Allosteric Approaches to the Targeting of Neuronal Nicotinic Receptor for Drug Discovery.

DISSERTATION

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Bitna Yi, M.S.

Graduate Program in Pharmacy

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Dissertation Committee:

Dennis McKay, Ph.D., Advisor

Popat N. Patil, Ph.D.

Anthony Young, Ph.D.

Karl A. Werbovetz, Ph.D.

R. Thomas Boyd, Ph.D.
ABSTRACT

Neuronal nicotinic receptors (nAChRs) are attractive targets for drug discovery. As ligand-gated ion channels mediating the effects of the endogenous neurotransmitter acetylcholine, nAChRs play an important role in numerous physiological and pathological conditions. Moreover, nAChRs exist in multiple subtypes (combinations of α2-α10 and β2-β4 subunits). Importantly, each subtype displays distinct functional properties and unique expression patterns, suggesting the possibility that individual nAChR subtypes may be involved in different physiological and pathological processes. From this consideration, the diversity of nAChRs may offer the opportunity for pharmacological intervention without perturbing other aspects of cholinergic signaling and thus provide promising prospects for nAChR drug discovery. Hence, the discovery of small molecules that modulate nAChR functions would be of great importance. As research tools, they would improve our understanding of the roles that nAChRs play in normal and pathological states. Furthermore, they hold clinical promise as therapeutic agents for diseases and disorders associated with nAChRs. The present study focused on allosteric modulation of nAChRs as a strategy to achieve subtle regulation of receptor activity in a potentially subtype-specific manner. The most important findings are twofold. First, novel chemical classes of benzamide analogs were identified and characterized as negative allosteric
modulators (NAM) of nAChRs by integrating \textit{in silico} methods, in combination with pharmacology, medicinal chemistry, computational modeling, and molecular biology. Second, the potential of nAChR NAMs as therapeutic agents was investigated in \textit{in vivo} mouse models of cocaine addiction. Given the considerable therapeutic advantages that allosteric modulators could provide, the present study contributes to nAChR drug discovery by 1) providing proof-of-concept for rational drug design, and 2) demonstrating the \textit{in vivo} effects of nAChR NAMs in a preclinical disease model.
Dedicated to my family and friends
for their continual support through my education
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VITA

2003-2007 ........................................... B.S. Pharmacy, The Sookmyung Women’s University, Seoul, South Korea

2007-2009 ........................................... M.S. Pharmacy, The Sookmyung Women’s University, Seoul, South Korea

2009-Current ........................................... Graduate Teaching Associate, College of Pharmacy, The Ohio State University, Columbus, Ohio, USA

PUBLICATIONS


Alkaloids from Grewia paniculata with Cytotoxic and Nicotinic Receptor Antagonistic Activities. J. Nat. Prod. 72(2), 243-249.


**TEACHING EXPERIENCE**

2013  Pharmacology for Physical Therapists (PT 7140, 2 CU)

College of Health Services and Professions, School of Rehabilitation and
Communication Sciences, Division of Physical Therapy, Ohio University.
Topics: Neuropharmacology.

2012 Pharmacology for Physical Therapists (PT 713, 2 CU)
College of Health Services and Professions, School of Rehabilitation and Communication Sciences, Division of Physical Therapy, Ohio University.
Topics: Neuropharmacology.

HONORS

2013 Maria Margarita Salaza-Bookaman Dissertation Award in Receptor Pharmacology
2013 Chauncey D. Leake Memorial Award in Pharmacology
2013 Uretsky Graduate Student Award
2012-2013 Patil Graduate Fellowship Award in Pharmacology

FIELDS OF STUDY

Major Field: Pharmacy
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LIST OF ABBREVIATIONS

ACh – Acetylcholine
CNS – Central Nervous System
MD – Molecular Dynamics
NAM – Negative Allosteric Modulator
nAChR – Nicotinic Acetylcholine Receptor
nh – Hill Coefficient
PNS – Peripheral Nervous System
SAR – Structure Activity Relationship
SBVS – Structure-based Virtual Screening
LBVS – Ligand-based Virtual Screening
CPP – Conditioned Place Preference
CPA – Conditioned Place Aversion
STATEMENT OF THE PROBLEM

With neuronal nicotinic receptors (nAChRs) as promising targets for drug discovery, a great deal of effort has been directed toward the discovery and development of drugs that target nAChRs. In particular, the binding sites of their endogenous neurotransmitter acetylcholine (i.e. orthosteric binding sites) have been the main focus of our drug discovery effort. The objective of the approach targeting the orthosteric binding sites is to discover drugs that interact with nAChRs through the same site to mimic or block the actions of the endogenous ligand acetylcholine. However, one potential problem associated with this approach is the difficulty of attaining drugs with high subtype-specificity due to the high degree of amino acid conservation among nAChR subtypes within the orthosteric binding sites. The lack of subtype-selectivity of nAChR drugs contributes to numerous unwanted side effects and prevents them from being used as therapeutic agents. Thus, targeting regulatory sites that are distinct from highly conserved orthosteric binding site have gained attention as an attractive approach to develop nAChR drugs. These sites that are topographically distinct from the orthosteric binding site are defined as allosteric binding sites. Since allosteric binding sites typically display sequence and structural divergence
among nAChR subtypes, they can represent promising molecular targets to develop subtype-selective nAChR drugs.

