquitously expressed in the brain (Champtiaux et al., 2002; Chefer et al., 1998; Marks et al., 2010; Vaupel et al., 1998; Whiteaker et al., 2000) where their activation on the neuron soma and terminals promotes release of GABA, serotonin, DA, norepinephrine and ACh, neurotransmitters that regulate mood and arousal and that are believed to contribute to the etiology of anxiety, depression and schizophrenia (Picciotto et al., 2001). The β2*nAChRs have also been implicated in contributing to rodent models of depression-like behavior with mecamylamine and partial agonists of β2*nAChRs showing anti-depressant-like efficacy (Mineur, Somenzi, & Picciotto, 2007; Rabenstein, Caldarone, & Picciotto, 2006; Rollema et al., 2009). Unlike our observations in the marble burying task, however, administration of DHβE blocks the antidepressant-like effects of the β2*nAChR partial agonists varenicline and sazetidine in the forced swim task (Caldarone et al., 2011), showing a dichotomy with the present results in the elevated plus maze which suggest that antagonism of β2*nAChRs promotes anxiolysis-like behavior.

It is possible that our findings in the CER task reflect changes in learning that are independent of fear and anxiety-like behavior. While it is possible that drug injection could result in state-dependent learning effects, we do not believe this was the case given that animals showed dose-dependent effects of nicotine and DHβE using a within-subject, Latin Square design. Place conditioning and drug discrimination studies clearly demonstrate that mice can physiologically detect the 0.1 mg/kg dose of nicotine (Brunzell et al., 2009; Chance, Murfin, Krynock, & Rosecrans, 1977; Le Foll, Justinova, Wertheim, Barnes, & Goldberg, 2008; Le Foll, Wertheim,
yet this dose did not reverse conditioned suppression as low doses did in these studies, suggesting that the effects of nicotine and DHβE on suppression ratios during CER were not due to a generalized decrement caused by state-dependent learning. It is also possible that the injection itself could have served as an occasion-setter to indicate that no shock would occur during these test sessions. This was not the case. Rather, saline injection led to a decrease in NON-CS responding during these test sessions, suggesting that the stress of injection led to a reduction in goal-oriented behavior as measured by lever pressing for saccharin. Several doses of nicotine, including a rewarding-like dose, reversed this suppression of NON-CS responding. Whereas it is possible that this behavior was stimulated by anxiolytic-like effects of nicotine, it is equally plausible that nicotine exposure promoted stimulus enhancing effects of the saccharin reinforcer as has been shown for an unconditioned stimulus light and a conditioned stimulus associated with an appetitive stimulus (Brunzell et al., 2006; Caggiula et al., 2001; Donny et al., 2000; Olasson, Jentsch, & Taylor, 2004). The present results also showed an interesting contrast to findings using Pavlovian fear conditioning without an operant component. Unlike our observations in the CER task, systemic administration of nicotine enhances freezing in a footshock-paired context with no effect on explicit cue conditioning (Gould, 2003; Gould & Wehner, 1999). These dichotomies may be due in part to the use of a more mild footshock and extended explicit cue CS training used during CER compared to traditional Pavlovian fear conditioning procedures. A significant difference in CS but not NON-CS lever pressing between mice trained to a 0.1 mA and 0.3 mA footshock suggests that the contextual fear did not contribute to CER behavior in these studies. In addition, systemic administration of DHβE alone does not affect either context or explicit cue CS-freezing
following fear conditioning (J. A. Davis & Gould, 2006), drawing a further contrast between these procedures. Together these findings suggest that basic Pavlovian fear conditioning and CER are modeling different behaviors. These data further suggest that CER, but not Pavlovian fear conditioning, is sensitive to inactivation of the high affinity $\beta_2*nAChRs$. Whereas the CER paradigm is a complex animal model that involves fear learning and operant behavior, this procedure benefits from subjects acting as their own controls both within and between sessions. The fact that mice showed similar effects in the marble-burying task and elevated plus maze, which do not have a learning components to them, supports the hypothesis that affective behavior was modified by nicotine and DH$\beta$E during CER.

Studies in human smokers reveal that multiple factors contribute to tobacco use; as well as the pleasure received from smoking, many report that they use tobacco to relieve anxiety or to relax (Berlin et al., 2003; Fidler & West, 2009; Livson & Leino, 1988; Perkins & Grobe, 1992; Shiffman et al., 1997; Shiffman & Waters, 2004; Skara et al., 2001). The first cigarette of the day results in an abrupt increase in nicotine plasma concentrations that smokers associate with the rewarding effects of the drug (Benowitz, 1988; Benowitz & Jacob, 1984; Fagerstrom et al., 1990). The nicotine ingested from a single cigarette is sufficient to occupy 80% of $\beta_2*nAChRs$ (Brody, Mandelkern, Costello, et al., 2009; Brody et al., 2006). During subsequent smoking episodes, smokers achieve smaller increases in nicotine that ought to preferentially favor desensitization of nAChRs (Benowitz, 1988; Benowitz & Jacob, 1984) if slice electrophysiology, Xenopus oocyte, tissue culture and synaptosome studies are predictive of how nAChRs function in vivo (Fenster et al., 1997; Grady et al., 2012; Kuryatov et al., 2011; R. A. Lester & Dani, 1995; Lu et al., 1999; Mansvelder et al., 2002; Pidoplichko et al., 1997). Nicotine reaches daily...
steady-state concentrations in the brains of human smokers between 200-400 nM. ACh is a major neuromodulator in brain that is thought to regulate anxiety-like behavior (DeBiasi, 2010; Power & McGaugh, 2002). As nicotine levels drop, populations of β2*nAChRs in brain regions that regulate anxiety become available for activation by ACh in response to stressful stimuli such as cigarette/tobacco cues (Carter et al., 2006; Lazev, Herzog, & Brandon, 1999; Littel & Franken, 2007; McDonough & Warren, 2001). Hence, in addition to smoking to activate their β2*nAChRs, tobacco users may also be titrating ACh signals via desensitization of the β2*nAChRs, particularly after the first cigarette of the day. Human imaging studies suggest that β2*nAChRs may be critical for nicotine’s ability to curb anxiety in smokers (Benowitz, 2008; Sharma & Brody, 2009) but presently available compounds that assess β2*nAChR occupancy in humans cannot differentiate between receptors in the activated or desensitized state.

To conclude, low dose nicotine and DHβE had similar effects on affective behavior in the CER, marble burying and elevated plus maze tasks. These studies support the hypothesis that nicotine may reduce negative affect and anxiety via desensitization of the high affinity β2*nAChRs. These data further suggest that antagonism of β2*nAChRs may be an effective strategy for promoting tobacco cessation or for relieving anxiety in non-tobacco users. The conclusions of these studies would be strengthened by evaluating whether β2*nAChRs are necessary for the anxiolytic-like effects of low-dose nicotine using mice with genetic deletion of β2*nAChRs (β2KO). Furthermore, if the anxiolytic-like effects of low-dose nicotine are promoted via inactivation of β2*nAChRs, then an anxiolytic-like dose of nicotine ought to block the behavioral effects of a high dose of nicotine.
Chapter 3 - Anxiolytic- and anxiogenic-like effects of nicotine are regulated via diverse action at β2*nAChRs

INTRODUCTION

Tobacco use is a major global risk factor for cancer and cardiopulmonary diseases (WHO, 2008). Many smokers indicate that they smoke to relieve anxiety and studies suggest that smokers experience anxiety more intensely than non-smokers (Fidler & West, 2009; Parrott, 1999; Perkins & Grobe, 1992). Stress is also a major precipitating factor in relapse to smoking (Shiffman et al., 1997) and for escalation of cigarette use (Skara et al., 2001). The exact mechanism of nicotine-mediated relief of anxiety experienced by smokers is not clearly understood.

Using a combination of genetic and pharmacological approaches, these studies sought to determine the role of β2 subunit containing nicotinic acetylcholine receptors (β2*nAChRs; *other subunits assemble with β2 to form a functional receptor) in the anxiolytic-like and anxiogenic-like effects of nicotine in the light-dark and elevated plus maze assays, rodent models with good predictive validity for FDA-approved pharmacological agents for treatment of anxiety in humans (Crawley & Goodwin, 1980; Wiley et al., 1995). Neurons in limbic system brain regions that regulate both affect and reward are enriched with β2*nAChRs (Clarke et al., 1985; Court & Clementi, 1995; Fujita et al., 2000; Grady et al., 2010; Kimes et al., 2008; Marks et al.,
suggesting that this nAChR subtype may serve a role in the relief of negative affect. In Experiment 1, β2*nAChR null mutant mice (β2KO) were compared to their wild type (WT) littermate controls across a range of doses of nicotine (0, 0.01, 0.05, 0.1 or 0.5 mg/kg i.p. by weight of freebase) on measures of latency, percent time in the light chamber and movements per second in the light and dark chambers. Preclinical studies demonstrate that low doses of nicotine promote anxiolysis-like behavior and high doses lead to anxiogenic-like behavior (Picciotto, Brunzell, & Caldarone, 2002). It is well known that high doses of nicotine promote anxiogenic-like behavior in a variety of rodent behavioral assays (Cheeta, Tucci, et al., 2001; File et al., 2000; File et al., 1998; Ouagazzal et al., 1999; Zarrindast et al., 2008), but it is not clear if this is due to activation or inhibition of nAChRs. Experiment 2 utilized a selective agonist of β2*nAChRs, 5-Iodo-A85380 (5I-A85380), to test if selective targeting of β2*nAChRs is sufficient to promote anxiolysis-like and anxiogenic-like behavior in the light-dark and elevated plus maze assays. Experiment 3 assessed if a pre-injection of the selective β2*nAChR antagonist DHβE would block or augment the anxiolytic-like effects of low-dose nicotine (0.05 mg/kg i.p.) or the anxiogenic effects of high dose nicotine (0.5 mg/kg i.p.).

In vitro and in vivo studies show that nicotine both activates and desensitizes nAChRs and that low concentrations may preferentially desensitize nAChRs, rendering them unavailable for further activation by nicotine or the endogenous neurotransmitter ACh (Buccafusco, Shuster, & Terry, 2007; Fenster et al., 1997; R. A. Lester & Dani, 1995; Mansvelder et al., 2002; Pidoplichko et al., 1997). Previously we showed that the administration of both low dose
nicotine and the selective β2*nAChR antagonist DHβE similarly reduced anxiety-like behaviors in various assays in mice, suggesting that the effective doses of nicotine may have exerted their effects via inhibition rather than activation of the high affinity β2*nAChRs. Experiments 4 and 5 therefore directly assessed if pre-injection of a low dose of nicotine (0.01 or 0.05 mg/kg i.p.) would effectively block or accumulate with doses of nicotine that promote anxiolytic-like (0.05 mg/kg i.p.) and anxiogenic-like (0.5 mg/kg i.p.) behavior in the light-dark and elevated plus maze assays.

MATERIALS AND METHODS

Subjects

64 adult male WT mice and 42 β2KO mice (Picciotto et al., 1995) backcrossed at least 10 generations on a C57BL/6J background were derived in a Virginia Commonwealth University (VCU) vivarium using heterozygous matings. Genotypes were confirmed as described previously (Salminen et al., 2004) using the following primers for the β2 nAChR subunit (β2 wild-type 5′, 5′-GCTTCAAACCTCTCCACGTC-3′; β2 wild-type 3′, 5′CGCCATAGAGTTGGAGCACC-3′) and the LacZ cassette reporter (LacZ 5′, 5′CACTACGTCTGAACGTCGAAAA-3′; and LacZ 3′, 5′-CGGGCAAATAATATCGGTGCG-3′). 121 C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME) for these experiments. Animals were group-housed (2-5 per cage), maintained in an AAALAC approved facility on a 12h light/dark cycle (lights on at 0600) and were provided ad libitum access to food and water throughout these studies. Animals purchased from Jackson labs were given 3 days to habituate to the vivarium prior to handling or experimentation. Mice were habituated to experimenter handling for 3 days and to the experimental room for 1 day prior to any
experimentation. Every effort was taken to minimize any pain and discomfort the animals may have experienced during these studies. All experiments were conducted during the light cycle and were approved by the VCU Institutional Animal Care and Use Committee and were in compliance with the Guide for Care and Use of Laboratory Animals.

**Apparatus**

Light-dark experiments were conducted in modified place conditioning chambers (Med Associates, St. Albans, VT). A small, enclosed, dark chamber with a black ceiling (L 16.8 cm x W 12.7 cm x H 12.7 cm) was directly adjacent to a larger, open, brightly-lit chamber (L 26.5 cm x W 12.7 cm x H 26.2 cm) that was illuminated by a single 23W fluorescent light bulb. A door between chambers (W 5 cm x H 5.9 cm) enabled mice to move freely throughout the apparatus. White and black plastic inserts (L 16.8 cm x W 12.7 cm) were placed in the stainless steel dropping trays in the respective light and dark chambers. Photocells placed 3 cm apart recorded mouse location and movement. Data were collected on a PC computer and calculated using Med Associates software. An elevated plus maze 68 cm above the floor was constructed of wood with white plastic flooring on two (5 x 30 cm) open arms that were perpendicular to two equivalent, white, plastic enclosed arms with 15.25 cm black Plexiglas wall enclosures. Experiments were conducted under fluorescent light illumination. Data was collected with a ceiling-mounted camera that was interfaced to a PC for collection of data using ANY-maze tracking software (Stoelting, Wood Dale, IL).

**Drugs**
Nicotine hydrogen tartrate (Sigma Aldrich, St. Louis, MO), the selective β2*nAChR antagonist, DHβE and the selective β2*nAChR agonist 5I-A85380 (Tocris, Bristol, United Kingdom) were diluted in 0.9% sterile saline vehicle for these experiments. Injections were delivered i.p. in volumes of 0.1 ml/30 g. Doses are presented as freebase.

**Behavioral Procedures**

*Experiment 1: Genetic Assessment of β2*nAChR contributions to Light-Dark Behavior*

On test day, the experimental room was dark other than illumination required for the light-dark apparatus. β2KO and WT mice received between-subject delivery of 0, 0.01, 0.05, 0.1 or 0.5 mg/kg i.p. nicotine (n = 8-13 for each dose and genotype) immediately prior to placement in the dark, enclosed chamber of the apparatus. Mice were evaluated for latency to leave the dark chamber, % time spent in the light chamber and movement counts per second in the light and dark chambers for a period of 10 min. Chambers were cleaned of debris and thoroughly wiped with 2% Nolvasan (Pfizer Animal Health, Madison, NJ) between trials. Mouse weights were taken approximately 1 h before testing.

*Experiment 2: Evaluation of the selective β2*nAChR agonist 5I-A85380 on Anxiety-like Behavior in the Elevated Plus Maze and Light-Dark assays*

C57BL/6J male mice received between-subject delivery of 0, 0.001, 0.0032, 0.01 or 0.032 mg/kg i.p. 5I-A85380 15 min prior to behavioral evaluation in the light-dark assay as described above or the elevated plus maze assay (n = 7-9 per dose). In the elevated plus maze assay, after between-subject delivery of 0, 0.001, 0.0032, 0.01 or 0.032 mg/kg i.p. 5I-A85380 15 min prior to experimentation, mice were evaluated for the number of entries made into the open arms, time
spent in the open arms, latency to explore the terminal 5 cm of the open arms and time spent in
the closed arms. The effect of drug administration on the number of entries made into the closed
arms was also evaluated to measure drug effects on locomotor activity.

Experiment 3: Pharmacological Assessment of β2*nAChR contributions to Light-Dark Behavior

Behavioral Procedures were as described for Experiment 1 except that 15 min prior to vehicle (0
mg/kg), low dose (0.05 mg/kg) or high dose (0.5 mg/kg) nicotine, WT mice received a pre-
injection of 0.9% saline or 2 mg/kg i.p. DHβE (n = 7-9 for each group). This dose of DHβE has
been shown previously to promote anxiolysis-like behavior in other assays without affecting
locomotor activity (Anderson & Brunzell, 2012), and to block nicotine conditioned place
preference (CPP), which requires activation of β2*nAChRs (Walters et al., 2006). The DHβE
given in the CPP study was delivered s.c., which would likely render it less potent than the dose
administered i.p. in these experiments.

Experiment 4: Evaluation of the effects of low dose nicotine pre-injection on Light-Dark
Behavior

Procedures were as described for Experiment 2 except that mice received pre-injection of 0, 0.01
or 0.05 mg/kg i.p. nicotine 10 min prior to low dose (0.05 mg/kg) or high dose (0.5 mg/kg)
nicotine. Control mice in Experiments 2 and 4 received two injections of vehicle. Mice from
Experiment 2 and 4 for were run concomitantly to conserve vehicle controls (n = 7-9 per group).

Experiment 5: Assessment of the effects of low dose nicotine pre-injection on Behavior in the
Elevated Plus Maze Assay
WT mice received a pre-injection of 0, 0.01 or 0.05 mg/kg i.p. nicotine 10 min before 0 or 0.5 mg/kg i.p. nicotine. After nicotine injection, mice were returned to home cage for 5 min prior to evaluation on an elevated plus maze (n = 10-11 per group).

Statistical Analysis

Two-way, between-subject ANOVA tests compared animals across genotypes and drug exposures in Experiments 1 and 2 and assessed interactions of pretreatment with nicotine condition in Experiment 5. One-way ANOVA was used in Experiments 3 and 4. Dunnett’s or Tukey’s post hoc tests assessed significant main effects and post hoc t-tests assessed significant interactions. For all analyses, P values ≤ 0.05 were reported as significant. Planned comparisons assessed genotypes independently of one another and assessed the stress of multiple injections by comparing behaviors of WT vehicle controls in Experiment 1 to WT mice that received VEH/VEH in Experiments 3 and 4.

RESULTS

Experiment 1: Genetic Assessment of β2*nAChR contributions to Light-Dark Behavior

Consistent with an anxiolytic-like phenotype, a main effect of nicotine treatment (F_{4,85} = 14.604, \( P < 0.001 \)) revealed that mice receiving a low dose of i.p. nicotine (0.05 mg/kg) spent more time in the light chamber compared to vehicle-injected controls (\( P < 0.05 \); Fig 3.1a). In contrast, mice administered a high dose of i.p. nicotine (0.5 mg/kg) spent significantly less time in the light chamber compared to vehicle controls (\( P < 0.001 \); Fig 3.1a), suggestive of anxiogenic-like behavior. Planned comparisons revealed that increased time spent in the light chamber following
Figure 3.1 - β2*nAChRs contribute to anxiolytic-like and anxiogenic-like effects of nicotine. a) A main effect of treatment on time spent in the light chamber revealed that acute nicotine treatment had a dichotomous effect on anxiety-like behavior. A low dose of nicotine (0.05 mg/kg i.p.) resulted in reduced anxiety-like behavior in wild type (WT) mice only, which spent more time in the light chamber than saline-treated WT mice. Mice receiving a high dose (0.5 mg/kg i.p.) of nicotine spent less time in the light chamber than saline-injected mice regardless of genotype, suggestive of an anxiogenic-like phenotype. There was a trend for increased time spent in the light chamber in β2 knock-out (β2KO) mice compared to WT mice receiving 0.5 mg/kg i.p. nicotine, suggesting that mice lacking the β2*nAChRs were protected from the anxiogenic-like effects of the high dose of nicotine. b) Unlike β2KO mice, which were not significantly affected by 0.5 mg/kg i.p. nicotine, WT mice receiving high dose nicotine required more time to enter the light chamber than saline-treated controls. There was also a non-significant trend for lower latencies in β2KO mice administered 0.5 mg/kg i.p. nicotine compared to WT mice receiving 0.5 mg/kg i.p. nicotine, further suggesting that β2KO mice were protected from the anxiogenic-like effects of a high dose of nicotine. c) WT mice administered 0.5 mg/kg i.p. nicotine explored the light chamber less than saline-treated WT control mice as measured by reduced movements per second spent in the light chamber. In contrast, light chamber exploration was not significantly affected by nicotine dose in β2KO mice. Compared to WT mice administered 0.5 mg/kg nicotine β2KO mice also showed higher movements per second in the light chamber, suggesting that activation of β2*nAChRs is important for regulating the anxiogenic-like effects of 0.5 mg/kg i.p. nicotine for this measure. d) Mice receiving 0.5 mg/kg i.p. nicotine moved less in the dark chamber compared to their saline-injected littermates independent of genotype, suggesting that other nicotinic receptor sub-types contribute to reduced locomotor activity in the dark chamber. Data are represented as means ± SEM. *P < 0.05
compared to saline-treated mice of same genotype; **$P < 0.05$, #$P < 0.09$ compared to WT mice receiving the same dose.
0.05 mg/kg i.p. nicotine was only observed in WT mice ($t_{20} = 2.293$, $P < 0.05$; Fig 3.1a) and not β2KO subjects compared to their saline controls ($t_{16} = 1.383$, $P = 0.186$), suggesting that β2*nAChRs are important for the expression of the anxiolytic-like effects of low dose nicotine. Both WT ($t_{19} = 4.485$, $P < 0.001$; Fig 3.1a) and β2KO mice ($t_{16} = 2.851$, $P < 0.05$; Fig 3.1a) spent less time in the light chamber following the 0.5 mg/kg i.p. nicotine dose, suggesting that nAChR receptor subtypes other than β2*nAChRs contribute to the anxiogenic effects of nicotine in this assay. There was a non-significant trend for β2KO mice to spend more time in the light chamber compared to WT mice receiving 0.5 mg/kg i.p. nicotine, however ($t_{15} = 2.088$, $P = 0.054$; Fig 3.1a), suggesting that β2*nAChRs contribute to nicotine’s anxiogenic effects as well. Similar genotype x treatment interaction trends were observed for the measure of latency ($F_{4,85} = 2.109$, $P = 0.087$), with main effects of genotype ($F_{1,85} = 6.533$, $P < 0.05$) and treatment ($F_{4,85} = 13.339$, $P < 0.001$). WT mice receiving 0.5 mg/kg i.p. nicotine required significantly more time to enter the light chamber than saline-injected mice ($t_{19} = 3.908$, $P = 0.001$; Fig 3.1b) with only trends for delayed light chamber entry in 0.5 mg/kg β2KO mice compared to their vehicle-injected counterparts ($t_{16} = 1.870$, $P = 0.08$; Fig 3.1b). Comparison across genotypes at the 0.5 mg/kg i.p. dose revealed a non-significant trend for β2KO mice to exhibit shorter latencies to enter the light chamber compared to their WT littermates ($t_{15} = 1.873$, $P = 0.081$; Fig 3.1b).

To assess locomotor activity in this assay, the effects of acute nicotine on movements per second were separately assessed in the dark and light chambers. Changes in exploration that were specific to the light chamber and not observed in the dark were interpreted as changes in anxiety-like behavior whereas effects of movements per second observed in both chambers implicates a likely locomotor component to the observed behavior. A significant interaction of genotype with
treatment was specific to movements per second in the light chamber \( (F_{4,85} = 3.020, P < 0.05) \), revealing that only WT mice showed a reduction in light chamber exploration at the highest dose of nicotine \((t_{19} = 4.592, P < 0.001; \text{Fig 3.1c})\). In addition to not having significantly reduced light chamber activity than β2KO controls \((t_{16} = 1.508, P = 0.151)\), β2KO mice receiving the high dose of nicotine showed increased movements per second in the light chamber than WT mice injected with 0.5 mg/kg nicotine \((t_{15} = 2.278, P < 0.05; \text{Fig 3.1c})\). In contrast to light chamber activity, there was no genotype x treatment interaction \((F_{4,85} = 2.146, P > 0.05)\) and no effect of genotype on dark chamber activity \((F_{1,85} < 1, P = 0.456)\). A significant effect of treatment \((F_{4,85} = 14.756, P < 0.001)\) revealed that 0.5 mg/kg mice, overall, moved less in the dark chamber compared to saline-injected mice independent of genotype \((P < 0.001; \text{Fig 3.1d})\). Present in both genotypes, these general reductions in locomotor activity were likely driven by nAChRs other than those that contain a β2 subunit.

