2012

INVESTIGATING THE ROLE OF $\alpha_6$ and $\alpha_4$ CONTAINING NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS IN NICOTINE AND COCAINE CONDITIONED PLACE PREFERENCE TESTS IN MICE.

Sarah Sanjukdar
Virginia Commonwealth University

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INVESTIGATING THE ROLE OF \( \alpha_6 \) and \( \alpha_4 \) CONTAINING NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS IN NICOTINE AND COCAINE CONDITIONED PLACE PREFERENCE TESTS IN MICE.

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

by

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April 2012
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<td>AAALAC</td>
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<td>CDC</td>
<td>center for disease control</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>CNS</td>
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<td>CP</td>
<td>caudate putamen</td>
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<td>CPP</td>
<td>conditioned place preference</td>
</tr>
<tr>
<td>CPA</td>
<td>conditioned place avoidance</td>
</tr>
<tr>
<td>Cre</td>
<td>cyclic AMP response element</td>
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<td>cyclic AMP response element binding protein</td>
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<td>conditioned stimulus</td>
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<td>dihydro-beta-erythroidine</td>
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<td>GABA</td>
<td>gamma-aminobutyric acid</td>
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<td>interpeduncular nucleus</td>
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<td>i.v.</td>
<td>intravenous</td>
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<td>ICSA</td>
<td>intra-cranial self administration</td>
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<tr>
<td>KI</td>
<td>knock in</td>
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<tr>
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<td>knock out</td>
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<tr>
<td>LDTg</td>
<td>laterodorsal tegmentum</td>
</tr>
<tr>
<td>MHb</td>
<td>medial habenula</td>
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<tr>
<td>mg/kg</td>
<td>milligrams/kilogram</td>
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<tr>
<td>nAChR</td>
<td>nicotinic acetylcholine receptor</td>
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<td>NE</td>
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<td>nicotine replacement therapy</td>
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<td>OT</td>
<td>olfactory tubercle</td>
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<td>prefrontal cortex</td>
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<td>picomole</td>
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<tr>
<td>PPTg</td>
<td>pedunculopontine tegmentum</td>
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<td>s.c.</td>
<td>subcutaneous</td>
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<tr>
<td>SC</td>
<td>superior colliculus (SC)</td>
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<td>sec</td>
<td>seconds</td>
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<td>SEM</td>
<td>standard error of the mean</td>
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<td>US</td>
<td>unconditioned stimulus</td>
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<td>VTA</td>
<td>ventral tegmental area</td>
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<td>WT</td>
<td>wildtype</td>
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INVESTIGATING THE ROLE OF α6 and α4 CONTAINING NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS IN NICOTINE AND COCAINE CONDITIONED PLACE PREFERENCE TESTS IN MICE.

By Sarah Susan Sanjakdar, Ph.D.

A Dissertation submitted in partial fulfillment of the requirements for the degree of Ph.D. at Virginia Commonwealth University.

Major Director: M. Imad Damaj, Ph.D.
Professor with the Department of Pharmacology and Toxicology

Neuronal nicotinic acetylcholine receptors modulate both cholinergic and non-cholinergic synaptic transmission. Our research concerns α6 and α4 neuronal nicotinic subunits because they often co-assemble with the β2 subunit, which has abundant expression in the CNS and previous work has demonstrated that β2* nAChRs are involved in nicotine and cocaine reward. α6β2* and α4β2* nAChRs are highly expressed in midbrain, which is known to be critical for the incentive salience associated with natural and artificial (drug) reward. Our goal was to assess the role of α6β2* and α4β2* nAChRs in nicotine and cocaine reward using an unbiased conditioned place preference (CPP) test in mice. Adult male C57BL/6J mice or male mice null for the α6 or α4 nicotinic receptor subunit were used. For CPP: On day 1, pre-conditioning scores were recorded; Days 2-4 mice underwent conditioning, where they were randomly assigned to either the black or the white compartment paired with drug, and the opposite chamber paired with saline; Day 5 was a drug-free test day where post conditioning scores were recorded. α-Conotoxin MII[H9A;L15A], a selective antagonist of α6β2* nAChRs, was given centrally either into the lateral ventricle or the nucleus accumbens on conditioning days, which tested for acquisition of CPP, or it was given only once into the lateral ventricle on test day which tested for expression of CPP. Antagonizing α6*nAChRs resulted in a significant attenuation of both nicotine and cocaine place preference. This was complemented with diminished nicotine and cocaine place preference in α6 KO mice compared to WT littermates. Studies with α4 KO mice showed significantly reduced nicotine place preference scores compared to WT littermates. In contrast, α4 KO and WT mice showed significant place preference for 20mg /kg cocaine, suggesting that the α4 subunit is not required for the reward-like effects of cocaine in our behavioral test. Our results implicate α6β2* and α4β2* nAChR involvement in nicotine and cocaine CPP, but only α6β2* nAChR involvement in cocaine CPP. Lithium conditioned place avoidance and food reward were not altered in α6 KO mice or by α-Conotoxin MII[H9A;L15A], thereby validating the specificity of hedonics of targeting α6* nAChRs in CPP. Our studies suggest that α6β2* and α4β2*nAChR should be further characterized for future nicotine cessation therapies, and α6β2* could provide a new target for treating cocaine addiction.
CHAPTER ONE

Introduction and Review of the Literature

1.1 Nicotine Dependence

Cigarette smoking is ever-present: there are 1.2 billion smokers on earth, and more than 5,500 billion cigarettes are produced a year, accounting for 96% of manufactured tobacco product sales globally (World Health Organization, 2004). Tobacco smoking is the leading cause of preventable death and disease (CDC, 2009). Over 50% of heavy smokers will eventually die of a disease attributed to tobacco use. In the United States over 400,000 deaths per year are associated with tobacco use, 40% of which are linked to cardiovascular disease. The decrease in life expectancy is mostly due to tobacco related vascular, neoplastic, and respiratory disease. Specifically, lung cancer is highly attributable to smoking and is the leading cause of cancer death among men and women. In the United States, about 21% of adults currently smoke cigarettes (CDC, 2009). In a given year, only 3% of smokers are actually successful in their cessation attempts, even though over 70% of smokers express desire to quit (Paolini et al., 2011).

Nicotine is the naturally occurring alkaloid which is thought to be a primary addictive component in tobacco (Castane et al. 2005). Nicotine alone produces reinforcement, and is easily absorbed through skin and mucous membranes (Goodman et al., 2011). Cigarettes produce CNS effects in a matter of seconds when smoked. Each puff of cigarette provides reinforcement, and for heavy smokers, this habit is reinforced hundreds of times daily.
Environmental cues, social settings, and the anticipation and physical act of smoking all become repeatedly associated with the rewarding effects of nicotine which contribute to the resilience of nicotine dependence illustrated by the high relapse rates in smokers who try to quit (Goodman et al., 2011). Several studies have investigated the relevance of environmental cues to smoking phenotypes. One study assessed brain activity in regions pertaining to attention, motivation, and reward as participants assigned to a fixed period of abstinence viewed a series of pictures of smoking-related cues and paraphernalia and reported their level of craving for smoking before, during, and after each session (McClernon et al., 2004). A greater yearning to smoke was correlated with stronger brain activity after viewing the smoking-related illustrations. To the contrary, smokers with fewer cravings had stable or decreased brain activity when viewing the same smoking-related images after a period of abstinence (McClernon et al., 2004). Additional studies have reported that in smokers, smoking-related cues (without nicotine exposure) can activate dopaminergic circuitry in the mesolimbic system (Due et al., 2002; Franklin et al., 2007). The mesolimbic system is one of the dopaminergic pathways in the brain known to be involved in modulating behavioral responses to stimuli that activate feelings of reward through the neurotransmitter dopamine. This pathway is part of the reward circuitry in the brain which has been shown to encode attention, expectancy of reward, and incentive motivation (Nestler et al, 2005).

These studies highlight the impact that smoking related cues have on smoking addiction, and show that smokers with a greater sensitivity to smoking-related cues may
have difficulty in abstaining from cigarette use and are more likely to relapse, in part due
to the craving triggered by environmental cues.

1.2 Pharmacological Intervention: Nicotine Cessation Aids

The current medications approved for treating tobacco dependence include five
nicotine replacement therapies (NRT): nicotine patch, nicotine gum, nicotine lozenge,
nicotine inhaler, and nicotine nasal spray. Two non-nicotine cessation aids have also been
approved (Hays and Ebbert, 2010). Bupropion sustained release (Zyban®) is an orally
administered atypical antidepressant and its primary pharmacological action is thought to
be norepinephrine (NE) and dopamine (DA) reuptake inhibition (Miller et al., 2002).
Bupropion inhibited nicotine induced overflow from rat striatal slices preloaded with DA
and overflow from rat hippocampal slices preloaded with NE (Miller et al., 2002).
Bupropion is also a non-competitive antagonist for $\alpha_3\beta_2$, $\alpha_4\beta_2$, and $\alpha_7$ nAChRs (Fryer
and Lukas, 1999; Slemmer et al., 2000). Varenicline (Chantix®) is an orally administered
$\alpha_4\beta_2$ nAChR partial agonist, an $\alpha_3\beta_4$ nAChR agonist, a weak partial agonist at $\alpha_3\beta_2$ and
$\alpha_6^*$ nAChRs, and a full agonist at $\alpha_7$ nAChRs (Mihalak et al, 2006) that has produced
slightly higher rates of successful smoking abstinence compared to other therapies. In one
current clinical assessment, varenicline produced abstinence rates of 36.7% versus only
7.9% for placebo one year after quitting smoking (Williams et al., 2007).

All medications approved for treating tobacco dependence have undergone rigorous
testing for effectiveness and safety, but limitations still exist. The primary limitation of
available medications is their overall low efficacy of successfully treating nicotine
dependence, and the undesirable side effects that ensue with use. Despite the availability
of several nicotine cessation aids, there remains an initial failure of smoking addicts to achieve abstinence and a high relapse rate among those who do initially achieve smoking abstinence. Estimated abstinence rates six months post-quitting range between 20-30% for all available therapies (Hays et al., 2011). Population-based studies show that adverse side effects associated with nicotine cessation aids are common. The main systemic adverse effects for NRT reported include sleep disturbance (insomnia and abnormal dreams), headache, nausea and/or vomiting, dizziness, gastrointestinal symptoms (dyspepsia, diarrhea, constipation), and cardiac palpitations (Hays et al., 2011). These effects could be explained in part, by the fact that nicotine is not a selective agonist at nicotinic receptors; many of the peripheral adverse effects of nicotine could be due to stimulating $\alpha_3\beta_4^*$ nAChRs, which are prevalent in the periphery ($*$ denotes the presence of other nicotinic subunits in the receptor subtype). The issue of receptor selectivity of the current available pharmacotherapies is another limitation to consider.

For bupropion, adverse effects that were reported included seizures, suicidal ideation, and several cardiovascular episodes (Hays and Ebbert, 2010). The occurrence of these adverse events was about 14 days after initiation of bupropion use, which indicates that doctors should be monitoring patients at the start of the treatment to help prevent these serious adverse effects from occurring or becoming fatal.

For varenicline, other than the reports of cardiovascular episodes (Singh et al., 2011) and gastrointestinal disturbances (Leung et al., 2011), there have also been several accounts of adverse neuropsychiatric effects. Data provided by the FDA indicates that by the end of year 2007, a total of 147 cases of suicidal thought or behavior were reported in
association with varenicline use. Warnings have been added to the prescribing information for both bupropion and varenicline because of the neuropsychiatric symptoms (FDA, 2009).

Considering the drawbacks and low efficacies of current pharmacotherapies, there is much progress to be made. Identification of the relevant nicotinic receptor subtypes involved in nicotine dependence is important for the discovery of new treatments. Therefore, developing and further refining the current therapies requires a better understanding of the physiology and the role of nicotinic subunits that co-express with the β2 subunit, which has been heavily studied and is known to be crucial to nicotine reward and reinforcement (Corrigall et al., 1994; Maskos et al., 2005; Picciotto et al, 1998; Pons et al., 2008; Walters et al., 2006).

1.3 Nicotinic Receptors: Composition, Distribution, and Subtypes

Nicotinic acetylcholine receptors (nAChRs) are members of the ligand gated ion channel family, which also include glycine, GABA_A, and 5-HT3 receptors. As their name indicates, nAChRs are stimulated by the endogenous acetylcholine neurotransmitter (ACh) or by exogenous nicotine (Goodman et al., 2011). Once nicotine binds, the receptor undergoes activation and desensitization at a rate that is dictated by the nicotinic subunits that make up the receptor. Activation and desensitization correspond to transitions between distinct structural states (open and closed channel state and positions in between). Nicotinic ligands influence the changes in the structural state of the receptor by stabilizing the position that they bind with high affinity (Champtiaux and Changeux, 2004). nAChRs mediate postsynaptic neurotransmission at neuromuscular junctions and in peripheral
ganglia. In the CNS, nAChRs mediate presynaptic neurotransmitter release, and they are located both pre- and post-synaptically (Goodman et al., 2011).

Historically, the electrical organs from the *Torpedo californica*, a species of electric ray, and *Electrophorus electricus*, a species of electric fish, have provided researchers a way to study nicotinic receptors due to the high levels of nicotinic receptors on the very excitable surface of the electric organ’s membrane. The nicotinic receptor was purified from these aquatic species and this ultimately led to the isolation of cDNAs for nicotinic receptor subunits, which provided a means for cloning the genes of these subunits from mammalian neurons (Numa et al., 1983), allowing for the expression of different subunit combinations in cellular systems where their functionality was assessed (Changeux, 2005; Karlin et al., 2002).

Nicotinic receptors exist as pentameric structures composed of either five alpha subunits (homomeric receptors) or a combination of alpha and beta subunits (heteromeric receptors). Of the sixteen genes identified that encode nAChR subunits, nine are expressed in the CNS. Identified subunits include $\alpha 7$ homopentamers, and $\alpha 2-\alpha 4$, $\alpha 6$, and $\beta 2$ and $\beta 4$ subunits which co-assemble into heteropentamers (Changeux and Edelstein, 1998 and 2005). $\beta 3$ and $\alpha 5$ subunits lack the amino acids that are required to form the ligand binding site, and are therefore considered structural accessory subunits (Ramirez-latorre et al., 1996; Groot-kormelink et al., 1998). Several functional subunit combinations have been identified, and not all alpha and beta subunit combinations result in a functional receptor. The assortment of subunit arrangement is vast and currently exceeds the ability of pharmacological agents to selectively distinguish between receptor subtypes.
As for nicotinic receptor distribution, different receptor subtypes confer different patterns of expression: High levels of $\alpha_4\beta_2^*$ nAChRs are ubiquitously expressed throughout the brain (Changeux and Edelstein, 1998) whereas high levels of $\alpha_3\beta_4^*$ and $\alpha_3\beta_2^*$ nAChRs are found in the peripheral ganglia (Ke et al., 1998). Previous work has established that $\beta_2^*$ nAChRs are critical for nicotine induced DA release, and nicotine reward and reinforcement (Corrigall et al., 1994; Maskos et al., 2005; Picciotto et al, 1998; Pons et al., 2008; Walters et al., 2006). In the CNS, $\alpha_3\beta_4^*$ nAChRs are located mainly in the medial habenula (MHb) and the interpeduncular nucleus (IPN), and are also found in lower levels in the pineal gland, adrenal medulla, dorsal medulla, hippocampus, and retina (Grady et al., 2009). Interestingly, data in human genetic studies implicate the CHRNA5/A3/B4 gene cluster in nicotine dependence (Beirut et al., 2007; Chen et al., 2009). This gene cluster codes for $\alpha_5$, $\alpha_3$, and $\beta_4$ nicotinic receptor subunits. These receptor subtypes have high expression levels in periphery, in autonomic ganglia and also in the adrenal and dorsal medulla (Flores et al., 1996) therefore targeting this receptor subtype would likely produce unwanted side effects, similar to the side effects that are faced with the current nicotine cessation aids. Research has shown increased nicotine intake in mice null for the $\alpha_5$ subunit, and re-expressing the subunit in the medial habenula rescued this effect (Fowler et al., 2011). Also while lower doses nicotine place preference scores do not differ between $\alpha_5$ KO or WT mice, $\alpha_5$ KO mice will maintain a significant place preference for higher doses of nicotine that is not observed in WT mice (Jackson et al., 2010). These results suggest an enhancement of reward in the absence of the $\alpha_5$ subunit.
α7* nicotinic receptors, usually expressed in a homomeric form, are also located throughout the brain, where they are found in brain regions involved in cognitive function, including the hippocampus, and layers I and VI of cortex (Clarke et al., 1985; Gotti et al., 2006; Wonnacott, 1986). They are also found in the ventral tegmentum, substantia nigra, and in some subcortical limbic regions. Brunzell and McIntosh (2012) showed that infusion of an α7 selective nAChR antagonist into the Acb shell and the anterior cingulate cortex resulted in increased motivation of rats to self-administer nicotine, whereas infusing an α7 selective agonist into the Acb shell resulted in a decrease of motivation to self-administer nicotine, implicating a role for α7 in nicotine reinforcement (Brunzell and McIntosh, 2012). Jackson et al. (2008) showed that α7 KO mice displayed reduced somatic signs of withdrawal, implicating a role of α7 in the physical signs of nicotine withdrawal.