This thesis focused on the discovery and development of small molecules that modulate nAChR functions potentially with improved subtype-selectivity through the allosteric approach. More specifically, the present study was aimed toward identifying novel chemical classes of drugs that bind to allosteric binding sites and antagonize nAChR functions (i.e. negative allosteric modulators; NAMs). The primary goals of this thesis are as follows:

1. To rationally design nAChR NAMs with desired properties through multidisciplinary and iterative approaches. The hypothesis is that the continuous flow of information obtained from various approaches (e.g. pharmacology, molecular biology, computational modeling, and medicinal chemistry) can be integrated to identify and optimize nAChR NAMs. Knowledge concerning the previously identified allosteric binding site as well as nAChR NAMs can be utilized through the application of multiple experimental methods and provide a rationale for discovering and designing novel chemical classes of nAChR NAMs.

2. To characterize pharmacological activities of nAChR NAMs and determine the chemical/structural features essential for nAChR antagonist activity. This can be achieved through structure-activity relationship (SAR) studies
on closely related analogs possessing the same core structure. The hypothesis is that analyses of SAR studies will enable the determination of chemical/structural features responsible for the analogs’ pharmacological activity on nAChRs.

3. To gain an understanding of the molecular mechanisms underlying the inhibitory effects of the discovered nAChR NAMs on receptor function. To this aim, a series of functional and binding studies will be performed with a new synthetic molecule (i.e. DCNR-I-235) that was reported as one of the nAChR NAMs in this study. The hypothesis here is that the effects of DCNR-I-235 on the efficacy and apparent affinity of the orthosteric agonist epibatidine can provide insight into the nature of the inhibitory effects of the nAChR NAM.

4. To evaluate the therapeutic potential of nAChR NAMs in preclinical disease models. nAChRs has a well-recognized role in addiction to numerous drugs of abuse including cocaine. Thus, in vivo effects of KAB-18, a nAChR NAM, were investigated in an animal model of cocaine addiction. The hypothesis is that KAB-18 can attenuate addictive properties of cocaine by blocking nAChR-mediated signaling, which are important for developing and maintaining cocaine addiction.
CHAPTER 1: INTRODUCTION

1.1. Nicotinic Acetylcholine Receptors.

In the central nervous system (CNS), cholinergic projection neurons and interneurons are widely distributed (Picciotto et al. 2012; Williams and Adinoff 2008). Projection neurons arise from multiple discrete sources (e.g. the pedunculopontine, the laterodorsal tegmental areas, the basal forebrain complex, and the medial habenula) and provide widespread innervation to nearly every region of the brain (Ren et al. 2011; Zaborszky 2002; Zaborszky et al. 2008). On the other hand, cholinergic interneurons are found in several brain areas including striatum, nucleus accumbens (NAc), and neocortex (Benagiano et al. 2003; von Engelhardt et al. 2007). In contrast to the projection neurons, these locally projecting neurons pose intensive innervation of neighboring neurons. The actions of ACh released from these cholinergic neurons are mediated by two classes of receptors: 1) the metabotropic G-protein coupled receptor, muscarinic acetylcholine receptors (mAChRs), and 2) the ligand-gated ion channels, nAChRs.