**Experiment 2: Evaluation of the selective β2*nAChR agonist 5I-A85380 on Anxiety-like Behavior in the Elevated Plus Maze and Light-Dark assays**

Significant effects of 5I-A85380 on elevated plus maze and light-dark behaviors revealed that selective targeting of β2*nAChRs was sufficient to support anxiolysis-like and anxiogenic-like phenotype. There were main effects of drug treatment for time spent in the open arms \((F_{4,35} = 4.254, P = 0.007)\), time spent in the closed arms \((F_{4,35} = 7.946, P < 0.001)\), number of entries into the open arms \((F_{4,35} = 5.131, P = 0.002)\), number of entries made into the closed arms \((F_{4,35} = 3.129, P = 0.027)\) and latencies to explore the terminal 5cm of the open arms \((F_{4,35} = 4.728, P = 0.004)\) of the elevated plus maze. Similar to nicotine, low dose 5I-A85380 supported anxiolysis-like behavior and mice injected with high dose 5I-A85380 showed anxiogenic-like behavior.
Compared to saline-injected controls, mice injected with 0.001 mg/kg i.p. 5I-A85380 spent more time in the open arms and made more entries into the open arms ($P < 0.05$; Fig 3.2a, $P < 0.05$; Figure 3.2c), whereas administration of a high 0.032 mg/kg i.p. dose of 5I-A85380 resulted in an increase in time spent in the closed arms of an elevated plus maze ($P < 0.05$; Figure 3.2b). Post hoc tests did not detect any dose-specific effects of 5I-A85380 administration on entries made into the closed arms ($P's > 0.2$; Table 3.1). Despite trends to show reduced latency to explore the terminal 5cm of the open arms at the lowest dose of 5I-A85380, post hoc tests did not reveal any dose-specific effects of 5I-A85380 administration on this measure ($P's > 0.1$; Figure 3.2d). There was a main effect of drug administration for % time spent in the light chamber ($F_{4,36} = 5.120, P = 0.002$) and latency to enter the light chamber ($F_{4,36} = 17.453, P < 0.001$). Consistent with the high dose of 5I-A85380 supporting anxiogenic phenotype, post hoc tests revealed that mice administered 0.032 mg/kg i.p. of the selective $\beta_2$*nAChR agonist 5I-A85380 spent less time in the light chamber ($P = 0.005$; Figure 3.3a) and showed increased latencies to enter the light chamber than saline-injected mice ($P < 0.001$; Figure 3.3b). A main effect of treatment was observed for light chamber ($F_{4,36} = 7.604, P = 0.002$), but not dark chamber activity ($F_{4,36} = 2.599, P > 0.05$) following selective agonism of $\beta_2$*nAChRs with 5I-A85830. Mice injected with 0.032 mg/kg i.p. 5I-A85380 showed reduced movements per second in the light chamber compared to saline-injected controls ($P < 0.001$; Figure 3.3c). An absence of a significant effect in the dark chamber suggests that changes in behavior observed during the light-dark assay following the administration of 0.032 mg/kg 5I-A85380 reflected increased anxiety-like behavior rather than reduced locomotor activity. In the light-dark assay, 5I-A85380 supported anxiogenic-like behaviors at high doses but did not support anxiolysis-like behavior.
Figure 3.2 - Administration of selective β2*nAChR agonist 5I-A85380 has bimodal effect on anxiety-like behavior in elevated plus maze assay.  

a-b) Similar to nicotine, the selective β2*nAChR agonist 5I-A85380 both reduced and increased anxiety-like behaviors, a) as mice receiving a low dose (0.001 mg/kg i.p.) of 5I-A85380 spent more time in the open arms of an elevated plus maze, b) whereas mice injected with a high dose (0.032 mg/kg i.p.) of 5I-A85380 spent more time in the closed arms than vehicle-injected controls.  

c) Mice injected with 0.001 mg/kg i.p. 5I-A85380 also made more entries into the open arms than saline-injected mice, indicative of anxiolytic-like behavior.  

d) Although a main effect of drug treatment was revealed for latency to explore the terminal 5cm of the open arms, post hoc tests did not detect any dose-specific effects for this measure.
Table 3.1 - Effect of 5I-A85380 administration on number of entries made into the closed arms.

<table>
<thead>
<tr>
<th>5I-A85380 mg/kg i.p.</th>
<th>0</th>
<th>0.001</th>
<th>0.0032</th>
<th>0.01</th>
<th>0.032</th>
</tr>
</thead>
<tbody>
<tr>
<td>Closed arm entries</td>
<td>23.1 ± 2.2</td>
<td>28.8 ± 4.1</td>
<td>25.9 ± 3.3</td>
<td>18.3 ± 3.1</td>
<td>15.3 ± 2.5</td>
</tr>
</tbody>
</table>

Post hoc tests did not detect any significant dose-dependent effects of 5I-A85380 administration on the number of entries made into the closed arms of an elevated plus maze, suggesting that changes in observed behaviors were not solely driven by regulation of general locomotor activity. Data are represented as Means ± SEM.
Figure 3.3 - Administration of the selective β2*nAChR agonist 5I-A85380 also results in anxiogenic-like behavior in the light-dark assay. a) Mice administered 0.032 mg/kg i.p. of the selective β2*nAChR agonist 5-Iodo-A85380 (5I-A85380) showed longer latencies to enter the light chamber and b) spent less time in the light chamber than mice receiving saline vehicle, suggestive of an anxiogenic-like effect. c) A main effect of drug treatment for movements per second in the light chamber revealed that mice injected with 0.032 mg/kg i.p. 5I-A85380 also showed lower light chamber exploration than saline-injected mice. d) There was no effect of drug treatment detected for dark chamber activity, suggesting that the behavioral effects of 0.032 mg/kg i.p. 5I-A85380 reflect increased anxiety-like behavior and not reductions in general locomotor activity. Data are reported as means ± SEM; *P < 0.05 compared to saline vehicle.
Table 3.2. Nicotine pre-injection time course.

<table>
<thead>
<tr>
<th>PRE NIC mg/kg nicotine i.p.</th>
<th>NIC mg/kg nicotine i.p.</th>
<th>Pretreatment time (min)</th>
<th>% Time light</th>
<th>Latency to enter light chamber (s)</th>
<th>n size</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Pre-Injection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO INJ VEH</td>
<td></td>
<td>-</td>
<td>28.3 ± 3.6</td>
<td>113.4 ± 43.3</td>
<td>(12)</td>
</tr>
<tr>
<td>NO INJ 0.05 NIC</td>
<td></td>
<td>-</td>
<td>38.3 ± 2.7^</td>
<td>50.0 ± 12.4</td>
<td>(10)</td>
</tr>
<tr>
<td>NO INJ 0.5 NIC</td>
<td></td>
<td>-</td>
<td>5.4 ± 3.1^</td>
<td>421.8 ± 68.8^</td>
<td>(9)</td>
</tr>
<tr>
<td>Vehicle Pre-Injection Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEH VEH</td>
<td></td>
<td>10</td>
<td>15.6 ± 4.5^</td>
<td>164.2 ± 82.6</td>
<td>(9)</td>
</tr>
<tr>
<td>Pre-Injection prior to an Anxiolytic dose of 0.05 mg/kg i.p. nicotine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05 NIC 0.05 NIC</td>
<td>5</td>
<td>21.0 ± 5.1</td>
<td>147.1 ± 88.84</td>
<td>(7)</td>
<td></td>
</tr>
<tr>
<td>0.05 NIC 0.05 NIC</td>
<td>10</td>
<td>21.5 ± 6.1</td>
<td>69.0 ± 43.3</td>
<td>(7)</td>
<td></td>
</tr>
<tr>
<td>Pre-Injection prior to an Anxiogenic dose of 0.5 mg/kg i.p. nicotine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05 NIC 0.5 NIC</td>
<td>5</td>
<td>7.0 ± 2.7^</td>
<td>196.7 ± 104.3</td>
<td>(7)</td>
<td></td>
</tr>
<tr>
<td>0.05 NIC 0.5 NIC</td>
<td>10</td>
<td>23.5 ± 4.6*</td>
<td>28.4 ± 10.9*</td>
<td>(7)</td>
<td></td>
</tr>
</tbody>
</table>

A nicotine pre-injection time course is shown for efficacy of pre-injection (PRE NIC) of a 0.05 mg/kg i.p. anxiolytic-like dose of nicotine to affect light-dark behaviors following a second anxiolytic-like dose, or a 0.5 mg/kg i.p. anxiogenic-like dose of nicotine (NIC) in this task. In mice receiving no pre-injection, 0.05 mg/kg i.p. nicotine (NO INJ/0.05 NIC) led to elevations in percent time spent in the light chamber \( (t_{20} = 2.096) \) compared to NO INJ/VEH controls. In contrast, mice receiving an anxiogenic-like dose of nicotine (NO INJ/0.5 NIC) showed significant increases in latency to enter the light chamber \( (t_{19} = 3.908) \) and overall reductions in time spent in this brightly-lit, exposed chamber compared to NO INJ/VEH controls \( (t_{19} = 4.485) \). The stress of two injections (VEH/VEH) also led to significant decreases in % time spent in the light chamber \( (t_{19} = 2.188) \). Despite the stress of multiple injections, mice pre-injected with 0.05 mg/kg i.p. nicotine prior to an anxiogenic dose of 0.5 mg/kg i.p. nicotine (0.05 NIC/0.5 NIC) showed significantly shorter latencies than NO INJ/0.5 NIC \( (t_{14} = 4.967) \), but only when injections were delivered 10 min and not 5 min prior to nicotine treatment. A time-course revealed that pre-injection of 0.05 mg/kg i.p. low dose nicotine promoted anxiolysis-like behavior only when given 10 min prior to subsequent nicotine injection and not at 5 min. 10 min pre-injection also led to elevations in time spent in the light chamber compared to NO INJ/0.5 NIC mice \( (t_{14} = 3.367) \). Data are represented as means ± SEM. \(^{\wedge}P < 0.05\) compared to NO INJ/VEH; \(^{*}P < 0.05\) compared to NO INJ/0.5 NIC.
Experiment 3: Pharmacological Assessment of β2*nAChR contributions to Light-Dark Behavior

The two injections required for this procedure increased anxiety-like behavioral measures (Table 3.2), but this did not preclude observation of anxiogenic or anxiolytic effects of nicotine in this task. Planned comparisons revealed that mice receiving saline pre-injection before 0.5 mg/kg i.p. nicotine (VEH/0.5 NIC) spent significantly less time in the light chamber than mice administered saline followed by another saline vehicle injection (VEH/VEH; \( t_{14} = 2.789, P < 0.05 \); Fig 3.4a). Latencies were not significantly affected (\( t_{14} = 1.396, P > 0.1 \); Fig 3.4b).

Experiment 3 evaluated whether pharmacological antagonism of β2*nAChRs would augment or reduce the anxiogenic-like effects of 0.5 mg/kg i.p. high dose nicotine and the anxiolytic-like effects of 0.05 mg/kg i.p. low dose nicotine. 0.5 mg/kg i.p. nicotine A pre-injection of DHβE prior to 0.5 mg/kg nicotine (DHβE/0.5 NIC) blocked the anxiogenic-like effects of 0.5 mg/kg nicotine and promoted an anxiolysis-like phenotype as measured by increased time spent in the light chamber (\( P = 0.007 \); Fig 3.4a) and decreased latencies to enter the light chamber (\( P < 0.05 \); Fig 3.4b) in these animals compared to VEH/0.5 NIC mice. Planned comparison analyses revealed that mice administered 0.05 mg/kg i.p. nicotine following saline pre-injection (VEH/0.05 NIC) spent more time in the light chamber (\( t_{14} = 2.131, P = 0.051 \); Fig 3.4c) compared to VEH/VEH mice. There was no significant interaction of antagonist pre-exposure with low dose nicotine treatment. Unlike anxiogenic-like effects of high dose nicotine, the anxiolytic-like effects of low dose nicotine were neither augmented nor blocked by pre-injection of DHβE.
Figure 3.4 - Selective antagonism of β2*nAChRs with pretreatment of dihydro-beta-erythroidine (PRE DHβE) blocks the anxiogenic-like effects of nicotine (NIC) in the light-dark assay.  

a) Consistent with Experiment 1, mice receiving saline pre-injection prior to a high dose nicotine (VEH/0.5 NIC) spent less time in the light chamber than mice given two injections of saline vehicle (VEH/VEH), suggestive of an anxiogenic-like effect at this dose. Although mice receiving a pre-injection of 2 mg/kg i.p. DHβE (DHβE/VEH) did not show anxiolytic-like behavior compared to VEH/VEH mice, a pre-injection of 2 mg/kg i.p. DHβE effectively blocked the anxiogenic-like effects of 0.5 mg/kg i.p. nicotine treatment (DHβE/0.5 NIC). DHβE/0.5 NIC mice spent more time in the light chamber and b) required less time to enter the light chamber than VEH/0.5 NIC mice.  

c-d) Despite the added stress of two injections, consistent with Experiment 1, pre-injection of saline vehicle prior to a low dose of nicotine (0.05 mg/kg i.p.) resulted in elevated time spent in the light chamber and reduced latencies to enter the light chamber following saline pre-injection. These effects were not further impacted by pre-injection of 2 mg/kg i.p. DHβE, suggesting that the anxiolytic-like effects of low dose nicotine are not mediated via activation of β2*nAChRs. Data are reported as means ± SEM; *P ≤ 0.05 compared to VEH/VEH; **P < 0.05 compared to VEH/0.5 NIC.
Planned comparisons between NO INJ/VEH and VEH/VEH mice revealed that multiple injections did not significantly affect light or dark chamber activity ($t_{19} = 0.335, P = 0.742; t_{19} = 1.681, P = 0.109$). Two-way ANOVA analyses detected a trend for a pre-injection (PRE DHβE) x nicotine (NIC) interaction in the light ($F_{1,26} = 3.316, P = 0.08$), but not the dark chamber ($F_{1,26} < 1, P = 0.336$) following pre-injections of vehicle (VEH) or 2 mg/kg i.p. DHβE (DHβE). Pre-injections of DHβE blocked the effects of 0.5 mg/kg i.p. nicotine as evidenced by significantly greater light chamber activity in DHβE/0.5 animals than VEH/0.5 NIC mice ($P = 0.005$; Fig 3.5a). Compared to VEH/VEH controls, animals injected with DHβE prior to saline vehicle (DHβE/VEH) did not show increased light chamber activity.

**Experiments 4 and 5: Evaluation of the effects of low dose nicotine pre-injection on nicotine-associated anxiogenic-like and anxiolytic-like behavior**

A time course testing the effect of nicotine pre-injection on the anxiogenic effects of nicotine in the light-dark assay showed that pre-injection with low dose nicotine dose-dependently blocked the anxiogenic effects of high dose nicotine when given 10 min, but not 5 min, prior to subsequent nicotine injection (Table 3.2). Data below are shown for 10 min pre-injections. ANOVA analyses revealed main effects of treatment condition for time spent in the light chamber ($F_{6,44} = 5.509, P < 0.001$) and latency to enter the light chamber ($F_{6,44} = 4.092, P = 0.002$). Pre-injection of saline vehicle prior to an anxiogenic 0.5 mg/kg i.p. dose of nicotine (VEH/0.5 NIC) resulted in a significant reduction of time spent in the light chamber compared to saline-injected controls receiving saline vehicle pre-treatment (VEH/VEH). Similar to pre-injection with DHβE, this effect was blocked by pretreatment with an anxiolytic-like dose of nicotine. 0.05 mg/kg nicotine prior to an anxiogenic dose of nicotine (0.05 NIC/0.5 NIC) resulted
in significant elevations of time spent in the light chamber and reduced latencies to enter the light chamber compared to VEH/0.5 NIC injected mice \((P's < 0.05; \text{Fig 3.6a,b})\). Mice pre-injected with vehicle prior to administration of the 0.05 mg/kg i.p. anxiolytic dose of nicotine showed a significant increase in time spent in the light chamber compared to VEH/VEH mice, but were unaffected by pre-injection with low-dose nicotine \((0.05 \text{ NIC}/0.05 \text{ NIC})\) for measures of % light chamber time and latency \((P's > 0.1; \text{Fig 3.6c,d})\). Together these data demonstrate that pre-injection of nicotine did not accumulate with experimental doses to produce its effects, but rather appeared to act like an antagonist when given at low doses 10 min prior to administration of an anxiogenic dose of nicotine.

Similar to pre-injections of the selective \(\beta2*\text{nAChR}\) antagonist DH\(\beta\)E, significant effects of treatment condition were observed following pretreatment of nicotine for movements per second in the light \((F_{6,44} = 4.22, P = 0.002)\) and dark chambers \((F_{6,44} = 4.30, P = 0.002)\). VEH/VEH mice showed greater exploration of the light chamber than VEH/0.5 NIC mice \((P = 0.07)\), an effect that was blocked by pre-injection of 0.05 mg/kg nicotine as evidenced by a non-significant trend between 0.05 NIC/0.5 NIC and VEH/0.5 NIC mice \((P = 0.07; \text{Fig 3.5c})\) with no evident difference between 0.05 NIC/0.5 NIC and VEH/VEH controls. Although post hoc tests did not detect differences between VEH/VEH controls and VEH/0.5 NIC mice for dark chamber activity \((P = 0.334)\), 0.05 NIC/0.5 NIC mice showed greater dark chamber activity than VEH/0.5 NIC mice \((P = 0.009; \text{Fig 3.5d})\).

In the elevated plus maze assay, planned comparison t-tests revealed that saline pre-injected mice receiving 0.5 mg/kg i.p. nicotine \((\text{VEH/0.5 NIC})\) spent significantly more time in the closed arms than vehicle pre-injected controls administered a second vehicle injection \((\text{VEH/VEH})\) \((t_{10} =\)
Figure 3.5 - Pre-injections of dihydro-beta-erythroidine (DHβE) and low dose nicotine prior to 0.5 mg/kg i.p. nicotine similarly affect activity in both dark and light chambers. a-b) Planned comparisons between mice from Experiment 1 given one saline injection (NO INJ/VEH) and mice administered two injections of saline vehicle (VEH/VEH) did not detect any significant differences for movements per second in either the light or dark chambers. Two-way ANOVA analyses detected a trend for a pre-injection (PRE DHβE) x nicotine (NIC) interaction in the light, but not the dark chamber following pre-injections of vehicle (VEH) or 2 mg/kg i.p. DHβE (DHβE). VEH/0.5 NIC mice showed reduced movements per second than VEH/VEH controls in the light chamber. Direct antagonism of β2*nAChRs blocked the behavioral effects of 0.5 mg/kg i.p. nicotine in both chambers, as DHβE pre-injected mice (DHβE/0.5 NIC) had higher movements per second in the light chamber than vehicle pre-injected mice (VEH/0.5 NIC). Compared to VEH/VEH controls, mice receiving pre-injections of DHβE prior to vehicle (DHβE/VEH) did not show increased light chamber activity, suggesting that the effects observed in DHβE/0.5 NIC were due to antagonism of 0.5 mg/kg i.p. nicotine. c-d) Similar to pre-injections of DHβE, pre-injections of low dose nicotine also attenuated the behavioral effects of high dose nicotine (VEH/0.5 NIC). c) Mice administered pre-injections of 0.05 mg/kg i.p. nicotine (0.05 NIC/0.5 NIC) showed a trend for higher movements per second in the light
chamber. d) 0.05 NIC/0.5 NIC mice showed higher dark chamber activity than VEH/0.5 NIC controls. Taken together, these data suggest that pre-injections of 0.05 mg/kg i.p. nicotine may have attenuated the anxiogenic-like effects of both 0.5 mg/kg i.p. nicotine and multiple injections by reducing the activity of β2*nAChRs. Data are reported as means ± SEM. *P ≤ 0.05 compared to VEH/VEH; **P < 0.05, # < 0.09 compared to VEH/0.5 NIC.
Figure 3.6 - Pretreatment with an anxiolytic-like low dose of nicotine (PRE NIC) blocks anxiogenic-like effects of high dose nicotine (NIC) in the light-dark assay. a) Similar to selective antagonism of β2*nAChRs, mice pretreated with an anxiolytic-like low dose of 0.05 mg/kg i.p. nicotine prior to 0.5 mg/kg i.p. nicotine (0.05 NIC/0.5 NIC) spent more time in the light chamber and b) required less time to enter the light chamber than mice pre-injected with saline vehicle prior to an anxiogenic dose of nicotine (VEH/0.5 NIC). c) Nicotine pretreatment did not significantly alter the effects of an anxiolytic-like dose of i.p. nicotine (0.05 NIC/0.05 NIC). d) Latencies to enter the light chamber were also not significantly affected by i.p. nicotine pretreatment; 0.05 NIC/0.05 NIC mice did not differ from VEH/0.05 NIC controls, demonstrating that the administration of two injections of low dose i.p. nicotine did not accumulate in this assay. Data are reported as means ± SEM; *P ≤ 0.05 compared to VEH/VEH; **P < 0.05 compared to VEH/0.5 NIC.
5.104, \( P < 0.001 \); Fig 3.7b). Two-way ANOVA analyses detected a main effect of anxiogenic nicotine injection (\( F_{1,54} = 12.027, P = 0.001 \)) and a trend for an interaction of nicotine pre-injection x anxiogenic nicotine dosing for time spent in the closed arms (\( F_{2,54} = 2.942, P = 0.061 \)). Student’s t-tests revealed that a pre-injection of 0.01 mg/kg i.p. nicotine blocked this effect of 0.5 mg/kg i.p. nicotine to increase time spent in the closed arms; 0.01 NIC/0.5 NIC mice spent less time in the closed arms compared to VEH/0.5 mice (\( t_{18} = 2.288, P < 0.05 \); Fig 3.7b). There was also a non-significant trend for 0.05 NIC/0.5 NIC mice to spend less time in the closed arms than VEH/0.5 NIC mice (\( t_{20} = 1.794, P = 0.088 \); Fig 3.7b). Planned comparisons also revealed that VEH/0.5 NIC mice spent less time in the open arms (\( t_{19} = 2.627, P < 0.05 \); Fig 3.7a), made fewer entries into the open arms (\( t_{19} = 3.012, P = 0.007 \); Fig 3.7c) and a non-significant trend for VEH/0.5 NIC mice to show increased latencies to explore the terminal 5cm of the open arms than VEH/VEH mice (\( t_{19} = 2.051, P = 0.054 \); Fig 3.7d). A main effect of nicotine pre-injection was also detected for time spent in the open arms (\( F_{2,54} = 4.235, P = 0.020 \)), and for number of entries made into both the open arms (\( F_{1,54} = 8.089, P = 0.006 \)) and into the closed arms (\( F_{1,54} = 82.547, P < 0.001 \)). There was no effect of nicotine administration on latency to explore the terminal 5cm of the open arms (\( F_{2,54} = 1.207, P = 0.307 \)). Nicotine pre-injection had no effect on the number of closed arm entries (\( F_{2,54} = 1.158, P = 0.322 \); Table 3.3), suggesting that the effects of low dose nicotine pre-treatment to reduce closed arm time following 0.5 mg/kg nicotine injection was not due to increased locomotor activity.

**DISCUSSION**

These pharmacological and genetic studies support the hypothesis that \( \beta^2 \cdot nAChRs \) are sufficient and necessary for expression of anxiolytic-like effects of low dose nicotine in the light-dark and
Figure 3.7 - Pre-injection of low-dose nicotine reverses anxiogenic-like effects of high dose nicotine during elevated plus maze assay. **a-d** Main effects of nicotine (NIC) administration revealed that mice injected with 0.5 mg/kg i.p. nicotine (0.5 NIC) spent less time in the open arms, more time in the closed arms and made fewer entries into the open arms of an elevated plus maze than saline-injected controls (VEH). **b** Vehicle pre-injected mice (PRE NIC) administered 0.5 mg/kg i.p. nicotine (VEH/0.5 NIC) spent significantly more time in the closed arms than VEH/VEH controls, suggesting that 0.5 mg/kg i.p. nicotine also promotes anxiogenic-like behavior in an elevated plus maze assay. Pre-injections of 0.01 mg/kg i.p. nicotine (0.01 NIC/0.5 NIC) blocked this effect, as 0.01 NIC/0.5 NIC mice spent less time in the closed arms than VEH/0.5 NIC mice. There was also a trend for 0.05 NIC/0.5 NIC mice to spend less time in the closed arms than VEH/0.5 NIC mice. These data suggest that pre-injections of low dose nicotine are also effective at blocking the anxiogenic-like effects of 0.5 mg/kg i.p. nicotine in an elevated plus maze assay. Data are reported as means ± SEM. *P ≤ 0.05 compared to VEH/VEH; **P < 0.05, # < 0.09 compared to VEH/0.5 NIC.
Table 3.3 - Effect of pre-injections of nicotine (PRE NIC) on number of closed arm entries made during the elevated plus maze assay.