Some nicotinic subtypes have a more conservative expression pattern in the CNS, such as α6β2* nAChRs which are predominantly expressed on catecholaminergic nuclei in midbrain, along with some expression in retinal regions (superior colliculus and lateral geniculate nucleus) (Champtiaux et al., 2003; Champtiaux et al., 2002; Grady et al., 2003; Klink et al. 2001; Salminen et al., 2007). The midbrain harbors the VTA which is part of the mesolimbic pathway, which is one of the dopaminergic pathways in the brain known to be involved in mediating behavioral motivational responses to stimuli that generate internal state of reward through dopaminergic neurotransmission (Koob et al., 2010). DA neurons begin in the VTA and project to the NAc and other areas including the OT, CP, PFC, and AMG. Drugs of abuse including nicotine and cocaine will result in increased
DA release in the ventral striatum (particularly the Acb) (Barrett et al., 2004; Stein et al., 1998). Nicotinic receptor subtypes that are located on terminals and are known to regulate nicotine stimulated DA release include: $\alpha_4\alpha_6\beta_2\beta_3$, $\alpha_6\beta_2\beta_3$, $\alpha_6\beta_2\alpha_4\beta_2$, and $\alpha_4\alpha_5\beta_2$ (Grady et al., 2007; Klink et al., 2001). The mesolimbic system is crucial for signaling the incentive salience of psychoactive drugs, and the effects that drugs have in these brain regions contribute to the behavioral phenotype underlying drug addiction (Koob, 2010).

### 1.4. Pharmacological and Genetic Approaches that Distinguish $\beta_2$* nAChR Subtypes

In order to characterize discrete nicotinic receptor subtypes, an array of pharmacological and genetic approaches have been discovered and developed. Some of the earliest studies involved the discovery of $\alpha$-toxins from venom of krait and cobra snakes, which have high affinities and low dissociation rates from the target receptor and act as high affinity antagonists. These toxins have historical value, as they were used to assay the first isolated cholinergic receptor in vitro (Changeux, 2005).

Many other toxins have evolved in marine vertebrate animals which enhance predation or protection from predation (Taylor et al., 2007). The marine animal of the genus *Conus* has provided a rich supply of biologically active pharmacological tools from their venom that target different voltage gated or ion gated channels (McIntosh et al., 1999). Recently, the nicotinic field has observed the discovery and characterization of many different $\alpha$-conotoxins which are small disulfide rich peptides that are derived from the genus *Conus* and target specific nAChR subtypes with high selectivity (Azam and McIntosh, 2009; McIntosh et al., 1999). One of the most important and exciting feature of
α-conotoxins, is their ability to discriminate between closely related members of nicotinic receptor subtypes, which has been extremely useful for further characterizing the distribution and pharmacological significance of these different receptor subtypes (Azam and McIntosh, 2009). These conotoxin peptides distinguished between different β2* nAChR subtypes, specifically it allowed for the discrimination between α4β2(α5) nAChRs and α3α6β2* nAChRs.

In 2004, α-conotoxin MII [H9A; L15A] was developed in Dr. Michael McIntosh’s Lab at the University of Utah (McIntosh et al., 2004). This conotoxin is a mutant analog of the original α-conotoxin MII, isolated from venom of cone snail in 1997, which was a 16 amino acid peptide with high affinity for α3β2 and α6β2* nicotinic receptors (Kulak et al., 1997). Since α-conotoxin MII could not differentiate between these two subtypes (Kuryatov et al., 2000), a series of α-conotoxin MII analogs with a higher selectivity for α6β2* nAChRs were developed, including α-conotoxin MII[H9A;L15A] (McIntosh et al., 2004). This was useful for selectively targeting the α6 subunit in brain regions that contained both α3 *and α6* nAChR subtypes.

α-Conotoxin MII[H9A;L15A] differs from the original α-conotoxin MII peptide sequence in the substitution of Histidine at position 9, and Leucine at position 15 for Alanine, hence the [H9A;L15A] in the naming of the compound. One of the main effects of these amino acid substitutions is significantly increasing the affinity for α6/α3β2β3, where the IC50 is approximately 2000-fold lower for α6/α3β2β3 versus α3β2 (refer to Table 1 for IC50 of α-conotoxin MII[H9A;L15A] at α3β2 vs. α6/α3β2β3 nAChRs)
(McIntosh et al., 2004). Another notable effect of the Alanine substitution in the peptide sequence is rapid binding kinetics and a more rapid recovery of the receptor from blockade. MII[H9A;L15A] has 100 fold lower activity at α6/α3β4* receptors, and has little to no activity on α2β2, α2β4, α3β4, α4β2, α4β4, and α7 (McIntosh et al., 2004). Overall, α-conotoxin MII[H9A;L15A] selectively blocks α6* nAChRs, with preference for the α6/α3β2β3 versus α6/α3β4 subunit combination.
Table 1. IC50 of α-Conotoxin MII[H9A;L15A] at α3β2 vs. α6/α3β2β3 nAChRs.

Activity of doubly substituted MII analogs
Numbers in parentheses are 95% confidence intervals.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>IC50</th>
<th>Ratioa</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Rat α3β2</td>
<td>Rat α6/α3β2β3</td>
</tr>
<tr>
<td>MII[S4A;H9A]</td>
<td>207 (156–274)</td>
<td>1.97 (1.31–2.07)</td>
</tr>
<tr>
<td><strong>MII[H9A;L15A]</strong></td>
<td><strong>4850 (3540–6630)</strong></td>
<td><strong>2.40 (1.68–3.43)</strong></td>
</tr>
<tr>
<td>MII[L10A;L15A]</td>
<td>17.2 (8.11–36.6)</td>
<td>1.80 (1.26–2.56)</td>
</tr>
<tr>
<td>MII[E11A;L15A]</td>
<td>50.1 (41.4–80.6)</td>
<td>0.415 (0.223–0.772)</td>
</tr>
</tbody>
</table>

*a IC50 α3β2/IC50 α6/α3β2β3.

McIntosh et al., 2004

(adapted from McIntosh et al., 2004)
The use of genetically engineered knock out (KO) and knock in (KI) mice provide another means of understanding nicotinic receptors. Genetically engineered mice provide a distinct way of exploring the subunit composition of native nicotinic receptors, and they allow scientists to re-asses and expand on results obtained from pharmacological studies. Furthermore, genetically engineered mice also permit scientists to explore nicotinic subunits for which there are no selective ligands available (such as the α4 subunit). Currently, KO mice lacking the nAChR subunits α2, α3, α4, α5, α6, α7, α9, β2, β3, and β4 have been generated (Champtiaux and Changeux, 2004) and have provided us with a better understanding of the contribution of specific nicotinic subunits to various aspects of nicotinic cholinergic transmission in vivo. KI mice provide a means to address the issue of sufficiency of a nicotinic subunit of interest, which differs from the KO approach which addresses the issue of necessity. KI mice involve the introduction of point mutations into a gene in order to generate mice expressing mutant forms of the nicotinic subunit of interest. Currently mice with mutant gain-of-function forms of α4, α6, and α7 have been generated (Tapper et al., 2004; Drenan et al., 2008; Orr-Urtreger et al., 2000). Studies with KI mice allow us to explore the relationships between the structural characteristics of nicotinic receptors and their function in vivo.

Mice null for the α6 nicotinic subunit were generated in 2002 in Jean-Pierre Changeux’s lab at Institute Pasteur, in France (Champtiaux et al., 2002). Transcription of the α6 gene was impaired by deletion of the first two exons of the gene. One of the issues that arise with the generation of KO mice is the possibility of developmental abnormalities. Therefore, it was essential to assess the viability of these mice. Null mutant mice for the
α6 subunit, as measured by a lack of α-Conotoxin MII binding, did not show any significant neurological or behavioral defects, were capable of breeding, and had normal body weight and brain size compared to wild type (WT) littermates (Champtiaux et al., 2002). Also, there were no developmental abnormalities in these mice, specifically when examining the visual and dopaminergic pathways, where the highest levels of α6 are expressed. Film autoradiography displayed no differences in the abundance of mRNA of α3, α4, α5, α7, β2, or β4 nAChR subunits, suggesting a lack of upregulation of other nicotinic subunits to compensate for the lack of the α6 subunit. These mice exhibited normal behavior in home cages, and had normal locomotor activity in both non-habituated and habituated settings (Champtiaux et al., 2002).

The binding profile of native nAChRs in α6 KO mice was characterized using several nicotinic ligands. Receptor autoradiography showed a decrease in nicotine, epibatidine, and cytisine binding, and a complete lack of α-conotoxin MII binding, at the superior colliculus (SC), the dorsal lateral geniculate nucleus (dLGN), and the midbrain regions of homozygous null mutant mice (Champtiaux et al., 2002). Intriguingly, displacement of epibatidine binding by α-conotoxin MII in the striatum showed an increase in α-conotoxin MII resistant areas in KO mice compared to WT mice (possibly α4β2* nAChRs), where there was no difference in the total binding, which suggests the possibility of developmental compensation (Champtiaux et al., 2002).

Mice null for the α4 nicotinic subunit were independently generated in 1999 in Dr. Jean-Pierre Changeux’s lab at Institut Pasteur, in France (Marubio et al., 1999), and also in 2000, in Dr. John Drago’s Lab at Monash University, in Australia (Ross et al., 2000).
The mice were evaluated for size and weight, fecundity, and overall behavior. α4 KO mice had a normal anatomy, were capable of reproduction, and portrayed normal locomotor behavior in their home cages.

The binding profile of native nAChRs in α4 KO mice was characterized using several nicotinic ligands. Receptor autoradiography showed a decrease in nicotine and epibatidine binding in most brain regions of α4 KO mice. However, epibatidine binding persisted in the medial habenula (MHB), superior colliculus (SC), and the interpeduncular nucleus (IPN), and some low levels in the substantia nigra (SN). Other studies have shown that α3β4* or α6β2* nAChR subtypes reside in these brain regions (Whiteaker et al., 2000).

Interestingly, the locomotor behavior of the Australian α4 KO mice in a novel environment revealed an increase of exploratory behavior compared to WT littermates (Ross et al., 2000). In addition, when assessing anxiety in these mice using the elevated plus maze test, α4 KO mice showed higher levels of basal anxiety compared to WT littermates, suggesting that the α4 subunit is mediates anxiety in mice. In contrast, this phenotype was not present in the α4 KO mice generated in France (Marubio et al., 1999; Marubio et al., 2003). This discrepancy could be due to a multitude of factors possibly pertaining to the differences in the methods and the background strains of mice used to generate these transgenic mice. The α4 KO mice generated in Australia, were derived from chimeras generated from BALB/C blastocytes, which were then mated with CF1 mice to create a heterozygous mouse (Ross et al., 2000). This heterozygous mouse was then crossed with C57BL/6J (B6) mice to generate the colony, and contains a total of four
different background strains of mice. This differs from the creation of the French α4 KO mice, which were derived from the 129 mouse strain mated with B6 mice. Interestingly, the anxiety responses in BALB/C, B6 and CD-1 mice exposed to a novel open space test were assessed in a study which found that of the three strains, the BALB/C mice displayed the most anxiety-like phenotype (Michalikova et al., 2010), which could be one possible explanation for the basal anxiety observed in the Australian α4 KO line.

In light of the knock-out study in mice suggesting a role of α4 in anxiety (Ross et al., 2000), one group conducted a genetic association study and found that the CHRNA4 rs1044396 polymorphism was associated with negative emotionality, where Caucasian subjects described themselves as being more anxious and emotionally unstable (using psychometric personality questionnaires) (Markett et al., 2011). Recently, another study reported cognitive phenotype of CHRNA4 rs1044396 SNP, characterized by T allele carriers inclination to preferentially process events in the attentional focus compared to events occurring outside the focus of attention (Greenwood et al., 2012). These genetic association studies help to bridge the gap between animal and human research; data pertaining to α4 suggest that this nicotinic subtype is involved in anxiety and cognition.

In summary, transgenic mice have proven to be a useful experimental approach to the study of nicotine dependence, capable of revealing phenotypic differences in various behavioral models of nicotine dependence. The most common criticism is that compensatory effects of other genes in transgenic mice may either mask the detection of the targeted gene’s phenotype (epistasis), or be confused for the phenotype of the null gene. Another issue to be aware of is the ‘hitch-hiking donor gene’ confound. Even after
12 generations of crossing with the B6 mouse strain about 1% of the mouse’s genome still remains from the strain that was originally used to derive the mouse (usually a 129 mouse strain) which could result in phenotypic differences. For these reasons, conducting research using both pharmacological and genetic manipulations is valuable.

1.5 Animal Behavioral Models of Reward: Understanding the Conditioned Place Preference Test

Humans consume psychoactive substances, in part, because they provide ‘rewarding’ and ‘pleasurable’ incentives. Drug reward entails a multifaceted interrelation of the physiological effects of drugs in the CNS associated with motivation and feeling, along with a learned association of drug-related environmental cues (Hyman et al., 2005). Generally, reward can be defined as the hypothetical internal state of pleasure or gratification (hedonia), which is achieved through the possession or utilization of appetitive stimuli (Sanchis-Segura et al., 2006). In the psychobiology of substance dependence, reward describes the gratifying or enjoyable effects of a drug (Sanchis-Segura et al., 2006). In this regard, reward refers to a subjective response related to the post-presentational effects of the appetitive stimuli, which later on become important features of the internalization of these incentives.

While there are a myriad of rewarding psychoactive substances with dissimilar mechanisms of action in the CNS, almost all of them will directly or indirectly activate the mesolimbic DA system (Di Chiara et al., 2004; Koob et al., 2010). The mesolimbic DA system normally reinforces behaviors that are vital to survival, including eating and sexual reproduction (Koob et al., 2010). Compared to natural reinforcers, drugs of abuse have
significantly greater effects on DA release in brain regions within the mesolimbic pathway, such as the Acb. One study showed that food increased DA levels in the Acb by 45%, while the psychostimulants, amphetamine and cocaine, increased DA levels by 500% (Hernandez and Hoebel, 1988). When the rewarding drug is consumed, DA is released in the CNS, which leads to the growth of synaptic connections in the neural pathways that involve the behavior correlated with or leading up to the reward.

Several animal models have been developed with the aim to objectively assess drug reward and reinforcement. Fundamental principles of learning and behavior have been assessed for many years, and made relevant in the study of drug reward and reinforcement. This is appropriate given that the development of drug dependence can be considered a learned trait, in the sense that internalization of the rewarding of effects of drugs and the association made with the environment related to the drug following repeated exposure, will result in changes in behavior (Bardo and Bevins, 2000).

One of many animal models developed to assess the reward like effects of drugs is the conditioned place preference (CPP) test. CPP is a well established test of drug induced conditioning that involves contextual cues which is an important aspect of learning and memory that underlies addiction, and have been proposed as useful at inferring the hedonic value (‘rewarding properties’) of a drug. CPP reflects a preference for a context due to the continuous association between the context and the stimulus, and there has been a persistent increase of publications that use this procedure (Tzschtzenteke, 2008). In a place conditioning test, the drug of interest is administered by the experimenter to the subject (mouse), and the drug’s effects, acting as the unconditioned stimulus (US), and a neutral
environment (acting as the neutral stimulus) are repeatedly paired. After repeated pairings, the previously neutral stimulus obtains the means to act as a conditioned stimulus (CS), and this is thought to be a Pavlovian type of learning (Cunningham, 1998). The CS will now be able to bring about approach or avoidance phenotype depending on the effect of the drug (US) (Bardo and Bevins, 2000). By using place conditioning to measure the approach or avoidance behavior, experimenters can gain more knowledge of the drug’s effects and its properties in an organism. Indeed, CPP is useful in depicting how drugs of abuse abnormally strengthen stimulus drug associations, which results in excessive value to the context or environment predictive of drug availability.

Although humans will portray some conditioned approach or avoidance behavior to certain stimuli associated with drug use, CPP is not principally meant to mimic human behavior. It is important to recognize that the dose and route of administration of drug is given to the subject by the experimenter, independent of the subject’s choice and will, which therefore separates it from human situations where drug is willingly and independently consumed. In this regard, CPP should be classified as a ‘test’ because it does not ‘model’ an aspect of human behavior and lacks discernible face validity. Rather, it is thought that CPP provides more insight to the characteristics of the drug than the subject’s behavior (Sanchis-Segura et al., 2006).

Interpretation of place conditioning results has been a topic of debate in the scientific community, and the various interpretations of CPP can be attributed in part to the various ways the test is conducted. CPP is thought to be useful for deducing the internal state of reward or hedonic value of a drug, and has also been suggested as useful for
measuring drug-seeking behavior (Bardo and Bevins, 2000; Sanchis-Segura et al., 2006). Another interpretation suggests that CPP measures the ‘conditioned approach’ of the individual subject (Mead et al., 2005). Although interpretation of CPP can be sometimes intangible, it remains a well established test of drug induced conditioning that heavily involves contextual cues, which involves synaptic transmission similar to that which underlies learning and memory in addiction. As discussed throughout this dissertation, several studies have investigated the impact of environmental cues on drug use and drug craving phenotypes, and cues have been shown to play an important role in addiction and relapse (Ehrman et al., 1992; McClernon et al., 2004; Reid et al., 1999).