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that mediate cholinergic signaling. Since 1914 when Sir Henry Dale first
postulated the existence of nAChRs, tremendous efforts have been made to characterize their biochemical properties and to understand their roles in various physiological and pathological conditions (Romanelli and Gualtieri 2003). At the molecular level, nAChRs are integral membrane proteins composed of five subunits. Each subunit is encoded by a family of genes (α1-α10, β1-β4, δ, γ, and ε) and contains a large extracellular amino-terminal domain, four transmembrane domains (TM1-TM4), and a short extracellular carboxy-terminal domain (Lukas et al. 1999). These subunits then symmetrically arrange around a central pore and form a functional receptor. nAChRs are cation-selective due to the negatively charged amino acids located at the lining of the channel pore. Thus, activation of nAChRs results in flow of monovalent and divalent cations (e.g. Na⁺, K⁺, Ca²⁺) (Changeux et al. 1987). Based on subunit composition as well as their general location, nAChRs can be sub-categorized as two types (Figure 1.1); the muscle-type nAChRs and the neuronal type nAChRs. The muscle type nAChRs are heteromeric receptors composed of α1, β1, γ, and δ (α1, β1, ε, and δ in the fetus) in a fixed subunit stoichiometry ratio of 2.1.1.1 and found at the neuromuscular junctions (Paterson and Nordberg 2000). On the other hand, the neuronal type nAChRs exist as multiple subtypes composed of different subunit combinations (combinations of α2-α10 and β2-β4); the subunits are defined as α subunits or β subunits based on the existence of a disulfide bridge between two vicinal cysteine residues (corresponding to C192 and C193 of the Torpedo receptor) (Romanelli and Gualtieri 2003). Homomeric neuronal
type nAChRs are formed solely by α subunits. In molecular biological studies, α7, α8, and α9 subunits have been shown to form functional homomeric nAChRs (Elgoyhen et al. 1994; Gerzanich et al. 1994). On the other hand, heteromeric nAChRs are composed of combinations of α and β subunit. Initially, it was believed that only one type of α subunit and one type of β subunit assemble to form a functional heteromeric nAChR with a stoichiometry of $\alpha_{(3)}\beta_{(2)}$ or $\alpha_{(2)}\beta_{(3)}$ (Albuquerque et al. 2009). However, it has been demonstrated that native nAChRs may contain more than a single type of α and β subunits (Dani 2001). Therefore, the complexity and diversity of nAChRs are considerable. Importantly, the molecular diversity of nAChRs may have physiological relevance, because the biochemistry and pharmacology of nAChRs are determined by the subunit combinations. Indeed, nAChR subtypes display unique functional properties (e.g. ion selectivity, ion conductance, mean channel open time, permeability to Ca$^{2+}$, desensitization rate, sensitivity to various agonists or antagonists) depending on subunit compositions (Papke 1993; Nelson et al., 2003; Jensen et al., 2005). For example, the homomeric α7 nAChR exhibits distinct properties as opposed to the muscle-type and heteromeric nAChRs (e.g. greater Ca$^{2+}$ permeability and rapid activation and desensitization kinetics) (Khiroug et al. 2002). Furthermore, each subtype displays unique spatial and temporal expression patterns. The existence of diverse subtypes with unique functional properties and expression patterns suggests the possibility that each subtype may play a distinct role in certain physiological processes (Goldman et al. 1987; Gotti et al. 2006b).
Figure 1. The Structure of nAChRs. nAChRs are ligand-gated ion channels composed of five subunits. (A) Side view of the membrane-embedded nAChRs. Activation of the nAChR leads to influx of cations (e.g. Na⁺ and Ca²⁺). (B) Top View of the most common subtypes of nAChRs. Muscle type nAChR is composed of α1, β1, γ, and δ (α1, β1, ε, and δ in the fetus). Homomeric neuronal nAChRs consist solely of α subunits, whereas heteromeric neuronal nAChRs contain α subunits and β subunits. The homomeric α7 nAChR and the heteromeric α4β2* nAChR are the two most predominate subtypes expressed in the central nervous system. The heteromeric α3β4 nAChR is abundantly expressed in ganglia of the autonomic nervous system. Binding sites for the endogenous ligand acetylcholine are located at the interface between subunits as illustrated with yellow dots. * denotes the potential inclusion of an additional, unspecified subunits (Gotti et al. 2006b; Lukas et al. 1999).
1.2. Nicotinic Acetylcholine Receptors as Promising Therapeutic Targets.

Due to the critical roles of ACh in numerous neural functions, nAChRs that mediate the physiological effects of ACh represent promising targets for drug discovery (Gotti et al. 2006a; Gregory et al. 2007). nAChRs are widely distributed throughout the brain. In particular, nAChRs are expressed at various cellular locations (e.g. somatodendritic, axonal, postsynaptic sites, and presynaptic terminals) and regulate neuronal communication both presynaptically and postsynaptically (Dani and Bertrand 2007). Activation of nAChRs located at presynaptic or preterminal locations stimulates the release of multiple neurotransmitters (e.g. ACh, dopamine, serotonin, glutamate, and γ-amino butyric acid) and thus enhance overall efficiency of circuits (McGehee and Role 1995). On the other hand, activation of post-synaptic nAChRs mediates fast synaptic transmission. Although not as pronounced as seen at the autonomic ganglia, the fast synaptic transmission through the post-synaptic nAChRs has been demonstrated at several brain regions such as the hippocampus and cortex (Dani et al. 2001) Frazier et al., 1998; (Arroyo et al. 2012). Furthermore, nAChR-mediated calcium influx has been shown to regulate intracellular signaling pathways (Dajas-Bailador and Wonnacott 2004). Through these actions, nAChRs play a fundamental role in a wide range of physiological processes (e.g. cognition, attention, and executive function, learning and memory, mood, reward, and sensory processing) (Gotti et al. 2006a). At the same time, abnormal nAChR signaling has been implicated in a number of diseases and disorders. For
example, genetic mutations in CHRNA4 (α4) and CHRN2 (β2) are linked to the specific form of epilepsy, autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) (Dani and Bertrand 2007). In addition, a polymorphism in the promoter region of the α7 gene, as well as reduced nAChR expression, has been associated with schizophrenia (Leonard and Bertrand 2001). Similarly, significant loss of high affinity nAChR sites is observed in the brain of patients with Alzheimer’s disease (AD) and Parkinson’s disease (PD) (Guan et al. 2000; Lange et al. 1993; Nagata et al. 1997). The reduced nAChR expression in AD and PD patients is correlated to the cognitive deficits and dementia. Nicotine dependence is another important human pathology associated with disrupted nAChR signaling. Inappropriate signaling and altered expression of nAChRs resulting from repeated and chronic nicotine exposure are believed to be the main mechanisms underlying compulsive tobacco use (Laviolette and van der Kooy 2004). Collectively, considering their physiological roles as well as their implications in a number of diseases and disorders, modulation of nAChR activity can offer rich and diverse therapeutic opportunities.