<table>
<thead>
<tr>
<th>NIC (mg/kg) i.p.</th>
<th>VEH</th>
<th>0.01</th>
<th>0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEH</td>
<td>21.1 ± 2.0</td>
<td>23.6 ± 3.9</td>
<td>25.3 ± 2.8</td>
</tr>
<tr>
<td>0.5</td>
<td>4.5 ± 1.6</td>
<td>5.3 ± 1.4</td>
<td>6.8 ± 1.5</td>
</tr>
</tbody>
</table>

All mice receiving 0.5 mg/kg i.p. nicotine (NIC = 0.5) made fewer closed arm entries than saline-injected mice (NIC = VEH), suggesting that 0.5 mg/kg i.p. nicotine resulted in locomotor suppression. Pre-injections of nicotine (PRE NIC) did not affect number of closed arm entries in 0.5 NIC mice, suggesting that attenuations in time spent in the closed arms in 0.01 NIC/0.5 NIC and 0.05 NIC/0.5 NIC mice are reflective of reduced anxiety-like behavior, not increased locomotor activity.
elevated plus maze tasks. These findings further suggest that β2*nAChRs are critically involved in the anxiogenic-like effects of high dose nicotine. Our previous work showed that a selective antagonist of β2*nAChRs had similar effects as low-dose nicotine to promote anxiolysis-like behavior in the CER, marble burying and elevated plus maze tasks (Anderson, 2012 #1095). Expanding upon this correlative data, which suggests that low dose nicotine exerts its anxiolytic-like effects via desensitization of the β2*nAChRs, the current experiments provide direct pharmacological evidence that a low dose of nicotine can effectively block the anxiogenic effects of a high dose of nicotine.

Similar to its bimodal effects on anxiety-like behaviors (Anderson & Brunzell, 2012; File et al., 1998; McGranahan et al., 2011; Picciotto et al., 2002; Varani et al., 2012), nicotine activates and desensitizes β2*nAChRs in a concentration-dependent manner. In vitro studies demonstrate that micromolar concentrations of nicotine activate β2*nAChRs whereas pretreatment with nanomolar concentrations of nicotine can preferentially desensitize β2*nAChRs, rendering them unavailable for subsequent stimulation by ACh or nicotine (Fenster et al., 1997; Grady et al., 2012; Kuryatov et al., 2011; R. A. Lester & Dani, 1995; Lu et al., 1999; Mansvelder et al., 2002; Pidoplichko et al., 1997), but see Liu et al. on α6α4β2*nAChRs (L. Liu et al., 2012). Low concentrations of nicotine have previously been shown to block the stimulatory effects of a nicotinic ganglionic agonist on mean arterial pressure (Buccafusco et al., 2007), suggesting that low doses of systemic nicotine are also capable of desensitizing nAChRs. The present studies similarly show that in vivo pre-administration of low dose nicotine can attenuate the behavioral effects of a nicotinic agonist. These findings are consistent with recent data showing that low dose nicotine and DHβE reverse conditioned inhibition and promote anxiolysis-like behavior in a
marble burying and elevated plus maze task (Anderson & Brunzell, 2012). Consistent with previous models of anxiety-like behavior (Anderson & Brunzell, 2012; McGranahan et al., 2011; Picciotto et al., 2002), treatment with a low dose of nicotine (0.05 mg/kg) resulted in anxiolytic-like behavior, whereas a high dose of nicotine (0.5 mg/kg) promoted anxiogenic-like behavior of WT mice in the light-dark assay. The genetic study in Experiment 1 expands upon previous data to show that β2*nAChRs promote anxiolysis-like behavior and contribute to anxiogenic-like behavior (Anderson & Brunzell, 2012; McGranahan et al., 2011; Picciotto et al., 2002). Unlike WT mice, β2KO mice failed to show elevated time spent in the light chamber when injected with low dose nicotine and showed attenuated reductions of time spent in the light chamber and increased latencies compared to WT controls following high dose nicotine. The selective removal of cholinergic input to the BLA, where α4β2*nAChRs prevail, results in reduced anxiety-like behavior (Power & McGaugh, 2002), further supporting the role for inactivation of α4β2*nAChRs in the promotion of anxiolysis-like behaviors. As with the β2KO studies performed herein, α4KO mice also failed to show nicotine-associated anxiolysis-like behavior in an elevated plus maze (McGranahan et al., 2011), suggesting that α4β2*nAChRs support the anxiolytic-like effects of nicotine, but the exact confirmation of these nAChRs and their neuroanatomical location(s) remains to be determined. Pharmacological tests revealed that activation of the β2*nAChRs is critical for nicotine’s anxiogenic-like effects, but not nicotine’s anxiolytic-like effects during the light-dark assay; pre-injection of the β2*nAChR selective antagonist, DHβE, blocked increases in latency to enter the light chamber and blocked decreases of time spent in the light chamber following 0.5 mg/kg nicotine but had no effect on low dose nicotine. Low-dose nicotine similarly blocked, rather than accumulated with the 0.5 mg/kg anxiogenic nicotine dose without significantly affecting the anxiolytic-like effects of 0.05 mg/kg nicotine.
nicotine. A pre-injection of a low dose of nicotine also blocked the anxiogenic-like effects of 0.5 mg/kg nicotine in the elevated plus maze. In contrast to studies demonstrating that DHβE blocks nicotine reward in mice and nicotine reinforcement in rats (Corrigall et al., 1994; Walters et al., 2006), pre-injection with DHβE did not significantly affect the anxiolytic-like effects of a low dose of nicotine in these studies, suggesting that activation of β2*nAChRs is not required for nicotine-associated anxiolysis. Consistent with recent work using other anxiety models (Anderson & Brunzell, 2012), a 0.1 mg/kg intermediate dose that supports nicotine CPP (Brunzell et al., 2009; Mineur et al., 2009) did not promote anxiolysis-like behavior in the light-dark assay. Pre-injection of 0.05 mg/kg nicotine 10 min prior to another 0.05 mg/kg dose did not accumulate to negate anxiolytic-like effects as was observed for 0.1 mg/kg nicotine. That intermediate doses of nicotine promote reward-like behavior and high doses support anxiogenesis is likely due to differences in neuroanatomical locale (Brunzell et al., 2010; Corrigall et al., 1994; Gould & Wehner, 1999; Power & McGaugh, 2002) as well as recruitment of nAChR subtypes other than β2*nAChRs (e.g. α3β4*nAChRs) at higher doses (Fenster et al., 1997; Nelson & Lindstrom, 1999; Petersen, Norris, & Thompson, 1984). Together these findings suggest that high doses of nicotine promote anxiogenesis via activation of β2*nAChRs and that low doses of nicotine promote anxiolysis via inhibition of β2*nAChRs.

As further evidence that β2*nAChRs contribute to anxiety-like behavior, 5I-A85380, like nicotine, promoted anxiolysis-like behavior at low doses and supported anxiogenic-like behavior at the highest dose tested. 5I-A85380 is 25,000 x more selective for β2*nAChRs than other nAChR subtypes (Mukhin et al., 2000), suggesting that β2*nAChRs are sufficient to support bimodal anxiety-like behavioral effects. The administration of 0.001 mg/kg 5I-A85380
promoted anxiolysis-like behavior in the elevated plus maze, whereas 0.032 mg/kg 5I-A85380 resulted in an increase in anxiety-like behavior in both the light-dark and elevated plus maze assays. Like ACh and nicotine, sub-activating concentrations of 5I-A85380 also preferentially desensitize β2*nAChRs (Wageman, Marks, & Grady, 2013). Therefore, it is possible that the anxiolytic-like effects of 5I-A85380 were also promoted via inactivation of β2*nAChRs.

High doses of nicotine that support anxiogenic-like behavior can also reduce locomotor activity (Clarke & Kumar, 1983; Jackson et al., 2010; Marubio et al., 2003; Salas et al., 2003; Tritto et al., 2004). Independent assessment of light and dark chamber activity revealed no difference in locomotor activity of WT compared to β2KO mice injected with vehicle in either chamber or in the dark chamber when injected with any dose of nicotine, suggesting that β2*nAChRs did not support gross changes in locomotor activity in this assay. Following 0.5 mg/kg i.p. nicotine, WT, but not β2KO mice showed reduced exploration of the light chamber, however, demonstrating that genetic deletion of β2*nAChRs protected against nicotine-induced increases in anxiety-like activity for this measure. The high dose of nicotine in these studies produced locomotor suppression of dark chamber activity independent of genotype, suggesting the contributions of nAChRs other than β2*nAChRs affected locomotor activity. β4 null mutants are less sensitive to the locomotor suppressive effects of 0.5mg/kg i.p. nicotine than WT mice and also show reduced anxiety-like behavior in the elevated-plus and staircase maze assays (Salas et al., 2003). Early studies showed that β2KO mice expressed elevated passive avoidance learning (Picciotto et al., 1995). β2KO mice in the present study showed partial attenuation of the anxiogenic effects of nicotine, suggesting that activation of other nAChRs, likely α3β4*nAChRs, contributed to behaviors observed following 0.5 mg/kg nicotine. Mice with a
deletion of the α4 subunit are less sensitive to the locomotor suppressive effects of high dose nicotine (Marubio et al., 2003) and administration of the α4β2*νAChR partial agonist, varenicline (Ortiz, O'Neill, Marks, & Grady, 2012), blocks nicotine-induced suppression of locomotor activity. Previous studies suggest that α4α6β2*νAChRs, which are more resistant to desensitization than α4β2*νAChRs (non-α6) (L. Liu et al., 2012), predominantly regulate the locomotor effects of nicotine (Drenan et al., 2010). DHβE pre-injection, which antagonizes α4β2*νAChR and α4α6β2*νAChRs also reversed “locomotor-suppressant” effects of 0.5 mg/kg nicotine in these studies. Both 0.01 NIC/0.5 NIC and 0.05 NIC/0.5 NIC mice showed reductions in time spent in the closed arms of an elevated plus maze compared to VEH/0.5 NIC mice. There was no effect of nicotine pretreatment on the number of entries made into the closed arms in mice administered 0.5 mg/kg nicotine, suggesting that the behavioral effects of pre-injections of 0.01 and 0.05 mg/kg nicotine reflect attenuation of anxiety-like behavior rather than blockade of locomotor suppression. The administration of 0.032 mg/kg of the highly selective β2*νAChR agonist 5I-A85380 resulted in reduced exploration of the brightly-lit chamber during the light-dark assay and increased time spent in the closed arms of an elevated plus maze without significantly affecting movements per second in the dark chamber or number of entries made in the closed arms of the elevated plus maze, suggesting that selective activation of β2*νAChRs promotes anxiogenic-like behavior without significantly affecting gross locomotor activity. Taken together, these data support the interpretation that the antagonist-like effects of pre-injections of nicotine and DHβE prior to 0.5 mg/kg nicotine reflect attenuation of anxiogenic-like behavior and not merely the locomotor suppressive effects of a high dose of nicotine.
The impact of nicotine on anxiety in humans appears to depend upon many variables including predisposition for anxiety and smoking histories. Individuals with diagnosis of anxiety disorder are 2X more likely to smoke than otherwise healthy smokers (Lasser et al., 2000). It is not known whether β2*nAChR expression correlates with anxiety-disorder diagnosis, but smokers, compared to non-smokers, show significant elevations in high-affinity β2*nAChR binding (Benwell et al., 1988; Breese et al., 1997; Cosgrove et al., 2009) and are more anxious overall than nonsmokers (Gilbert et al., 2008; Tsuda et al., 1996). Nicotine patch compared to placebo alleviated negative affect in smokers, but not non-smokers in a picture-attention task (Gilbert et al., 2008). Nicotine patch has been shown to reduce the functional connective strength between the amygdala with insular and anterior cingulate cortices in smokers, but to increase this connectivity in non-smokers (Sutherland et al., 2013). Similar reductions in the activation of amygdalar networks were observed in human smokers following pre-injection with the partial α4β2*nAChR agonist varenicline (Sutherland et al., 2013). These findings are particularly germane considering that human imaging studies show increased activity in both the amygdala and pre-frontal cortex in subjects presenting with trait anxiety (Britton et al., 2011; Sehlmeyer et al., 2011) and provide a mechanistic explanation to the hypothesis that people suffering from anxiety-related disorders may be using cigarette smoking as a means of self-medication. For individuals who are motivated to smoke to relieve anxiety, it appears that low doses of nicotine may be sufficient to support nicotine intake. This is highly relevant to smokers given current policy measures being considered to reduce nicotine in cigarettes (Pearson, Abrams, Niaura, Richardson, & Vallone, 2013) as even low doses of nicotine are capable of binding nearly 80% of β2*nAChRs in brain (Brody, Mandelkern, Costello, et al., 2009).
The light-dark and elevated plus maze assays are animal models with good predictive validity for the anxiolytic effects of drugs. Using these assays, these genetic and pharmacological data show that the anxiolytic-like effects of nicotine are regulated via inhibition of $\beta_2*n$AChRs and suggest that activation of $\beta_2*n$AChRs contribute to increased anxiety-like behavior. These preclinical studies further demonstrate the efficacy of low doses of nicotine to promote anxiolytic-like behavior and to prevent increases in anxiety-like behaviors resulting from activation of the cholinergic system with high doses of nicotine. Whereas the precise confirmation of nicotinic receptors regulating this behavior remains to be determined, these preclinical studies indicate that partial agonists or negative allosteric modulators of $\beta_2*n$AChRs may be helpful therapeutic strategies for the treatment of smoking cessation in smokers with anxiety-related co-morbidities. Taken together, these data collectively support the hypothesis that low doses of nicotine promote anxiolysis-like behavior in mice via inactivation of the high affinity $\beta_2*n$AChRs. Further studies are necessary to determine the subunits assemble with $\beta_2$ to support the anxiolytic and anxiogenic effects of nicotine. $\beta_2*n$AChRs can be further divided into two subclasses that are sensitive or insensitive to $\alpha$-conotoxin MII. To test if nicotine is acting in part via $\alpha_6\beta_2*n$AChRs to exert anxiolytic-like effects, the anxiety phenotype of mice with a gain of function single point L9’S mutation in the $\alpha_6$ subunit (L9S) and with genetic deletion of $\alpha_6*n$AChRs ($\alpha_6KO$) could be used to determine if cholinergic persistent activation or genetic deletion of $\alpha_6*n$AChRs are sufficient to promote anxiogenic and/or anxiolytic-like behavior.
Chapter 4 – Assessment of the contribution of $\alpha_6\beta_2\ast$nAChRs to anxiety-like behavior

INTRODUCTION

Many smokers indicate that they smoke to relieve anxiety. Although it has proved difficult to detect that anxiety measures provoke smoking episodes throughout the day, a number of studies suggest that smokers experience anxiety more intensely than non-smokers (Fidler & West, 2009; Parrott, 1999; Perkins & Grobe, 1992). Reports of stress are correlated with escalation of cigarette use (Byrne et al., 1995; Byrne & Mazanov, 2003; Finkelstein et al., 2006; Siqueira et al., 2000; Skara et al., 2001) and stress is a major precipitating factor in relapse to smoking (Shiffman et al., 1997). Studies also show that craving and exposure to smoking-related cues underlie maintenance of smoking behavior (Chandra, Scharf, & Shiffman, 2011; Shiffman et al., 2013; Shiffman, Dunbar, Scholl, & Tindle, 2012). This is consistent with escalation models of drug use and dependency, wherein the positive effects of drugs of abuse contribute to their initial use, but avoidance of the negative effects of withdrawal promotes escalated drug use, leading to dependency (Koob & Volkow, 2010). The mechanism of nicotine-mediated relief of anxiety and elevated anxiety-phenotype experienced by smokers is not clearly understood.

Preclinical studies assessing anxiety phenotype in rodents show that nicotinic agonists, including nicotine, produce a bimodal effect on anxiety-like behavior with low doses promoting anxiolytic phenotypes and high doses promoting anxiogenic phenotypes (Anderson & Brunzell, 2012;
Cheeta, Irvine, et al., 2001; Cheeta, Tucci, et al., 2001; 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). Anxiolytic effects of nicotinic antagonists in these behavioral measures suggest that nicotine may promote anxiolysis via desensitization of nAChRs (Anderson & Brunzell, 2012; Dawson et al., 2013; Newman et al., 2002; Newman et al., 2001; Roni & Rahman, 2011). Once activated, the heteromeric nicotinic receptors revert to a stable desensitized state that renders them incapable of further activation for upwards of 90 min (Fenster et al., 1997; R. A. Lester & Dani, 1995; Mansvelder et al., 2002; Pidoplichko et al., 1997). In vitro studies reveal that low concentrations of nicotine in the nM range that are subthreshold for activation of most nicotinic receptor subtypes can result in preferential desensitization of these receptors (Fenster et al., 1997; Grady et al., 2012; Kuryatov et al., 2011; Lu et al., 1999). Although nicotine concentrations can vary in temporal waves, the steady-state concentration of nicotine in the brains of smokers is thought to be 200-400 nM (Rose et al., 2010), within a range that would preferentially desensitize the β2 subunit containing nAChRs (β2*nAChRs; *denotes that other subunits assemble with β2 to form a functional receptor) which have been implicated in promoting the anxiolytic-like effects of nicotine (Anderson & Brunzell, 2012; McGranahan et al., 2011). Capable of activation by 300 nM nicotine (Drenan et al., 2010; Kuryatov & Lindstrom, 2011; L. Liu et al., 2012; Zhao-Shea et al., 2011), and slower than other subtypes to desensitize (Grady et al., 2012; Kuryatov & Lindstrom, 2011), the α4α6β2*nAChRs appear to be an exception to this rule.

Previous studies show that, like low doses of nicotine, the β2*nAChR-selective antagonist, dihydro-beta-erythroidine (DHβE) significantly attenuates conditioned inhibition in a CER task.
increases time spent in the open arms of an elevated plus maze and decreases marble-burying behavior without affecting locomotor activity (Anderson & Brunzell, 2012). Although largely driven by the availability of an α4 subunit, DHβE antagonizes α4β2*nAChRs and α6β2*nAChRs with similar potency (Grady et al., 2010; Papke et al., 2008). Compared to the more ubiquitous expression of α4β2*nAChRs, α6β2*nAChRs have a more selective expression pattern in the brain. Enriched on the soma and axon terminals of catecholaminergic DA and NE neurons as well as in the visual circuitry (Champtiaux et al., 2002; Marks et al., 2010; Whiteaker et al., 2000), the α6β2*nAChRs are not highly expressed in brain regions such as the amygdala, cingulate cortex and lateral septum (Champtiaux et al., 2002; Clarke et al., 1985; Marks et al., 2010; Whiteaker et al., 2000) that are well-known for their contributions to anxiety and fear behaviors. Recent studies, however, indicate that the anxiolytic-like efficacy of a low dose of nicotine was reduced in mice with a selective genetic deletion of the α4 subunit from VTA DA neurons, suggesting that the anxiolytic efficacy of nicotine is mediated in part by α4β2*nAChRs on DA neurons in the VTA. It is not clear if VTA α4α6β2*nAChRs contributed to this phenotype. Given their high affinity for activation by nicotine and their resistance to desensitization, it is possible that activation, rather than inhibition, of this subclass of β2*nAChRs supports anxiolytic effects of nicotine (L. Liu et al., 2012; McGranahan et al., 2011). Albeit at low levels, α6β2*nAChRs are expressed in the hippocampus, a brain region where excessive cholinergic signaling is implicated in supporting anxiety-like behavior (Luo, Wei, Niu, Wang, & Wang, 2013; Mineur et al., 2013). Inhibition of α6β2*nAChRs in the locus coeruleus (LC) could also dampen neuroanatomical signals which
support fight or flight responses that are downstream of this NE-enriched nucleus (Fu, Matta, James, & Sharp, 1998; Fu, Matta, McIntosh, & Sharp, 1999).

To test the contributions of $\alpha 6\beta 2^n$AChRs to anxiety phenotype, these studies assessed the anxiety-like behaviors of mice with a genetic gain of function or loss of function to their $\alpha 6\beta 2^n$AChRs. Gain of $\alpha 6\beta 2^n$AChR function mice were generated by a single leucine to serine point mutation in the M2 pore-forming region of the $\alpha 6$ subunit (L9S), which renders their $\alpha 6\beta 2^n$AChRs highly hypersensitive to both nicotine and ACh as compared to wild type (WT) littermates (Drenan et al., 2010; Drenan, Grady, et al., 2008). If stimulation of $\alpha 6\beta 2^n$AChRs is sufficient to support anxiogenesis, then L9S mice ought to show increased latency to enter and less total time spent in the light chamber of a light-dark apparatus and spend less time in the open arms of an elevated plus maze. If stimulation of $\alpha 6\beta 2^n$AChRs is necessary for anxiogenesis, then mice with a null mutation of their $\alpha 6$ subunit ($\alpha 6$KO) ought to display an anxiolysis phenotype in these tasks, showing shorter latencies to enter and spending more time than their WT littermates in the open, brightly-lit regions of the experimental apparatus during performance of these tasks. Conversely, if activation of $\alpha 6\beta 2^n$AChRs is sufficient to promote anxiolysis, then L9S mice ought to show less anxiety-like behavior in these tasks. As these tasks are sensitive to horizontal movement, locomotor activity was assessed across genotypes during bright light and low light conditions. These studies also compared footshock sensitivities and HPA-axis responses following novel cage exposure observed in L9S, $\alpha 6$KO and WT mice.
MATERIALS AND METHODS

Subjects

A total of 148 mice participated in these studies: 43 L9S and 53 WT littermates; 26 α6KO mice and 26 WT littermates. All mice were derived in a Virginia Commonwealth University breeding colony. A single allele for the L9S transgene produces the L9S genotype and hypersensitive α6β2*nAChR phenotype (Drenan, Grady, et al., 2008) so that breedings to C57BL/6J mice (Jackson labs, Bar Harbor, ME) resulted in 50% L9S transgenics and 50% non-transgenic or WT offspring. α6KO mice were derived from heterozygous matings of mice backcrossed at least 10 generations on a C57BL/6J background. Offspring were group-housed (2-5 per cage), maintained in the animal colony (20 - 22 °C) with a 12h light/dark cycle (lights on at 0600) and had ad libitum access to both food and water throughout these studies. All mice were habituated to experimenter handling for 3 days and the experimental room for 1 day prior to any experimentation. All experiments were approved by the VCU Institutional Animal Care and Use Committee and were in compliance with the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 2010).

Apparatus

Behavioral Procedures

Assessment of α6*nAChR contributions to Anxiety-Like Behaviors
Light-dark assay

Light-dark experiments were conducted in modified place conditioning chambers (Med Associates, St. Albans, VT). A small, enclosed, dark chamber with a black ceiling (L 16.8 cm x W 12.7 cm x H 12.7 cm) was adjacent to a larger, open, brightly-lit chamber (L 26.5 cm x W 12.7 cm x H 26.2 cm) that was illuminated by a single 23W fluorescent light bulb. Manual opening of a door (W 5 cm x H 5.9 cm) at the beginning of the test provided mice with free access to explore both the dark and light chambers. White and black plastic inserts (L 16.8 cm x W 12.7 cm) were placed in the stainless steel dropping trays in the respective light or dark chambers to increase the brightness of the light chamber and reduce the glare of light that might reach the dark chamber from the light chamber. Data was collected using Med Associates software.

Elevated plus maze assay

A wooden elevated plus maze 68 cm above the floor had white plastic flooring on two (5 x 30 cm) open arms that were perpendicular to two equivalent arms with the exception that these closed arms had 15.25 cm black Plexiglas wall enclosures. Experimentation took place under overhead fluorescent light illumination.

Open field locomotor assay

Locomotor tests took place in a polycarbonate cage (33 cm x 21 cm x 9 cm high). The polycarbonate cage was placed within a white plastic enclosure that surrounded all 4 walls and the floor. Testing took place under overhead room lighting conditions or with overhead lights off and infrared illumination provided by 500 mA intensity infrared lights emitting an 830 nM
frequency (Wisecomm, Cerritos, CA). A ceiling-mounted camera was interfaced to a PC for collection of elevated plus maze and locomotor data using ANY-maze tracking software by Stoelting (Wood Dale, IL).

Assessment of $\alpha_6^n$AChR contributions to acute foot shock sensitivity

Flinch, vocalize and jump experiments were conducted in mouse operant chambers (21.6 cm x 17.8 cm x 12.7 cm; Med Associates, St. Albans, VT). A 2.24 watt incandescent house-light was positioned 9.5 cm from the floor of the operant chamber. The floor consisted of steel rods (0.32 cm in diameter placed 0.79 cm apart) connected to a Med Associates shock generator/scrambler.