When a substance is administered in conjunction with the psychoactive drug during the pairing sessions of US to CS, and disrupts the ability of the psychoactive drug to induce CPP, it is interpreted as preventing the acquisition of the reward-like properties of the psychoactive drug. The antagonist is most likely acting by blocking the reward-like effect of the drug in the CNS during the conditioning sessions, thereby preventing the effect that results in the association of the drug to the context; animals do not feel motivated or have reason to find the context desirable or preferable at the end of the CPP procedure, therefore no CPP is observed. Alternatively, when a substance is administered only on test day of CPP after the completion of the conditioning pairing sessions of the US to the CS, and successfully disrupts drug induced place preference, it is identified as blocking the expression of CPP. In this manner, the substance was able to stop place preference from being expressed after the animal had undergone conditioning with the drug. The substance could be acting by blocking DA release that occurs when the animal
is in contact with the environment or context associated with the psychoactive drug, thereby reducing the motivational or preferable attributes of the context.

Some issues must be addressed in order to correctly interpret CPP results. One issue is to assess the specificity of hedonics of the substance being tested or the nicotinic subunit being targeted via genetic manipulations; does the substance or genetic manipulation cause an overall state of anhedonia? Does it impair all forms of memory or cause general confusion in the animal? It is important to examine the effect of experimental manipulations on the overall associative process (memory recollection), and to also evaluate possible locomotor effects, when a substance is administered to the animals on days when preference scores are being collected.

Considering that CPP is a sensitive test, it is rudimentary to realize that the parameters of the procedure will significantly affect the outcome of experimental results. The room where CPP is conducted must be maintained at a stable room temperature of 68-72°F, have dim lighting, and a fan should provide ambient noise to drown out any extraneous noise occurring outside the room. On the whole, more apparent preference is realized when the drug (US) is given just before exposure to the neutral context or environment. In fact, studies have shown that exposing the drug (US) after exposure to a neutral context will result in conditioned place avoidance (CPA) instead of CPP (Font et al., 2006; Fudala et al., 1990). This could be due to the negative effects of the drug at the end of the pharmacokinetic curve, or the withdrawal or negative effects that ensue after the reward like effects of the drug have passed. Also, in general with more pairing sessions of US to CS, more robust and persistent conditioned preference can be achieved. Another
important factor is handling the mice prior to the initiation of the CPP procedure, as our lab has published on habituation to the handling techniques influencing the results of nicotine CPP (Grabus et al., 2006). These are some of the parameters to consider, especially when working with drugs such as ethanol, nicotine, or cannabinoids that produce place preference scores in animals that are not as robust compared to preference scores for opiates and psychostimulants (Cunningham et al., 1993; Tzschentke, 2007). The route of administration of a drug, the species, and the genetic background of the animals are also important aspects to consider when conducting CPP.

Many groups have observed that nicotine induced place preference in rodents (Berrendero et al., 2002; Brunzell et al., 2009; Calcagnotti and Schechter, 1994; Castañé et al., 2002; Fudala et al., 1985; Grabus et al., 2006; McGranahan et al., 2010; Walters et al., 2006), while other groups have reported that nicotine only causes a place aversion (Jorenby et al., 1990) or has no effect on place preference (Belluzzi et al., 2005; Clarke et al., 1987). Several parameters (addressed in the paragraph above) including species used and the different protocols for conditioning in different laboratories could account for the discrepancies in the results of these studies. Figure A illustrates a typical dose effect curve for nicotine CPP using our protocol, which gives results that can be replicated in our lab. Notice the inverted U shape of the nicotine place preference scores, where the effective dose range for inducing place preference for nicotine appears to be relatively narrow. The subcutaneous dose of 0.5 mg/kg nicotine repeatedly produces the most robust preference scores in adult male C57BL/6J mice in our lab (p=0.0006; F=6.532).
Adult male B6 mice display significant place preference for nicotine at doses between 0.25, 0.5, and 0.75 mg/kg (s.c.). The group that received 0.5 mg/kg nicotine had significantly greater preference scores than both saline and 1mg/kg nicotine groups (*** = p<0.001 compared to saline; * = p<0.05 compared to saline; # = p < 0.05 compared to 0.5 mg/kg nicotine).
1.6 The Role of α6 Containing nAChRs in Nicotine Reward and Reinforcement.

In 1986, DeNoble and Mele discovered that rats could be trained to self-administer nicotine, and that this reinforcement was blocked by mecamylamine, but not hexamethonium, implicating a role for the neuronal nicotinic receptors in nicotine reward and reinforcement (DeNoble and Mele, 2006). Since then, studies have established that β2* nAChRs are critical for nicotine induced DA release, and nicotine reward and reinforcement (Corrigall et al., 1994; Maskos et al., 2005; Picciotto et al, 1998; Pons et al., 2008; Walters et al., 2006). Of the various nicotinic subunits that co-assemble with β2, α6 is of particular interest. α6β2* nAChRs are predominantly expressed on catecholaminergic nuclei in midbrain, where they are located pre-synaptically in the NAc (ventral striatum) and post-synaptically in the VTA. They are also located in the caudate-putamen (dorsal striatum), substantia nigra, locus coeruleus, and the superior colliculus and lateral geniculate nucleus (two retinal regions) (Champtiaux et al., 2002; Klink et al., 2001) (Whiteaker et al., 2000). The high expression of α6β2* nAChRs within dopaminergic circuitry renders this subtype an attractive target for probing nicotine reward.

Several studies have demonstrated a critical role of α6* nAChR involvement in nicotine induced DA release, reward, and reinforcement. About thirty percent of nicotine-stimulated DA release in striatum is mediated by α6β2* nicotinic receptors (Grady et al., 2002). Furthermore, using fast-scan cyclic voltammetry, DA neurotransmission stimulated by nicotine was observed to be distinctively governed by α6β2* nAChRs in the Acb (Exley et al., 2008). Brunzell et al. (2010) has also shown that antagonism of α6β2* nAChRs in the NAc shell significantly reduces motivation to self administer nicotine, and Gotti et al.
(2010) showed that α6β2* nAChRs in the VTA mediate nicotine’s effects on DA release, locomotion, and reinforcement.

Pons et al. (2008) showed that α6 KO mice (and α4 or β2 KO mice) displayed decreased self-administration of nicotine compared to WT counterparts, and nicotine self-administration was restored by re-expression of α6 (or α4 or β2) in the VTA. Additionally, Drenan and colleagues (2008) showed that α6 gain-of-function mutant mice were hyperactive compared to WT littermates, and had augmented nicotine-stimulated DA release from presynaptic terminals. Our lab has shown that injecting a selective α6*nAChR antagonist, α-Conotoxin MII [H9A;L15A], into the lateral ventricle resulted in a decreased expression of nicotine place preference in mice (Jackson et al., 2009). This study from our lab has showed that α-conotoxin MII [H9A; L15A], a selective α6β2* nAChR antagonist, did not affect the analgesia, locomotion, or body temperature changes after acute exposure to nicotine. α-Conotoxin MII [H9A; L15A] blocked conditioned place aversion and anxiety related behavior associated with affective nicotine withdrawal signs but had no effect on somatic signs or on hyperalgesia, which comprise the physical signs of nicotine withdrawal (Jackson et al., 2008).

Of nicotinic subtypes located on dopaminergic terminals that include α6, some are made up of α6β2β3* subunits and others comprise α4α6β2β3* subunits (Zoli et al., 2012). These different receptor subtypes have different binding properties and therefore different sensitivities to nicotine, with α4α6β2β3* nAChRs exhibiting the greatest sensitivity to nicotine (EC50 = 230 nM), with high affinity for nicotine and ACh binding and a slower desensitization profile (Grady et al., 2012; Salminen et al., 2007). Drenan et al. (2010)
showed that gain of function α6 KI mice, which typically displayed hyperactivity and displayed enhanced nicotine stimulated DA release in synaptosomal preparations, had normal behavior and had fewer and less sensitive α6* nicotinic receptors, and a decrease in DA release when the α4 subunit was removed. This implicates a vital role for α4α6β2* nAChRs in the behaviors of the α6 gain of function KI mice and in the cholinergic control of DA neurotransmission (Drenan et al., 2010).

Another set of studies conducted by Exley et al. (2008; 2011) focused on the examining the contributions of α6* and α4* nicotinic receptors in nicotine stimulated DA neurotransmission and nicotine reinforcement. This lab found using cyclic volatometry, that the majority of nicotine stimulated DA release in the Acb was mediated by α6β2* nAChRs (Exley et al., 2008). In addition, using an intracranial self administration (ICSA) model they observed that α6 KO mice readily self administered nicotine (ICSA) into the VTA similar to WT at a dose of 100ng but for lower dose of 10ng they self administered to a lesser extent than WT. α4 KO mice transiently self administered nicotine in early training sessions (the first 3 sessions) but all following sessions did not result in self-administration of nicotine into the VTA compared to WT mice (Exley et al., 2011). Exley et al. (2011) were also interested in the effect of nicotine on dopaminergic neuronal activity in VTA, knowing that nicotine modifies DA neuron excitability (VTA) and also modulates DA release (terminals in striatum). Systemic administration of 30ug/kg nicotine resulted in increased firing rate of DA neurons in the VTA in both α6 KO and WT mice (Exley et al., 2011). In α4 KO mice the increases in firing rate were delayed and attenuated compared to WT mice, and also lacked burst firing activity of neurons in response to
Targeted re-expression of α4 restored the ICSA and nicotine sensitive bursting properties of VTA DA neurons. They also found that at terminals in the Acb, both the α4 and the α6 subunits were necessary to maintain nicotine-sensitive cholinergic regulation of DA release (Exley et al., 2011). To summarize, this study proposed a critical of α4* nAChRs in nicotine induced DA neuron activity and in ICSA of nicotine into the VTA, whereas α6* nAChRs did not seem necessary in mediating these nicotine induced effects. It is important to keep in mind that the parameters under which the study was conducted were very site specific (VTA only), and nicotine affects a whole system differently than in an isolated system or brain region. This study also showed both α4 and α6 nicotinic subunits are important for gating DA neurotransmission in the Acb (Exley et al., 2011).

Smoking dependence is due to complex behavioral traits that are influenced by genetics (Rose et al., 2009). Recent genetic association studies have reported variation in the CHRNA6 gene was associated with tobacco dependence in Caucasians (Hoft et al., 2009). Another study found associations between CHRNA6 and CHRNB3 and subjective responses to smoking (Zeiger et al., 2008). This study found two CHRNB3 SNPs (rs4950 and rs13280604) that were significantly associated with subjective response factors to initial tobacco use. CHRNB3 gene codes for β3, which is an accessory subunit that been shown to be expressed very often with α6* nAChR subtypes (Cui et al., 2003; Gotti et al., 2006).

In our hands, using CPP, which heavily involves conditioning and cues, we hypothesized that both α6β2* nAChRs in the Acb are involved in mediating the effects of nicotine place preference. It is of interest to determine the significance of the several
nicotinic receptor subtypes containing α6 and β2 nicotinic subunits to nicotine reward by exploring α6* nAChRs and also α4* nAChRs using the pharmacological and genetic tools available to us.

1.7 The Role of α4 Containing nAChRs in Nicotine Reward and Reinforcement

It is well known that the α4 subunit is most often co-expressed with the β2 subunit and that α4β2* nAChRs have the highest affinity for nicotine and display the most abundant binding to nicotine and nicotinic agonists in the CNS (Changeux, 2005). α4β2* nAChRs are highly expressed in the midbrain (Klink et al., 2001), and previous work has illustrated the necessity of β2* nAChRs for nicotine reward and reinforcement in rodents (Corrigall et al., 1994; Maskos et al., 2005; Picciotto, 2003; Pons et al., 2008; Walters et al., 2006). One study observed a loss of nicotine reinforcement in α4 KO mice, and reintroduction of the missing subunit in the VTA of the KO mouse rescued this phenotype (Pons et al., 2008). Another study showed that α4 KO mice displayed a decrease for nicotine ICSA into the VTA, and systemic administration of 30ug/kg nicotine did not result in increased firing rate of DA neurons in this brain region (Exley et al., 2011). This study also found that at terminals in the Acb, both the α4 and the α6 subunits were necessary to maintain nicotine-sensitive cholinergic regulation of DA release. Studies have also shown that DA levels in the striatum of α4 and β2 KO mice do not increase in response to nicotine, which supports the notion that α4β2* nAChRs are necessary for DA release, which is a crucial component of drug reward and reinforcement (Marubio et al., 2003; Picciotto et al., 1998). Interestingly the α4 KO mice generated by Changeux
colleagues had increased basal DA levels in the striatum compared to WT counterparts (Marubio et al., 2003). This suggests that α4* nAChRs may play a role in the tonic control of DA in mesostriatal regions of the brain. Although there were elevated basal DA levels in striatum of α4 KO mice, nicotine failed to induce an increase in striatal DA (Marubio et al., 2003). Another study showed that a single point mutation, Leu9’→Ala9’, of the α4 subunit in mice rendered α4* nAChRs hypersensitive to nicotine, and illustrated that activation of these receptors by low doses of nicotine was sufficient for nicotine reward as measured by CPP, tolerance as measured by hypothermia, and sensitization as measured by locomotor activity (Tapper et al., 2004).

One key study engineered mice where the α4 subunit was deleted only in dopaminergic neurons (McGranahan et al., 2011). This was accomplished by first generating mice where exon 5 (codes for channel of the receptor) of the α4 gene was ‘floxed’ (flanked on either side by loxP recognition sequences). These lox-only mice were then bred to KI mice that expressed Cre-recombinase 5’ to the DA transporter gene, which consequently selectively eliminated α4 subunit expression from dopaminergic neurons (α4-DA mice) (McGranahan et al., 2011). These mice progressed our understanding of the role of α4* nAChRs specifically in dopaminergic pathways in the brain that are heavily implicated in drug reward. Using these α4-DA mice, the study revealed that α4* nAChRs specifically on dopaminergic neurons were necessary for nicotine place preference, but not for cocaine place preference. They also demonstrated that α4* nAChRs on dopaminergic neurons were necessary for the anxiolytic effects of nicotine in the elevated plus maze test,
and were also involved in the sensitivity to the locomotor depressing effects of nicotine (McGranahan et al., 2011).

Recently, Cahir et al. (2011) reported that mice null for the α4 nicotinic subunit showed similar place preference scores to WT littermates for 0.5 mg/kg nicotine (i.p.). They also reported that the same dose of nicotine used caused significant locomotor depression in WT but hyperactivity in α4 KO mice; overall results proposed a role for α4 in the locomotor depressant effects but not in the reward like effects of nicotine (Cahir et al., 2011). Data from this study is in disagreement with other studies that proposed that α4* nAChR are important for nicotine reward (Mcgranahan et al., 2011; Pons et al., 2008; Tapper et al., 2004). When reviewing the study more closely, several factors could account for this discrepancy. This study injected 0.5 mg/kg nicotine i.p. and used a biased design for place conditioning. The study failed to include a control group of mice that received only saline injections, which is important so that preference scores of mice that received only saline can be compared to the preference scores of mice that received nicotine. It also ensures that stress from handling and injections did not affect place conditioning scores in the study. This is also an important control considering the biased design they used for CPP where they paired drug treatment to the context that was initially less preferred by mice. Also, because they used a biased design, the initial preference for the compartments that mice were paired to (baseline preference scores) were not taken into consideration when calculating final preference scores. One study observed that mice given saline on both sides of a CPP compartment in a biased study resulted in higher preference scores for the initially less preferred side (Cunningham, 2003).
considered habituation or ‘disinhibition’ of the mice to the initially less preferred side of the CPP compartment because they acclimated to the environment over conditioning sessions with CPP. These are some factors that could explain why Cahir et al. (2011) did not find different nicotine CPP in α4 KO mice compared to WT littermates.

Taking into account all the current evidence implicating α4* nAChRs in nicotine reward and DA neurotransmission, we hypothesized that α4β2* nAChRs in the Acb are critical mediators of the conditioned hedonic effects of nicotine-associated cues which we assessed in the conditioned place preference test.

1.8 Involvement of the Nicotinic-Cholinergic System in Cocaine Reward and Reinforcement.

Cocaine is a powerful psychostimulant that inhibits dopamine transporters leading to a sustained elevation of DA levels in several brain regions including the Nucleus Accumbens (NAc); a process that is considered critical to development of addiction (Nestler, 2005). The cocaine induced short-term buildup of DA results in a state of euphoria which is thought to motivate repeated use thereby altering behavior and intensifying stimulus drug associations (Di Chiara, 1999). Repeated exposure to cocaine results in alterations in genetic activity and nerve cell structure that last for months (Nestler, 2005), and this contributes to relapse in individuals who are exposed to cocaine related cues in surrounding environment, affecting incentive salience (Ehrman et al., 1992; Reid et al., 1998). Incentive salience is the importance given to the drug, due to its reinforcing effects, that motivate or encourage one to seek the drug at all costs. The reactivity to cocaine cues can persist in patients who have abstained from cocaine use for
many months (Rohsenow et al., 1990). The use of cocaine cues to induce cocaine craving in patients has been a useful model used to assess the efficacy of medications for their ability to reduce cocaine craving. Reid at al. (1998) showed that nicotine enhances cue-induced cocaine craving, which coincides with other studies reporting co-morbidity of cigarette smoking in cocaine addicts (Budney et al., 1993). One study reported that patients found that mentholated cigarettes can prolong the hedonic state induced by cocaine, and can even alleviate the craving for cocaine when cocaine is not available to them (Sees and Clark, 1991). Reid et al. (1999) also showed using cocaine cues to induce craving for cocaine, that administering a 2.5 mg tablet dose of mecamylamine (a non-selective nicotinic antagonist) to patients reduced the reports of cocaine craving (Reid et al., 1999).