1.3. Challenges in Nicotinic Receptor Drug Discovery.

The diversity of nAChRs represents major possibilities as well as challenges in the development of therapeutic agents that target nAChRs. As mentioned earlier, the pentameric nAChR displays enormous diversity and complexity due to the existence of multiple genes encoding receptor subunits.
Currently, it is estimated that as many as 25 nAChR subtypes with distinct functional properties and unique expression patterns are active in humans (Puskar et al. 2011; Romanelli et al. 2007). On one hand, the receptor diversity could provide abundant prospects in drug discovery. Given the possibility that each subtype contributes to distinct physiological processes, modulation of specific functions without affecting other aspects of cholinergic neurotransmission could be possible by selectively targeting certain subtypes. Indeed, recent studies with genetically engineered mice elucidated the roles of different nAChR subtypes in a specific disease or disorder (e.g. α4β2 nAChR in nicotine dependence, α7 nAChR in schizophrenia) (Guan et al. 1999; Picciotto et al. 1998; Tapper et al. 2004). With the improved understanding of the link between individual subtypes and specific physiological functions, subtype-selective modulation of nAChRs may provide useful therapeutic strategies that will maximize the desired effect and minimize the unwanted side effects.

In order to exploit nAChR diversity, a great deal of effort has been directed to discover and develop drugs that selectively target different receptor subtypes. However, this attempt has been hampered by the highly homologous nature of nAChR subtypes. In particular, orthosteric binding sites (sites of ACh binding) exhibit considerably high sequence homology. A group of five aromatic amino acids (i.e. Trp 56, Trp61, Trp87, Trp150, and Tyr199) interacting with ACh is completely conserved in all nAChR subunits (the numbers used are based on the
first transcribed muscle type nAChRs). In addition, amino acid residues surrounding the orthosteric binding sites also display high sequence conservation (i.e. Cys193, Cys 194, and Tyr 94; 75-100 % amino acid identity across nAChR subunits). It is believed that the evolutionary pressure to retain the site and sequence for the neurotransmitter ACh contributed to the high conservation of the orthosteric binding sites of nAChRs (Grigoriadis et al. 2009). Due to this extensive level of sequence homology, traditional drug discovery approaches that target the orthosteric binding sites has only resulted in nAChR ligands with limited subtype-specificity and undesired cross-reactivity (Jensen et al. 2005). The lack of subtype-selectivity of nAChR drugs often leads to multiple cholinergic side effects and thus prevents them from being used as therapeutic agents. In order to develop subtype-selective nAChR ligands, less conserved ‘non-orthosteric’ binding sites (i.e. allosteric binding sites) will be more promising molecular targets as opposed to the highly homologous orthosteric binding sites.

Allosteric binding sites are regulatory sites that are topographically distinct from the orthosteric binding sites (Christopoulos 2002). Being away from the binding sites for the endogenous ligand ACh, the allosteric binding sites are under reduced evolutionary pressure to preserve sequence homology and thus typically exhibit greater sequence divergence among nAChR subtypes. To date, multiple allosteric binding sites on nAChRs have been reported including three major sites that are present on all nAChR subtypes (Moaddel et al. 2007): the
ethidium binding site, the quinacrine binding site, and the central luminal site(s) (Figure 1.2). The ethidium binding site is positioned at the extracellular domain of the receptor, 46 Å above the transmembrane region (Pratt et al. 2000) (Figure 1.2). The antipsychotic medications chlorpromazine and clozapine have been shown to bind at this site (Arias et al. 2001; Fryer and Lukas 1999; Hernandez et al. 2000; Park et al. 2001). The quinacrine binding site has been identified at the interface between the α subunit and the lipid membrane, 7-12 Å below the extracellular-transmembrane domain interface (Moaddel et al. 2007) (Figure 1.2). As the name indicates, this site is the primary binding domain for the antimalarial agent quinacrine (Figure 1.3). The luminal binding site is located at the internal lumen of the ion channel. This site represents a high affinity non-competitive inhibitor binding domain (Figure 1.2). Many non-competitive inhibitors of nAChRs (e.g. mecamylamine, bupropion, phencyclidine, and barbiturates) have been shown to inhibit the receptor activity through their interactions with the luminal site (Arias et al. 2001; Fryer and Lukas 1999; Hernandez et al. 2000) (Figure 1.3). In addition to these sites, our laboratory also identified and characterized a novel allosteric binding site on nAChRs (Henderson et al. 2010; Pavlovicz et al. 2011) (Figure 1.2). This site was initially identified as a potential binding site for the subtype-selective ligand KAB-18 through a combination of homology modeling, blind docking, and MD simulation studies (Figure 1.3). The proposed binding mode between KAB-18 and nAChRs occurring at this site was then validated through the use of the site-directed mutagenesis approach (Henderson et al.
This site is located at the interface between α and β subunits, adjacent to the orthosteric site (i.e. ~10 Å from the orthosteric site) (Henderson et al. 2010; Pavlovicz et al. 2011). In contrast to the orthosteric site where principal ligand-binding elements were provided by the α subunit, the major amino acids compromising this novel site originate from the β subunit: Thr58, Glu60, Ser97, Ser133, Ser138, Ser142, Phe118, and Ser137 (Henderson et al. 2012). Therefore, we named this site the ‘β subunit site’. Importantly, the ‘β subunit site’ exhibits sequence and structural divergence among nAChR subtypes. As revealed by subunit sequence analyses, most amino acid residues positioned within the ‘β subunit site’ (e.g. Thr58, Ser133, Ser138, Ser142, Phe118, and Ser137) show less than 26 % sequence identity across nAChR subunits. Two other amino acids that play an important role in interactions between ligands and the ‘β subunit site’ (e.g. Glu60 and Ser97) also share a limited degree of sequence conservation (i.e. 30 ~ 60% amino acid identity across nAChR subunits). Therefore, as a novel molecular target bearing sequence and structural diversity, the ‘β subunit site’ has significant implications for drug discovery.
Figure 1.2. A presentation of binding sites for competitive and non-competitive ligands of nAChRs. Side section (A) and Top (B) views of nAChRs showing binding sites for competitive and non-competitive ligands of nAChRs. The orthosteric sites where the endogenous ligand ACh and competitive ligands bind are situated at a region at about 30 Å above the cell membrane (Boido et al. 2003). A binding site for the non-competitive channel blocker mecamylamine is positioned within the central transmembrane domain of the ion channel. Binding sites for the non-competitive antagonists ethidium and quinacrine are located at the extracellular portion of the receptor 46 Å above the cell membrane and lipid-protein interface 7-12 Å below the extracellular-transmembrane, respectively (Moaddel et al. 2007; Pratt et al. 2000). The ‘β subunit site’ is located at the interface between α and β subunits adjacent to the orthosteric binding sites (Henderson et al. 2010; Henderson et al. 2012; Pavlovicz et al. 2011).
Figure 1.3. Chemical structures of some nAChR ligands. Acetylcholine is the endogenous ligand of nAChRs. Nicotine is a competitive agonist that activates nAChRs by binding to the acetylcholine binding site (i.e. orthosteric site). Mecamylamine is a non-competitive inhibitor that blocks the ion channel of nAChRs. Ethidium, quinacrine, and KAB-18 are nAChR non-competitive antagonists that inhibit nAChR function by acting at sites distinct from the orthosteric site (i.e. allosteric sites) (Henderson et al. 2012; Moaddel et al. 2007; Pavlovicz et al. 2011; Pratt et al. 2000). Asterisk (*) indicates chiral centers.
1.4. Allosteric Modulation as an Approach for nAChR Drug Discovery.