Assessment of $\alpha_6^n$AChR contributions to HPA axis activity

Exposure to a novel cage is a stressor to mice that is demonstrated to activate the HPA-Axis (Hennessy, 1991; Hennessy & Foy, 1987). Mice were thus exposed to a novel cage prior to having blood drawn for assessment of corticosterone levels. Clean polycarbonate cages (33 cm x 21 cm x 9 cm high) with filtered cage tops and 1/8 corn cob bedding like that in the home cage were used for these studies. Novel cage exposure took place under standard fluorescent room lighting conditions.

Drugs

Injections of 0.9% sterile saline were delivered i.p. in volumes of 0.1 ml/30 g. Mouse weights were taken approximately 1 h prior to behavioral assays.
Behavioral Procedures

Assessment of α6*nAChR contributions to Anxiety-Like Behaviors

Light-dark assay

On test day, the experimental room was dark other than illumination required for the light-dark apparatus. In order to assess α6β2*nAChR contributions to anxiety-like behavior following exposure to a stressor, adult male L9S, α6KO mice and their corresponding WT controls (n = 13-14 per genotype) received i.p. sterile 0.9% saline immediately prior to evaluation in the light-dark assay. A separate cohort of L9S and WT mice received no injection immediately prior to evaluation in the light-dark apparatus (n = 8 per genotype). The light-dark assay was conducted as described in Chapter 3. Latency to enter the light chamber, % time spent in the light chamber and movement counts per second spent in the light and dark chambers were separately calculated as described in Chapter 3. Between animals, feces, urine and animal dander were removed and all apparatus were thoroughly wiped with 2% Nolvosan between trials (Pfizer Animal Health, Madison, NJ).

Elevated-plus maze

Saline-injected L9S, α6KO mice and their corresponding WT controls (n = 12-15 per genotype) were returned to their home cage for a 5 minute post-injection period before placement on the center of an elevated plus maze apparatus facing the closed arms. Behavior was evaluated for a period of 10 minutes. Subjects were scored for 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 (the extreme 5 cm) of the open arms. Time spent in the open arms, entries made in the open arms and distance traveled in the open arms were separated into two 5 min time bins.

Locomotor assessment

Untreated L9S, α6KO mice and their corresponding WT controls (n = 6-8 per genotype) were removed from their home cage and placed into the polycarbonate cage facing one of the corners. Behavior was evaluated for a period of 15 minutes. Total distance traveled, latency to enter the central zone (6 cm x 6 cm), central zone entries and time spent in the corners (6 cm x 6 cm) were evaluated. Experimentation took place under infrared lighting or overhead fluorescent lighting conditions (n = 6-8 per genotype and lighting condition).

Assessment of α6*nAChR contributions to acute foot shock sensitivity

Non-injected L9S (n = 10), WT (n = 15), α6KO mice (n = 11) and their WT controls (n = 11) were evaluated for footshock thresholds for 4 responses in Med Associates mouse operant chambers as described previously (Brunzell & Kim, 2001; Kim, DeCola, Landeira-Fernandez, & Fanselow, 1991). Briefly, mice received footshocks 1 sec in duration every 30 sec. Mice were scored for flinching, scrambling, vocalization and jumping responses to footshock. A flinch was defined as an observable reaction to the shock (e.g., typically an orienting response of the head, usually directed at the grid floor). A scramble was defined as movement of front or hind paws in response to the shock. A vocalization was noted when the foot shock resulted in any audible vocalization by the mouse. A jump was recorded when a leaping response to the shock was
observed. The initial footshock intensity was 0.1 mA and was increased in increments of 0.1 mA every 30 sec. The test ended when each of the four responses measures were observed or when 1 mA was reached. A value of 1 mA was recorded in the event that a mouse did not record a visible jump response during the assay. Experimenters were blinded to the genotype of the subject during testing.

Assessment of α6*nAChR contributions to HPA axis activity

Novel cage exposure

Untreated L9S, α6KO mice and their corresponding WT controls were exposed to a clean, novel polycarbonate cage for a period of 15 (n = 8-10 per genotype) or 30 (n = 9-10 per genotype) min. Subjects were then removed and were euthanized via rapid decapitation. Control mice (n = 5-6 per genotype) were removed from their home cage immediately prior to decapitation. Trunk blood was collected in 1.5 mL centrifuge tubes containing 15 μl of heparin, which were immediately placed on ice. Subsequently, samples were centrifuged at 4° C at a speed of 15,000 rpm for 15 minutes. Serum was removed and stored at -80° C until evaluation of serum corticosterone levels. Mice were sacrificed between 1000 and 1700 hours and were counterbalanced across genotype and cage exposure time to minimize the impact of circadian fluctuations of serum corticosterone levels on test results.

Determination of serum corticosterone via ELISA
Serum corticosterone was determined using the Corticosterone HS EIA kit by Immunodiagnostic Systems (Scottsdale, AZ). The assay was performed according to manufacturer instructions and curve-fitting was determined using ReaderFit software (Hitachi Solutions America Ltd, USA).

Statistical Analysis

A 2-Way ANOVA comparing saline-injected and non-injected mice returned no significant effects of injection for any behavioral measure in the light-dark assay and hence these groups were combined for subsequent analyses ($F’s < 1$). Student’s t-tests were used to determine genotype-specific effects in the light-dark assay. A repeated measures ANOVA analyzed between-subject effects of genotype and within-subject repeated measures for exploratory behavior across 5 min time bins (within subject repeated measures) in the elevated plus maze. Latency to explore the terminal 5 cm of the open arms was analyzed via Student’s t-tests. A two-way (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 Student’s t-tests to identify the specific nature of the interaction. Serum corticosterone values were analyzed using a 2 x 3 (genotype x exposure time) ANOVA. Planned comparisons assessed genotype-specific effects on basal serum corticosterone levels obtained from control L9S, WT, $\alpha$6KO and their corresponding WT mice. Planned comparisons analyzing genotype effects of HPA-axis activity in animals with 15 and 30 min of novel cage exposure were assessed via Student’s t-tests using a Bonferroni correction. Student’s t-tests were used to detect genotype-specific threshold differences for each response in the Flinch, Scramble, Vocalize and Jump test. For all analyses, $P$ values $< 0.05$ were reported as significant.
RESULTS

Assessment of $\alpha_6*nAChR$ contributions to Anxiety-Like Behaviors

Light-dark assay

There was an effect of genotype for time spent in the light chamber ($t_{41} = 3.837, P < 0.001$; Fig 4.1a) revealing that L9S mice spent less time in this more aversive area than their WT counterparts. Despite similar trends, there was no effect of genotype on latency to enter the light chamber ($t_{41} = 1.395, P = 0.171$; Fig 4.1b). L9S mice further showed less movements per second in both the light ($t_{41} = 4.387, P < 0.001$; Fig 4.1c) and dark chambers ($t_{41} = 5.151, P < 0.001$; Fig 4.1d), suggesting that decreases in locomotor activity may have contributed to these findings. In contrast, there were no significant differences between WT and $\alpha_6$KO mice for these measures ($t_{25} = 1.924, 1.206, 0.398, 0.109; P’s > 0.05$; Fig 4.1e-h).

Elevated plus maze

Repeated measures ANOVAs detected significant time bin x genotype interactions for time spent in the open arms ($F_{1,27} = 6.114, P < 0.05$) and distance traveled in the open arms of an elevated plus maze ($F_{1,27} = 7.729, P < 0.05$). Post-hoc t-tests revealed that L9S mice spent less time in the open arms ($t_{27} = 2.341, P < 0.05$; Fig 4.2a) and traveled shorter distances on the open arms during the first 5 minutes ($t_{27} = 2.745, P < 0.05$; Fig 4.2b), but not the second 5 minutes compared to WT littermates ($t_{27} = 1.424, P = 0.166$; Fig 4.2a, $t_{27} = 0.899, P = 0.377$; Fig 4.2b). Additionally, t-tests revealed that L9S mice required more time to explore the terminal 5 cm of the elevated plus maze compared to their WT controls ($t_{27} = 2.812, P = 0.009$; Fig 4.2d). A
Figure 4.1 - Mice with gain of function $\alpha_6^\ast nAChRs$ (L9S) express anxiogenic-like phenotype in the light-dark assay. 

a) Mice with a single point L9S mutation in $\alpha_6^\ast nAChRs$ (L9S) spent less time in the light chamber compared to wild type (WT) controls, suggestive of an anxiogenic-like phenotype in this assay. 

b) Although latencies did not significantly differ between genotypes, L9S mice showed reduced exploration of both the c) light and d) dark chambers than WT controls, suggesting that changes in locomotion may have contributed to genotype differences in light-dark activity. 

e-h) Mice with genetic deletion of $\alpha_6$ subunit ($\alpha_6$KO) did not significantly differ from WT mice in any of these measures, suggesting that increased activation, but not inhibition of $\alpha_6^\ast nAChRs$ promotes anxiety-like behavior in the light-dark assay. Data are represented as means $\pm$ SEM. *$P < 0.05$ vs. WT.
Figure 4.2 - L9S mice also express increased anxiety-like behavior in an elevated plus maze assay. During the first 5 minutes of elevated plus maze activity, wild type (WT) controls a) spent more time in the open arms, b) traveled a greater distance on the open arms than mice expressing gain of function α6*nAChRs (L9S). d) Finally, WT mice required less time to explore the terminal 5 cm of the open arms than L9S animals. e-h) As in the light-dark assay, there were no differences between the behavior of WT and α6KO mice on an elevated plus maze. These data suggest that increased cholinergic activation of α6*nAChRs is also anxiogenic in the elevated plus maze. Data are represented as means ± SEM. *P < 0.05 vs. WT.
repeated measures ANOVA revealed that mice made fewer entries into the open arms of the elevated plus maze during the second 5 minutes independent of genotype ($F_{1,27} = 13.04, P = 0.001$). These data suggest that L9S mice showed higher levels of anxiety-like behavior than WT controls during the elevated plus maze assay. Repeated measures ANOVAs revealed that mice spent more time in the open arms, traveled greater distances and made more entries into the open arms during the first 5 minutes of the elevated plus maze assay 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 of genotype were detected between WT and α6KO mice evaluated in the elevated plus maze for measures of time spent in the open arms, distance traveled in the open arms or number of entries made in the open arms ($F_{1,23} < 1, P = 0.423; F_{1,23} = 1.052, P = 0.316; F_{1,23} = 1.087, P = 0.308$). Furthermore, α6KO did not show different latencies to explore the terminal 5cm of the open arms compared to WT mice ($t_{23} = 0.240, P = 0.814$).

**Locomotor activity**

Two-way ANOVA revealed a genotype x light condition interaction for latency to enter the central zone and total distance traveled during a locomotor test for L9S mice compared to their WT littermates ($F_{1,26} = 10.630, P = 0.003; F_{1,26} = 4.356, P < 0.05$). Post-hoc tests revealed that L9S mice showed higher levels of anxiety-like behavior than WT mice under fluorescent lighting conditions, having longer latencies to enter the central zone of the arena ($t_{12} = 3.218, P = 0.007$; Fig 4.3a). WT mice showed greater distance traveled than L9S mice under infrared, but not fluorescent lighting conditions ($t_{14} = 2.296 P < 0.05$; Fig 4.3d), suggesting that the increased latencies to enter the center zone expressed by L9S mice under fluorescent lighting conditions
Figure 4.3 - Mice with L9S gain of function α6*nAChRs are more sensitive to lighting conditions than wild type (WT) mice regarding anxiety-like but not locomotor activity. L9S gain of function mice (L9S) showed higher levels of anxiety-like behavior during a locomotor activity test than wild type (WT) mice.  

- **a)** Under full room lighting, (Room lights on +), L9S had higher latencies to enter the central zone of the locomotor chamber.  
- **b)** L9S mice also made fewer center zone entries and **c)** spent more time in the corners of a locomotor activity chamber than WT mice, suggestive of increased anxiety-like behavior.  
- **d)** Compared to L9S mice, WT mice showed greater distance traveled under infrared lighting conditions. There were no differences between WT and L9S mice for distance traveled under fluorescent lighting conditions, suggesting that differences in open field activity expressed by L9S mice under full room lighting conditions reflect increased anxiety-like behavior.  
- **e-h)** Both WT and α6-null mutant (α6KO) mice **g)** spent more time in the corners and **h)** showed greater distance traveled under infrared lighting conditions. However, no effects of genotype were detected between WT and α6KO mice during an open field locomotor assay. Data are represented as means ± SEM. *P < 0.05 vs. WT, **P < 0.05 compared to Room lights on +.
did not reflect an overall decrease in locomotor activity of L9S mice. Main effects of genotype revealed that L9S made fewer number of entries into the central zone and spent more time in the corners of the locomotor apparatus compared to WT mice ($F_{1,26} = 10.630, P = 0.003$; Fig 4.3b, $F_{1,26} = 4.356, P < 0.05$; Fig 4.3c), suggestive of an anxiogenic-like phenotype. There were no main effects of genotype between WT and α6KO mice for any of the behavioral measures in the locomotor assay ($F_{1,27} = 1.645, P = 0.211$; $F_{1,27} = 1.698, P = 0.204$; $F_{1,27} < 1.645, P = 0.862$; $F_{1,27} = 1.292, P = 0.266$). Unexpectedly, main effects of lighting condition revealed that all mice spent more time in the corners under infrared lighting conditions than under fluorescent lighting ($F_{1,27} = 19.48, P < 0.001$; Fig 4.3g). WT and α6KO mice also traveled greater distances ($F_{1,27} = 5.817, P = 0.023$; Fig 4.3h) under infrared lighting conditions than under normal room lighting conditions independent of genotype.

**Assessment of α6*nAChR contributions to foot shock sensitivities**

Student’s t-tests revealed dichotomous effects of genotype on footshock thresholds that depended upon the measure in L9S mice. Compared to WT controls, L9S mice had lower thresholds to vocalization ($t_{23} = 2.849, P = 0.009$; Fig 4.4a) but higher thresholds for the jump response ($t_{23} = 2.856, P = 0.009$; Fig 4.4a). There were no significant effects of genotype detected for footshock thresholds for the flinch ($t_{19} = 0.641, P = 0.529$) or scramble responses ($t_{19} = 0.195, P = 0.848$). α6KO mice did not differ from WT controls in any measure of footshock threshold in this assay ($t_{20}’s < 1, P’ s > 0.5$; Fig 4.4b).

**Assessment of α6*nAChR contributions to HPA axis activity**
Figure 4.4 - Mice expressing hypersensitive $\alpha_{6^*}n$AChRs (L9S) show different footshock thresholds than wild type (WT) mice in flinch, scramble, vocalize and jump assay. a) L9S mice had lower footshock thresholds for vocalization but increased thresholds before expressing a jump response than WT mice. b) Animals with genetic deletion of $\alpha_6$ subunit ($\alpha_6$KO) did not significantly differ from WT mice in any of these measures, suggesting that increased activation, but not deletion of $\alpha_6*n$AChRs affects the thalamic response to a footshock stimulus. Data are represented as means ± SEM. *$P < 0.05$ vs. WT.
Student t-tests did not detect baseline differences in serum corticosterone levels across genotypes at the zero time point showing that basal HPA-axis activity was not different between L9S and non-transgenic WT mice ($t_8 = 0.031, P = 0.976$; Fig 4.5a) or between α6KO mice and their WT controls ($t_{10} = 0.464, P = 0.653$; Fig 4.5b). ANOVA analyses revealed main effects of time ($F_{2,40} = 32.620, P < 0.001$) and genotype for corticosterone levels in L9S ($F_{1,40} = 5.629, P < 0.05$). Planned comparison t-tests showed that serum corticosterone levels were lower in L9S mice after 30 ($t_{17} = 3.011, P = 0.008$; Fig 4.5a), but not 15 min novel cage exposure ($t_{15} = 1.496, P = 0.155$; Fig 4.5a). Post-hoc Dunnett’s tests revealed elevated corticosterone levels in mice exposed to the novel cage for 15 ($P < 0.001$; Fig 4.5a) or 30 min ($P < 0.001$; Fig 4.5a) compared to controls independent of genotype. In assessment of corticosterone levels of α6KO mice, there was a main effect of exposure time ($F_{2,44} = 20.700, P < 0.001$), revealing that serum corticosterone levels were higher in mice after 15 and 30 min of novel cage exposure compared to control mice independent of genotype ($P$’s $< 0.001$; Fig 4.5b). As in other assays, no differences in serum corticosterone levels were detected between WT and α6KO mice after 15 ($t_{16} = 0.085, P = 0.933$; Fig 4.5b) or 30 min of novel cage exposure ($t_{18} = 1.187, P = 0.251$; Fig 4.5b).

**DISCUSSION**

In the present studies, gain-of-function L9S mice showed increased anxiety-like behavior compared to WT controls in the light-dark, elevated plus maze and open field locomotor assays, suggesting that stimulation of α6β2*nAChRs is sufficient to promote anxiogenic-like behavior. In contrast, anxiety-like behavior did not differ between WT animals and mice with a genetic
Figure 4.5 - L9S mice show a blunted HPA-axis response following 30 minutes of exposure to a novel cage.  a) Compared to controls (0), serum corticosterone levels were elevated after 15 or 30 min of exposure to a novel cage independent of genotype. After 30 min of novel cage exposure, compared to WT mice, L9S mice showed lower serum corticosterone levels, reflective of a blunted HPA-axis response.  b) Compared to controls, serum corticosterone levels were elevated in all mice after 15 and 30 min exposure to a novel cage independent of genotype. As in other assays, serum corticosterone levels did not significantly differ between WT and α6KO mice at any time point tested.  These data suggest that activation, but not deletion of α6β2*nAChRs regulates the HPA-axis response to novel cage exposure.  Data are represented as means ± SEM.  *P < 0.05 vs. WT; **P < 0.05 vs. 0 min novel cage exposure time.
deletion of the α6 subunit (α6KO) in these measures, revealing that α6β2*nAChRs are not necessary for expression of anxiety-like behavior.

An accumulation of data suggest that cholinergic hyperactivity promotes anxiety- and depression-like behavior in rodent animal models (Kolasa, Fusi, Garattini, Consolo, & Ladinsky, 1982; Luo et al., 2013; Mineur et al., 2013; Power & McGaugh, 2002). Reduced AChE activity is inversely correlated with elevated trait anxiety and is thought to contribute to depression in humans (Saricicek et al., 2012; Sklan et al., 2004). Candidate brain areas for this include the amygdala, where cholinergic lesions reduces anxiety-like behavior (Power & McGaugh, 2002) and the hippocampus, where local reductions in AChE function increases cholinergic activation along with anxiety and depression phenotype (Luo et al., 2013; Mineur et al., 2013).

Additionally, reductions in serum AChE were shown to be inversely correlated with increased trait anxiety in humans, and in fMRI human imaging studies, activation of the anterior cingulate cortex and the amygdala is implicated in anxiety-like behavior (Sutherland et al., 2013). α6β2*nAChRs are not greatly enriched in these brain areas, suggesting that neuroanatomical systems outside of the amygdala circuitry contribute to anxiety-like behaviors. The administration of the anxiolytic buspirone to mice led to reduced acetylcholine levels in both the dorsal and ventral striatum, suggesting that reduced cholinergic tone in these brain regions may in part attenuate anxiety-like behavior (Kolasa et al., 1982).

α6β2*nAChRs are also enriched on the soma of NE projection neurons in the LC. Exposure to stressors activates the LC, resulting in norepinephrine release at thalamic nuclei involved with processing noxious stimuli as well as the PVN, leading to increases in serum cortisol (Condes-
Although smokers in abstinence require shorter times to report pain following exposure to a cold pressor task that is negatively correlated with expression of $\beta_2*nAChRs$, abstinent smokers express higher levels of antinociception following a social stress test that corresponded with increased levels of NE compared to non-smokers (Cosgrove et al., 2010; Girdler et al., 2005; Pomerleau, Turk, & Fertig, 1984). L9S mice showed both lower footshock thresholds for the vocalization response and higher thresholds for the jump response than WT mice. It is possible that exposure to subsequent footshocks led to activation of NE projection neurons in the LC, resulting in NE release in the thalamus and higher thresholds for the jump response in L9S mice (Condes-Lara, 1998; Voisin et al., 2005; C. Zhang et al., 1998). These higher thresholds required for the jump response are similar to the increased antinociception shown by smokers after stress exposure. Considering that monoamine reuptake inhibitors with greater selectivity at NE or serotonin transporters are typically more efficacious at reducing pain-stimulated behaviors, such as acid-induced stretching, than reuptake inhibitors with preferential activity at DA transporters, the differences in footshock thresholds observed in L9S mice in these experiments may have been mediated via activation of $\alpha_6\beta_2*nAChRs$ on NE neurons, rather than on DA neurons (Aoki et al., 2006; Ardid et al., 1992; Leventhal et al., 2007; Rojas-Corrales, Casas, Moreno-Brea, Gibert-Rahola, & Mico, 2003; Rosenberg, Carroll, & Negus, 2013).

Recent studies in transgenic mice with their $\alpha_4$ subunit specifically knocked down in VTA DA neurons suggest that mesolimbic DA neurons support the anxiolytic-like effects of nicotine in the elevated plus maze tasks (McGranahan et al., 2011). The selective deletion of the $\alpha_4$ subunit from DA neurons resulted in an attenuation of the anxiolytic efficacy of 0.01 mg/kg i.p. nicotine in an elevated plus maze. The results of these studies suggest that persistent activation of
α6β2*nAChRs promotes, rather than attenuates anxiety-like behavior. If low doses of nicotine in these studies exerted their effects via desensitization of α4β2*nAChRs, as has been proposed elsewhere (Anderson & Brunzell, 2012), the present data in L9S mice suggest that low doses of nicotine may in part mediate their anxiolytic-like effects via inhibition of α4α6β2*nAChRs.

Previous studies demonstrate that low doses of nicotine and broad-spectrum antagonists of nAChRs promote anxiolysis-like behavior in rodents in a number of preclinical models with predictive validity for anxiolysis, suggesting that low dose nicotine may reduce anxiety-like behavior via inactivation of the high affinity β2*nAChRs (Anderson & Brunzell, 2012; McGranahan et al., 2011; Newman et al., 2002; Roni & Rahman, 2011; Varani et al., 2012). Varenicline and DHβE administration results in reduced time to eat palatable food in a novel environment in the novelty induced hypophagia assay, reversal of suppressed operant responding in the presence of conditioned stimulus paired with a footshock during a conditioned emotional response assay, reduced digging in a marble burying task and increased exploration of the open arms of an elevated plus maze (Anderson & Brunzell, 2012; Dawson et al., 2013; Turner et al., 2010). Both drugs have similar affinities for β2*nAChRs comprised of either α4 or α6 subunits, suggesting that α4β2*, α6β2*, and or α4α6β2*nAChRs may contribute to these effects (Grady et al., 2010; Papke et al., 2008). The present studies in α6KO and L9S mice reveal that although α6β2*nAChRs do not appear to be necessary for expression of anxiety-like behavior in the light-dark task, elevated plus maze, or open field assay, α6β2*nAChRs may contribute to anxiety-like behavior when stimulated by exogenous compounds such as nicotine under conditions in which endogenous cholinergic tone is elevated.
One theory suggests that expression of β2*nAChRs contributes to anxiety and depression phenotype (Picciotto et al., 2002). Chronic nicotine self-administration results in up-regulation of α4β2*nAChRs in mice (Even et al., 2008; Metaxas et al., 2010; Nashmi et al., 2007; Nuutinen, Ahtee, & Tuominen, 2005; Pakkanen, Jokitalo, & Tuominen, 2005; Pauly, Marks, Robinson, van de Kamp, & Collins, 1996; Perez et al., 2008; Pietila, Lahde, Attila, Ahtee, & Nordberg, 1998; Sparks & Pauly, 1999; Turner et al., 2011; C. Xiao et al., 2009), rats (Abdulla et al., 1996; Barrantes, Rogers, Lindstrom, & Wonnacott, 1995; Collins, Romm, & Wehner, 1990; el-Bizri & Clarke, 1994; Flores, Davila-Garcia, Ulrich, & Kellar, 1997; Nguyen, Rasmussen, & Perry, 2003, 2004; Perez et al., 2008; Walsh et al., 2008; F. Wang, Chen, Steketee, & Sharp, 2007; Yates, Bencherif, Fluhler, & Lippiello, 1995) non-human primates (Kassio et al., 2001; McCallum et al., 2006; Perez et al., 2012; Perez et al., 2013; Slotkin, Pinkerton, Auman, Qiao, & Seidler, 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). This is consistent with preclinical models which suggest that selective inhibition of β2*nAChRs promotes anxiolysis-like behavior and anti-depressant activity (Anderson & Brunzell, 2012; Dawson et al., 2013; Mineur et al., 2013). In contrast to the α4β2*nAChRs, a preponderance of the evidence suggests that chronic nicotine results in a down-regulation of α6β2*nAChRs (Perez et al., 2008; Perez et al., 2012; Perez et al., 2013) (but see (Parker et al., 2004)). More recent studies showed reduced binding of the E11A analog of the α6/α3*nAChR antagonist α-conotoxin MII, believed to have greater affinity for α4α6β2*nAChRs than α6(non-α4)β2*nAChRs, in rats following chronic oral nicotine self-administration. Compared to oral consumption of saccharin, chronic oral nicotine self-administration in mice resulted in a reduction in α-conotoxin MII binding in WT but not in
α4KO mice, suggesting that reductions in α6β2*nAChRs appear to be predominantly of the α4α6β2*nAChR subtype, whereas upregulation of α6(non-α4)β2*nAChRs is reported 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., 2013; C. Xiao et al., 2009). Future studies in L9S mice bred to α4KOs could help clarify if α4α6β2* or α6β2*nAChRs contribute more or less to this phenotype. Basal or nicotine exposure-dependent differences in nAChR expression may in part account for affective behavior observed during nicotine withdrawal. Intracerebroventricular infusions of the selective α6/α3β2*nAChR antagonist α-conotoxin [H9A;L15A] at doses that had no effect on elevated plus maze activity on their own attenuated reductions in open arm time expressed by mice in spontaneous withdrawal from nicotine (Jackson et al., 2009). This lack of anxiolytic efficacy of α-conotoxin [H9A;L15A] in nicotine-naïve mice is consistent with results of the present genetic studies wherein α6KO mice did not show any significant differences in either anxiety-like behavior or responses to external stimuli compared to WT controls. In light of the anxiogenic behavior exhibited by L9S mice in these present studies, the anxiolytic efficacy of α-conotoxin [H9A;L15A] in mice experiencing spontaneous withdrawal from nicotine suggests that activation of α6β2*nAChRs on DA neurons by ACh during spontaneous withdrawal may contribute to the increased anxiety and sensitivity to external stressors reported by smokers in abstinence (Cosgrove et al., 2010; Jackson et al., 2009; Swan, Ward, & Jack, 1996).