There have been several behavioral studies that investigated the role of nicotine and nicotinic receptors in cocaine dependence in rodents. Horger et al. (1992) observed increased self-administration of cocaine in rats that were pre-exposed to nicotine. Champtiaux et al. (2005) showed that DHβE (β2* nAChR antagonist) microinjected into the VTA prevented cocaine locomotor sensitization. This effect was not seen with microinjections of MLA (α7* nAChR antagonist), which implicates β2* nAChR involvement in cocaine sensitization (Champtiaux et al., 2006). Zachariou et al. (2001) found that β2 KO mice displayed decreased place preference for 5mg/kg cocaine compared to WT littermates, and also observed a decrease in place preference for cocaine with co-treatment of 1mg/kg mecamylamine in B6 mice. Interestingly, Picciotto and colleagues could train β2 KO mice to self administer cocaine (Picciotto et al., 1998); this could be
explained by the possibility that these mice compensated due to the lack of β2 in the system by increased self administration of cocaine in order to achieve the rewarding and reinforcing effects of the drug.

Other studies observed a dose-dependent decrease of cocaine self-administration with pre-treatment of non-selective nAChR antagonists, mecamylamine or MRZ 2/621 (Levin et al. 2000; Blokhina et al. 2005). A recent study by Levine et al. (2011) showed that pre-treatment of mice with nicotine increased the response to cocaine as observed by a 98% increase in locomotor sensitization and 78% increase in CPP. This effect was not observed when reversing the order of drug administration (cocaine had no effect on nicotine sensitization and reward) (Levine et al., 2011). Previous research has also shown that drugs of abuse, including psychostimulants like cocaine, enhance release of ACh in the Acb and increase responsiveness of cholinergic neurons during acute and repeated drug exposure (Fiserová et al., 1999; Nestby et al., 1997).

These studies, along with several others have linked nAChRs to cocaine reward, reinforcement, and sensitization (Champtiaux et al., 2006; Horger et al. 1992; Levine et al., 2011; Reid et al. 1998; Reid et al., 1999; Zachariou et al., 2001; Zanetti et al., 2007; Levin et al. 2000; Blokhina et al. 2005; Fiserová et al., 1999; Nestby et al., 1997).

Our research targeted α6* and α4* nAChRs subtypes in the investigation of cocaine reward because these receptors are often co-expressed with the β2 subunit, and β2* nAChRs are known to be crucial for nicotine reinforcement and reward (Maskos et al., 2005; Picciotto et al., 1998; Walters et al., 2006) and have also been shown to play a role in cocaine reward (Zachariou et al., 2001). Previous research has shown that most, if not
all, DA terminals express nicotinic receptors, with β2 identified as the common subunit expressed (Salminen et al., 2007; Zoli et al., 2012). However, implications of the variety of nAChR subtypes expressed on DA terminals are not yet fully understood. Recent studies have shown that α6β2* nAChRs are expressed on dopaminergic neurons that play major roles in addiction to nicotine. Indeed, α6β2* nAChRs subtypes have high expression in catecholaminergic nuclei in midbrain regions thought to mediate drug reward, play a major role in presynaptic DA release (Grady et al., 2002; Whiteaker et al., 2000) and mediate nicotine reward and reinforcement in rodents (Brunzell et al., 2010; Gotti et al., 2010; Jackson et al., 2009; Pons et al., 2008). Of equal relevance, α4β2* nAChRs are also highly expressed in the midbrain (Klink et al., 2001), and previous work has illustrated the sufficiency of α4* nAChRs (Tapper et al., 2005) and the necessity of α4β2* nAChRs for nicotine reward and reinforcement (McGranahan et al., 2011; Pons et al., 2008) and nicotine induced DA release in rodents (Drenan et al., 2010; Marubio et al., 2003). Elucidating the role of nicotinic cholinergic receptors in psychostimulant induced behavioral reward, and investigating novel pathways involved in cocaine reward will pave a path for the development of potentially successful treatments for cocaine addiction in the future.

The goal of this dissertation was to investigate the role of α6β2* and α4β2* nAChR subtypes in the acquisition and expression of nicotine and cocaine place preference, because CPP is a well established test of drug induced conditioning involving contextual cues which is an important aspect of learning and memory underlying addiction. As previously discussed, α6β2* and α4β2* nAChRs have been implicated in nicotine reward
and reinforcement, and to date β2* nAChRs have been implicated in cocaine reward, reinforcement, and sensitization. We set out to characterize the nicotinic subtype (α6β2*, α4α6β2*, and/or α4β2*), and the neuro-anatomical locus (Acb) contributing to nicotine and cocaine reward-like effects using pharmacological antagonism of α6β2* nAChRs and genetic deletion of the α6 and α4 subunits in mice. Our hypothesis was that the α6β2* nAChRs in the Acb are critical for nicotine and cocaine place preference, whereas α4β2* nAChRs are critical for nicotine place preference, but are not involved in cocaine place preference.
CHAPTER TWO
Materials and Methods

2.1 Drugs

For studies involving nicotine, (-)-nicotine hydrogen tartrate salt [(-)-1-methyl-2-(3-pyridyl)pyrrolidine (+)-bitartrate salt] was purchased from Sigma Chemical Co. (St. Louis, Mo), and was dissolved in physiological 0.9% sodium chloride (saline). All doses are expressed as the free base of the drug and were always injected subcutaneously at a volume of 10 ml/kg body weight. Nicotine was prepared fresh for every experiment and refrigerated for no longer than one week. It was kept in a glass vile wrapped in aluminium to protect the nicotine from degradation.

For studies involving cocaine, cocaine was provided by the National Institute for Drug Abuse (NIDA), was dissolved in saline, and was always injected intraperitonealy at a volume of 10 ml/kg body weight, and stock solution was also kept refrigerated to maintain the integrity of the compound. A fresh solution of cocaine was prepared for each CPP experiment.

α-Conotoxin MII [H9A;L15A] was provided by Dr. Michael McIntosh at the University of Utah. Conotoxin was dissolved in small aliquots of saline in an 18°C to 20°C freezer until use. The doses used in the studies were calculated based on IC50 values of the compound at nAChRs (refer to Table 1) from McIntosh et al. (2004).

For studies involving lithium, lithium chloride was purchased from Sigma Chemical Co. (St. Louis, Mo), and was dissolved in physiological saline. Lithium was
always injected intraperitoneally at a volume of 10 ml/kg body weight, and stock solution was kept refrigerated to maintain the integrity of the compound.

2.2 Food

Food used to induce place preference in mice included ‘Reese’s’ peanut butter chips and cheesecake. The Reese’s peanut butter chips are available at any grocery store, and the ingredient label on the package reads: ‘partially defatted peanuts, sugar, partially hydrogenated vegetable oil (palm kernel oil and soybean oil), corn syrup solids, dextrose, reduced minerals whey (milk), contains 2% or less of salt, vanillin (artificial flavor), and soy lecithin.’ The cheesecake was purchased from Shockoe Espresso Mill Mountain Coffee and Tea in Richmond, Virginia.

2.3 Animals

Animals used in the pharmacological experiments were male B6 mice from the Jackson Laboratory (Bar Harbor, ME) that were 8-10 weeks old, unless otherwise noted. In order to minimize biological variability due to estrous cycling, none of the experiments involved female mice. Animal maintenance and research were conducted in accordance with the guidelines provided by the NIH Committee on Laboratory Animal Resources. Animals were housed, 4 per cage, in temperature- and humidity-controlled housing rooms, and a 12 hr light-dark cycle (lights on from 7 a.m. to 7 p.m.). Experiments were performed during the light cycle. The facility was licensed by the United States Department of Agriculture and accredited by the Association for Assessment and Accreditation of
Laboratory Animal Care (AAALAC), and protocols were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University (Richmond, VA).

For studies involving genetically modified mice, B6 provided the background strain for our $\alpha_6$ and $\alpha_4$ KO and WT mice. These KO mouse lines are backcrossed to the B6 strain every year to maintain vigor, and have been backcrossed for at least 10 generations. Healthy viable mice null for the $\alpha_6$ nicotinic subunit were provided by Dr. Uwe Maskos at Institut Pasteur (Paris, France) (Champtiaux et al., 2002). Viable mice null for the $\alpha_4$ subunit were provided by Dr. Henry Lester at the California Institute of Technology, with the permission of Dr. John Drago who generated them at Monash University, in Australia (Ross et al., 2000). KO mice used in the study were generated from HETxHET breeders and from confirmed KOxKO breeders.

2.4 Place Conditioning Involving Nicotine, Cocaine, or Lithium

The unbiased place conditioning test is well established in our lab and was used for the last several years in pharmacological and genetic studies (Damaj et al., 2010; Grabus et al., 2006; Jackson et al., 2009; Merritt et al., 2008; Walters et al., 2006). CPP is a test that reflects a preference for a context due to the repeated association between the context and the drug (refer to section 1.3). Animals can be trained readily and the procedure has been well established in various strains of mice our laboratory uses (e.g. ICR mice and B6 mice). The procedure also has the ability to test compounds, such as lithium, for place conditioned avoidance (CPA). CPP (or CPA) is conducted in a room dedicated solely to this procedure, in dim lighting, and with ambient sound from fans to drown out extraneous
background noise. Each mouse was handled for one minute each, for 3 days prior to CPP testing and mice were also brought into the CPP room on a cart and allowed to acclimate to room lighting and room temperature for at least thirty minutes, if not longer when possible, before each CPP session. The boxes were wiped down after every session with water and 1% Triton disinfectant.

Place conditioning chambers and software were purchased from Med Associates (St. Albans, VT). The conditioning apparatus consisted of three distinct compartments that were separated by doors that could slide up. The black and white compartments (each 16.8 cm long) served as the context that was paired to the psychoactive drug. Each side had distinct visual (color) and tactile (flooring) cues to allow the animal to learn to associate the environment with the treatment it was paired to. The black compartment had stainless steel grid rod flooring consisting of 3.2 mm rods placed on 7.9 mm centers. The white compartment had a 6.35×6.35-mm stainless steel mesh floor. The small gray compartment (7.2 cm long) was gray in color with a smooth PVC floor, and served as a thoroughfare between the two sides. All chambers had hinged, clear porous polycarbonate lids that were closed during testing. Infrared photobeam strips that were located within each chamber provided the data that was collected by an attached computer.

Place conditioning was conducted in three phases: preconditioning day, conditioning days, and post-conditioning day. On preconditioning day, mice are allowed to move freely among all three compartments for 15 min (900 sec), after a 5 minute habituation period in the gray compartment. Time spent on each side is recorded and these data are used to separate the animals into groups of equal bias. Days 2-4 are conditioning
days, where animals got either a saline or psychoactive drug injection, and were then confined to either the white or black compartment for 20 minutes for the morning session. 4 hours later, mice were injected with the alternate treatment and placed in the opposite compartment of the chamber for 20 minutes during the afternoon session. Groups were counterbalanced equally in order to ensure that some mice get drug in the morning while others got it in the afternoon, and some mice got drug injections paired to the white compartment while others had drug paired to black. Day 5 was a drug-free post-conditioning test day when mice are allowed to move freely among the CPP chamber and time spent in each side was recorded. Scores were calculated by subtracting the time spent in drug paired side on pre-conditioning day from time spent in drug paired side on post-conditioning day.

2.5 Conditioned Place Preference for Food Reward.

For studies involving food conditioned place preference. The procedure followed the general outline stated in the above paragraph, with a few minor modifications. No injections were given to these mice during conditioning days, they were simply exposed to the food, be that the food pellet that made up their usual diet, peanut butter chips, or room temperature cheesecake. Peanut butter chips and cheesecake were always placed in a small plastic weigh tray, and the mice were exposed to the same tray every day, which was placed in the exact same corner of the CPP chamber for every conditioning session in order to minimize any novelty to the procedure to allow them to make the association of the food exposure to the context in the chamber. The mice were also food restricted for 4 hours before each conditioning session. There was one extra day of conditioning, giving a total
of 4 conditioning days, in order to attain statistically significant food induced place
preference in mice. Also each conditioning session was 40 minutes long instead of the
usual 20 minute session for place conditioning with drug.

2.6 Intracerebroventricular Surgeries and Injections

Antagonists selective for \(\alpha_6\beta_2\) nAChRs that can be administered systemically are
still being developed and are not readily available for use. Consequently, we used \(\alpha\)-
Conotoxin MII [H9A;L15A] which is a peptidic compound that cannot cross the blood
brain barrier and was administered centrally via intracerebroventricular (i.c.v.) injections.
Mice undergoing i.c.v. surgeries were anesthetized with an i.p. injection of 45 mg/kg
sodium pentobarbital. An incision was made at the scalp of the mouse midway between
the eyes and ears, to expose cranial sutures. The skin that was cut resulted in a flap that
was put back into place at the end of the surgery to keep the site free of debris. Using the
stereotaxic apparatus, a site of injection was made, through the dura mater, with the
following coordinates: -0.6mm AP; +1.3 mm ML, with respect to bregma, and -2.1 mm
DV from the skull’s surface. After the surgery, animals were returned to clean home cages
and were allowed to recover for 20-24 hours.

On CPP test day, i.c.v. injections were made directly into the skull using a
Hamilton syringe. Sterile saline soaked cotton swabs were used to lightly nudge the flap
of skin to expose the injection site, while gently restraining mouse by holding the nape of
the neck. 5 \(\mu\)l of \(\alpha\)-Conotoxin MII [H9A,L15A] or saline was given, with the syringe
being held in place for 30 seconds to allow some time for drug diffusion into the lateral
ventricle. Following the injection period, animals were returned to home cages and allowed
5 minutes to recover from the light restraint required for injection before being placed into the CPP chamber. After the 5 minute habituation period, time was recorded for 15 minutes as the mouse freely explored all compartments of the CPP chamber.

2.7 Intracranial Cannula Implantation and Infusions

For cannulation surgeries, mice were anesthetized with an injection of 45 mg/kg sodium pentobarbital (i.p.). Once a mouse was readied for surgery, an incision was made to expose skull of mouse. Using the stereotaxic apparatus, the mouse’s head was leveled, and a site of cannula implantation was found with the following coordinates for the lateral ventricle: -0.6mm AP; +1.3 mm ML, with respect to bregma, and −2.1mm DV from the skull’s surface, the following coordinates for the nucleus accumbens (Acb): +1.25mm AP; ±0.75 mm ML, with respect to bregma, and −4.3mm DV from the skull’s surface, and the following coordinates for the cingulate cortex: +1 mm AP; ±0.5 mm ML, with respect to bregma, and -2.0mm DV from the skull’s surface. A guide cannula was adhered to the skull using dental glue which was then reinforced with dental cement. The cannulas used in our studies were 26 gauge, with an 8 mm pedestal height for the bilateral Acb cannulas and a 5 mm pedestal height for the lateral ventricle and cingulate cortex cannulas. These pedestals had a 3.5 mm diameter. These cannulas fit 33 gauge internal cannulas for injections. A dummy cannula was inserted to maintain integrity of the guide. After completion of surgeries, animals were returned to clean home cages and were allowed to recover for 5 days before behavioral testing. At the end of the experiment, each brain was collected to evaluate accurate cannula placement.
For lateral ventricle infusions: during the three conditioning days of the CPP procedure, before both morning and afternoon conditioning sessions, mice received unilateral infusions of α-Conotoxin MII [H9AL15A], or saline, 5 minutes before injection with psychoactive drug of interest or saline (i.p.). Infusions were conducted using a micro-infusion pump at a rate of 25nl/sec (for 2 minutes total, 3 µl total volume) through a sterile 33 gauge internal cannula extending 0.1mm beyond the guide, which is attached to a Hamilton syringe via PE50 tubing.

For Acb infusions: Before both morning and afternoon conditioning sessions, mice received bilateral infusions of α-Conotoxin MII [H9AL15A] or saline. Infusions were done using a micro-infusion pump at a rate of 16.7nl/sec for 30 seconds (0.5 µl total volume) in a similar fashion to lateral ventricle infusions (described above).

For cingulate cortex infusions: during the three conditioning days of the CPP procedure, before both morning and afternoon conditioning sessions, mice received unilateral infusions of α-Conotoxin MII [H9AL15A], or saline, 5 minutes before injection with psychoactive drug of interest or saline (i.p.). Infusions were carried out using an internal connected to a micro-infusion pump via Hamilton syringe and PE50 tubing. Drug (or saline) was infused at a rate of 16.7nl/sec for 30 seconds.