nAChRs are sophisticated allosteric macromolecules that have an oligomeric quaternary structure with multiple topographically distinct but interacting domains (Bertrand and Changeux 1999). Through allosteric interactions, the binding of agonist at the extracellular domain of the receptor leads to the opening of the channel, which occurs in the transmembrane domain 50 Å away from the agonist binding site (Lena and Changeux 1993; Unwin 2005). Due to their allosteric nature, nAChRs possess great potential as molecular targets for drug discovery. That is, in addition to competitive agonist/antagonist, nAChR activity can also be modulated by allosteric ligands. Compared to competitive ligands which act through orthosteric sites, allosteric ligands bind to receptors through interactions with spatially distinct sites. The binding of allosteric ligands then causes changes in receptor conformations and alters the agonist-induced receptor activity, resulting in positive or negative cooperativity (i.e. positive allosteric modulator, PAM or negative allosteric modulator, NAM, respectively) (Hogg et al. 2005).

As opposed to the traditional orthosteric approach, allosteric modulation of nAChRs can offer unparalleled opportunities for drug discovery and development. First, allosteric modulation can be an attractive approach to develop subtype-selective nAChR ligands (Taly et al. 2009). As mentioned previously, the orthosteric binding sites display high sequence homology. Thus,
it has been difficult to attain nAChRs ligands with high subtype-specificity through the orthosteric approach. On the other hand, allosteric binding sites typically display sequence and structural diversity among receptor subtypes. With the allosteric approach, this diversity can be exploited to develop ligands that selectively target specific nAChR subtypes. The identification of the structurally diverse ‘β subunit site’ as well as subtype-selective nAChR NAMs acting through this allosteric site demonstrates the successful utilization of the allosteric approach to enhance ligands’ subtype-selectivity (Henderson et al. 2010; Henderson et al. 2011; Mahasenan et al. 2011; Pavlovicz et al. 2011). In our previous studies, the ‘β subunit site’ has been shown to exhibit sequence and structural diversity among nAChR subtypes (Henderson et al. 2012). Furthermore, several nAChR NAMs have been shown to selectively antagonize α4β2 nAChRs principally through their interactions with non-conserved amino acids within the ‘β subunit site’ (Henderson et al. 2010; Henderson et al. 2012). Given the fact that clinical development of many nAChR drugs have been discontinued due to the side effects associated with their non-selective actions, the discovery of drugs that target specific nAChR subtypes would be of great importance (Arneric et al. 2007). Second, the allosteric approach can allow a more physiologically relevant signaling modulation as opposed to the orthosteric approach. That is, allosteric modulators can preserve the temporal characteristics of endogenous signaling, because they selectively alter receptor activity only in the presence of endogenous ligands with little or no intrinsic
activity on their own (Gregory et al. 2007). This aspect of mechanisms may be of particular importance for PAMs and allow them to produce better therapeutic outcomes compared to the orthosteric agonist. While drugs with intrinsic agonist activity often induce receptor desensitization or inappropriate tonic signal, PAMs can circumvent these problems while potentiating the endogenous neurotransmitter mediated signaling (Christopoulos 2002). Third, allosteric modulation can impart multiple modes of target modulation, leading to a vast repertoire of functional properties of the receptors. Binding of allosteric modulators induces global changes in nAChR structures both on their own and in the presence of the bound orthosteric agonist. These conformational changes engendered by allosteric modulators can then affect multiple factors relevant for receptor activity (e.g. orthosteric ligand binding affinity, orthosteric ligand signaling efficacy, and receptor activation/desensitization kinetics). Since allosteric modulators can simultaneously affect these factors with various degrees and directions of modulation, fine-tuning the receptor activity would be possible with allosteric modulators (Christopoulos and Kenakin 2002). In addition to the therapeutic benefits, the allosteric approach can also provide unprecedented potential for drug discovery and development processes by expanding the chemical space for new chemical entities. As interactions between allosteric modulators and nAChRs are not limited to the orthosteric binding site, targeting the unexplored allosteric binding sites can remarkably widen the chemical diversity of drug candidates (Ivetac and McCammon 2010). Supporting
this claim, a wide variety of structurally diverse allosteric modulators of nAChRs have been reported over the last decade (Arneric et al. 2007; Grønlien et al. 2007; Pandya and Yakel 2011; Williams et al. 2011).