Considering the anxiogenic-like phenotype expressed by L9S mice observed in various behavioral models, the blunted HPA-axis response in L9S mice following 30 min novel cage exposure was surprising, since novel cage exposure results in elevated serum corticosterone, a
biomarker of increased anxiety (Armario et al., 2012; Hennessy, 1991; Hennessy & Foy, 1987). However, these results are similar to the HPA-axis dysregulation observed in abstinent smokers following exposure to stress assays such as the Trier Social Stress Test (Childs & de Wit, 2009; Kudielka & Wust, 2010; Swan et al., 1996). The HPA-axis response in humans diagnosed with depression is also muted following social stress, further suggesting that L9S mice might express a depression-like phenotype, which warrants further experimentation to determine the effect of the L9’S mutation on depression-like behavior. This is not surprising, since depression is also associated with increased cholinergic tone (Saricicek et al., 2012).

Taken together, these studies suggest that activation of α6β2*nAChRs promotes anxiety-like behavior. They do not eliminate the possibility that the anxiolytic-like efficacy of low doses of nicotine are in part via inactivation of α6(non-α4)β2*nAChRs. They further suggest that L9S mice may provide insight into the neurocircuitry that regulates the rewarding and reinforcing effects of nicotine, but also how activation of α6β2*nAChRs may contribute to the symptoms of nicotine withdrawal and possibly depression-like behavior.
INTRODUCTION

As of 2009, those aged 65 years or older comprised approximately 13% of the total population of the United States (Administration on Aging, 2009). Anxiety disorders overall are lower in the elderly than in the general population (Flint, 1994). While reductions in nicotinic acetylcholine receptors (nAChRs) are a part of the normal human aging process (Mitsis et al., 2009), pathologic reductions in nAChRs are associated with cognitive declines and the emergence of psychological co-morbidities, such as increased anxiety (Brodaty et al., 2012; Sabbagh et al., 2006; Wolitzky-Taylor et al., 2010).

nAChRs are pentameric, ligand-gated ion channels that promote neuronal activity. nAChRs containing the α4 and β2 subunits (α4β2*nAChRs; *denotes possible assembly with other subunits) have a high affinity for the endogenous ligand, ACh. The activation and expression of α4β2*nAChRs is neuroprotective (Zanardi et al., 2007); increased age-dependent neurodegeneration is observed in mice with a genetic null mutation of β2*nAChRs (Zoli et al., 1999).

Brain areas that may regulate anxiety include the hippocampus, amygdala, anterior cingulate cortex and lateral septum. The hippocampus, amygdala and anterior cingulate cortex are
predominantly excitatory nuclei, whereas the lateral septum is a GABA-ergic projection nucleus. In addition to vasopressin inputs from the amygdala that promote anxiety-like behavior, the lateral septum receives a convergence of excitatory afferents from brain areas known to regulate motivational salience, such as dopamine from the ventral tegmental area and glutamate from the hippocampus and anterior cingulate (Beckstead, 1979; Buchanan, Thompson, Maxwell, & Powell, 1994; Caffé, van Leeuwen, & Luiten, 1987; Calderazzo, Cavalheiro, Macchi, Molinari, & Bentivoglio, 1996; Canteras, Simerly, & Swanson, 1995; De Vries & Buijs, 1983; Lindvall, 1975; Numan & Numan, 1996; Risold & Swanson, 1997a, 1997b). Exposure to both restraint and chronic social stress results in lower levels of phosphorylated, or activated extracellular regulated kinase (pERK) in the lateral septum, and thus reduced pERK in the lateral septum may serve as a cellular biomarker for increased anxiety (Sheehan et al., 2003; Singewald et al., 2009).

Activation of nAChRs promotes growth factors (Belluardo, Mudo, Blum, et al., 1999; Belluardo et al., 2000; Belluardo, Mudo, Caniglia, et al., 1999; Ying et al., 2002), supports release of neurotransmitters that regulate adenylyl cyclase and results in an increase in intracellular calcium, which can indirectly activate ERK via the Raf/Ras/MEK/ERK pathway. In its active form, pERK is capable of phosphorylating several downstream substrates leading to activation of RSK, c-Fos, MSK and the transcription factor cAMP response element-binding protein (CREB) (Nakayama et al., 2001). Inactivation of α4β2*nAChRs on GABA interneurons in the ventral tegmental area promotes activation of dopamine neurons via disinhibition, leading to increased pERK (Graupner, Maex, & Gutkin, 2013; Mansvelder et al., 2002; Valjent et al., 2005). Thus, reduced cholinergic tone promoted via α4β2*nAChRs can result in dopamine release in the lateral septum, leading to elevated pERK and increased lateral septum activity.
These studies used genetic and pharmacologic manipulations to study the age-dependent effects of reduced basal expression of α4β2*nAChRs on anxiety-like behavior in mice using numerous ethological tests. Mice heterozygous for a null mutation in their α4 nAChR subunit (α4HET) have a 50% reduction in α4β2*nAChR expression (Gotti et al., 2008). Using the light-dark assay, the elevated plus maze, and the open field test, these studies assessed anxiety-like behavior in 6-8 month (Adult) and 22-24 month (Aged) wild type (WT) and α4HET mice. Adult and aged mice received a challenge dose of the selective α4β2*nAChR antagonist dihydro-beta-erythroidine (DHβE) to further determine how inhibition of α4β2*nAChRs affects anxiety-like behavior during adulthood and aging. Immunoprecipitation assays on brain samples taken from the lateral septum, amygdala, anterior cingulate cortex and hippocampus confirmed reduced α4β2*nAChR expression in these brain areas during adulthood and assessed if there was a decline of α4β2*nAChR expression during aging. Following a challenge injection of either saline or a behaviorally relevant dose (3 mg/kg i.p.) of DHβE, the lateral septum and anterior cingulate cortex were extracted from mice for western blot analysis of levels of total ERK and pERK and total CREB and pCREB of Adult and Aged WT, α4HET and α4 knockout (α4KO) mice.

MATERIALS AND METHODS

Animals

68 WT-Adult, 17 WT-Aged, 70 α4HET-Adult, 23 α4HET-Aged, 30 α4KO-Adult and 20 α4KO-Aged male mice back-crossed against the C57BL/6 background over ten generations were
used for these studies. Mice were generated in a breeder colony at Virginia Commonwealth University. Animals received *ad libitum* access to food and water and were group-housed (2-5 per cage) with 1/8 inch corncob bedding in an environmentally controlled, AALAC-approved vivarium with a 12h light/dark cycle (lights on 0600). To promote hydration and nutrition, the diets of aged mice were supplemented with Clear H₂O Diet Gel (Pharmaserv, Framingham, MA). Mice were habituated to experimenter handling for 3 days and the experimental room at least one day prior to any training or testing. All experiments were conducted with approval of the Institutional Animal Care and Use Committee at Virginia Commonwealth University in compliance with the *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 2010).

**Drugs**

Dihydro-beta-erythroidine (DHβE) (Tocris, Bristol, UK) was dissolved in 0.9% sterile saline. DHβE doses are expressed as hydrogen bromide salt. Injections were delivered i.p. in volumes of 0.1 ml/30 g. After DHβE injection, animals were returned to their home cages for 15 minutes before evaluation in the light-dark assay. Weights were taken approximately 1 h prior to behavioral assays.

**Apparatus**

*Light-Dark Apparatus*

Light-dark experiments were conducted in modified place conditioning chambers (Med Associates, St. Albans, VT) so that a small, enclosed, dark chamber with a black ceiling (L 16.8 cm x W 12.7 cm x H 12.7 cm) was directly adjacent to a larger, open, brightly-lit chamber (L
26.5 cm x W 12.7 cm x H 26.2 cm) that was illuminated by a single 23W fluorescent light bulb. Mice had free access to both light and dark chambers of the apparatus via a door (W 5 cm x H 5.9 cm) that was opened at the beginning of the test. The stainless steel dropping trays had plastic inserts (L 16.8 cm x W 12.7 cm) in the respective light or dark chambers to enhance the brightness of the light chamber and reduce brightness and glare in the dark chamber. Data were collected on a PC computer and calculated using Med Associates software.

Open Field
Open field experiments were conducted in a polycarbonate cage (33 cm × 21 cm × 9 cm high) under ambient room lighting conditions provided by overhead fluorescent lights. The apparatus was covered with clear Plexiglas to permit tracking but to prevent escape of mice. Holes drilled in this lid allowed ventilation during the test. During experiments when additional illumination was provided by a 23W fluorescent light bulb (No injection/bright room lighting), the polycarbonate cage was placed within a white plastic enclosure that surrounded all 4 walls and the floor.

Elevated Plus Maze
An elevated plus maze raised 68 cm above the floor had two arms with white plastic flooring that were enclosed with 15.25 cm black Plexiglas walls (5 x 30 cm) that were perpendicular to two equivalent (5 x 30 cm) exposed arms also with white flooring. Experimentation took place under overhead fluorescent light illumination. For both the open field and elevated plus maze assays, data was collected by a ceiling-mounted camera that was interfaced to a PC for collection of data using ANY-maze tracking software (Stoelting, Wood Dale, IL).
Experimental Procedures

*Experiment 1: Behavioral assessment of α4β2* nAChR expression and aging on anxiety-like behavior.*

*Light-dark assay*

Untreated Adult and Aged α4HET and WT mice (n = 9-15 per age group and per genotype) were brought to the experimental room at least one hour prior to testing. The room was dark with the exception of lighting required for the apparatus. Mice were started in the dark chamber of the apparatus with the door closed. This enabled the use of latency to enter the light chamber as a measure of anxiety-like behavior while preventing the potential confound of freezing that occurs when rodents are started in the light chamber. Mice moved freely throughout the apparatus for a period of 10 min. Latency and % time spent in the light chamber were calculated as described in Chapter 3. Movement counts per second spent in the light and dark chambers were separately calculated over 1 min time bins.

*Open field locomotor assay*

Untreated Adult and Aged α4HET and WT mice (n = 6-7 per age group/genotype) were placed in the open field facing the left corner of the apparatus farthest from the experimenter and were allowed to freely explore the entire apparatus for 15 min (No injection/bright room lighting). Latency to first entry into the center zone (6 x 6 cm), the number of entries made into the center zone and time spent in the corners (6 x 6 cm each corner) were evaluated. A separate cohort of α4HET-Adult, α4HET-Aged, WT-Adult and WT-Aged mice (n = 5-9 per age and genotype)
received i.p. delivery of 0.9% sterile saline as a stressor immediately before evaluation in the open field locomotor assay in novel polycarbonate cages containing corncob bedding (Saline injection/normal room lighting).

**Elevated plus maze**

Untreated Adult and Aged α4HET and WT mice were placed on the center of an elevated plus maze apparatus facing one of the closed arms (n = 6-11 per age group/genotype). Mice were evaluated for number of open arm entries, time spent in the open arms and latency to explore the terminal zones (the extreme 5 cm) of the open arms for a period of 10 min as described previously (Anderson & Brunzell, 2012). Between animals, feces, urine and dander were removed and behavioral apparatus was cleaned with 2% Nolvosan (Pfizer Animal Health, Madison, NJ).

**Experiment 2: Assessment of α4β2*nAChR expression on anxiety-like behavior following pharmacological antagonism of β2*nAChRs.**

α4HET-Adult and WT-Adult mice received between-subject delivery of 0 or 3 mg/kg of the selective β2*nAChR antagonist DHβE i.p. (n = 18 per genotype/dose) 15 minutes prior to evaluation in the light-dark assay as described above.

**Experiment 3: Serum corticosterone determination by ELISA**
Immediately following completion of the open field locomotor assay described above in Experiment 1, mice underwent rapid decapitation between 1000 and 1700 hours and were counter-balanced by age and genotype to minimize the effect of circadian fluctuations of serum corticosterone levels on test results. Trunk blood was collected in 1.5 mL centrifuge tubes containing 15 µl of heparin, which were immediately placed on ice. Samples were centrifuged at 4°C at 13000 g for 15 minutes. Serum was decanted and stored at -80°C until thawed on ice for corticosterone ELISA determined using the Corticosterone HS EIA kit by Immunodiagnostic Systems (Scottsdale, AZ). The assay was performed according to manufacturer instructions and serum corticosterone concentrations were determined using ReaderFit curve-fitting software (Hitachi Solutions America Ltd, USA).

Experiment 4: nAChR immunoprecipitation: Assessment of age-related expression of α4*nAChRs

Brains from α4HET-Adult, α4HET-Aged, α4KO-Adult, α4KO-Aged, WT-Adult and WT-Aged mice (n = 6-8 per genotype and age) were harvested following rapid decapitation. Brains were removed, placed in ice-cold PBS and sliced in a plastic matrix chilled with dry ice. Coronal slices were floated in ice-cold PBS and anterior to posterior hole punches were taken of the amygdala (1.20 mm; approx. -1.06 to -1.94 from Bregma, with the dorsal hippocampus as the primary anterior and posterior landmarks), anterior cingulate cortex (1.50 mm; approx. +1.94 to +0.14 from Bregma, with the anterior commissure as the primary landmark) and septum (1.50 mm; approx. +1.18 to +0.14 from Bregma, with the anterior commissure aligning with the lateral ventricles as the anterior landmark, and crossing midline as the posterior landmark).
anterior dorsal hippocampus was hemi dissected with the right lobe retained for processing. Tissue samples were fast frozen in ice-cold isopentane and stored at -80° C until the expression of α4 nAChR subunits was determined via immunoprecipitation.

**nAChR antibody generation**

All nAChR antibodies were targeted against the cytoplasmic loop region of mouse nAChR subunits as previously published (David et al. 2010). The immunoprecipitation (IP) efficacy and specificity of the antibodies has previously been tested with recombinant receptors expressed in HEK-293 cells, and by comparing the IP results in the SCG of α5β2 and α5β4 double KO mice (which express pure α3β4 and α3β2 receptors, respectively) with polyethylene glycol precipitation of all solubilized receptors. We furthermore took advantage of nAChR-KO mice in order to exclude false-positive reactions (see supplemental Fig. 1, David et al. 2010).

**Immunoprecipitation of [³H]-epibatidine labeled receptors**

Receptors were solubilized in 2 % Triton X-100 lysis buffer: 50 mM Tris-HCl pH = 7.5, 150 mM NaCl, 2 % Triton X-100, supplemented with one complete mini protease inhibitor cocktail tablet (Roche) per 10 ml buffer. Following one ultrasound pulse of 5 seconds duration at 30 % energy level, samples were left for 2 hours at 4°C and thereafter centrifuged at 16000 g for 15 min at 4°C. 130 µl clear supernatant from were incubated with 20 µl 10 nM [³H]-epibatidine and 5 µg antibody in 30 µl phosphate-buffered saline (PBS: 10 mM Na2HPO4, 1.8 mM KH2PO4, 2.7 mM KCl, 140 mM NaCl, pH = 7.4) on a shaking platform at 4°C overnight. Unspecific binding was determined by adding 300 µM nicotine to half of the samples.
Heat-killed, formalin-fixed Staphylococcus aureus cells carrying protein A (Standardized Pansorbin-cells, Calbiochem) were centrifuged at 2300 g for 5 min at 4°C. Pansorbin-pellets were washed twice with IP-High (50 mM Tris-HCl pH = 8.3, 600 mM NaCl, 1 mM EDTA, 0.5 % Triton X-100), once in IP-Low (50 mM Tris-HCl pH = 8.0, 150 mM NaCl, 1 mM EDTA, 0.2 % Triton X-100), and re-suspended with IP-Low. 20 µl of this suspension of Pansorbin cells were added to the above mentioned cocktail containing the antibody, solubilized receptors, and [³H]-epibatidine for 2 hours at 4°C on a shaking platform. Samples were centrifuged at 2300 g for 5 min at 4°C and washed twice with IP-High and once with IP-Low at 2300 g for 1 min at 4°C. Pellets were re-suspended in 200 µl 1 M NaOH and subjected to liquid scintillation counting. All protein quantifications were performed using the BCA Protein Assay Reagent Kit (Pierce) following the manufacturer’s instructions.

Experiment 5: Assessment of α4β2*nAChR-associated changes in intracellular signaling:

Regulation of ERK and CREB in the lateral septum and anterior cingulate cortex.

Western blot analysis

Adult and Aged α4HET (n=43), α4KO (n=33) and WT mice (n=51) were placed in a novel cage x 15 minutes after administration of 3 mg/kg DHβE or 0.9% sterile saline i.p. Brains were then harvested via rapid decapitation and brain sections were collected as described previously (Brunzell et al., 2003). For collection of the lateral septum, the medial portion of the septal region was removed via a 0.75 mm brain punch and discarded prior to collection of the septal region with a 1.5 mm punch. Tissue samples were fast frozen on dry ice and stored at -80º C until the expression of ERK, pERK, CREB, pCREB and alpha tubulin were determined via
western blot analysis. Samples were sonicated while on ice in a 1% SDS homogenization buffer containing 50 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM PMSF supplemented with a protease inhibitor cocktail tablet (Roche, Indianapolis, IN) and Phosphatase Inhibitor Cocktails 2 and 3 (Sigma, St. Louis, MO) at a 1:100 dilution. Samples were centrifuged at 13000 g for 15 min. The supernatant fluid was removed and stored at -80° C. Protein concentrations were determined in duplicate using Bio-Rad DC protein detection reagent (Bio-Rad, Hercules, CA). 10µg of protein was loaded per lane to determine immunoreactivity for ERK, pERK, CREB, pCREB and alpha tubulin. Protein was run on a 4-15% gradient SDS-polyacrylamide gel (Bio-Rad, Hercules, CA) and then transferred to a nitrocellulose membrane in chilled Tris-glycine buffer for 55 minutes at 100 V.

All incubation steps were performed on a rocker. Membranes were rinsed in Tris-buffered saline (TBS), then blocked in Odyssey Blocking Buffer (1:5 in 1X PBS) for 1.5 h at room temperature. Membranes were washed briefly in TBS-T and incubated overnight at 4° C in primary antibody diluted in 3% BSA in TBS-T. Primary antibodies to detect ERK and pERK (threonine 183/tyrosine 185) were used at a dilution of 1:5000 in mouse and rabbit respectively (MAB1576; R&D Systems, Minneapolis, MN; CS4376; Cell Signaling Technology, Beverly, MA) CREB in rabbit (CS9197) at 1:500, pCREB in mouse (CS9196) at 1:1000 (Cell Signaling Technology, Beverly, MA) and alpha tubulin in chicken (SAB3500023; Sigma, St. Louis, MO) diluted 1:5000 was used as a loading control. Blots were washed for 30 min in TBS-T and incubated for 50 min at room temperature in 1:15000 affinity-purified anti-rabbit IgG IRDye fluorescent (800CW) for pERK, 1:20000 anti-mouse IgG IRDye fluorescent (680LT) for ERK, 1:15000 affinity-purified anti-mouse IgG IRDye fluorescent (800CW) for pCREB, 1:20000 affinity-
purified anti-rabbit IgG IRDye fluorescent (680LT) for CREB and 1:20000 affinity-purified anti-chicken IgG IRDye fluorescent (680LT) for alpha tubulin (LI-COR Biosciences, Lincoln, NE) secondary antibody in 3% BSA in 0.01% SDS/PBS-T. After exposure to secondary antibodies, blots were protected from light to prevent degradation of the fluorescent signal of the secondary antibody. Blots were scanned on an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Integrated densities were analyzed and quantified using ImageJ software (NIH). Relative protein expression values were normalized to alpha tubulin in these experiments, and each subject was further normalized to % of WT-Adult saline-injected subjects on each blot.

**Statistical Analysis**

2 x 2 ANOVA assessed genotype x age or genotype x treatment effects in behavioral and corticosterone assays. For the light-dark assays, % time spent in the light was initially analyzed using a repeated measures ANOVA, with a within-subject factor of 5 min time bins. 3 x 2 ANOVA analyzed genotype x age or genotype x treatment effects on α4 nAChR subunit expression or levels of ERK, pERK, CREB, pCREB and alpha tubulin. Tukey’s post hoc tests were used to identify main effects of genotype. Significant interactions were followed with post hoc Student’s t-tests to determine the nature of the interaction. For Experiment 1, detection of a significant genotype x age x time bin interaction for movements per second in either the light or dark chamber were followed by a repeated measures ANOVA for each age group, to determine the genotype-specific nature of the interaction. In Experiment 2, a repeated measures ANOVA was performed for each genotype after detection of a significant effect of drug treatment. Planned comparisons to evaluate the effect of a stressful saline-injection on anxiety-like behavior.
in the light-dark and open field assays between WT-Adult and α4HET-Adult mice were performed via Student’s t-tests. Planned comparisons to assess age-dependent changes in α4 nAChR subunit expression in WT mice were performed using Student’s t-tests. For all analyses, $P$ values < 0.05 were reported as significant.

**RESULTS**

*Basal expression of α4β2*nAChRs affects age-dependent changes in anxiety-like behavior*

*Light-Dark Assay*

There was a significant genotype x age interaction for movements per second in the light ($F_{1,49} = 13.096$, $P = 0.001$), but not the dark chamber ($F_{1,49} = 2.444$, $P = 0.124$). Post hoc analyses revealed that α4HET-Adult mice showed significantly more exploration of the light chamber than their age-matched WT littermate controls ($F_{1,27} = 4.384$, $P < 0.05$; Fig 5.1a). The absence of any genotype effect on dark chamber activity suggests that this difference was not due to general changes in locomotor activity. Interestingly, this genotypic effect was reversed in Aged mice. α4HET-Aged mice showed reduced movements per second in the light chamber compared to Aged-WT mice ($F_{1,22} = 12.070$, $P = 0.002$; Fig 5.1b). A significant genotype x age interaction was also observed for latency to enter the light chamber ($F_{1,49} = 10.670$, $P = 0.002$), revealing that α4HET-Aged mice showed significantly higher latencies to enter the light chamber than their WT-Aged counterparts ($t_{22} = 2.930$, $P < 0.01$; Fig 5.2a). This observation was not evident in Adult mice. α4HET mouse latencies increased with age as evidenced by a significant difference between α4HET-Adult and α4HET-Aged mice ($t_{27} = 2.847$, $P < 0.01$; Fig 5.2a). As with exploratory behavior of the light chamber, there was a non-significant trend for
WT-Aged mice to require less time to explore the light chamber than WT-Adult controls ($t_{22} = 1.839, P = 0.079$; Fig 5.2a).