2.8 Histology

To assess accurate cannula placement, methylene blue dye was injected centrally, followed by cervical dislocation, decapitation, and harvesting of brain. Whole brain tissue was then fixed in a formalin/formaldehyde solution for 48 hours before being sliced at thickness of 50-60µm in a cryostat. Tissue slices were then stained with Nissl using a
sequence of steps involving decrement concentrations of ethanol in distilled water to hydrate tissue slices, followed by staining with cresyl violet, then dehydrating the tissue slices using incremental concentrations of ethanol. Each site of injection was then reconstructed and marked on a worksheet of mouse bran coronal slice image for assessment, and any sites that were not in the target area were not included in the final data set.

2.9 Statistical Analyses

All CPP results are expressed as mean preference scores ± standard error of the mean. Preference scores are measured in seconds and reflect the time spent in the drug paired side pre-conditioning (baseline) subtracted from the time the mice spend in the drug paired side during post conditioning day. Statistical analyses of all CPP studies were performed with an analysis of variance test (ANOVA) followed by a post-hoc analysis with Student Newman-Keuls test when appropriate. P-values of <0.05 were considered to be statistically significant. All data were graphed and statistical analyses performed using GraphPad Prism version 5.00 for Windows; GraphPad Software; San Diego California USA.
CHAPTER THREE

Results: The Role of α6 Containing nAChRs in Nicotine Conditioned Place Preference

3.1 Nicotine Place Preference in α6 Knock Out Mice

Previous work has shown that α6β2* nAChRs are involved in nicotine induced DA neurotransmission in Acb (Exley et al., 2008; Drenan et al., 2008), and that α4β2* and α6β2* nAChR mediate nicotine reinforcement and reward in mice (Pons et al., 2008; Jackson et al., 2009; Brunzell et al., 2010; Gotti et al., 2010; Drenan et al., 2010; Exley et al., 2011). Using our CPP procedure, α6 KO male mice were conditioned with 0.25, 0.5, or 1 mg/kg nicotine (s.c.) for three days and preference scores were assessed on test day. Figure 3.1 illustrates the capacity of nicotine to induce CPP in α6 KO mice and their WT littermates. As mentioned previously (section 1.5), the dose of 0.5 mg/kg nicotine (s.c) induced significant CPP in WT mice, which is the dose that normally produces the most robust place preference in our hands (F(7, 63) = 4.803; p = 0.0003). However, this dose failed to produce a CPP response in α6 KO mice. In contrast, at the highest dose of nicotine tested, 1 mg/kg nicotine, we were surprised to see a conditioned place preference for nicotine in α6 KO mice that was significantly higher than α6 WT littermates.

Subsequently, in order to determine which receptor subtype was contributing to CPP observed in α6 KO mice at 1 mg/kg nicotine, we assessed the effect of dihydro-beta-erythroidine (DHβE), a relatively selective β2* nAChR antagonist, on place preference induced by 1 mg/kg nicotine in α6 KO mice. 2mg/kg DHβE (s.c.) was administered 5
minutes before 1 mg/kg nicotine injection on conditioning days of the CPP procedure.

Figure 3.2 illustrates the effect of DHβE on nicotine induced place preference for 1 mg/kg nicotine in α6 KO mice. Pre-treatment with DHβE followed by nicotine exposure on conditioning days resulted in significant attenuation of nicotine place preference in α6 KO mice. α6 KO mice that received a pretreatment of saline before nicotine exposure had significant CPP compared to saline controls and compared to the nicotine group receiving DHβE (F(7, 72) = 6.005; p = 0.0003).

Overall, these results suggest that α6β2* nAChRs mediate nicotine place preference at doses that, when given s.c., typically produce robust place preference in mice. However, at higher doses of nicotine, this effect is overcome, and this is mediated by β2* nAChRs, as indicated by the datum that illustrates that DHβE, a β2* nicotinic receptor antagonist, attenuates 1 mg/kg nicotine place preference in α6 KO mice.
Figure 3.1 Nicotine Induced Place Preference in α6 KO Mice.

Robust place preference scores are observed in α6 WT mice for 0.5 mg/kg nicotine (s.c.), whereas α6 KO mice failed to show significant preference for nicotine at 0.5 mg/kg. However, nicotine place preference occurs in α6 KO mice at a higher dose of 1mg/kg nicotine, which does not produce place preference in WT counterparts. Place preference scores for nicotine 0.5 mg/kg in α6 WT mice and nicotine 1mg/kg in α6 KO mice were significantly greater than all other treatment and genotype groups (*p<0.05 compared to respective saline control group; $p<0.05$ compared to WT 0.5 mg/kg nicotine; #p<0.05 compared to KO 1 mg/kg nicotine). Results are expressed as mean preference scores ±SEMs.
Figure 3.2 The Effect of DHβE on Nicotine Induced Place Preference in α6 KO Mice.

Nicotine induced place preference occurred in α6 KO mice at 1 mg/kg nicotine (**p<0.01 compared to saline groups), and had significantly higher scores than α6 WT littermates that received 1mg/kg, and α6 KO and WT mice that were treated with DHβE (#p<0.05 compared to α6 KO-1mg/kg nicotine group). α6 KO mice failed to show significant preference for 1 mg/kg nicotine when given a pre-injection of 2 mg/kg DHβE (s.c.). Results are expressed as mean preference scores ±SEMs.
3.2 The Effect of α-Conotoxin MII [H9A; L15A] on the Expression of Nicotine Place Preference.

Our KO data clearly suggest that α6β2* nAChRs mediate nicotine place preference. However recognizing that there are drawbacks and possible developmental compensations that can occur in transgenic mice (refer to section 1.4), it was important for us to confirm our results using a pharmacological approach. We assessed the role of α6β2* nicotinic receptors in the expression of nicotine induced CPP using the α6β2* nAChR selective antagonist, α-Conotoxin MII [H9A; L15A]. Mice received a one time unilateral injection of 5µl of 1.5, 4.5, or 6pmol α-Conotoxin MII [H9A; L15A] into the lateral ventricle (i.c.v.) on the post-conditioning test day, which was after the mice were conditioned for 3 days with 0.5 mg/kg nicotine (s.c.). Figure 3.3 depicts the effect of α-Conotoxin MII [H9A; L15A] on the expression of nicotine place preference in male B6 mice. We observed a dose dependent decrease in the expression of nicotine place preference with α-Conotoxin MII [H9A; L15A] injections (F(5, 38) = 2.504; p = 0.0455). Mice that received a saline injection on test day portrayed significant place preference for nicotine. In contrast, mice receiving 1.5 pmol α-Conotoxin MII [H9A; L15A] had a slightly lower, but significant place preference for nicotine, compared to saline control groups. Conversely, Mice that received 4.5 and 6 pmol α-Conotoxin MII [H9A; L15A] did not show place preference for nicotine. This datum suggests that pharmacological blockade of α6β2* nAChRs results in a decrease of nicotine place preference, and therefore a decrease in the reward-like effects of nicotine.
It was imperative to ensure that the effect of α-Conotoxin MII [H9A; L15A] was not simply due to a locomotor impairment of the mice on test day, therefore locomotor counts were reviewed. Table 2 shows that α-Conotoxin MII [H9A; L15A] did not have an effect on the locomotor activity of mice on test day.

Due to the nature of the inverted U shaped dose effect curve observed in nicotine CPP in B6 mice, it was also important to confirm that α-Conotoxin MII [H9A; L15A] was not acting by enhancing the effects of nicotine, which would result in a shift to the descending end of the inverted U shaped dose effect curve for nicotine CPP. We therefore tested the effect of α-Conotoxin MII [H9A; L15A] on place preference induced by a lower dose of nicotine, 0.25 mg/kg (s.c.). If the conotoxin acted by enhancing the effects of nicotine we would then be able to unmask this effect and would expect scores similar to 0.5 mg/kg nicotine, which as previously mentioned, typically produces the most robust CPP scores in our lab. A decrease in CPP would suggest that the conotoxin is acting by blocking the reward like effects of nicotine that produce CPP. Figure 3.4 demonstrates that α-Conotoxin MII [H9A; L15A] does not enhance place preference induced by 0.25 mg/kg nicotine, and therefore does not act by enhancing the effects of nicotine but rather by blocking the effects of nicotine that result in CPP.
Figure 3.3 The Effect of α-Conotoxin MII [H9A; L15A] on the Expression of Nicotine Place Preference.

Injection of α-Conotoxin MII [H9A; L15A] into the lateral ventricle on test day of CPP resulted in a dose dependent decrease in the expression of nicotine place preference. Both Saline-nicotine 0.5 mg/kg group and 1.5 pmol MII-nicotine 0.5 mg/kg group had significantly higher place preference for nicotine compared to the saline control groups (*p<0.05 compared to saline groups). Nicotine groups that were exposed to either 4.5 or 6pmol MII resulted in attenuated place preference scores for nicotine. Results are expressed as mean preference scores ±SEMs.

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(Jackson et al., 2009)
Table 2. Locomotor scores on Test Day for Expression of Nicotine Place Preference.

This table shows that α-Conotoxin MII [H9A;L15A] did not affect locomotor activity on test day. Locomotor scores were assessed by the number of interruptions of the photocell beams in the CPP compartments.

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<th>Treatment groups (s.c.-i.c.v.)</th>
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<td>486.1 ± 75.1</td>
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Figure 3.4

Injection of α-Conotoxin MII [H9A; L15A] on test day of CPP did not result in an enhancement of the expression of nicotine place preference, but rather a decrease in the already low preference score induced by 0.25 mg/kg nicotine (s.c.). Results are expressed as mean preference scores ±SEMs.

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**Figure 3.4. The Effect of α-Conotoxin MII [H9A; L15A] on the Expression of Low Dose Nicotine Induced Place Preference.**
Table 3

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<th>Treatment groups</th>
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<td>0.25 Nicotine – Saline</td>
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<td>0.25 Nicotine - 6 pmol MII</td>
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Table 3. Locomotor scores on Test Day for Expression of Low Dose Nicotine Place Preference.

This table shows that α-Conotoxin MII [H9A;L15A] did not affect locomotor activity on test day. Locomotor scores were assessed by the number of interruptions of the photocell beams in the CPP compartments.
3.3 The Effect of Intra-Ventricular, Intra-Accumbal, and Intra-Cingulate Cortex Infusions α-Conotoxin MII [H9A; L15A] on the Acquisition of Nicotine Place Preference.

We next evaluated the effect of α-Conotoxin MII [H9A; L15A] on the acquisition of nicotine place preference. This differs from exploring the expression of nicotine place preference, in that the conotoxin was given during the conditioning days of the procedure along with nicotine administration, instead of administering α-Conotoxin MII [H9A; L15A] only on test day, thereby targeting different neuromechanisms that mediate reward memory, learning, and association of an US to CS (Sanchis-Segura and Spanagel, 2006). Figure 1.5 illustrates the effect of α-Conotoxin MII [H9A; L15A] on the acquisition of nicotine place preference (Jackson et al., 2009). Mice that received saline infusions via micro-infusion pump into the lateral ventricle followed by 0.5 mg/kg nicotine (s.c.), showed a significant place preference for nicotine on test day. On the other hand, mice that were infused with α-Conotoxin MII [H9A; L15A] (i.c.v.) had attenuated place preference scores for nicotine on test day in a dose related manner ($F_{(4, 27)} = 7.526; p = 0.0010$). Indeed, mice that received infusions of 3µl of 12pmol α-Conotoxin MII [H9A; L15A] (i.c.v.) on nicotine conditioning days had significantly decreased acquisition of nicotine place preference compared to the nicotine group that received only saline infusions. These results propose a critical role of α6β2* nAChRs in the acquisition of nicotine place preference.

Intra-ventricular infusions of α-Conotoxin MII [H9A; L15A] result in a diffusion of the conotoxin into the entire brain. To begin determining which brain regions mediate the
effect of nicotine place preference, we examined the role of the Acb, due to its location in the mesolimbic system. The Acb is part of the ventral striatum, which receives a large dopaminergic input from the VTA has been shown to be involved in drug reward. It is thought that $\alpha_6\beta_2^*\text{nAChRs}$ are located pre-synaptically in the Acb where they are involved in DA neurotransmission (Exley et al., 2008). 6pmol and 12pmol of $\alpha$-Conotoxin MII [H9A; L15A] was infused into the Acb on nicotine conditioning days, and place preference induced by nicotine was then recorded on test day. Figure 1.6 shows the effect of intra-accumbal $\alpha$-conotoxin MII [H9A; L15A] infusions on the acquisition of nicotine place preference. The nicotine group the received intra-accumbal saline infusions had a significant place preference compared to the nicotine group that received intra-accumbal infusions of 3pmol $\alpha$-Conotoxin MII [H9A; L15A] ($F_{(4, 35)} = 7.38; p = 0.0003$), which had a significant decrease in nicotine place preference. Overall these results propose that the Acb is an important region in the brain for the acquisition of nicotine place preference.

As a neuroanatomical control to assess whether the decrease in place preference could be attributed to damage to cortex caused by insertion of the cannula guide, we investigated the effect of $\alpha$-Conotoxin MII [H9A; L15A] infusions into the cingulate cortex on nicotine place preference. The cingulate cortex is a brain region (dorsal to the Acb) that also receives afferents from the VTA, and also expresses $\alpha_6\beta_2^*\text{nAChRs}$ (Champtiaux et al., 2002; Whiteaker et al., 2000). The effect of intra-cingulate cortex infusions of $\alpha$-Conotoxin MII [H9A; L15A] is depicted in figure 1.7, where there was no effect on nicotine induced place preference. Mice receiving either 12pmol $\alpha$-Conotoxin MII [H9A; L15A] or saline infusions into the cingulate cortex during conditioning days for
nicotine displayed significant place preference for nicotine compared to saline control
groups ($F_{(3,18)} = 4.472; p = 0.0379$). These results show that the obligatory lesioning of
cortex inflicted by insertion of the cannula guide does not affect place preference induced
by nicotine, and shows the selectivity of effect in the Acb. Based on our data showing that
intra-accumbal but not intra-cingulate cortex infusion of $\alpha$-conotoxin MII [H9A; L15A]
results in a decrease in the acquisition of nicotine place preference, we propose that $\alpha 6\beta 2^*$
nAChRs in the Acb are critical for the acquisition of nicotine place preference.
Unilateral infusions of α-Conotoxin MII [H9A; L15A] into the lateral ventricle on nicotine conditioning days resulted in a dose dependent decrease in the acquisition of nicotine place preference. The saline-nicotine 0.5 mg/kg group had significantly higher place preference for nicotine compared to saline control groups (***p<0.01 compared to saline groups) and compared to the 12 pmol MII-nicotine 0.5 mg/kg group (# p<0.001 compared to saline-nicotine 0.5 mg/kg group). Results are expressed as mean preference scores ±SEMs.
Figure 3.6. Bilateral Intra-Accumbal α-Conotoxin MII [H9A; L15A] Infusions Affect the Acquisition of Nicotine Place Preference.

Bilateral intra-accumbal infusions of α-Conotoxin MII [H9A; L15A] on nicotine conditioning days resulted in dose dependent decrease in the acquisition of nicotine place preference. Saline-nicotine 0.5 mg/kg group had significantly high place preference scores for nicotine compared to the 3pmol MII-nicotine 0.5 mg/kg group (*** p < 0.001 compared to saline groups, and # p < 0.01 compared to saline-nicotine 0.5 mg/kg group). Results are expressed as mean preference scores ±SEMs.

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Figure 1.7

Figure 1.7. The Effect of Unilateral Intra-Cingulate Cortex Infusion of α-Conotoxin MII [H9A;L15A] on the Acquisition of Nicotine Place Preference.

Unilateral intra-cingulate cortex infusions of α-Conotoxin MII [H9A; L15A] had no effect on the acquisition of nicotine place preference. Both saline-nicotine 0.5 mg/kg and 12pmol MII- nicotine 0.5 mg/kg groups had significant place preference scores for nicotine (*p < 0.05 compared to saline groups).

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CHAPTER FOUR

The Role of α6β2* nAChRs in Cocaine Conditioned Place Preference

4.1. Cocaine Place Preference in α6 KO and α6 WT Mice

Previous work has shown that β2* nAChRs are involved in cocaine place preference (Zachariou et al., 2001). Since α6β2* nAChRs are often co-expressed with the β2 subunit, and due to the neuroanatomical distribution of α6β2* nAChRs in catecholaminergic nuclei in midbrain, which is a brain region known to mediate the appetitive and rewarding effects of many psychoactive drugs, we chose to investigate if α6β2* nAChRs mediated cocaine place preference. Using our CPP procedure, α6 KO, HET, and WT male mice were conditioned with 20 mg/kg cocaine (i.p.) for three days and preference scores were assessed on test day. Figure 4.1 illustrates the capacity of cocaine to induce CPP in α6 KO mice and their HET and WT littermates. We see a genotype dependent effect where α6 WT mice displayed significant cocaine place preference, whereas place preference for cocaine was abolished in α6 KO counterparts (F(4, 32) = 5.826; p = 0.0030). The α6 HET mice, although having higher scores than α6 KO mice, did not portray significant place preference for cocaine. These results indicate that the α6 nicotinic subunit is important for place preference induced by cocaine. This is the only other nicotinic subunit, other than the β2* nicotinic subtype, reported to have a role in cocaine reward.
Figure 4.1 Cocaine Place Preference in α6 KO and WT Mice.