For the past 10 years, the main research interest of our laboratory has been in discovery of drugs that allosterically modulate the nAChR functions. In particular, we have utilized diverse approaches from the conventional analog chemistry in concert with structure-activity relationship (SAR) studies to the modern strategies of computer-aided drug design (CADD) (e.g. molecular modeling, ligand-based virtual screening, structure-based virtual screening, and 3D-quantitative SAR analysis) in an iterative manner. In contrast to the traditional ‘trial-and-error’ drug discovery approach, a combination of multiple approaches have provided valuable insights into many aspects of the drug discovery process and enabled rational drug design. Our work on nAChR allosteric modulators started with methyllycaconitine (MLA), a potent competitive nAChR antagonist found in many Delphinium species (larkspurs). Our hypothesis was that synthesizing simpler MLA analogs would lead to the identification of more druggable yet equally potent nAChR antagonists. This hypothesis was tested by simplifying the MLA core structure, which led to ring E analogues of MLA containing the succinimidoylanthranilate side chain (Bergmeier et al. 1999; Bergmeier et al. 2004; Bryant et al. 2002a). In nicotine-stimulated neurosecretion assays using adrenal chromaffin cells, these ring E analogues of
MLA were shown to non-competitively interact with nAChRs and inhibit nAChR activity (Bergmeier et al. 1999; Bryant et al. 2002a; Bryant et al. 2002b). Since the initial identification and characterization of these analogs as non-competitive antagonists of nAChRs, our research efforts have focused on discovery of nAChR NAMs. The continued commitment to nAChR drug discovery employing integrated and multidisciplinary approaches led to the creation of the McKay NAM library. This small focused chemical library now contains ~ 250 molecules, from 4 distinct chemical scaffolds. Furthermore, the allosteric binding site for these NAMs (e.g. ‘β subunit site’) was identified and insight into molecular interactions within the ‘β subunit site’ that are critical for the NAMs' binding to the nAChRs was gained (Henderson et al. 2010; Henderson et al. 2012; Pavlovicz et al. 2011). The identification of several novel chemical classes of nAChR NAMs, through exploitation of previously generated knowledge on the known NAMs as well as their binding site, proved the application of a highly interdisciplinary approach to be useful in identifying and designing drugs.

One of the primary goals of this dissertation is to identify new chemical classes of nAChR NAMs. Based on the hypotheses that knowledge on the previously identified nAChR NAMs as well as their binding site (i.e. the ‘β subunit site’) can be exploited to discover novel chemical classes of nAChR NAMs, multiple approaches (e.g. pharmacology, molecular biology, computational modeling, and medicinal chemistry) were utilized to direct the design of nAChR
drugs with desired properties (i.e. selectivity and potency). The physicochemical features of drugs essential for nAChR NAM activity were determined through synthesis of structurally related analogs and evaluation of their activity on nAChRs. Furthermore, molecular mechanisms of actions of the discovered nAChR NAMs were characterized. The research effort toward the discovery and characterization of novel chemical classes of nAChR NAMs is discussed in Chapters 3 and 4 of this dissertation.

1.5. Nicotinic Receptor Antagonists for the Treatment of Addiction.

The mesolimbic dopaminergic system is the brain circuitry that controls motivation, emotion, and reward. An important component of this system is the dopaminergic neurons originating from the ventral tegmental area (VTA) that send projection to the nucleus accumbens (NAc), amygdala, and frontal cortex. From a biological point of view, the mesolimbic dopaminergic system is of great importance due to its essential roles in survival of individuals and species. Behaviors related to survival functions (e.g. eating, drinking, social interactions, and sexual activity) are known to produce feelings of pleasure by stimulating the mesolimbic system (Fattore et al. 2010; Kelley and Berridge 2002). Due to these emotional values, the basic survival behaviors have positive reinforcing effects and could be actively and persistently pursued. From a clinical perspective, the mesolimbic dopamine system also has implications for understanding drug abuse because of the common effects that addictive drugs have on this system.
Through various mechanisms, all drugs of abuse including psychostimulants, nicotine, opioids, cannabinoids, and ethanol stimulate the mesolimbic dopamine system upon acute administration (Heidbreder and Hagan 2005; Nestler 2001b). In addition to the acute responses, repeated exposures to abused drugs also induce chronic neuronal adaptations in the mesolimbic dopamine system (e.g. increased expression of AMPA receptors in the VTA, long-term depression (LTD)-like state in NAc neurons) (Nestler 2001a). On the basis of these common effects of addictive drugs, it is believed that the mesolimbic dopamine system plays a key role in drug-reinforced behaviors.