There was a significant genotype x age x time bin interaction ($F_{1,49} = 4.701, P < 0.05$) for time spent in the light chamber. A subsequent two-way ANOVA detected a significant interaction of genotype x age for this measure during the first 5 minutes ($F_{1,49} = 12.359, P = 0.001$) but not the second five minutes of the 10 min light-dark assay ($F_{1,49} < 1, P = 0.351$). Consistent with other light-dark measures, post hoc tests showed that WT-Aged mice spent more time in the light chamber than WT-Adult mice ($t_{22} = 2.206, P < 0.05$; Fig 5.2b), whereas α4HET-Aged mice spent less time in the light chamber than both α4HET-Adult mice ($t_{27} = 2.819, P < 0.01$; Fig 5.2b) and their WT-Aged counterparts ($t_{22} = 3.350, P < 0.01$; Fig 5.2b).

Open Field Test

Two-way ANOVA analyses revealed significant genotype x age interactions for latency to enter the central zone ($F_{1,27} = 15.068, P = 0.001$), number of entries made into the central zone ($F_{1,22} = 5.757, P = 0.025$) and time spent in the corners of the open field locomotor assay under bright lighting conditions ($F_{1,22} = 5.757, P = 0.025$). α4HET-Aged mice required longer to enter the central zone ($t_{10} = 3.606, P < 0.01; t_{11} = 3.736, P < 0.01$; Fig 5.3a), made significantly fewer entries into the central zone ($t_{10} = 4.622, P = 0.001; t_{11} = 2.336, P < 0.05$; Fig 5.3b) and spent more time in the corners than both α4HET-Adult mice and WT-Aged mice, respectively ($t_{10} = 3.501, P < 0.01; t_{11} = 3.274, P < 0.01$; Fig 5.3c). These differences in open field behaviors are also graphically represented by group occupancy plots in Figure 5.4, with blue representing the lowest center zone group occupancy. In contrast to these age-dependent effects observed in
Figure 5.1 - Basal expression of α4β2*nAChRs regulates anxiety-like behavior in an age-dependent manner. a) Compared to age-matched wild type (WT) mice, exploration of the light chamber was higher in 6-8 month (Adult) mice with 50% basal expression of α4β2*nAChRs (α4HET), suggestive of an anxiolytic-like phenotype. b) In contrast, light chamber activity was reduced in 22-24 month (Aged) α4HET mice compared to WT-Aged mice, suggesting that anxiety phenotype increases as α4HET mice age. c-d) There were no effects of genotype on movements per second spent in the dark chamber, suggesting that the age- and genotype-specific differences in behavior were reflective of changes in anxiety-like behavior. Data are represented as means ± SEM.
Figure 5.2 - Age-dependent changes in anxiety-like behavior regulated by basal expression of α4* nAChRs during the light-dark assay in mice. a) Compared to 6-8 month (Adult) mice of the same genotype, anxiety-like behavior was increased in 22-24 month (Aged) mice with heterozygous expression of α4* nAChRs (α4HET) and reduced in Aged wild type (WT) mice, as
measured by increased latencies to enter the light chamber compared to both α4HET-Adult and age-matched WT mice. Additionally there was a non-significant trend for WT-Aged mice to show lower latencies to enter the light chamber than WT-Adult controls ($P = 0.079$). b) WT-Aged mice spent more time in the light chamber than WT-Adult mice, while α4HET-Aged mice spent less time in the light chamber than both α4HET-Adult and WT-Aged mice during the first 5 minutes of the light-dark assay, suggesting that basal expression of α4*nAChRs differentially regulates age-dependent changes in anxiety-like behavior in this assay. c) No age or genotype-specific effects were observed for time spent in the light chamber during minutes 6-10, suggesting that all mice habituated to the apparatus after 5 minutes. Data are represented as means ± SEM. *$P < 0.05$; **$P < 0.01$ vs. Adult mice of same genotype; ††$P < 0.01$ vs. WT-Aged mice.
α4HET mice, WT-Aged mice did not differ from WT-Adult mice in center zone latency time ($t_{12} = 0.025$, $P = 0.980$; Fig 5.3a), center zone entries ($t_{12} = 0.253$, $P = 0.805$; Fig 5.3b), or time spent in the corners during the open field locomotor assay ($t_{12} = 0.343$, $P = 0.738$; Fig 5.3c).

Although two-way ANOVA did not reveal any significant genotype x age interactions for latency to enter the center zone ($F_{1,22} = 2.760$, $P = 0.111$), number of entries made into the center zone ($F_{1,22} < 1$, $P = 0.932$) or time spent in the corners during the open field locomotor assay under normal lighting conditions (Saline injection/normal room lighting; $F_{1,22} < 3.216$, $P = 0.087$), planned comparison t-tests revealed that α4HET-Adult mice spent significantly less time in the corners than WT-Adult controls ($t_{13} = 2.873$, $P < 0.05$; Table 5.1). There was also a non-significant trend for α4HET-Adult mice to make more entries into the center zone than age-matched WT mice ($t_{13} = 2.072$, $P = 0.059$; Table 5.1). Data from 2 subjects were excluded due to inconsistent video tracking. Data from an additional mouse was excluded due to total distance traveled being 4 standard deviations from the mean.

**Elevated Plus Maze**

Two-way ANOVA tests revealed main effects of age for number of open arm entries ($F_{1,29} = 5.937$, $P < 0.05$), time spent in the open arms ($F_{1,29} = 4.839$, $P < 0.05$) and latency to explore the terminal 5cm of the open arms of an elevated plus maze ($F_{1,29} = 14.652$, $P = 0.001$). Aged mice made fewer entries into the open arms, spent less time in the open arms and had higher latencies to explore the terminal zone of the open arms of an elevated plus maze compared to Adult mice independent of genotype (Table 5.2). There was no effect of genotype or interaction of genotype with age in the elevated plus maze task.
Figure 5.3 - Age-dependent increases in anxiety-like behavior also observed in α4HET mice during open field test. a) Unlike wild type (WT) mice, whose behaviors did not differ between
6-8 (Adult) and 22-24 month (Aged) mice, anxiety-like behavior was increased in Aged α4 heterozygous (α4HET) mice during an open field test. α4HET-Aged mice had higher latencies to enter the center zone, b) made fewer entries into the center zone and c) spent more time in the corners than both α4HET-Adult controls and age-matched WT mice. Data are represented as means ± SEM. **P < 0.01 vs. α4HET-Adult mice; †P < 0.05; ††P < 0.01 vs. WT-Aged mice.
Figure 5.4 - Group occupancy plots for 6-8 (Adult) and 22-24 month (Aged) wild type (WT) and α4 heterozygous (α4HET) mice during open field locomotor test. α4HET-Aged mice made the fewest number of entries into the center zone as represented by the color blue (lowest occupancy) and spent the most time in the corners of the open field apparatus, represented as the color red (highest occupancy) among all groups tested.
Table 5.1 - Mice with reduced expression of α4β2*nAChRs (α4HET) show less anxiety-like behavior in open field locomotor task following i.p. saline.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age</th>
<th>Latency to enter center (s)</th>
<th>Center zone entries</th>
<th>Time spent in corners (s)</th>
<th>n size</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Adult</td>
<td>71.68 ± 42.3</td>
<td>16.50 ± 2.8</td>
<td>364.54 ± 33.3</td>
<td>8</td>
</tr>
<tr>
<td>WT</td>
<td>Aged</td>
<td>37.64 ± 16.4</td>
<td>10.20 ± 1.5</td>
<td>249.34 ± 48.0</td>
<td>5</td>
</tr>
<tr>
<td>α4HET</td>
<td>Adult</td>
<td>15.19 ± 5.0</td>
<td>25.71 ± 3.3#</td>
<td>248.51 ± 14.8*</td>
<td>9</td>
</tr>
<tr>
<td>α4HET</td>
<td>Aged</td>
<td>94.40 ± 34.6</td>
<td>14.33 ± 3.5</td>
<td>248.00 ± 21.7</td>
<td>7</td>
</tr>
</tbody>
</table>

After i.p. delivery of 0.9% sterile saline, mice were allowed to explore a novel polycarbonate housing cage under normal fluorescent lighting conditions that was not surrounded by a white enclosure and x 15 min. α4 heterozygous (α4HET) 6-8 month (Adult) mice spent less time in the corners and showed trends for more entries made in the center zone than age-matched wild type (WT) controls, showing that the reduced expression of α4β2*nAChRs protects against increased anxiety-like behavior following an i.p. saline injection. Key: WT = wild type; α4HET = α4 heterozygous; Adult = 6-8 months; Aged = 22-24 months. All data represented as Mean ± SEM. *P < 0.05, #P = 0.059 compared to WT-Adult.
Pharmacological antagonism of α4β2*nAChRs increases anxiety-like behavior in α4HET, but not WT mice

Planned comparison t-tests revealed that Saline-treated α4HET mice showed shorter latencies compared to saline-injected WT mice, suggesting that reduced expression of α4β2*nAChRs reduced the stress of injection during this assay ($t_{36} = 2.788$, $P < 0.01$; Fig 5.5a). Despite this basal reduction in anxiety-like behavior observed in α4HET mice, a genotype x treatment interaction was observed for latency ($F_{1,72} = 7.547$, $P = 0.008$), revealing that α4HET mice administered 3 mg/kg i.p. DHβE required more time to enter the more aversive light chamber compared to α4HET saline-injected controls ($t_{36} = 2.335$, $P < 0.05$; Fig 5.5a). There was also a significant genotype x treatment interaction for percentage of time spent in the light chamber during the first 5 minutes ($F_{1,72} = 3.984$, $P = 0.05$). Despite the appearance of DHβE injection to increase time spent in the light chamber in WT mice and for α4HET mice to spend less time in the brightly-lit chamber than saline-injected α4HET animals, post hoc tests did not detect any significant effects of treatment on either genotype in this measure ($t_{36} = 1.461$, $P = 0.153$; $t_{36} = 1.363$, $P = 0.181$; Fig 5.5b). No significant treatment x genotype interaction was observed during the final 5 minutes of the light-dark assay ($F_{1,72} < 1$, $P = 0.754$).

A repeated measures ANOVA revealed a main effect of time bin ($F_{9,72} = 17.134$, $P < 0.001$) and a non-significant trend for a main effect of DHβE treatment on movements per second in the light chamber ($F_{1,72} = 3.344$, $P = 0.072$). Post hoc tests revealed that unlike WT mice, which were unaffected by DHβE treatment ($F_{1,36} < 1$, $P = 0.865$; Fig 5.6a), α4HET mice receiving DHβE showed reductions in movements per second in the brightly illuminated chamber compared to saline-injected controls ($F_{1,36} = 5.238$, $P < 0.05$; Fig 5.6b). Although between
Table 5.2 - Aged mice express higher levels of anxiety-like behavior than Adult mice on an elevated plus maze independent of genotype.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age</th>
<th>Open arm entries</th>
<th>Time in open arms (s)</th>
<th>Latency to explore terminal 5cm of open arms (s)</th>
<th>n size</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Adult</td>
<td>4.56 ± 0.90</td>
<td>32.70 ± 7.94</td>
<td>303.00 ± 7.94</td>
<td>10</td>
</tr>
<tr>
<td>WT</td>
<td>Aged</td>
<td>2.29 ± 0.75</td>
<td>15.17 ± 4.54</td>
<td>572.40 ± 19.75</td>
<td>7</td>
</tr>
<tr>
<td>α4HET</td>
<td>Adult</td>
<td>4.18 ± 1.05</td>
<td>55.45 ± 18.88</td>
<td>392.15 ± 69.49</td>
<td>11</td>
</tr>
<tr>
<td>α4HET</td>
<td>Aged</td>
<td>1.67 ± 0.71</td>
<td>10.25 ± 7.07</td>
<td>600.00 ± 0.00</td>
<td>6</td>
</tr>
</tbody>
</table>

22-24 month (Aged) mice expressed higher levels of anxiety-like behaviors than 6-8 month (Adult) mice, suggesting that Aged mice are more anxious than Adult mice independent of genotype during an elevated plus maze assay. Key: WT = wild type; α4HET = α4 heterozygous; Adult = 6-8 months; Aged = 22-24 months. All data represented as Mean ± SEM.
Figure 5.5 – Dihydro-beta-erythroidine (DHβE) administration results in anxiogenic-like behavior in α4HET mice. a) Despite the fact that α4HET saline-injected mice showed lower latencies than vehicle-treated wild type (WT) controls, latencies were increased in α4HET mice receiving 3 mg/kg i.p. dihydro-beta-erythroidine (DHβE) than their saline-injected (DHβE=0) α4HET counterparts. b) Although there was a treatment x genotype interaction for time spent in the light chamber ($F_{1,72} = 3.984, p = 0.05$), post hoc tests did not detect any specific effects of DHβE administration on time spent in the light chamber. Taken together, these data suggest that although lower basal expression of α4β2*nAChRs may protect against the anxiogenic-like effects of a saline vehicle injection, further antagonism of α4β2*nAChRs promotes an anxiogenic-like response in α4HET mice. Data are represented as means ± SEM. *$P < 0.05$ vs. saline-injected mice of same genotype (DHβE=0); ††$P < 0.01$ vs. saline-injected WT mice (DHβE=0).
Figure 5.6 - α4HET mice with lower expression of α4nAChRs are more sensitive to anxiogenic-like effects of dihydro-beta-erythroidine (DHβE).  a-b) Exploration of the light chamber was reduced in α4 heterozygous (α4HET) mice administered 3 mg/kg i.p. of the selective β2nAChR antagonist dihydro-beta-erythroidine (DHβE) compared to saline-injected (SAL) α4HET mice.  c-d) Compared to saline injection, administration of 3 mg/kg i.p. DHβE did not affect movements per second in the dark chamber in either genotype.  These data suggest that the observed behavioral effects of DHβE treatment in α4HET mice reflect increases in anxiety-like behavior and not changes in general locomotion.  Data are represented as means ± SEM.
subject ANOVA tests revealed a trend for a treatment x genotype interaction for movements per second spent in the dark chamber \((F_{1,72} = 3.060, P < 0.085)\), post hoc tests did not reveal any significant between subject effects of drug treatment for movements per second in the dark chamber for WT \((F_{1,36} = 1.129, P > 0.1; \text{Fig 5.6c})\) or \(\alpha 4\text{HET}\) mice \((F_{1,36} = 1.946, P > 0.1; \text{Fig 5.6d})\).

*Expression of \(\alpha 4\beta 2^*\text{nAChRs does not regulate HPA-axis activity after 15 min exposure to an open field locomotor assay}*

Two-way ANOVA analyses did not reveal any significant effects of genotype, age or interactions of genotype x age for serum corticosterone levels following the open field assays conducted under either bright \((F_{1,26} < 1, P = 0.504; F_{1,26} < 1, P = 0.605; F_{1,26} < 1, P = 0.434; \text{Table 5.3})\) or normal room lighting conditions \((F_{1,23} = 1.054, P = 0.315; F_{1,23} < 1, P = 0.852; F_{1,23} < 1, P = 0.353; \text{Table 5.3})\).

*\(\alpha 4\) nAChR subunit expression*

nAChR immunoprecipitation confirmed that \(\alpha 4\) nAChR subunit expression was significantly reduced in \(\alpha 4\text{HET}\) compared to WT mice. Two-way ANOVA analyses detected main effects of genotype for \(\alpha 4\) nAChR subunit expression in the amygdala \((F_{2,19} = 32.278, P < 0.001)\), cingulate cortex \((F_{2,19} = 33.652, P < 0.001)\), hippocampus \((F_{2,19} = 13.802, P < 0.001)\) and lateral septum \((F_{2,19} = 22.963, P < 0.001)\). Tukey’s post hoc tests revealed that compared to WT mice, \(\alpha 4\) nAChR subunit expression was significantly reduced in \(\alpha 4\text{HETs} (P’s < 0.05)\) and essentially absent in \(\alpha 4\text{KO}\) mice in all brain areas evaluated \((P’s < 0.001)\). Planned comparisons revealed a
Table 5.3 - No effects of genotype or age on serum corticosterone levels observed after 15 minutes of exposure to a novel cage.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age</th>
<th>Serum corticosterone (ng/ml)</th>
<th>n size</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No injection/bright room lighting</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>Adult</td>
<td>303.5 ± 27.5</td>
<td>7</td>
</tr>
<tr>
<td>WT</td>
<td>Aged</td>
<td>286.3 ± 15.4</td>
<td>7</td>
</tr>
<tr>
<td>α4HET</td>
<td>Adult</td>
<td>305.2 ± 21.1</td>
<td>7</td>
</tr>
<tr>
<td>α4HET</td>
<td>Aged</td>
<td>330.9 ± 22.5</td>
<td>7</td>
</tr>
<tr>
<td><strong>Saline injection/normal room lighting</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>Adult</td>
<td>299.2 ± 32.6</td>
<td>10</td>
</tr>
<tr>
<td>WT</td>
<td>Aged</td>
<td>289.6 ± 44.2</td>
<td>5</td>
</tr>
<tr>
<td>α4HET</td>
<td>Adult</td>
<td>295.1 ± 29.8</td>
<td>8</td>
</tr>
<tr>
<td>α4HET</td>
<td>Aged</td>
<td>342.0 ± 33.6</td>
<td>7</td>
</tr>
</tbody>
</table>

Untreated mice were allowed to explore a polycarbonate cage that was brightly lit and surrounded by a white plastic enclosure x 15 min (No injection/bright room lighting). A separate cohort of mice received i.p. saline immediately prior to exploration of a novel polycarbonate housing cage that was not surrounded by a white plastic enclosure under normal fluorescent lighting conditions x 15 min (Saline injection/normal room lighting). Key: WT = wild type; α4HET = α4 heterozygous; Adult = 6-8 months; Aged = 22-24 months. All data represented as Mean ± SEM.
Figure 5.7 - Trends observed for an age-dependent increase in the amygdalar expression of the $\alpha_4$ nAChR subunit in wild type (WT) mice. Two-way ANOVA analyses detected that the expression of the $\alpha_4$ nAChR subunit was higher in wild type (WT) mice than both $\alpha_4$ heterozygous ($\alpha_4$HET) mice and mice with genetic deletion of the $\alpha_4$ subunit ($\alpha_4$KO) in all brain areas evaluated. a) A non-significant trend for increased $\alpha_4$ subunit expression in the amygdala in 22-24 month (Aged) WT mice compared to 6-8 month (Adult) WT mice ($P = 0.073$) likely contributed to reduced amygdalar $\alpha_4$ subunit expression in $\alpha_4$HET-Aged mice compared to WT-Aged mice. Data are represented as means ± SEM (n = 3-4 experiments per genotype and age). †$P < 0.05$ vs. aged-matched WT.
non-significant trend for increased amygdalar α4 nAChR subunit expression in Aged WT mice compared to Adult WT controls ($t_4 = 3.423$, $P = 0.073$; Fig 5.7a).

**Effects of Age and α4β2*nAChR expression on regulation of ERK and CREB**

ANOVA analyses detected significant age x genotype interactions for levels of pERK42 ($F_{2,52} = 4.795$, $P = 0.012$) and pCREB ($F_{2,52} = 4.446$, $P = 0.017$) in the lateral septum following saline vehicle injection. Compared to WT-Adult mice, post hoc tests revealed that pERK42 was significantly higher in α4HET-Adult mice ($t_{22} = 2.766$, $P < 0.05$; Fig 5.8a) with a trend for elevated pERK42 in WT-Aged mice ($t_{25} = 2.033$, $P = 0.052$; Fig 5.8a). pERK42 was lower in α4HET-Aged mice compared to α4HET-Adult mice ($t_{15} = 2.152$, $P < 0.05$; Fig 5.8a). Compared to WT-Adult controls, pCREB was significantly higher in both WT-Aged mice ($t_{25} = 3.902$, $P < 0.001$; Fig 5.8a) and α4HET-Adult mice ($t_{22} = 2.228$, $P < 0.05$; Fig 5.8a). Age-dependent changes in pERK42 or pCREB were not observed in α4KO mice. No significant age x genotype interactions were detected for total ERK42 ($F_{2,48} = 1.280$, $P > 0.2$), ERK44 ($F_{2,46} < 1$) or total CREB in the lateral septum ($F_{2,53} < 1$; Fig 5.8b).

**Regulation of ERK and CREB phosphorylation observed in the lateral septum in α4HET mice following antagonism of α4β2*nAChRs**

A trend for a genotype x treatment interaction ($F_{2,97} = 2.868$, $P = 0.062$) revealed that levels of pERK44 were reduced in α4HET mice administered DHβE compared to saline-injected α4HET mice ($t_{30} = 2.232$, $P < 0.05$; Fig 5.9a), whereas injections of DHβE did not affect pERK44 in WT or α4KO mice ($t_{41} = 0.8309$, $P = 0.411$; $t_{26} = 0.044$, $P = 0.965$). A main effect of genotype ($F_{2,96} = 2.868$, $P = 0.0034$) as well as a similar trend for an interaction of genotype x treatment ($F_{2,96} =$
2.413, \( P = 0.094 \) revealed that pCREB levels in the lateral septae of saline-injected \( \alpha 4 \)HET mice were significantly higher than saline-treated WT controls (\( t_{36} = 3.266, P = 0.002 \); Fig 5.9a). Post hoc t-tests also identified a non-significant trend for reduced pCREB in \( \alpha 4 \)HET mice receiving DH\( \beta \)E compared to \( \alpha 4 \)HET mice administered saline (\( t_{30} = 2.029, P = 0.051 \); Fig 5.9a). As with pERK, DH\( \beta \)E injections did not affect pCREB in WT or \( \alpha 4 \)KO mice, compared to their saline-injected counterparts (\( t_{40} = 0.365, P = 0.717 \); \( t_{26} = 0.306, P = 0.762 \)). A trend for a genotype x treatment interaction (\( F_{2,92} = 2.780, P = 0.067 \)) revealed that DH\( \beta \)E–injected \( \alpha 4 \)HET mice showed significant reductions in total ERK 42 compared to saline-injected \( \alpha 4 \)HET mice (\( t_{29} = 2.384, P < 0.05 \); Fig 5.9b). A trend for a main effect of treatment for total ERK 44 (\( F_{1,92} = 2.780, P = 0.067 \)) was likely due to similar trends for reduced total ERK 44 in DH\( \beta \)E–injected \( \alpha 4 \)HET mice, suggesting that reduced ERK phosphorylation in the lateral septum following DH\( \beta \)E injections may have been due to downregulation of total ERK protein, rather than regulation of ERK phosphorylation.