α6 KO mice show significantly decreased place preference for 20 mg/kg cocaine compared to α6 WT mice, which displayed significant place preference for cocaine at this dose (***p<0.01 compared to saline groups; # p<0.01 compared to α6 WT-cocaine 20 mg/kg group).

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</tr>
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<td>R squared</td>
<td>0.3761</td>
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</table>
4.2. The Effect of α-Conotoxin MII [H9A; L15A] on the Expression of Cocaine Place Preference.

Our KO data encourages the concept that α6β2* nAChRs are important mediators of cocaine place preference. Once again, being aware of possible developmental compensations that can occur in transgenic mice (refer to section 1.4), it was important for us to validate our results by using a pharmacological approach that studies a system with an unaltered gene pool. We assessed the role of α6* nicotinic receptors in the expression of cocaine CPP using α-Conotoxin MII [H9A; L15A]. Following the three days of conditioning with 20 mg/kg cocaine (i.p.), mice received a one time unilateral injection of 6pmol or 12pmol α-Conotoxin MII [H9A; L15A] into the lateral ventricle (i.c.v.) on post-conditioning test day. Figure 4.2 illustrates the effect of α-Conotoxin MII [H9A; L15A] on the expression of cocaine place preference in male B6 mice. We observed a dose dependent decrease in the expression of cocaine place preference with i.c.v. injections of α-Conotoxin MII [H9A; L15A] (F(5, 67) = 3.873; p = 0.0041). Mice that received a saline injection on test day portrayed a significant place preference for cocaine. In contrast, mice receiving 6pmol α-Conotoxin MII [H9A; L15A] had lower scores and did not display significant place preference for cocaine. Conversely, mice that received 12 pmol α-Conotoxin MII [H9A; L15A] resulted in a significant attenuation of cocaine place preference compared to the saline-cocaine 20 mg/kg group. This datum suggests that pharmacological blockade of α6β2* nAChRs results in a decrease of cocaine place preference, and therefore a decrease in the psychosomatic effects of cocaine that result in place preference.
It was important to assess the effect of \( \alpha \)-Conotoxin MII [H9A; L15A] on locomotion, in order to confirm that there was no locomotor impairment in these mice on test day. Locomotor scores were assessed by the number of interruptions of the photocell beams in the CPP compartments. Table 4 shows that \( \alpha \)-Conotoxin MII [H9A; L15A] did not have an effect on the locomotor activity of the mice on test day.
Figure 4.2 The Effect of Intracerebroventricular α-Conotoxin MII [H9A; L15A] Injection on the Expression of Cocaine Place Preference.

Unilateral injection of α-Conotoxin MII [H9A; L15A] into the lateral ventricle on test day of CPP resulted in a dose dependent decrease in the expression of cocaine place preference. The saline-cocaine group had significantly higher place preference scores compared to the cocaine group that received 12pmol MII (*p<0.05 compared to saline groups; #p<0.05 compared to saline-cocaine group).
Table 4

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<th>Treatment groups (i.p.-i.c.v.)</th>
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<td>Saline - Saline</td>
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<tr>
<td>Saline - MII[H9A;L15A], 6 pmol</td>
<td>489.12 ± 107.30</td>
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<tr>
<td>Saline - MII[H9A;L15A], 12 pmol</td>
<td>478.00 ± 26.14</td>
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<td>Cocaine – Saline</td>
<td>543.73 ± 79.42</td>
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<tr>
<td>Cocaine - MII[H9A;L15A], 6 pmol</td>
<td>472.00 ± 65.49</td>
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<td>Cocaine- MII[H9A;L15A], 12 pmol</td>
<td>495.10 ± 83.91</td>
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Table 4. Locomotor scores on Test Day for Expression of Cocaine Place Preference.

This table shows that α-Conotoxin MII [H9A;L15A] did not affect locomotor activity on test day. Locomotor scores were assessed by the number of interruptions of the photocell beams in the CPP compartments.
4.3. The of Intra-Cerebroventricular, Intra-Accumbal, and Intra-Cingulate Cortex Infusions of $\alpha$-Conotoxin MII [H9A; L15A] on the Acquisition of Cocaine Place Preference.

We determined the effect of $\alpha$-Conotoxin MII [H9A; L15A] on the acquisition of cocaine place preference. Figure 4.3 illustrates the effect of unilateral intra-cerebroventricular infusion of $\alpha$-Conotoxin MII [H9A; L15A] on the acquisition of cocaine place preference. Mice that received saline infusions into the lateral ventricle followed by 20 mg/kg cocaine (i.p.), displayed significant place preference for cocaine on test day. On the other hand, mice that were infused with $\alpha$-Conotoxin MII [H9A; L15A] (i.c.v.) had significantly attenuated place preference scores on test day ($F_{(4, 35)} = 9.619; p < 0.0001$). Indeed, mice that received infusions of 12 pmol and 24 pmol $\alpha$-Conotoxin MII [H9A; L15A] (i.c.v.) on conditioning days had significantly decreased acquisition of cocaine place preference compared to the cocaine group that received only saline infusions. These results propose a critical role of $\alpha_6\beta_2^*$ nAChRs in the reward like effects of cocaine that prompt place preference.

Since intra-cerebroventricular infusions of $\alpha$-Conotoxin MII [H9A; L15A] resulted in a diffusion of the conotoxin through the ventricles to the entire brain, we examined a more specific brain region implicated in drug reward and reinforcement, the Acb, which is part of the ventral striatum and which receives a large dopaminergic input from the VTA. $\alpha$-Conotoxin MII [H9A; L15A] was infused into the Acb on cocaine conditioning days, and place preference scores were recorded on test day in a drug free state. Figure 2.4 shows the effect of intra-accumbal $\alpha$-conotoxin MII [H9A; L15A] infusions on the
acquisition of cocaine place preference. The cocaine group that received intra-accumbal saline infusions had significantly greater place preference compared to the cocaine groups that received intra-accumbal infusions of 3pmol or 30pmol α-Conotoxin MII [H9A; L15A] (p<0.0001; F=11.08). However significant cocaine place preference persisted in the cocaine groups infused with 3pmol or 30pmol α-Conotoxin MII [H9A; L15A], as indicated by having significantly higher scores compared to saline controls. Therefore there appears to be a significant but partial reduction for cocaine preference that is mediated by α6β2* nAChRs in Acb, suggesting α6β2* nAChRs in other brain regions, or other substrates are contributing to the effect of cocaine on CPP in mice. Overall these results propose that α6β2* nAChRs in Acb are important, but are not the only factors, mediating the acquisition of cocaine place preference.

Next, the effect of α-Conotoxin MII [H9A; L15A] infusions into the cingulate cortex for cocaine place preference was assessed. The effect of intra-cingulate cortex infusions of α-Conotoxin MII [H9A; L15A] is depicted in figure 2.5, where cocaine induced place preference persevered. Mice receiving either saline or 12pmol α-Conotoxin MII [H9A; L15A] infusions into the cingulate cortex during conditioning days for cocaine displayed significant place preference scores compared to saline control groups (p<0.0001; F=32.02). These results show that the unavoidable lesioning of cortex inflicted by insertion of the cannula guide does not affect place preference induced by cocaine. Based on the results showing that intra-accumbal but not intra-cingulate cortex infusion of α-Conotoxin MII [H9A; L15A] results in a decrease in the acquisition of cocaine place preference, we suggest that α6β2* nAChRs in the Acb are critical for cocaine place
preference, but are not the sole substrates mediating the reward like effects of cocaine.
Unilateral infusions of α-Conotoxin MII [H9A; L15A] into the lateral ventricle resulted in a decrease in the acquisition of cocaine place preference. The saline-cocaine 20 mg/kg group had significant place preference scores compared to saline control groups (***p<0.001 compared to saline groups) and compared to the 12 pmol MII-cocaine 20 mg/kg and 24 pmol MII- cocaine 20 mg/kg groups (# p<0.01 compared to saline- cocaine 20 mg/kg group). Results are expressed as mean preference scores ±SEMs.
Figure 4.4 The Effect Bilateral Intra-Accumbal Infusions of α-Conotoxin MII [H9A; L15A] on the Acquisition of Cocaine Place Preference.

Bilateral intra-accumbal infusions of α-Conotoxin MII [H9A; L15A] resulted in a decrease in the acquisition of cocaine place preference. The saline-cocaine 20 mg/kg group had significant place preference scores compared to the 3pmol MII-cocaine 20 mg/kg and 30pmol MII- cocaine 20 mg/kg groups (***p < 0.001 compared to saline groups; **p<0.01 compared to saline groups; #p < 0.05 compared to saline-cocaine 20 mg/kg group). Results are expressed as mean preference scores ±SEMs.
Figure 4.5 The Effect of Intra-Cingulate Cortex Infusion of α-Conotoxin MII [H9A;L15A] on Cocaine Place Preference.

Unilateral intra-cingulate cortex infusions of α-Conotoxin MII [H9A; L15A] had no effect on the acquisition of cocaine place preference. Both saline-cocaine 20 mg/kg and 12pmol MII- cocaine 20 mg/kg groups demonstrated significant place preference scores (**p < 0.001 compared to saline groups).

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CHAPTER FIVE

The Role of α4 Containing nAChRs in Nicotine and Cocaine Conditioned Place Preference

5.1 Nicotine Place Preference in α4 KO and α4 WT Mice

Previous work has shown that α4* nAChRs are critical for nicotine induced DA release (Marubio et al., 2003; Exley et al., 2011; Drenan et al., 2010), and that α4β2* nAChR mediate nicotine reinforcement and reward in mice (Tapper et al., 2006; Pons et al., 2008; Mcgranahan et al., 2011). Since there are currently no ligands selective for α4* nAChRs, we used transgenic mice to investigate the role of α4* nAChRs in nicotine place preference. Using our CPP procedure, α4 KO and α4 WT male mice were conditioned with 0.25, 0.5, or 1 mg/kg nicotine (s.c.) for three days and preference scores were assessed on test day. Figure 5.1 illustrates the capacity of nicotine to induce CPP in α4 KO mice and their WT littermates. The dose of 0.5 mg/kg nicotine (s.c.) induced significant CPP in α4 WT mice, which is the dose that normally produces the most robust place preference in our hands ($F_{(7, 45)} = 4.328; p = 0.0014$). However, this dose failed to produce a CPP response in α4 KO mice. This was not due to a shift in the curve, as lower (0.25 mg/kg) and higher (1 mg/kg) doses of nicotine did not induce any place preference in α4 KO mice. This datum supports previous data in the nicotinic field suggesting that α4* nAChRs are necessary for nicotine reward.
Figure 5.1

Robust place preference scores are observed in α4 WT mice for 0.5 mg/kg nicotine (s.c.), whereas α4 KO mice failed to show significant preference for nicotine at any of the doses tested. (*p<0.05 compared to saline groups; #p<0.05 compared to α4 WT 0.5 mg/kg nicotine; ^p<0.1 compared to α4 WT 0.5 mg/kg nicotine). Results are expressed as mean preference scores ±SEMs.

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5.2. Assessing Cocaine Place Preference in α4 KO and α4 WT Mice.

As we assessed the role of α6β2* nAChRs in both nicotine and cocaine place preference (Chapters 3 and 4), and since previous data suggests a role of β2* nAChRs in cocaine reward (Zachariou et al., 2001) which is the subunit that is predominantly co-expressed with α4, we decided to examine the involvement of α4* nAChRs in cocaine reward. Using our CPP procedure, α4 KO and α4 WT male mice were conditioned with various doses of cocaine (i.p.) for three days and preference scores were assessed on test day. Figure 5.2 illustrates the capacity of cocaine to induce CPP in α4 KO mice and their WT littermates. We did not see a genotypic effect on cocaine preference across various doses of cocaine. Both α4 KO and WT mice displayed similar cocaine place preference scores across several doses of cocaine. These results indicate that α4* nAChRs are not important for place preference induced by cocaine. These results complement the McGranahan et al. study (2011) which illustrated that of α4* nAChRs on DA neurons are involved in nicotine but not cocaine reward.
Place preference was induced by various doses of cocaine in α4 KO and WT mice. There were no genotypic differences in the preference scores for cocaine, suggesting that α4 is not necessary for cocaine place preference.
5.3 The Effect of \( \alpha \)-Conotoxin MII [H9A; L15A] on Cocaine Place Preference in \( \alpha 4 \) KO and \( \alpha 4 \) WT Mice.

We assessed the role of \( \alpha 4 \)* nAChRs in cocaine reward, and found that both \( \alpha 4 \) KO and \( \alpha 4 \) WT mice have similar place preference for cocaine. We decided to test the effect of \( \alpha \)-Conotoxin MII [H9A; L15A] in cocaine CPP in \( \alpha 4 \) KO and WT mice to confirm the role of \( \alpha 6\beta 2 \)* nAChRs in these mice. Figure 5.3 illustrates the effect if intra-ventricular infusion of \( \alpha \)-Conotoxin MII [H9A; L15A] on cocaine place preference in \( \alpha 4 \) KO and WT mice. Both \( \alpha 4 \) KO and WT mice displayed significant place preference for 20mg/kg cocaine when receiving an infusion of saline. However \( \alpha \)-Conotoxin MII [H9A; L15A] caused a significant decrease in cocaine place preference in \( \alpha 4 \) KO and WT mice (\( F_{(5, 23)} = 6.506; p = 0.0013 \)). This datum confirms the role of \( \alpha 6\beta 2 \)* nAChRs in cocaine place preference, and also suggests that \( \alpha 6\beta 2 \)* nAChRs are the main receptor subtypes mediating the effects of cocaine and do not require the \( \alpha 4 \) subunit.
Figure 5.3

α-Conotoxin MII [H9A; L15A] infusions into the lateral ventricle resulted in a decrease in the acquisition of cocaine place preference in α4 KO and WT mice. Both α4 KO and α4 WT saline-cocaine 20 mg/kg groups had significant place preference scores compared to saline control groups (*p<0.05 compared to saline groups) and compared to both α4 KO and WT 12 pmol MII-cocaine 20 mg/kg groups (#p<0.05 compared to α4 WT saline-cocaine 20 mg/kg group; $p<0.05 compared to α4 KO saline-cocaine 20 mg/kg group). Results are expressed as mean preference scores ±SEMs.
CHAPTER SIX
Assessing the Specificity of Hedonics of the Effects of α-Conotoxin MII [H9A; L15A] and the α6 Nicotinic Subunit in Place Conditioning.

6.1 Lithium Induced Conditioned Place Avoidance in α6 KO and α6 WT Mice.

The study of drug reward and reinforcement involves fundamental principles of learning and behavior. This is appropriate given that the development of drug dependence can be considered a learned trait, in the sense that internalization of the rewarding of effects of drugs and the association made with the environment related to the drug following repeated exposure, will result in changes in behavior. CPP is considered a Pavlovian type of learning, where the US is taught to be associated to the CS via the appetitive effects of the US and therefore involves memory formation and recollection. To allow a more accurate interpretation of our CPP results, it was necessary to assess the specificity of hedonics of α-conotoxin MII [H9A; L15A] and the inactivation of the α6 nicotinic subunit. We accomplished this by examining the effect of α-conotoxin MII [H9A; L15A] and α6 KO mice on the associative process in place conditioning (memory recollection) that is not specific to reward, such as memory specific to aversion.

Consequently, we examined the effect of lithium induced place avoidance in α6 KO and α6 WT mice using the same unbiased place preference protocol used throughout our studies for nicotine and cocaine CPP. Several studies have shown that lithium is aversive to rodents (Risinger and Cunningham, 2000; Tenk et al., 2006). Figure 6.1 illustrates the effect of lithium on place conditioning in α6 KO and α6 WT mice. Both α6 KO and α6
WT displayed an avoidance of the context that was associated with 150mg/kg lithium ($F_{(3, 35)} = 3.447; p = 0.028$). Therefore the deletion of α6 subunit did not have an effect on the ability of these mice to associate and recall the association of aversive stimuli to the context that it was paired with ($F_{(3, 35)} = 3.447; p = 0.028$).
Figure 6.1. Lithium Induced Conditioned Place Avoidance in α6 KO and WT Mice.
Both α6 KO and WT mice displayed conditioned place avoidance induced by 150 mg/kg lithium (i.p.). (Post hoc Newman–Keuls analysis did not find any significance between groups).

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6.2. α-Conotoxin MII [H9A; L15A] and Lithium Induced Conditioned Place Avoidance.

In order to rule out any possible confound of α-conotoxin MII [H9A; L15A] affecting the associative process in place conditioning (memory recollection) that is not specific to reward, we tested the effect of α-conotoxin MII [H9A; L15A] on lithium induced CPA, illustrated in Figure 6.2. We found that 50 mg/kg and 150 mg/kg lithium (i.p.) resulted in significant place avoidance that was not altered by an i.c.v. injection of either saline or α-conotoxin MII [H9A; L15A] (F(7, 38) = 3.957; p = 0.0036). We used 6pmol and 12pmol of α-conotoxin MII [H9A; L15A] because these were the doses used in several of our nicotine and cocaine CPP expression and acquisition studies. These results suggest that α-conotoxin MII [H9A; L15A] is not attenuating nicotine and cocaine place preference by acting on the associative memory process itself, but rather on the associative process pertaining to the reward-like effects induced by nicotine or cocaine.
Figure 6.2 The Effect of α-Conotoxin MII [H9A; L15A] on the Expression of Lithium Induced Conditioned Place Avoidance.