Cholinergic systems are critical components of the mesolimbic dopamine system (Mesulam 1996) (Figure 1.4). In the striatum, there are cholinergic interneurons. These neurons are tonically active and provide the primary source of ACh to the dorsal and ventral striatum (Williams and Adinoff 2008). In addition to the striatal cholinergic interneurons, the pedunculopontine tegmental nucleus (PPT) and laterodorsal tegmental nucleus (LDT) from brain stem mesopontine nuclei also provide cholinergic input to the VTA, influencing output of dopaminergic transmission (Wonnacott et al. 2005). mAChRs and nAChRs that are expressed in the striatum mediate the cholinergic signaling originated from these projection neurons and interneurons.
Figure 1.4. A schematic of the cholinergic signaling in the mesolimbic dopamine system. The ventral tegmental area (VTA) and nucleus accumbens (NAc) are major components of the mesolimbic dopamine system that are strongly implicate in addictive properties of abused drugs. Cholinergic neurons originated from the laterodorsal tegmental nucleus (LDT) or pedunculopontine tegmental nucleus (PPT) influence the mesolimbic dopamine system through nAChR expressed on multiple neuronal populations within the system (e.g. dopaminergic, GABAergic, and glutamatergic neurons). Tonically active cholinergic interneuron in the striatum also regulates the activity of the mesolimbic dopamine system. nAChR subtypes expressed in the VTA include \((\alpha4)2(\beta2)3\), \(\alpha4\alpha6\alpha5/\beta3(\beta2)2\), \((\alpha4)2\alpha5(\beta2)2\), and \((\alpha7)5\) nAChRs. nAChR subtypes expressed in the NAc include \((\alpha4)2(\beta2)3\), \((\alpha4)2\alpha5(\beta2)2\), \((\alpha6)2(\beta2)2\beta3\), and \(\alpha4\alpha6(\beta2)2\beta3\) nAChRs (Wannacott et al., 2005).
As the neurotransmitter receptors that mediate the cholinergic signaling, nAChRs are critically involved in regulating the mesolimbic dopamine system’s activity. In previous molecular biology studies, it has been demonstrated that manipulations of nAChR-mediated signaling with pharmacological approaches change dopaminergic transmission in the system. For example, activation of nAChRs with nicotine has been shown to increase the firing rate of dopamine neurons in the mesolimbic dopamine system and stimulate the dopamine release (Dajas-Bailador and Wonnacott 2004; Picciotto et al. 1998). Conversely, blockage of nAChR activity with the non-selective nAChR antagonist mecamylamine decreased the firing rate of mesolimbic dopamine neurons. This indicates the contribution of nAChR-mediated signaling to dopamine transmission under physiological conditions (Grenhoff et al. 1986). With well-established roles in regulating the brain reward system, nAChRs are involved in drug addiction. The prevalence of cigarette smoking would be the most obvious example showing the roles of nAChRs in the development and maintenance of addictive behaviors. The nAChR agonist nicotine contained in tobacco changes functions and expression patterns of nAChRs within the mesolimbic dopamine system and thereby drives ongoing smoking. In addition to nicotine addiction, nAChRs can also contribute to dependence on a wide range of drugs. Indeed, previous studies demonstrated that manipulations of nAChR activity through pharmacological or genetic approaches affect the addictive properties of cocaine, amphetamine, alcohol, cannabis, and morphine (Feng et al. 2011; Horger et al.)
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Kamens et al. 2010; Kim et al. 2011; Solinas et al. 2007; Zachariou et al. 2001). For example, nicotine increased self-administration behavior of cocaine and alcohol (Horger et al. 1992; Lê et al. 2003). Conversely, it has been shown that nAChR antagonists (e.g. mecamylamine and 18-MC) decreased reinforcing properties of ethanol, cocaine, methamphetamine, and morphine in place preference or self-administration paradigms (Rezvani et al. 1997; Glick et al. 2000; Lê et al. 2000; Zachariou et al. 2001). Furthermore, genetically engineered mice lacking α7 and β2 nAChR subunits have shown decreased place preference to cocaine and alcohol intake, respectively (Kamens et al. 2010; Zachariou et al. 2001). Epidemiological studies have also shown that tobacco is frequently co-abused with other forms of addictive drugs, further suggesting the involvement of nAChRs in multiple drug addictions (Miller and Gold 1998; Smith et al. 2011). Collectively, these data suggest that nAChRs may prove valuable therapeutic targets for drug addiction. That is, therapeutic agents targeting nAChRs can be useful in the treatment of multiple forms of substance abuse disorders.