**DISCUSSION**

In these studies, we evaluated the effects of reduced basal expression of \( \alpha 4 \beta 2^* \)nAChRs on age-dependent anxiety-like behaviors in mice. During the light-dark assay, a genetic reduction of \( \alpha 4 \beta 2^* \)nAChRs promoted anxiolysis-like behavior in untreated 6-8 month old \( \alpha 4 \)HET-Adult mice compared to controls, as measured by increased exploration of a brightly-lit chamber and shorter latencies to enter that chamber compared to age-matched WT controls. Adult \( \alpha 4 \)HET mice also spent less time in the corners and showed trends for increased entries into the center
Figure 5.8 - Opposing age- and genotype-specific changes in anxiety-like behavior are associated with regulation of ERK and CREB phosphorylation in the lateral septum.  a) Compared to 6-8 month (Adult) WT controls, reductions in anxiety-like behavior observed in
α4HET-Adult mice were associated with elevated phosphorylation of ERK 42 (pERK42) in the lateral septum. Conversely, the increased anxiety-like behavior observed in 22-24 month (Aged) α4HET mice was associated with reduced pERK42. There was also a non-significant trend for increased pERK42 in WT-Aged mice ($P = 0.052$) compared to WT-Adult controls. These data suggest that the age- and genotype-dependent changes in anxiety-like behavior may have been regulated via phosphorylation of ERK in the lateral septum. Similar trends were observed for pCREB. Compared to WT-Adult animals, pCREB was significantly higher in both WT-Aged mice and α4HET-Adult mice. Age-dependent changes in pERK42 or pCREB were not observed in α4KO mice, suggesting that the changes in pERK and pCREB require α4β2*nAChRs. b) No significant age x genotype interactions were detected for the expression of total ERK42, ERK44 or total CREB in the lateral septum. Data are represented as means ± SEM. *$P < 0.05$ vs. α4HET-Adult mice; **$P < 0.001$; †$P < 0.05$ vs. WT-Adult mice.
Figure 5.9 - Increased anxiety-like behavior following i.p. DHβE in α4HET mice is associated with reduced ERK and CREB phosphorylation in the lateral septum.  

a) Consistent with results from the aging study, the dose of DHβE (DHβE) that promoted anxiogenic-like behavior in α4HET mice in the light-dark assay (3 mg/kg i.p.) also regulated phosphorylation of ERK44 (pERK44) in the lateral septum, as α4HET mice receiving DHβE expressed lower levels of pERK44 than saline-injected (SAL) α4HET animals. Compared to wild type (WT) SAL mice, phosphorylation of CREB (pCREB) was higher in α4HET mice. A non-significant trend for reduced CREB phosphorylation (pCREB) in DHβE-injected α4HET mice was also observed ($P = 0.051$). The administration of DHβE did not affect anxiety-like behavior, or levels of either pERK or pCREB in WT mice, suggesting that 3 mg/kg i.p. DHβE may have promoted anxiogenic-like behavior in α4HET mice by reducing ERK and CREB phosphorylation in the lateral septum.  

b) DHβE-injected α4HET mice showed reduced levels of total ERK 42 than vehicle-injected α4HET animals. Data are represented as means ± SEM. *$P < 0.05$ vs. α4HET i.p. saline (SAL) mice; †$P < 0.05$, ††$P < 0.01$ vs. WT SAL mice.
Figure 5.10 Representative western blots from aging and DHβE challenge studies.
Key: Genotype- WT = Wild type, HET = α4HET, KO = α4KO. Age: Adult = 6-8 months old, Aged = 22-24 months old at time of harvest.
zone of a novel cage than age-matched WT mice. These genetic studies complement pharmacological studies suggesting that inhibition of α4β2*nAChRs promotes anxiolysis-like behavior during the conditioned emotional response, light-dark, elevated plus maze, novelty induced hypophagia and marble burying assays (Anderson & Brunzell, 2012; Dawson et al., 2013; Newman et al., 2002; Newman et al., 2001; Roni & Rahman, 2011; Turner et al., 2010). In contrast to these observations in Adult mice, reduced basal expression of α4β2*nAChRs promoted anxiogenic-like behavior in 22-24 month Aged mice. α4HET-Aged mice showed increased anxiety-like behaviors in both the light-dark and open field locomotor assays compared to α4HET-Adult mice and aged-matched WT controls. An opposing effect was observed in WT-Aged mice, which exhibited reduced anxiety-like behavior compared to WT-Adult controls in the light-dark and compared to α4HET-Aged mice in both the light-dark and open field assays. These genotype-specific effects in Aged mice were not likely due to global differences in locomotor activity, since there were no differences between aged WT and α4HET mice in either dark chamber activity during the light-dark assay or distance traveled in the open field locomotor assay. Injections of the selective β2*nAChR antagonist DHβE increased anxiety-like behavior in α4HET, but not WT mice during the light-dark assay. Antagonism of β2*nAChRs did not significantly affect dark chamber activity in α4HET mice, suggesting that the resultant behaviors reflected anxiogenic-like behavior in the light-dark assay. These data suggest that while reduced basal expression of α4β2*nAChRs may promote an anxiolytic-like phenotype in α4HET-Adult mice, it renders them more sensitive to age-dependent increases in anxiety-like behaviors.

The results of the aging studies were surprising in the context of previous work showing that α4β2*nAChR receptor expression decreases with aging in brain areas that support anxiety,
including the hippocampus (Gahring et al., 2005; Mitsis et al., 2009; S. W. Rogers et al., 1998). In these studies WT mice showed a decrease in anxiety that would be consistent with reduced expression of α4β2*nAChRs. However, our immunoprecipitation studies showed trends for increases in α4 subunit expression in WT mice with aging. The previous studies analyzed the number of neurons staining positive for the expression of the α4 nAChR subunit via immunohistochemistry and nicotinic agonist binding assays to detect changes in the number of high affinity α4β2*nAChR binding sites. Unfortunately, we did not have the animals to perform agonist binding studies to quantify the number of available binding sites across age groups. It is possible for the expression of α4 subunits to remain relatively unchanged or even increase with a coincident reduction in the number of receptors. This would favor the assembly of the lower affinity α4(3)β2(2)nAChRs and reduce, rather than increase, the α4β2*nAChR-mediated response of these brain regions to nicotine and ACh. It is possible that aged WT mice have an increase in expression of the α4(3)β2(2) low sensitivity receptors and hence show a phenotype that is more like a4HET adults (Moroni et al., 2006; Zwart & Vijverberg, 1998). If this is true in a4HETs as well as WT mice, then cholinergic tone at these receptors could explain the dichotomy in effects of aging on anxiety-like behavior in WT vs. a4HET mice, with a4HET-Aged mice showing elevations in anxiety that resembled a4HET-Adult animals following antagonism of α4β2*nAChRs. Our immunoprecipitation studies may have also detected α4 subunits sequestered in the endoplasmic reticulae of neurons, which would also result in an increase in the expression of α4 subunit protein without a corresponding increase in functional receptors (H. A. Lester et al., 2009; Wanamaker & Green, 2007).
Little is known regarding the neuroanatomical circuitry wherein $\alpha 4\beta 2*nAChRs$ exert their effects on anxiety-like behavior. Elevated cholinergic tone in the hippocampus is associated with increased anxiety and depression-like behavior in mice and humans (Luo et al., 2013; Mineur et al., 2013; Saricicek et al., 2012). Excitotoxic lesions of cholinergic inputs to the basolateral amygdala reduce anxiety-like behavior in rats (Power & McGaugh, 2002), and fMRI studies show that amygdalar activation is associated with negative affect in humans (Sutherland et al., 2013). The lateral septum is a predominantly GABA-ergic projection area that receives a convergence of excitatory input from brain regions known to be important for determining the salience of external stimuli such as the ventral tegmental area, hippocampus, amygdala and anterior cingulate cortex (Risold & Swanson, 1997a, 1997b) (for review, see (Sheehan et al., 2004)). Studies in rodents show that electrical stimulation of the lateral septum protects against stress-induced ulceration, and that administration of the anxiolytic chlordiazepoxide results in increased neuronal activity in the lateral septum (E. Thomas et al., 2005; E. Thomas & Yadin, 1980; E. Thomas et al., 1991; Yadin & Thomas, 1996).

These studies explored the effect of nAChR expression and aging on ERK and CREB in the lateral septum and found that these pathways were regulated in a fashion that correlated with anxiety-like behavior. ERK is an intracellular signaling protein that is known to be critical in neuronal plasticity related to learning and memory, and the rewarding properties of drugs of abuse, including nicotine (Brunzell et al., 2009; Sweatt, 2001; Valjent et al., 2006; Valjent et al., 2004). pERK is reduced in the lateral septum following restraint stress (Sheehan et al., 2003), whereas the overexpression of Pyk-2, an activator of the Raf/Ras/MEK/ERK pathway, in the lateral septum promotes an anti-depression-like phenotype in rats (Sheehan et al., 2003). The
opposing effects of increased lateral septum activity following the administration of anxiolytic drugs and reductions of pERK in the lateral septum after restraint stress suggest that increased pERK in the lateral septum may be an important molecular mechanism underlying the reduced anxiety-like behaviors observed in these studies. Reduced anxiety-like behaviors in α4HET-Adult mice were associated with increased pERK in the lateral septum compared to both WT-Adult and α4HET-Aged mice. Likewise, WT-Aged mice showed reduced anxiety-like behaviors and trends for increased pERK compared to WT-Adult controls. Unlike previous studies, wherein antagonism of β2*nAChRs reduced anxiety-like behavior (Anderson & Brunzell, 2012; Dawson et al., 2013), we did not observe significant effects of DHβE administration in WT mice on anxiety-like behavior during the light-dark assay. It is possible that the 3 mg/kg DHβE dose used in these studies is on the descending arm of the dose-response curve for DHβE in the light-dark assay. We observed anxiogenic-like behavior and associated reductions in pERK in the lateral septum in α4HET mice following the administration of 3 mg/kg DHβE, suggesting that while reduced expression of α4β2*nAChRs may promote anxiolysis-like behavior via activation of ERK in the lateral septum, further antagonism of α4β2*nAChRs in mice with already reduced α4β2*nAChRs has the opposite effect, increasing anxiety-like behavior and reducing pERK in the lateral septum. The effects of DHβE on ERK phosphorylation in α4HET mice may have been due to downregulation of total ERK, rather than regulation of kinases or phosphatases upstream of ERK. We also observed age- and genotype-specific changes in pCREB in the lateral septum following an i.p. saline challenge. CREB is a transcription factor that is activated by several intracellular kinases, including ERK. The overexpression of CREB in the nucleus accumbens, another GABA-ergic nucleus, reduced anxiety-like behaviors in both the open field (Barrot et al., 2002) and elevated plus maze assays
(Barrot et al., 2005). In these present studies, we observed increased levels of pCREB in both WT-Aged and α4HET-Adult Adult mice compared to WT-Adult controls. However, pCREB was not reduced in the lateral septae of α4HET-Aged mice, suggesting that activation of CREB by pERK in the lateral septum may be an important for the promotion of anxiolysis-like behavior, but that other downstream targets of pERK may be critical in regulating the increased anxiety-like behaviors observed in Aged α4HET mice.

Although the incidence of anxiety-related disorders is typically lower in healthy, elderly subjects than young adult human subjects (Flint, 1994), it is elevated in those with co-morbidities associated with abnormal neurodegenerative processes such as AD or dementia (Wolitzky-Taylor et al., 2010). β2*nAChRs protect against age-related neurodegeneration and hypergliosis in mice (Zoli et al., 1999) as well as damage resulting from excitotoxic lesions (Zanardi et al., 2007). In vitro studies show that activation of β2*nAChRs and the ERK pathway inhibits neuronal toxicity associated with AD (Kawamata & Shimohama, 2011; Kihara et al., 1998), although the role of α7nAChRs cannot be excluded (Kawamata & Shimohama, 2011). In the present studies, anxiogenic-like behaviors observed in α4HET-Aged mice were associated with reduced pERK in the lateral septum. Furthermore, direct antagonism of β2*nAChRs increased anxiety-like behavior and reduced pERK in the lateral septum in α4HET mice, but not WT mice. Taken together these data suggest that impaired ERK signaling in the lateral septum of α4HET-Aged mice may have accelerated age-related neurodegeneration, contributing to increased anxiety-like behaviors. These present studies are consistent with human studies showing that anxiety-like behavior decreases in an age-dependent manner, while elderly subjects with advanced neurodegeneration associated with pathologic reductions of nAChRs actually
demonstrate increased anxiety. Finally, these studies suggest that pathological reductions of α4β2*nAChRs in non-elderly human subjects may be a biomarker of the onset of neurodegenerative processes such as AD.
Chapter 6 – Concluding discussion

In these experiments, we first demonstrated that low doses of nicotine (0.01, 0.032 and 0.05 mg/kg) and a selective antagonist of β2*nAChRs, DHβΕ, reduced anxiety-like behavior in mice using four independent behavioral models with predictive validity for evaluating the anxiolytic-like properties of drugs, namely the conditioned emotional response assay, the marble burying task, the elevated plus maze assay, and the light-dark assay. The administration of 0.001 mg/kg of the selective β2*nAChR agonist 5I-A85380 also promoted anxiolysis-like behavior in the elevated plus maze assay. Conversely, injections of a high dose of nicotine (0.5 mg/kg) and 5I-A85380 (0.032 mg/kg) promoted an anxiogenic-like phenotype in the light-dark and elevated plus maze assays. These results are consistent with previous studies showing that nicotine has a bimodal effect on anxiety-like behavior (Anderson & Brunzell, 2012; Cheeta, Irvine, et al., 2001; Cheeta, Tucci, et al., 2001; File et al., 2000; File et al., 1998; Irvine et al., 1999; McGranahan et al., 2011; Ouagazzal et al., 1999; Varani et al., 2012). Additionally, anxiolytic-like doses of nicotine, DHβΕ or 5I-A85380 did not significantly affect locomotor activity, suggesting that the observed behaviors reflected an attenuation of anxiety-like behavior rather than increased locomotion.

Pre-injections of both anxiolytic-like doses of nicotine (0.01 and 0.05 mg/kg i.p.) and a dose of DHβΕ previously shown to block nicotine place preference and reduce anxiety-like behavior (2 mg/kg i.p.) blocked the anxiogenic-like effects of 0.5 mg/kg i.p. nicotine during the light-dark
and elevated plus maze assays (Anderson & Brunzell, 2012; Grabus et al., 2006; Walters et al., 2006). Based upon previous pharmacokinetic studies in mice, the concentrations of nicotine achieved in the brains of mice following the anxiolytic-like doses of nicotine used in these studies (0.01 – 0.05 mg/kg i.p.) would likely be between 25-125 nM (Crooks, Li, & Dwoskin, 1995; Petersen et al., 1984; Zhou et al., 2010). These concentrations are sub-threshold of the EC₅₀ values of nicotine for the high affinity β²*nAChRs (Fenster et al., 1997; R. A. Lester & Dani, 1995; Mansvelder et al., 2002; Pidoplichko et al., 1997), but are concentrations of nicotine that in in vitro studies result in preferential desensitization of α₄β²*nAChRs and α₆β²*nAChRs (Fenster et al., 1997; Grady et al., 2012; Kuryatov et al., 2011; Lu et al., 1999), but not α₄α₆β²*nAChRs (L. Liu et al., 2012). Presently available radioligands to study nAChR binding in human SPECT studies are not selective for subclasses of β²*nAChRs and are unable to determine if nAChRs are in an activated or desensitized state. While we did not confirm the activity of nAChRs in awake, behaving animals after injections of low doses of nicotine, the fact that both low doses of nicotine and DHβE displayed anxiolytic-like efficacy across behavioral models and similarly blocked the anxiogenic-like effects of a high dose (0.5 mg/kg) of nicotine suggests that low doses of the anxiolytic properties of low dose nicotine were promoted via inhibition, rather than activation, of β²*nAChRs.

Although compounds highly selective for β²*nAChRs were used in these experiments, pharmacological studies are limited. We therefore performed genetic studies in β²KO mice with a null mutation of the β2 or α₄HET mice with a 50% reduction of the α₄ subunit. The anxiolytic-like behavior observed in WT mice receiving 0.05 mg/kg i.p. nicotine in the light-dark assay was absent in β²KO mice, confirming that β²*nAChRs are required for the anxiolytic-like
efficacy of low doses of nicotine. 6-8 month α4HET-Adult mice showed reduced anxiety-like behavior compared to WT-Adult mice in the light dark and open field assays, suggesting that reduced cholinergic tone mediated via α4β2*nAChRs is required for the attenuation of anxiety-like behavior. L9S mice with hypersensitive α6β2*nAChRs showed an anxiogenic-like phenotype across several behavioral assays, whereas genetic deletion of the α6 subunit did not significantly affect the expression of anxiety-like behavior in α6KO null mutant mice, suggesting that activation of α6β2*nAChRs is sufficient for the promotion of anxiety, but that inhibition of α6β2*nAChRs is not required for the expression of anxiolytic-like behavior.

Although the pharmacologic and genetic studies suggest that the low doses of nicotine used in these studies were selective for β2*nAChRs, the use of L9A and L9S mice with hypersensitive α4β2*nAChRs or α6β2*nAChRs would confirm if these nAChR subclasses are sufficient to promote the anxiolytic efficacy of nicotine. If the anxiolytic effects of nicotine are promoted by sub-activating concentrations of nicotine that favor desensitization of β2*nAChRs, then the anxiolytic-like behaviors observed in WT mice should be absent in L9A or L9S mice. We could also compare the efficacy of pre-injections of nicotine to dose-dependently block CPP to 0.09 mg/kg i.p. nicotine in WT, L9S and L9A mice. Pre-injections of low-dose nicotine ought to block CPP in WT, but not in either the L9S or L9A mice, since low doses of nicotine would activate α4β2*nAChRs in L9A mice or α6β2*nAChRs in L9S mice, but not in WT animals. Using localized optogenetic activation of nAChRs in Chat-CRE mice co-expressing channel rhodopsin, we could activate cholinergic neurons in the amygdala, hippocampus or lateral septum to directly determine if activation of nAChRs in these brain regions promotes an anxiety
phenotype. Lesions of cholinergic neurons projecting to the amygdala attenuated fear response in rats (Power & McGaugh, 2002). Genetic deletion of the prototoxin Lynx2, an endogenous negative allosteric modulator of α4β2* nAChRs highly expressed in the amygdala, results in increased anxiety-like behavior in mice (Tekinay et al., 2009), suggesting that increased cholinergic tone in the amygdala promotes anxiety-like behavior. Local infusions of the AChE inhibitor physostigmine and siRNA knock-down of AChE in the hippocampus resulted in elevated anxiety-like and depression-like behavior (Mineur et al., 2013). Additionally, exposure to the synthetic compound bisphenol a results in elevated anxiety-like behavior and reduced activity of hippocampal AChE (Luo et al., 2013), suggesting that elevated cholinergic tone in the hippocampus also increases anxiety-like behavior in rodents. Reduced levels of pERK are observed in the lateral septum following restraint stress and chronic social stress, and our studies reveal that increased anxiety-like behavior is associated with reduced pERK in the lateral septum (Sheehan et al., 2003; Singewald et al., 2009). These three brain regions form part of the circuitry that regulates emotive responses to external stimuli. Once we identify a neuroanatomical locus wherein optogenetic activation of cholinergic neurons promotes an anxiogenic phenotype, we would test whether local infusions of nicotine and/or DHβE would attenuate these effects. Using in vivo electrophysiology technology, we could also measure the neuronal activity of the amygdala, hippocampus or lateral septum during CER. This would provide tremendous insight into what neuronal pathways are involved when mice are responding for a reinforcer in the presence or absence of an aversive CS, or what neuronal pathways are upregulated or downregulated following the presentation of a footshock, for example.
β2KO mice were also less sensitive than WT mice to the anxiogenic-like effects of 0.5 mg/kg i.p. nicotine, suggesting that they were protected from the behavioral effects of 0.5 mg/kg i.p. nicotine. Locomotor suppression was observed in both WT and β2KO mice during the light-dark assay following 0.5 mg/kg nicotine. An anxiogenic-like dose of 5I-A85380 did not significantly affect measures of locomotion, further confirming that activation of β2*nAChRs in mice promotes anxiogenic-like behavior and that activation of some other subclass of nAChRs likely contributed to the locomotor depressive effects of high dose nicotine, possibly α3β4*nAChRs (Jackson et al., 2010; Marubio et al., 2003; Salas et al., 2003). We want to pharmacologically and genetically confirm that the 0.032 mg/kg dose of 5I-A85380 is selective for β2*nAChRs by assessing if the anxiogenic-like effects of this dose of 5I-A85380 can be blocked by DHβΕ and if these effects are present in β2KO mice. Administration of 0.5 mg/kg nicotine to β4KO mice during the light-dark and elevated plus maze assays would further determine if the anxiogenic and/or locomotor depressive effects of this dose of nicotine are in part mediated via α3β4*nAChRs.

Interestingly, a dose of nicotine shown to promote conditioned place preference (0.1 mg/kg i.p.) (Brunzell et al., 2009; McGranahan et al., 2011; Mineur et al., 2009), which requires the activation of β2*nAChRs (Brunzell et al., 2010; Corrigall et al., 1994; Gotti et al., 2010; Grabus et al., 2006; Jackson et al., 2009; X. Liu et al., 2003; Maskos et al., 2005; McGranahan et al., 2011; Picciotto et al., 1998; Pons et al., 2008; Tapper et al., 2004; Walters et al., 2006), did not affect anxiety-like behavior in either the light-dark or CER assays. Unlike the opposing effects of nicotine and nAChR antagonists that are observed for the reward-like and reinforcing properties of nicotine, pre-injections of anxiolytic-like doses of nicotine and DHβΕ did not
accumulate with anxiogenic-like effects of 0.5 mg/kg nicotine or block the anxiolytic efficacy of low doses of nicotine, further confirming that low doses of nicotine promote anxiolysis via inactivation of β2*nAChRs.

Pre-injections of 0.01 mg/kg nicotine were more effective at blocking the anxiogenic-like effects of high dose nicotine than pretreatment with 0.05 mg/kg nicotine during the elevated plus maze assay. This leftward shift in the “antagonist-like” efficacy of nicotine is possibly reflective of nAChRs being in a “smoldering” state, wherein their conversion to an irreversible Bound and Desensitized state or to the Bound and Activated state is concentration dependent (Kuryatov et al., 2011; Williams, Wang, & Papke, 2011). Lower nicotine concentrations prior to the delivery of 0.5 mg/kg nicotine promoted by pre-injections of 0.01 mg/kg nicotine would favor desensitization, whereas higher concentrations following 0.05 mg/kg pretreatment would be more likely to promote activation, leading to increased anxiety-like behavior associated with the administration of 0.5 mg/kg nicotine.

We also observed age-dependent reductions in anxiety-like behavior in 22-24 month WT-Aged mice compared to Adult 6-8 month controls during the light-dark assay. In contrast to these findings, α4HET-Aged mice showed higher levels of anxiety-like behavior than α4HET-Adult mice and WT-Aged mice in both the light-dark and open field assays. The administration of DHβE promoted anxiogenic-like behavior in α4HET-Adult, but not WT-Adult mice during the light-dark assay, suggesting that the age- and genotype-specific changes in anxiety-like behavior may have been due to further reductions in α4β2*nAChRs. However, the immunoprecipitation assays performed herein determined that there were no further age-dependent reductions in the
expression of the α4 subunit in the amygdala, anterior cingulate cortex, lateral septum or dorsal hippocampus. These findings contrast with previous studies demonstrating age-dependent reductions in α4β2*nAChRs in mice (Gahring et al., 2005; S. W. Rogers et al., 1998). It is possible that the antibodies used to detect α4β2*nAChRs in the former studies were less specific than the antibodies used in these experiments. Also, previous studies were performed using immunohistochemistry and [3H]nicotine binding assays, which determined the total number of cells expressing α4β2*nAChRs and/or nicotine binding sites, whereas the immunoprecipitation experiments detected the expression of α4 subunit protein present in each sample. A large proportion of nAChR subunits remain sequestered in the endoplasmic reticulum, and are not expressed on the cell membrane (H. A. Lester et al., 2009; Wanamaker & Green, 2007). Another possibility is that there was an age-dependent reduction in the expression of the β2 subunit, which would favor the assembly of α4β2*nAChRs in a lower-affinity α4(3)β2(2) stoichiometry, resulting in a reduction in α4β2*nAChR-mediated cholinergic tone (Anand et al., 1991; Buisson & Bertrand, 2001; Cooper et al., 1991; Covernton & Connolly, 2000; Gotti et al., 2008; Khiroug et al., 2004; Moroni et al., 2006; Nelson et al., 2003; Zwart & Vijverberg, 1998). Unfortunately, we did not have sufficient animals to perform either immunoprecipitation assays to determine the expression of the β2 subunit or binding studies to detect potential age-dependent changes in the number of nicotine binding sites. We also did not detect any age or genotype-specific differences in serum corticosterone levels following 15 min exposure to a novel cage. These data suggest that basal expression of α4β2*nAChRs regulated anxiety-like behavior in Adult animals, but that some factor other than age-dependent changes in nAChR regulation contributed to age-associated reductions in anxiety-like behavior in Aged WT mice and the inverse elevations in anxiety-like behavior observed in Aged α4HETs.
Extracellular regulated kinase is a cellular signaling protein that is important for synaptic plasticity related to learning and memory and the addictive properties of drugs of abuse (Brunzell et al., 2009; Brunzell et al., 2003; S. Davis et al., 2000; Schafe et al., 2000; Schafe et al., 2008; Sweatt, 2001; Valjent et al., 2006; Valjent et al., 2004). In the present studies, elevated levels of pERK in α4HET-Adult and WT-Aged mice in the lateral septum were associated with reduced anxiety-like behavior compared to their counterparts of the same genotype. Additionally, significant decreases in pERK in the lateral septum were associated with the anxiogenic-like behavior observed in α4HET-Adult mice following 3 mg/kg i.p. DHβE. These data are consistent with previous studies showing reduced pERK in the lateral septum following exposure to restraint stress or chronic social stress (Sheehan et al., 2003; Singewald et al., 2009). We did not observe any effects of age or DHβE administration on pERK in α4KO mice, suggesting that the changes of pERK in the lateral septum observed in these aging and pharmacological studies were mediated via α4β2*nAChRs. They further suggest that ERK phosphorylation in the lateral septum may regulate the age-related changes in anxiety-like behaviors that were observed in Aged WT and α4HET mice in these studies.