Unilateral injection of α-Conotoxin MII [H9A; L15A] (i.c.v.) had no effect on lithium chloride induced place aversion in B6 mice. All groups exposed to 50 mg/kg or 150 mg/kg lithium (i.p.) displayed significant place aversion compared to saline controls (*p<0.05 compared to saline groups).
6.3 Establishing Food Reward in Mice.

Since our data are suggesting an effect of $\alpha_6\beta_2^*\text{nAChRs}$ on both nicotine and cocaine reward, another important question to address was whether the effect of $\alpha$-Conotoxin MII [H9A; L15A] or genetically deleting $\alpha_6$ subunit caused a general decrease in reward (anhedonia) including the natural incentives for food and sex. We chose to assess the effect of $\alpha$-Conotoxin MII [H9A; L15A] or the genetic deletion of the $\alpha_6$ subunit on food reward. In order to accomplish this, we first had to establish a successful protocol that resulted in place preference for food. Along with the minor modifications, including food restriction for 4 hours before each conditioning session, 4 conditioning days, and 40 minute long conditioning (previously described in section 2.3 and 2.4), we had to determine which food would allow for place preference to occur.

Based on previous literature that reported using palatable foods high in sugar and fat (Spiteri et al., 2000; Wise, 2006), we decided to induce food reward using peanut butter chips or cheesecake, which are both high in fat and sugar. Figure 6.3 illustrates place preference for palatable foods. Mice displayed significant place preference for cheesecake, with scores significantly greater than those for the basic food pellet that makes up their regular diet ($F(2,40) = 3.336; p = 0.0463$ ). There was a trend for increased place preference with peanut butter chips, but scores were not significantly greater than food pellet scores. These results indicate that with minor modifications to our CPP protocol, we can induce place preference for cheesecake; therefore cheesecake was used for subsequent experiments that assessed the effect of $\alpha$-Conotoxin MII [H9A; L15A] or the genetic deletion of the $\alpha_6$ subunit on natural reward.
Figure 6.3. Establishing Food Reward in Mice.

Using a slightly modified CPP protocol, we were able to induce place preference for palatable foods in B6 mice. Cheesecake induced significant place preference scores compared to both food pellet (*p<0.05 compared to food pellet).
6.4 Food Reward in α6 KO and α6 WT Mice

To determine if the genetic deletion of the α6 subunit caused a general decrease in reward including natural reward such as food and sex, we assessed food reward induced by cheesecake in α6 KO and WT mice illustrated in figure 6.4. After four days of conditioning, both α6 KO and α6 WT displayed similar place preference scores for the context associated with cheesecake ($F_{(3,18)} = 3.620; p = 0.0381$). These results show that the deletion of α6 subunit does not result in general anhedonia or a learning/memory deficit, thereby suggesting that the phenotypic effects of nullifying the α6 nicotinic subunit is specific to the reward-like effects of nicotine and cocaine, and not specific to food reward.
Figure 6.4. Food Reward in α6 KO Mice

After four days of conditioning, both α6 KO and α6 WT displayed similar place preference scores for the context associated with cheesecake ($F_{(3,18)} = 3.620; p = 0.0381$). Student Newman-Keuls post hoc test did not result in any significant comparisons.
CHAPTER SEVEN

Discussion

Nicotine is the main psychoactive constituent of tobacco and a major contributor to
tobacco dependence and addiction. There are many components to drug addiction, one of
them being reward, which motivates repeated exposure to a drug thereby altering behavior
and intensifying stimulus drug associations (Di Chiara et al., 1999). Nicotine acts in the
brain through nAChRs, and the predominant nAChR subtypes in mammalian brain are
those containing α4 and β2 subunits. The α4β2 nAChRs regulate many of the addiction-
related actions of nicotine and all current FDA-approved anti-smoking agents target this
subtype. These smoking cessation aids have only been modestly effective in maintaining
abstinence, and have many undesirable side effects. Identification of relevant nAChR
subtypes with a more conservative distribution in the brain involved in drug reward is
essential to finding more effective treatments for smoking and drug addiction. Our
research targets nAChRs subtypes that contain α4 and α6 subunits because they often co-
assemble with the β2 subunit which has abundant expression in the central nervous system
(CNS), and has previously been demonstrated to be crucial for nicotine reinforcement and
reward (Corrigall et al., 1994; Maskos et al., 2005; Picciotto et al., 1998; Pons et al., 2008;
Walters et al., 2006) and has also been shown to mediate cocaine reward (Zachariou et al.,
2001).
7.1 α6β2* nAChRs are Critical for Nicotine Conditioned Place Preference

α6β2* nAChRs are complex heteromeric subtypes highly expressed on dopaminergic neurons that play major roles in addiction to nicotine. Indeed, α6β2* nAChRs subtypes have high expression in catecholaminergic nuclei in midbrain regions thought to mediate drug reward, play an important role in presynaptic dopamine release (Grady et al., 2002; Whiteaker et al., 2000) and have been reported to mediate nicotine reward and reinforcement in rodents (Pons et al, 2008; Jackson et al., 2009, Brunzell et al., 2010; Gotti et al., 2010; Drenan et al., 2008).

To complement previous studies implicating α6β2* nAChRs in nicotine reward and reinforcement, and to determine the role of α6β2* nAChRs using a behavioral test that uses drug induced associations to contextual cues, we first examined the ability of nicotine to induce place preference in mice null for the α6 subunit. Using our CPP procedure the dose of 0.5 mg/kg nicotine (s.c) induced significant CPP in WT mice, which is the dose that normally produces the most robust place preference in our hands. However, this dose failed to produce a CPP response in α6 KO mice, which suggests that removal of the α6 subunit results in the elimination of the reward-like effects of nicotine that stimulate the association to the visual and tactile contexts paired to it, that ultimately result in place preference in mice. This data is supported in the literature by a study showing that α6 KO mice fail to self administer nicotine compared to WT littermates, but re-expression of the subunit in the VTA in the α6 KO mice reinstated the phenotype to self administer nicotine (Pons et al, 2008).
When examining place preference for various doses of nicotine in α6 KO mice, we observed a significant increase in place preference for 1mg/kg nicotine, which was significantly higher than α6 WT littermates. We did not expect to see this effect, and we predicted that it could be mediated by α4β2* nAChRs. α4β2* nAChRs have high levels of expression in the midbrain (Klink et al., 2001), and previous work has illustrated the necessity of β2* nAChRs for nicotine reward and reinforcement in rodents (Corrigall et al., 1994; Picciotto et al., 1998; Maskos et al., 2005; Pons et al., 2008, Walters et al., 2006). We found that pre-treatment with DHβE, a selective β2* nAChR antagonist, followed by nicotine exposure on conditioning days resulted in significant attenuation of nicotine place preference in α6 KO mice, which suggests that place preference for the high dose of nicotine in α6 KO mice is mediated by β2* nAChRs. We can speculate that these findings could be attributed in part to the increase in α-conotoxin MII resistant areas in striatum of KO mice compared to WT mice, which are most likely representing α4β2* or α4β2(α5)* nAChRs, which suggests the possibility of developmental compensation (Champtiaux et al., 2002). This information ties into the common criticism that compensatory effects of other genes in transgenic mice may either mask the detection of the targeted gene’s phenotype, or be confused for the phenotype of the null gene.

In addition, we must take into consideration that nAChR subtypes may contribute to the same function in the brain; suggesting the concept of receptor redundancy between various nAChR subtypes. Thereby the removal of one nicotinic subtype from the system might not be enough to produce a detectable insufficiency in phenotype. This concept can be used to explain why α6 KO, α3 KO, and β4 KO mice do not show any signs of
developmental alterations in the visual system where high levels of these receptor subtypes are usually expressed (Champtiaux et al., 2002). In contrast, removing the α3 subunit from the autonomic ganglia, where it is expressed in high levels in combination with β4 results in autonomic dysfunction rendering these mice unviable (Xu et al., 1999). This deleterious effect is not observed in β4 KO mice, even though nicotine induced currents in ganglion cells were 98% diminished, verifying that the β4 subunit was not being replaced by β2 or another nicotinic subunit (Xu et al., 1999). Instead, it appears that the residual β2 in the system was sufficient to maintain the changes in the phenotype below the point of detection.

Our KO data suggest that α6β2* nAChRs mediate nicotine place preference. However, pharmacological data is also necessary to study a system with an unaltered gene pool. Therefore we assessed the role of α6β2* nicotinic receptors in the expression of nicotine induced CPP using the α6β2* nAChR selective antagonist, α-Conotoxin MII [H9A; L15A]. We observed a dose dependent decrease in the expression of nicotine place preference, suggesting that pharmacological blockade of α6β2* nAChRs results in a decrease of nicotine place preference, and therefore a decrease in the reward-like effects of nicotine which was not due to shift to the descending part of the inverted U shaped curve typical for nicotine CPP. We found similar results of dose dependent decrease in the acquisition of nicotine place preference, where α-Conotoxin MII [H9A; L15A] was given i.c.v. on nicotine conditioning days, further supporting the critical role of α6β2*nAChR in nicotine place preference. These findings are in agreement with previous work implicating
a role for α6β2* in the rewarding and reinforcing effects of nicotine (Pons et al, 2008; Jackson et al., 2009, Brunzell et al., 2010; Gotti et al., 2010; Drenan et al., 2008).

Although the literature is in agreement that α6β2* mediates nicotine induced DA neurotransmission, reward, and reinforcement, there is some divergence when it comes to which α6β2* nAChR population in the brain is important for mediating nicotine’s effects. Some studies implicate a role for α6β2* in the Acb in nicotine reinforcement and nicotine induced DA neurotransmission and release (Brunzell et al, 2010; Grady 2007 Exley 2008) whereas others show that the VTA is primarily involved (Pons et al, 2008, Gotti et al, 2010). In the Gotti et al (2010) paper, infusion of α-Conotoxin MII in the Acb did not affect the increase in DA levels induced by systemic nicotine, whereas when it was infused into the VTA, they observed a significant decrease of nicotine induced DA levels. They did remark that perhaps under their experimental set up, α-Conotoxin MII had limited diffusion and may not have reached nAChRs in the Acb that were outside the area that the cannula permitted the α-Conotoxin MII to access (Gotti et al, 2010). Therefore α6β2*nAChRs may not have been inhibited, explaining why the α-Conotoxin MII infusions appeared ineffective.

The Pons et al., (2008) study showed that β2, α4, and α6 KO mice failed to self-administer nicotine as WT counterparts did. However this phenotype was rescued when the missing subunit was re-expressed in the VTA using a lentiviral vector. Although the mice that re-expressed the missing subunit self-administered nicotine, it is not definite that this phenotype was due to re-expressing the missing subunit specifically and exclusively in the VTA. It is likely that the lentiviruses traveled down the projections from the VTA to
the mesolimbic terminals and re-expressed the missing subunit in those brain regions, which could suggest that other regions in addition to the VTA are responsible for the rescue of nicotine reinforcement observed in these transgenic mice.

The Exley at al. (2011) study reported that the majority of nicotine stimulated DA release in the Acb was mediated by α6β2* nAChR (Exley et al, 2011). Furthermore they observed that α6 KO mice readily self administered nicotine (ICSA) into the VTA similar to WT, and systemic administration of 30ug/kg nicotine resulted in increased firing rate of DA neurons in the VTA in both α6 KO and WT mice (Exley at al., 2011). These results propose that α6* nAChRs in the VTA do not seem necessary in mediating nicotine induced DA neuron firing or reinforcement, which is not in agreement with findings from Pons et al., 2008 or Gotti et al., 2010. This is where it is important to be aware of the different parameters under which the study was conducted, in terms of how nicotine was administered (systemic vs. intracranial), the species (rat vs. mouse), and the doses of nicotine that were used. The effects of nicotine were studied in a very site specific manner (intra-VTA infusions of nicotine only), and nicotine affects a whole system differently than in an isolated system or brain region. Also, the α6 KO mice self administered nicotine at a dose of 100ng, but did not do it as readily for 10ng nicotine. A dose effect curve would be useful to understand the relevance of the nicotine doses used under these experimental parameters.

When we infused 3pmol α-Conotoxin MII [H9A; L15A] into the Acb on nicotine conditioning days we observed a significant decrease in nicotine place preference. Our results coincide with several studies including the Brunzell et al. (2010) study which
observes that antagonism of α6β2* nAChRs in the Acb shell significantly reduces motivation to self administer nicotine, and the Exley et al. (2008) study showing that α6β2 responses dominate in the Acb, which suggests the importance of α6β2* nAChRs during early exposure in the acquisition of nicotine reward and reinforcement. Overall, our data and data from other labs have implicated a critical role for α6β2* nAChRs in the Acb in nicotine reward and reinforcement.

All of our pharmacological studies used α-conotoxin MII [H9A; L15A] to investigate the role of α6β2* nAChRs in nicotine (and cocaine) place preference. The development of these α-conotoxins have benefited the nicotinic field tremendously because of the selectivity that these peptidic compounds have at specific nAChR subtypes for which there were previously no selective ligands. One of the unanswered questions about these α-conotoxins is their biological stability in a system and half-life in vivo. There could be possible reduction or scrambling of the compound soon after exposure to extracellular environments such as blood. Work is being done to improve the stability of these compounds in a biological system, and one way to do this is to find a way protect the disulfide bonds against any reduction or scrambling in vivo. Another limitation, is that local levels of α-conotoxin MII [H9A; L15A] may affect its selectivity in the brain, and there is always the possibility of unwanted diffusion. Ultimately, the development of antagonists that are selective for α6β2* nAChRs and can be administered systemically will help us progress in finding more efficacious treatments for tobacco addiction. Both genetic and pharmacological approaches have their limitations, but combining the results of these two approaches seems to confirm the role of α6β2* nAChRs in nicotine reward.
7.2 α4* nAChRs are Critical for Nicotine Conditioned Place Preference.

It is well known that the α4 subunit is most often co-expressed with the β2 subunit and that α4β2* nAChRs have the highest affinity for nicotine and display the most abundant binding to nicotine and nicotinic agonists in the CNS (Changeux, 2005). α4β2* nAChRs are highly expressed in the midbrain (Klink et al., 2001), and previous work has illustrated the necessity of β2* nAChRs for nicotine reward and reinforcement in rodents (Corrigall et al., 1994; Picciotto et al., 1998; Maskos et al., 2005; Pons et al., 2008, Walters et al., 2006).

Using our CPP procedure the dose of 0.5 mg/kg nicotine (s.c) induced significant CPP in α4 WT mice, which is the dose that normally produces the most robust place preference in our hands. However, this dose failed to produce a CPP response in α4 KO mice. This datum supports previous data in the nicotinic field suggesting that α4* nAChRs are necessary for nicotine reward, reinforcement, and striatal DA release (Pons et al., 2008; Exley et al., 2011, Marubio et al., 2003, Tapper et al., 2004).

In contrast to the above-mentioned studies and contrary to our results, a recent report found that mice null for the α4 nicotinic subunit showed similar place preference scores to WT littermates for 0.5 mg/kg nicotine (i.p.) (Cahir et al., 2011). Their CPP study used a different route of administration (i.p. vs. s.c.), used a biased design where initial baseline preference scores were not included when calculating final preference scores on test day, and they did not include a saline control. This was the same group generated
mice null for the α4 nicotinic subunit in 2000 in Australia (Ross et al., 2000) and reported observations of higher levels of basal anxiety in α4 KO mice compared to WT littermates in the elevated plus maze test. This is also where our α4 KO mice originate (although they have been backcrossed for 10 generations onto B6 background in the United States) so it was important for us to determine if the α4 KO mice we used for CPP that showed a decrease for nicotine place preference, did have higher levels of basal anxiety, which would perhaps be a confound in our study. We tested naïve α4 KO and WT mice in elevated plus maze, and found no differences in the basal anxiety levels and locomotor activity of these mice. This can be explained by the fact that these α4 KO mice have been backcrossed to B6 mice for several generations since the observation of basal anxiety in the α4 KO mice that were first generated. Therefore the anxiety could stem from the background Balb/c strain that was used to create these mice; the Balb/c strain has been shown to have higher anxiety compared to B6 (Michalikova et al., 2010). This is further supported by the generation of other α4 KO mice that used 129 background strain crossed with B6 (Marubio et al., 1999), and another line of α4 KO mice that used B6 as their background strain crossed with B6 (McGranahan et al., 2011) which did not show changes in basal anxiety levels.

Recently, several studies have implicated both α4 and α6 in nicotine’s effects on dopaminergic circuitry. α4α6β2β3* nAChRs display the greatest sensitivity to nicotine (EC50 = 230 nM), with high affinity for nicotine and ACh binding (Salminen et al., 2007). Enhanced nicotine induced DA release in the α6 KI mice, was reduced when the α4 subunit was removed from their system, indicating that α4α6β2* nAChRs are key players
in the cholinergic control of DA neurotransmission. Another study that coincides with those results showed that at terminals in the Acb, both the α4 and the α6 subunits were necessary to maintain nicotine-sensitive cholinergic regulation of DA release (Exley et al., 2011). We found that removal of the α4 subunit or α6 subunit, or antagonism of α6β2* resulted in a decrease in place preference for doses of nicotine that produce robust place preference in normal WT mice; our data proposes a critical role for α4β2*, α6β2* nAChRs, and while our data do not directly verify this, they also suggest that α4α6β2* nAChRs are critical for nicotine place preference.

As the field progresses in understanding the contribution of the various nicotinic receptors to the effects of nicotine in the CNS, several ideas have been discussed. Mansvelder et al., (2002) Exley et al., (2008) among many others have shown that nicotine disrupts basal ACh activity at DA neurons. Mansvelder and McGehee (2000) have reported that nicotine desensitizes α4β2*nAChRs involved in GABAergic transmission thereby causing disinhibition of DA neurons, while activating α7* nAChR involved in glutamatergic transmission thereby facilitating excitation of DA neurons which increases DA neuron activity and DA release in the mesolimbic system resulting in the reward-like effects of nicotine. In the mesostriatal and mesolimbic DA system, α4β2* nAChRs are expressed in cell bodies and axon terminals of midbrain and striatal DA and GABA neurons. Although there have been recent reports that α6β2* nAChRs may be located on GABAergic terminals in the VTA (Yang et al., 2011), it is well established that they are predominantly expressed on DA neurons in the mesolimbic system, and are involved in nicotine reward, reinforcement, and DA neurotransmission. The current compilation of
studies along with our results pertaining to α6β2* and α4β2* nAChRs have added to our understanding of the contribution of the various nicotinic receptors in nicotine reward and reinforcement.

7.3 α6* nAChRs, but not α4* nAChRs, are Critical for Cocaine Conditioned Place Preference.

Several studies have shown that nicotinic agonists and antagonists modulate cocaine reward, reinforcement, and sensitization (Champtiaux et al., 2006; Horger et al., 1992; Levine et al., 2011; Reid et al., 1998; Reid et al., 1999; Zachariou et al., 2001; Zanetti et al., 2007). Our research targeted α6* and α4* nAChRs subtypes in the investigation of nicotinic receptor modulation of cocaine reward because they are often co-expressed with the β2 subunit, and β2* nAChRs are known to be crucial for nicotine reinforcement and reward (Maskos et al., 2005; Picciotto et al., 1998; Walters et al., 2006) and have also been shown to modulate cocaine reward (Zachariou et al., 2001).

Using our CPP procedure, we saw a genotype dependent effect where cocaine preference was reduced in α6 HET mice (which express half the amount of α6β2* nAChRs), and totally eliminated in α6 KO mice compared to α6 WT counterparts. To complement this data, we also observed a dose dependent decrease in the expression of cocaine place preference with i.c.v. injections of α-Conotoxin MII [H9A; L15A], and a decrease in the acquisition of cocaine place preference as well. When we targeted the Acb, we observed a significant but partial reduction for cocaine preference that was mediated by α6β2* nAChRs in Acb, which suggests that α6β2* nAChRs in other brain regions are contributing to the effect of cocaine on CPP, or there is the possibility that there are other
substrates involved. Overall these results propose that α6β2* nAChRs in Acb are important, but are not the only brain regions/substrates mediating the acquisition of cocaine place preference. Our results expand on the study implicating a role for β2 in cocaine place preference (Zachariou et al., 2001), by suggesting that α6 is the subunit co-expressing in the nicotinic subtype mediating the reward like effects of cocaine. Our results implicating α6β2* nAChRs in Acb in cocaine reward can be explained by the mechanisms underlying the reports of psychostimulants enhancing the release of ACh in the Acb and increasing responsiveness of cholinergic neurons during acute and repeated drug exposure (Nestby et al., 1997).

α4β2* nAChRs are also highly expressed in the midbrain (Klink et al., 2001), and previous work has illustrated the sufficiency of α4* nAChRs (Tapper et al., 2004) and the necessity of α4β2* nAChRs for nicotine reward and reinforcement (McGranahan et al., 2011; Pons et al., 2009) and nicotine induced DA release in rodents (Drenane et al. 2010; Marubio et al. 2003). McGranahan et al. (2011) reported that while α4* nAChRs specifically on dopaminergic neurons were necessary for nicotine place preference, they were not for required for cocaine place preference. Along the same line, we found that both α4 KO and WT mice displayed similar cocaine place preference scores across several doses of cocaine, suggesting that α4* nAChRs are not required for cocaine reward in the place preference test.

We confirmed the role of α6β2* nAChRs in cocaine place preference by exposing α4 KO mice to the α6β2* nAChRs selective antagonist and then testing them for the acquisition of cocaine place preference. We found that α-Conotoxin MII [H9A; L15A]
caused a significant decrease in cocaine place preference in α4 KO and WT mice, which confirms the role of α6β2* nAChRs in cocaine place preference, and also suggests that α6β2* nAChRs are the main receptor subtypes mediating the effects of cocaine and do not require the α4 subunit.

Progress has reported that a nicotinic component is involved in the rewarding and reinforcing effects of cocaine (Champtiaux et al., 2006; Horger et al. 1992; Levine et al., 2011; Reid et al. 1998; Reid et al., 1999; Zachariou et al., 2001; Zanetti et al., 2007; Levin et al. 2000; Blokhina et al. 2005; Fiserová et al., 1999; Nestby et al., 1997)). Indeed the Pedunculopontine tegmentum (PPTg) and laterodorsal tegmentum (LDTg) fibers supply heavy cholinergic input to the mesolimbic system that is robustly involved in excitation of DA neurons (Lanca et al., 2000). α7*nAChRs in midbrain located on glutamatergic terminals projecting from cerebral cortex, α4β2* nAChRs located on GABAergic terminals and DA cell bodies in midbrain, and α4* and α6* nAChRs on dopaminergic terminals in midbrain neurons are all capable of responding to PPTg/LDTg derived ACh (Calabresi et al., 1989). In the mesolimbic system, α4β2* nAChRs are expressed in cell bodies and axon terminals of midbrain and striatal DA and GABA neurons. In contrast, α6β2* nAChR expression is predominantly restricted to DA cell bodies and axon terminals, and are therefore more exclusively involved in mediating DA neurotransmission when targeted in the whole system. Given this information, we can speculate that a possible mechanism explaining our cocaine results would be that interfering with the cocaine induced PPTg/LDTg excitation and cholinergic activitation of α6β2* nAChRs on DA neurons in the mesolimbic system by inhibiting or removing the α6 subunit ultimately
results in the disruption of an important neuromechanism involved in the attainment of the reward like euphoric effect of cocaine.

7.4 Lithium Conditioned Place Avoidance and Food Reward are not altered by pharmacological or genetic manipulations of α6* nAChRs.

The study of drug reward and reinforcement involves fundamental principles of learning and behavior. CPP is considered a Pavlovian type of learning, where the US is trained to be associated to the CS via the appetitive effects of the US. The association of US with CS involves memory formation and memory recollection. It was necessary to assess the specificity of hedonics of α-conotoxin MII [H9A; L15A] and the inactivation of the α6 nicotinic subunit.

We tested the effect of α-conotoxin MII [H9A; L15A] on lithium induced CPA, and found that lithium induced a conditioned place avoidance that was not altered by exposure to α-conotoxin MII [H9A; L15A]. Furthermore, lithium induced a significant CPA in α6 KO mice. Overall, our results show that α-conotoxin MII [H9A; L15A] decreased nicotine and cocaine place preference without having an effect on overall memory or causing confusion in the mouse as indicated by the ability of the mice to associate the context paired with the aversive properties of lithium and recall this memory on test day of CPP.

We also addressed the involvement of the α6 subunit in natural reward, specifically examining place preference for cheesecake. We found that cheesecake was able to induce similar place preference profiles in α6 KO mice and WT littermates, suggesting that
inactivation of the α6 subunit does not result in a general decrease in reward (anhedonia) specifically pertaining to the natural incentive for food. Previous work has shown that the α-conotoxin MII compound had no effect on rats responding to a cue-only stimulus (Brunzell et al., 2010), or on the rate that rats were self-administering food in the Gotti et al. (2010) study. It is important to note however, that the rats in the Gotti et al. study were severely food restricted, and this may have heavily influenced the outcome of their assessments. Since α-conotoxin MII [H9A;L15A] is an analogue of α-conotoxin MII, this helps to support our results showing the lack of involvement of α-conotoxin MII [H9A;L15A] on food or other non-psychoactive drug stimuli.

7.5 Future Directions.

Our data suggests a critical role for α6β2* and α4β2* nAChR in nicotine reward, but only α6β2* were found to be required for cocaine conditioned place preference. To expand on these studies, it would be beneficial to investigate the α6β2* nAChR component in cocaine induced striatal DA release, which would further support the involvement of α6β2* nAChRs in cocaine reward. Also, investigating the role of α6β2* nAChRs in other behavioral models of reward, such as intracranial self-stimulation or self-administration, would be useful to confirm our CPP results. It would also be interesting to determine the role of α6β2* nAChRs in cocaine locomotor sensitization, which underlies synaptic plasticity and long term potentiation of synapses that modify neural circuitry affected by cocaine. As other measures of assessing the specificity of hedonics for our CPP results, it would be of interest to assess α-conotoxin MII [H9A;L15A] and α6 and α4
KO mice in fear conditioning which also uses contextual cues and learning similar to the contextual learning used for CPP.

\( \alpha 6\beta 2^* \) nAChRs very often co-assemble with the \( \beta 3 \) subunit. Determining its relevance in nicotine and cocaine CPP would further characterize the subunits that make up the discrete nicotinic receptor subtype that mediate nicotine and cocaine reward. The \( \beta 3 \) subunit is encoded by a gene adjacent to the gene for \( \alpha 6 \), and its expression is pertinent to functional \( \alpha 6^* \) nAChRs (Cui et al, 2003). Also, populations of \( \beta 3 \) nAChRs have been identified as sensitive to \( \alpha \)Conotoxin MII and have been shown to modulate striatal DA release (Cui et al, 2003). In the absence of specific antagonists/agonists for the native \( \beta 3^* \) subtypes, \( \beta 3 \) KO mice should be used. CPP experiments with \( \beta 3 \) KO mice should be conducted across a range of nicotine doses (0.1-2.0 mg/kg).

7.6 Concluding Remarks.

Tobacco smoking is a prevalent addiction that constitutes the leading cause of preventable death and disease. Cigarettes produce CNS effects in a matter of seconds when smoked. Each puff of cigarette provides reinforcement, and for heavy smokers, this habit is reinforced hundreds of times daily. Environmental cues, social settings, and the anticipation and physical act of smoking all become repeatedly associated with the rewarding effects of nicotine which contribute to the resilience of nicotine dependence illustrated by the high relapse rates in smokers who try to quit. Several studies have investigated the relevance of environmental cues to smoking phenotypes and highlight the impact that they have on smoking addiction and the tendency of addicts to relapse.
Current nicotine cessation therapies only produce up to a 30% successful abstinence rate, and are beset with adverse systemic and some neuropsychiatric side effects. The low efficacy and adverse side effects can be partly explained by the lack of selectively targeting relevant nicotinic receptors that are involved in smoking addiction. Most current therapies are not targeted towards one specific nAChR subtype, and many will act at α4β2* nAChRs which have a ubiquitous expression profile in the CNS. Our goal was to target α6 and α4 subunits in our research because they are often co-expressed with the β2 subunit which has been established as a critical subunit in nicotine reward and reinforcement. The relevance of contextual cues in addiction have been confirmed by several human and animal studies, which is why we chose to conduct our assessments of these receptor subtypes in nicotine and cocaine reward using conditioned place preference which involves the association of visual and tactile cues to the drug. Our results coincide with other studies that implicate a critical role for α6β2* and α4β2* nAChRs in nicotine reward and reinforcement. Given the neuroanatomical distribution of α6β2* nAChRs on catecholaminergic neurons and the behavioral assessments involving this receptor subtype, these studies are suggesting that inhibition of α6β2* may be a valuable approach for smoking cessation treatments. Our studies also provide the first evidence for an important role of these subtypes in behavioral effects of cocaine.

The role of α6β2* nAChRs extends beyond addiction to neurological diseases including Parkinson’s disease. α6β2* nAChRs are expressed not only in the mesolimbic system, but also found in the nigrostriatal dopaminergic system which is primarily involved in movement; the loss of dopamine neurons in this pathway is one of the main
features of Parkinson’s disease. Studies have shown that there is a decreased occurrence of Parkinson’s disease with smoking (Gorell et al., 2004; Ritz et al., 2007, Thacker et al, 2007). One study found that nicotine pretreatment resulted in protection against nigrostriatal damage in rats and monkeys, which may be linked to α4α6β2* nAChRs (Huang et al, 2009) Data suggest that drugs targeting α6* nAChRs may be beneficial for the treatment of smoking addiction, Parkinson’s disease, and other disorders with movement and locomotor deficits.

To conclude, It is important to remember that nicotine dependence is due to complex behavioral traits that are influenced by genetics and environment. Nicotine and other drugs are not consumed for the sole purpose of pleasure and euphoria, but can be used for milder forms of gratification including stress relief, anxiety relief, improved cognition, mood, and performance, reduction of fatigue, decreased appetite, and for social purposes among many others. In fact, smokers report that they smoke for many of these reasons (Brandon, 1999). Therefore as the field progresses, the ideal combination of psycho and pharmacotherapies would be those that are geared towards the individual’s genetic background and the lifestyle they lead which governs when, why, and how often they smoke. Hopefully new and improved nicotine cessation therapies that target α6* nAChRs will be developed in the future to bring about improved abstinence rates in people struggling with addiction but striving to be tobacco free.
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This is to certify that the dissertation prepared by Sarah Susan Sanjakdar entitled
INVESTIGATING THE ROLE OF $\alpha_6$ and $\alpha_4$ CONTAINING NICOTINIC
ACETYLCHELONE RECEPTORS IN NICOTINE AND COCAINE CONDITIONED
PLACE PREFERENCE TESTS IN MICE.
has been approved by his or her committee as satisfactory completion of the thesis or
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RESEARCH INTERESTS:
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RESEARCH EXPERIENCE:
Fall 2007-Spring 2008:  
We determined the effect of nicotine during adolescence on locomotor sensitization to cocaine in adulthood in mice. Previous studies have shown that adolescence is a time of altered sensitivity to the effects of nicotine and other drugs of abuse. We wanted to determine the effects of early exposure to nicotine on the various effects of drugs, locomotion, sensitization, and reward during adulthood

Fall 2009-Spring 2010:  
Our objective was to explore the involvement of nicotinic acetylcholine (nAChRs) receptors in both nicotine and cocaine induced behavioral reward in mice using the conditioned place preference test. We targeted nAChR subtypes that are often co-expressed with the β2 nicotinic subunit, due to its abundant expression in the central nervous system. Of the various subunits co-expressing with β2, α6 is of particular interest. α6β2* nAChRs are complex heteromeric subtypes that have high expression in catecholaminergic nuclei in midbrain regions, which play a major role in presynaptic dopamine release, and mediate nicotine reward and reinforcement in rodents.

Fall 2010-Spring 2011:  
Recently, human genetic studies have implicated the CHRNA5/A3/B4 gene cluster, coding
for the α5, α3, and β4 subunits respectively, in nicotine dependence. They have high levels of expression in the medial habenula and interpeduncular nucleus, and exist mainly as α3β4(α5) or α3β4(β3). We assessed the contribution of α3β4* nAChRs in nicotine dependence, using the nicotine conditioned place preference test in mice.

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- Sanjakdar S, McIntosh JM, Damaj MI. The Role of α6-Containing Nicotinic Acetylcholine Receptors in Cocaine Reward and Locomotor Sensitization.
- Sanjakdar S, Jackson KJ, McIntosh JM, Damaj MI. The Role of α3β4-Containing Nicotinic Acetylcholine Receptors in Nicotine Reward and Withdrawal in Mice.
MEETING PRESENTATIONS:
· S.S. Sanjakdar, K.J. Jackson, D.H. Brunzell, U. Maskos, J.M. McIntosh, M. I. Damaj. The Role of $\alpha 6^*$ and $\alpha 3\beta 4^*$ nAChRs in Nicotine Reward. Society for Neuroscience 2009, Chicago, IL (poster).
· S.S. Sanjakdar, K.J. Jackson, J.M. McIntosh, M. I. Damaj. Role of $\alpha 5^*$ and $\alpha 3\beta 4^*$ Nicotinic Receptor Subtypes in the Rewarding Properties of Nicotine Using Conditioned Place Preference in Mice. Society for Research on Nicotine and Tobacco 2010, Baltimore, MD (poster).
· S. S. Sanjakdar, K.J. Jackson, M.J. McIntosh, M.I. Damaj. The Role of $\alpha 3\beta 4^*$ nAChRs in Nicotine Dependence. Society for Neuroscience, 2010, San Diego, CA (poster).
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