The preponderance of evidence suggests that pERK serves a neuroprotective role (Al Rahim et al., 2008; Hetman, Kanning, Cavanaugh, & Xia, 1999; Karmarkar, Bottum, Krager, & Tischkau, 2011; Kihara et al., 1998; Yim et al., 2009), but can also promote neuronal death (Chen, Rusnak, Lombroso, & Sidhu, 2009), showing that dysregulation of ERK signaling is deleterious to cellular health, including neurons. pERK is regulated either by changes in the activity of MEK, the only known kinase of ERK, or by phosphatases that inactivate ERK. The phosphatase STEP can inactivate ERK, and outside of the striatum, STEP expression is highest in the lateral
septum, suggesting its importance as a regulator of pERK in this brain region (Boulanger et al., 1995; Lombroso et al., 1993). STEP expression is increased in the cortex in various transgenic rodent models of AD and in the PFC of humans diagnosed with AD, suggesting that its increased expression may contribute to premature neurodegeneration (Chin et al., 2005; Kurup et al., 2010; Xu, Kurup, Nairn, et al., 2012; Y. Zhang et al., 2010). Perhaps there was an age-dependent increase in STEP activity in α4HET animals leading to decreased ERK phosphorylation in the lateral septum and elevated anxiety-like behavior. Additional western blot analyses could be performed to quantify the expression of STEP in the lateral septum of Aged and Adult WT and α4HET mice. If the western blots indicate that STEP is elevated in the lateral septum of α4HET–Aged mice, one could then knock-down STEP in the lateral septum of α4HET–Aged mice via siRNA to reverse the anxiety phenotype.

The studies conducted herein demonstrate that pERK in the lateral septum is inversely associated with anxiety-like behavior. We are unable to determine whether the changes in pERK are downstream of α4β2*nAChRs or are the result of reduced anxiety. Data from the Aged mice suggests the latter, since α4β2*nAChRs are not differentially expressed in the hippocampus, anterior cingulate cortex, amygdala or lateral septum compared to Adult mice. However, we did observe reduced pERK and increased anxiety-like behavior in α4HET mice after the administration of DHβE, suggesting that basal expression of α4β2*nAChRs may have regulated pERK in Adult animals. Compared to WT-Adult controls, α4HET-Adult mice showed elevated levels of pERK in the lateral septum. These mice would likely have a 50% reduction of α4β2*nAChRs on the terminals of GABA interneurons in the VTA. Compared to WT mice, ACh release in the VTA would favor disinhibition of VTA DA neurons in α4HET mice,
facilitating DA release in the lateral septum, leading to increased pERK in that brain region, supporting a reduction in anxiety (Graupner et al., 2013; Mansvelder et al., 2002; Mineur et al., 2009; Pidoplichko et al., 1997). Determining the effects of infusions of the MEK inhibitor U1026 into the lateral septum on anxiety would directly assess if reduced ERK phosphorylation in this brain region promotes anxiety-like behavior. Additionally, with new technology available in our lab, we would also be able to determine in vivo electrophysiological changes in the brains of awake, behaving mice in real time following MEK inhibition. The administration of the benzodiazepine chlordiazepoxide activates the lateral septum and inhibits activity within the amygdala (Yadin & Thomas, 1998), suggesting that the opposing activities of these two brain regions may be coordinated to regulate the emotive response to an aversive stimulus. Therefore, this technology would also provide us the ability to evaluate how infusions of U1026 into the lateral septum may affect neuronal activity in the amygdala. It is unknown if the nAChRs affected by nicotine and/or nicotinic antagonists in the lateral septum are pre or post-synaptic. Mice receiving nicotine or DHβE challenge could be sacrificed and probed for pERK immunoreactivity using immunohisotochemistry, which would also allow dual-labeling of α4β2*nAChRs, to help determine if nAChR ligands are regulating pERK levels pre or post-synaptically.

Human SPECT imaging studies and in vivo studies in rodents show that reduced levels of AChE promote both anxiety and depression-like behavior (Luo et al., 2013; Mineur et al., 2013; Saricicek et al., 2012). Reduced serum AChE is associated with increased trait anxiety and SPECT imaging studies in humans suggest that lower levels of AChE activity are associated with depression (Saricicek et al., 2012; Sklan et al., 2004). In contrast to the lateral septum, which
has low levels of AChE staining, AChE staining in the amygdala is dense (Paxinos & K.P.J., 2003). As stated above, elevated levels of ACh favor desensitization of α4β2*nAChRs in the presence of nanomolar concentrations of nicotine (Graupner et al., 2013). This would suggest that higher endogenous levels of ACh might be sustained in the lateral septum than the amygdala, favoring enhanced nicotine-mediated desensitization of α4β2*nAChRs in the lateral septum in comparison. However, changes in endogenous AChE activity would have a greater potential to regulate anxiety-like behavior directly within the amygdala, as increased ACh levels would support increased amygdalar activity, potentially leading to increased fear and anxiety-like responses to aversive stimuli. Human PET imaging studies measuring levels of AChE activity by metabolism of [14C] MP4A, a radioactive substrate of AChE, show reduced AChE activity in the amygdala of subjects with mild cognitive deficits, a population that also demonstrates increased anxiety and amygdalar activity (Herholz et al., 2000; Herholz et al., 2004; Irie et al., 1996; Iyo et al., 1997; Y. D. Li et al., 2013; Ota et al., 2010). Combined with activation of the lateral septum, desensitization of α4β2*nAChRs in the amygdala could potentially coincide to enhance the anxiolytic effects of initial nicotine exposure.

Human studies measuring displacement of the selective β2*nAChR agonist 2-F-A85380 by nicotine show that there is enough nicotine in a single cigarette to occupy 80% of β2*nAChRs in the brain of a smoker for hours (Brody, Mandelkern, Costello, et al., 2009; Brody et al., 2006). Although higher spikes are possible, steady-state concentrations of nicotine achieved in the brains of human smokers are estimated to be between 200-400 nM, concentrations that ought to favor desensitization of β2*nAChRs, suggesting the likelihood that many of the β2*nAChRs would be desensitized for hours (Fenster et al., 1997; Grady et al., 2012; Kuryatov et al., 2011;
However, slice electrophysiology studies show that nanomolar concentrations of nicotine activate $\alpha_4\alpha_6\beta_2*nAChRs$, and that this nAChR subclass shows slower rates of desensitization than other $\beta_2*nAChRs$ (L. Liu et al., 2012). Nonetheless, anxious individuals smoking for the first time may experience more relief from stressors than non-anxious subjects following initial nicotine exposure due to desensitization of a large population of $\alpha_4\beta_2*nAChRs$ in brain areas such as the amygdala or lateral septum. As nicotine levels drop, $\alpha_4\beta_2*nAChRs$ in these brain regions would return to the resting state, where they are activated in the presence of stressful stimuli (Carter et al., 2006; Lazev et al., 1999; Littel & Franken, 2007; McDonough & Warren, 2001). Additionally, human imaging studies suggest that $\beta_2*nAChRs$ may be critical for nicotine’s ability to curb anxiety in smokers (Benowitz, 2008; Sharma & Brody, 2009). Thus, hours after consumption of the first cigarette, people with an underlying propensity for anxiety-related disorders may be motivated to smoke again in order to return their $\alpha_4\beta_2*nAChRs$ to a desensitized state.

Unfortunately, in addition to being a leading risk factor for the development of most chronic diseases, repeated nicotine use leads to upregulation of nAChRs, especially $\beta_2*nAChRs$ and $\alpha_7*nAChRs$. Human PET imaging studies suggest that nicotine-facilitated DA release is increased in the NAcc of smokers, likely due to upregulation of $\beta_2*nAChRs$ (Baker et al., 2013; Brody, Mandelkern, Olmstead, et al., 2009; Brody et al., 2004; Domino et al., 2013; Domino et al., 2009; Lomazzo et al., 2011; Metaxas et al., 2010; Moretti et al., 2010; Perez et al., 2008; Perez et al., 2013; Takahashi et al., 2008; C. Xiao et al., 2009). Studies in rodents show that chronic nicotine administration results in a downregulation of the high affinity $\alpha_6\alpha_4\beta_2*nAChRs$
on DA neurons, whereas the expression of $\alpha4\beta2*nAChRs$ on GABA interneuron terminals is increased (Baker et al., 2013; Lomazzo et al., 2011; Metaxas et al., 2010; Moretti et al., 2010; Perez et al., 2008; Perez et al., 2013; C. Xiao et al., 2009). This dichotomous up and downregulation of subclasses of $\beta2*nAChRs$ has the potential to affect not only the rewarding effects of nicotine use, but also anxiety. Upregulation of $\alpha4\beta2*nAChRs$ in the amygdala would enhance the nAChR-mediated cholinergic tone in this brain region, increasing anxiety in response to ACh release in the amygdala when these receptors are in the Resting and Unbound state. Similarly, upregulation of $\alpha4\beta2*nAChRs$ in the lateral septum would facilitate greater release of anxiogenic neuropeptides such as CRF, if these nAChRs are positioned on the terminals of CRF projection neurons. Taken together with data showing that repeated administration of nicotine results in a downregulation of AChE activity in the brains of rats, circulating levels of ACh would increase, facilitating greater nicotine-mediated desensitization of $\alpha4\beta2*nAChRs$. Furthermore, as nicotine levels drop, populations of $\beta2*nAChRs$ in brain regions that regulate anxiety such as the amygdala, cingulate cortex and septum become available for activation by increased levels of ACh in response to stressful stimuli such as cigarette/tobacco cues (Carter et al., 2006; Lazev et al., 1999; Littel & Franken, 2007; McDonough & Warren, 2001), contributing to escalation of tobacco use in order to alleviate the negative consequences of upregulation of $\alpha4\beta2*nAChRs$ in these brain regions (Koob & Volkow, 2010).

This would also suggest that smokers might be more sensitive to the anxiety-relieving properties of nicotine. In fact, human imaging studies show that the nicotine patch alleviates negative affect in smokers, but not non-smokers, in a picture attention task (Gilbert et al., 2008) and
reduces the functional connectivity between the amygdala and the insular and cingulate cortices in smokers, but increases it in non-smokers (Sutherland et al., 2013). Similar effects are observed in smokers following administration of the selective α4β2*nAChR agonist varenicline (Sutherland et al., 2013), suggesting that the nicotine delivered via the application of a nicotine patch may be inactivating α4β2*nAChRs in the amygdala in smokers. Furthermore, elevated cued fear conditioning responses in human subjects with trait anxiety correlate with increased activation of the amygdala and anterior cingulate cortex, brain regions also important regulators of fear conditioning in rodents (Britton et al., 2011; Craske et al., 2008; Delgado et al., 2006; Lang & McTeague, 2009; Sehlmeyer et al., 2011; Waters et al., 2009). Thus, when in the resting state, upregulation of α4β2*nAChRs in the amygdala and/or lateral septum could contribute to enhanced perception of environmental stressors, signaling subsequent smoking episodes, contributing to the perceived anxiety relief provided by nicotine use (Parrott, 1999). In fact, heightened perception of stress is linked with escalation of smoking behavior from occasional to daily use (Byrne et al., 1995; Byrne & Mazanov, 2003; Finkelstein et al., 2006; Siqueira et al., 2000). Studies suggest that smokers are more anxious than non-smokers, perhaps due to upregulation of nAChRs in the cortex or striatum. Unfortunately, human imaging studies have not determined if the expression of β2*nAChRs is regulated in the amygdala of smokers (Benwell et al., 1988; Breese et al., 1997; Cosgrove et al., 2009; Gilbert et al., 2008; Staley et al., 2006; Staley et al., 2005; Tsuda et al., 1996).

To test the hypothesis that reduced elevated cholinergic tone due to reduced AChE activity would render one more sensitive to the initial anxiolytic-like effects of low doses of nicotine, one could first evaluate the anxiolytic-like efficacy of between-subject delivery of 0, 0.01 or 0.032
mg/kg i.p. nicotine. After the conclusion of the behavioral test, the mice would then be sacrificed and the amygdala and hippocampus would be harvested. These tissues could then be assayed for AChE activity. From this, one could determine if there is a negative correlation between AChE activity and initial sensitivity to the anxiolytic-like efficacy of nicotine. We would then determine if the administration of physostigmine and/or siRNA knock-down of AChE in either the amygdala or hippocampus would result in a leftward shift in the anxiolytic-like efficacy of nicotine.

Although the results of the genetic studies conducted in Chapter 5 suggest that reduced basal expression of α4β2*nAChRs promotes anxiolytic-like behavior in Adult mice, they also demonstrate that α4HET mice are sensitive to age-dependent increases in anxiety-like behavior. This is consistent with human studies, showing that pathological reductions in nAChRs are associated with elevated prevalence of anxiety-related disorders in geriatric subjects with advanced cognitive deficits and AD (Brodaty et al., 2012; Mitsis et al., 2009; Sabbagh et al., 2006). A preponderance of the research concerning cognitive deficits and AD is centered around the homomeric α7nAChRs. These studies suggest that α4β2*nAChRs may also be important modulators of molecular changes related to age-dependent changes in anxiety. Additionally, the pharmacological studies revealed that selective antagonism of β2*nAChRs promoted anxiety-like behavior in α4HET mice in the light-dark assay, but the administration of DHβE resulted in reduced anxiety-like behavior in WT mice in a number of assays for anxiety-like behavior. These results are consistent with increased cholinergic tone improving affect in individuals with advanced cognitive deficits that are associated with pathological reductions in nAChRs (S. L. Rogers et al., 1998; Sabbagh et al., 2006) and promoting increased anxiety and depression-like
behavior in younger humans and rodents (Mineur et al., 2013; Saricinek et al., 2012). The administration of repeated injections of physostigmine or nicotine to Aged α4HET mice might reverse age-dependent changes in anxiety-like behavior observed in these studies. We could further assess if optogenetic stimulation of cholinergic neurons in the lateral septum would promote anxiolysis-like behavior in Aged α4HET mice.

The activation of α6β2*nAChRs regulates nicotine-induced release of DA in the dorsal striatum and NAcc, and is thought to chiefly be responsible for the reinforcing effects of nicotine (Brunzell et al., 2009; Drenan et al., 2010; Drenan, Grady, et al., 2008; Pons et al., 2008). Persistent activation of α4α6β2*nAChRs promoting sustained DA release in the NAcc is thought to be a mechanism by which nanomolar concentrations of nicotine may support cigarette smoking throughout the day and may also reduce anxiety in smokers (L. Liu et al., 2012). Additionally, the anxiolytic effects of 0.01 mg/kg i.p. nicotine observed in the elevated plus maze were absent in mice with a selective deletion of the α4 subunit from VTA DA neurons, suggesting that activation of α4α6β2*nAChRs by nanomolar concentrations of nicotine may also promote anxiolysis in mice in this behavioral model (McGranahan et al., 2011). However, the genetic studies performed herein demonstrate that, compared to WT controls, L9S mice with a gain-of-function single point L9’S mutation in the M2 pore-forming region of the α6 subunit showed an anxiogenic-like phenotype across multiple behavioral assays, whereas no behavioral differences were observed between α6KO mice and their WT counterparts. These data suggest that persistent activation of α6β2*nAChRs by nicotinic agonists would heighten rather than attenuate anxiety-like behavior. Taken together with data demonstrating that antagonism of α6β2*nAChRs attenuates both anxiety-like behavior and conditioned place aversion during
nicotine withdrawal, these present data highlight that $\alpha_6\beta_2*nAChRs$ may be important for both the reinforcing effects of nicotine as well as the relief from stress reported by smokers during abstinence periods (Jackson et al., 2009). The discrepancy between L9S mice expressing an anxiogenic phenotype and the lack of an anxiety phenotype in $\alpha_6KO$ animals may have been due to the fact that the paradigms used in these studies were not stressful enough to uncover an anxiolytic phenotype in $\alpha_6KO$ mice. Future studies will evaluate if genetic deletion of $\alpha_6\beta_2*nAChRs$ will promote anxiolysis in the light-dark, elevated plus maze or open field locomotor assays following restraint stress compared to WT control animals. The suppression of HPA-axis activity following 30 min exposure to a novel cage observed in L9S mice could be reflective of reduced HPA-axis responses observed in human smokers exposed to a social stress paradigm or with the depression-like behaviors exhibited by humans and mice with reduced AChE activity (Childs & de Wit, 2009; Kudielka & Wust, 2010; Mineur et al., 2013; Saricicek et al., 2012; Swan et al., 1996). Further studies would include evaluation of spontaneous nicotine withdrawal in L9S mice. I predict that L9S mice would display more observable signs of nicotine withdrawal than WT mice. Furthermore, I predict that there would be a left-ward shift in the dose of chronic nicotine required to elicit observable signs of spontaneous withdrawal. I also expect that there would a left-ward shift in the ability of $\alpha$-conotoxin MII to attenuate the reduced time spent in the open arms of an elevated plus maze in L9S mice. Considering that HPA-axis suppression is also expressed in individuals diagnosed with depression, L9S mice could be further evaluated for immobility time in the forced swim or tail suspension tests to determine if they also express a depression-like phenotype in addition to an anxiety phenotype.
With the exception of the conditioned emotional response and marble burying experiments, wherein animals received within-subject delivery of multiple doses of nicotine and/or DHβE, these experiments evaluated the effects of acute administration of nAChR ligands on anxiety-like behavior. While acute administration is informative regarding mechanisms underlying initial sensitivity to nicotine’s effect on anxiety-like behavior, tobacco use is typically chronic, leading to nAChR upregulation and enhanced DA release in the NAcc (Baker et al., 2013; Brody, Mandelkern, Olmstead, et al., 2009; Brody et al., 2004; Domino et al., 2013; Domino et al., 2009; Lomazzo et al., 2011; Metaxas et al., 2010; Moretti et al., 2010; Perez et al., 2008; Perez et al., 2013; Takahashi et al., 2008; C. Xiao et al., 2009). Chronic administration of nicotine in mice promotes anxiolysis before withdrawal, but leads to anxiogenic-like behavior during withdrawal (Jackson et al., 2009; Koob & Volkow, 2010; Turner et al., 2013). The α4β2*nAChR partial agonist sazetidine-a and the selective α6β2*nAChR antagonist α-conotoxin [H9A;L15A] attenuated these anxiogenic-like effects of nicotine withdrawal (Damaj, Kao, & Martin, 2003; Jackson et al., 2009; Turner et al., 2013). Additionally, mice in spontaneous withdrawal from nicotine are less sensitive to the anxiogenic-like effects of a high dose of nicotine (0.5 mg/kg), which attenuates the anxiogenic-like behavior promoted by spontaneous nicotine withdrawal (Damaj et al., 2003).

In light of these data and the results from these present acute studies, we will first administer mice either 0.01 or 0.032 mg/kg i.p. nicotine prior to evaluation of anxiety-like behavior in the elevated plus maze assay. These mice will then be administered chronic saline or nicotine via osmotic mini-pumps. During nicotine withdrawal, I predict that mice showing more anxiolytic-like behavior after an acute dose of nicotine would be more sensitive the anxiogenic-like effects
of nicotine withdrawal. Furthermore, we would observe a left-ward shift in the dose response curve of sazetidine-a to attenuate anxiogenic-like behavior associated with nicotine withdrawal in mice more sensitive to the anxiolytic properties of acute nicotine.

Due to their expression throughout the CNS, antagonism or partial agonism of α4β2*nAChRs has the potential to promote many unwarranted side-effects in addition to possible relief of anxiety and smoking behavior. With the exception of the partial agonist/preferential α4β2*nAChR desensitizer sazetidine-a, chronic administration of nicotinic agonists and antagonists results in upregulation of α4β2*nAChRs in *in vitro* and *in vivo* systems, which could result in heightened negative affect during periods of withdrawal from the drug (Baker et al., 2013; Lomazzo et al., 2011; Metaxas et al., 2010; Moretti et al., 2010; Perez et al., 2008; Perez et al., 2013; C. Xiao et al., 2009). Although the expression of α6β2*nAChRs is more selective, the paucity of ligands selective for α6β2*nAChRs that cross the blood-brain barrier makes it difficult to evaluate the contributions that this nAChR subclass makes to nicotine’s effects on anxiety. Efforts to develop ligands that selectively affect α4β2*nAChRs or α6β2*nAChRs are hampered by the inability to express native α6*nAChRs in *in vitro* assays utilizing *xenopus* oocytes without the use of concatomers (Kuryatov & Lindstrom, 2011). A therapeutic alternative would be negative allosteric modulators (NAM) of nAChRs. NAMs of α4β2*nAChRs would reduce cholinergic activation of nAChRs, rather than block it or exogenously promote partial agonism of nAChRs. Thus, NAMs have the potential to be less likely to promote unwarranted side effects than partial agonists or nAChR antagonists. The non-selective NAM UCI-30002 potently blocks both electrical stimulus and nicotine-evoked nAChR-mediated currents, effectively crosses the blood-brain barrier, and dose-dependently reduces
intravenous nicotine self-administration (Yoshimura et al., 2007). Although the $\alpha_4\beta_2 > > \alpha_3\beta_4/\alpha_7$ selectivity of several molecules has been evaluated using *in vitro* systems, a negative allosteric modulator selective at $\alpha_4\beta_2$ nAChRs has not yet been identified (Henderson et al., 2012; Henderson et al., 2010; Pandya & Yakel, 2011). It is not known whether chronic administration of NMs of nAChRs would affect nAChR expression, but the fact that chronic administration of sazetidine-a does not increase the expression of nAChRs suggests that upregulation of nAChRs may also be absent with chronic administration of nAChR NMs, reducing the negative effects of withdrawal that may accompany upregulation of $\alpha_4\beta_2$ nAChRs.

In light of the data presented in these studies, it is critical to understand how high affinity $\beta_2$ nAChRs regulate changes in anxiety, whether their contributions are connected with tobacco use or with changes resulting in age-dependent increases in anxiety. These studies also suggest that it would be beneficial to determine if dysregulation of the basal cholinergic tone underlies the tobacco use and/or anxiety disorder of the individual. Furthermore, since chronic anxiety can contribute to an acceleration of cell death, including neuronal death, and the emergence of anxiety in the geriatric population may signal the onset of cognitive degeneration and/or AD, it is important to develop diagnostic tools to identify susceptible populations and select therapeutic intervention strategies that address the needs of the individual. Presently, the selective $\beta_2$ nAChR agonist 5I-A85380 is the best available tool to determine expression levels of $\beta_2$ nAChRs via SPECT scans. Unfortunately, it does not distinguish between the subclasses of $\beta_2$ nAChRs, and its utility is limited. Also, it cannot determine if nAChRs are in an active or desensitized state. The radioactive ligand of AChE [$^{14}$C] MP4A is presently used to determine endogenous AChE activity via PET scans, but the use of PET scans to determine basal AChE
activity in the general population is cost prohibitive. However, combined with the emerging molecular tools being presently employed in preclinical studies, improved methods to detect the functional status of nAChRs and basal AChE activity in in vivo human assays may be developed. By determining the specific dysregulated neurotransmitter system that may underlie symptoms of anxiety and/or habitual tobacco use, a more precise pharmacological treatment regimen can be prescribed that better meets the needs of the individual.


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Vita

Rev. Shawn Matthew Anderson, OSB was born on May 20, 1969, in Philipsburg, PA. He graduated from Clearfield Area High School in 1987, and subsequently received a B.S. in Pharmacy degree from Duquesne University in 1992. He then worked as a hospital pharmacist over the next 8 years. In 2001, he entered Saint Vincent Monastery as a Novice and took Solemn Monastic vows on July 11, 2005, the feast of Saint Benedict, Father of Western Monasticism. He received a Masters of Divinity Degree from Saint Vincent Seminary and was later ordained a priest of the Latin Catholic Rite in 2007. In 2008, he entered the Department of Pharmacology and Toxicology of Virginia Commonwealth University and in 2009 joined the laboratory of Dr. Darlene H. Brunzell. Upon acceptance of this dissertation, he will be awarded the degree of Doctor of Philosophy on December 14, 2013, the feast of St. John of the Cross.