Chapter 5 of this dissertation describes a study designed to investigate the *in vivo* effects of KAB-18, a nAChR NAM previously identified by our laboratory, in drug addiction. Among many drugs of abuse, a special focus was given to the psychostimulant cocaine, a powerfully addictive stimulant that induces one of the most debilitating forms of addiction. Since no pharmacotherapy is available for
the treatment of cocaine addiction despite the staggering impact that it places on our society, the development of therapeutic agents that can be used to treat cocaine addiction would be of great importance. In order to evaluate the therapeutic potential of KAB-18 in cocaine addiction, the *in vivo* effects of KAB-18 on addiction-relevant behavioral responses to cocaine were investigated. Considering the key modulatory functions of nAChRs in the mesolimbic dopamine system, it was hypothesized that blockage of nAChR-mediated signaling with KAB-18 would decrease the addictive properties of cocaine.
CHAPTER 2: MATERIALS AND METHODS

2.1. In vitro Studies.

2.1.1. Materials.

Dulbecco’s Modified Eagle Medium (DMEM), penicillin, streptomycin, and L-glutamine were obtained from Invitrogen Corporation (Grand Island, NY). Calcium 5 NW dye was purchased from Molecular Devices (Sunnyvale, CA). (±)-Epibatidine dihydrochloride hydrate, (-)-nicotine hydrogen tartrate, and polyethylenimine were obtained from Sigma-Aldrich (St. Louis, MO). (±)-[5,6,-bicycloheptyl-³H]epibatidine (specific activity, 55.5 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). M-PER buffer and Bio-Rad DC assay kit were obtained from Peirce (Rockford, IL) and Bio-Rad (Castle Hill, Australia), respectively. Whatman GF/B filters were purchased from Brandel Inc (Gaithersburg, MD). All other reagents were obtained from Fisher Scientific (Pittsburgh, PA). All test compounds were purchased from Chembridge (San Diego, CA) or synthesized in the Werbovetz laboratory or the Coleman laboratory at The Ohio State University. All molecules purchased from Chembridge were obtained at ≥ 90 % purity as determined by ¹H NMR analyses as stated by the supplier. All compounds prepared at The Ohio State University were > 95% pure as determined by elemental or high-performance liquid chromatography (HPLC)
analysis. To aid solubilization in the biological assay buffer, all compounds were tested as hydrochloride or oxalate salts. For pharmacological activity evaluation, all compounds were initially dissolved in 100% DMSO (0.01 M stocks). Further dilutions of compounds were made with HEPES-buffered Krebs (HBK) solution (155 mM NaCl, 4.6 mM KCl, 1.2 mM MgSO₄, 1.8 mM CaCl₂, 6 mM glucose and 20 mM HEPES, pH 7.4).

2.1.2. Cell Cultures.

HEK tsA201 cells stably expressing either human α4β2 nAChRs (Hα4β2 nAChRs) or human α3β4 nAChRs (Hα3β4 nAChRs) were maintained as previously reported and used to characterize compounds’ pharmacological activity on nAChRs (obtained from Professor Jon Lindstrom, University of Pennsylvania, Philadelphia, PA) (Henderson et al. 2010; Henderson et al. 2011). Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 10 mM L-glutamine, 0.7 mg/ml genetecine (G418), 100 units/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml zeocin and were maintained in an atmosphere of 5% CO₂ at 37°C. For calcium accumulation assay, the cells were plated into 96-well plates previously coated with poly-L-ornithine at a density of 2.0 - 2.3 x 10⁵ cells per well. The plated cells were used for the calcium accumulation assay within 24-48 hours when cells reached 100 % confluence to form a monolayer. For the binding assays with [³H]epibatidine, cells were grown to confluence in 100-mm dishes and harvested with M-PER Mammalian Protein Extraction
Reagent according to the manufacturer’s instructions. Protein concentrations were determined using the Bio-Rad DC assay.

2.1.3. Calcium Accumulation Assay.

Compounds’ pharmacological activities on nAChRs were determined with calcium accumulation assays, using a procedure previously reported by our laboratory (Henderson et al., 2011). At the beginning of the experiment, cells were rinsed with 100 μl of HBK buffer, and incubated with 50% Calcium 5 NW dye for 1 hour (protected from light, room temperature). Plates were then placed into a fluid handling integrated fluorescence plate reader (FlexStation, Molecular Devices, Sunnyvale CA). During the assay, treatment solutions were added to the cells and changes in intracellular calcium levels were simultaneously measured at 1.5 s intervals (excitation, 485 nm; emission, 525 nm). The assay was structured with one-pretreatment period and two treatment periods. During the pretreatment period, cells were incubated with 40 μl of HBK buffer and fluorescence signal was monitored for 20 sec. Recordings from the pretreatment period were used to establish resting levels of fluorescence signal. At the start of the first treatment period, 40 μl of treatment solutions containing test compounds were added to the cells and changes in fluorescence signal were monitored for 40 sec. At the start of the second treatment period, 40 μl of treatment solutions containing the agonist epibatidine with test compounds were added to the cells and changes in fluorescence signal were monitored for an additional 60 sec. To
construct a full concentration-response curve, functional effects of test
compounds were tested at 6 concentrations in the range of 0.1 – 100 μM. For
quantification of test compounds’ pharmacological activity, control-sham group
and control-agonist group were included in the assay. For the control-sham
group, 40 μl of HBK buffer was added to the cells at the start of the first and
second treatment period. For the control-agonist group, 40 μl of HBK buffer was
added to the cells at the start of the first treatment period, followed by the
addition of treatment solution containing epibatidine at the start of the second
treatment period. Quantification of test compounds’ functional activity was
determined by their ability to inhibit the agonist-induced increases in fluorescence
signal. Curve fitting was performed using Prism software (Graphpad, Sand
Diego, CA) with the equation for a single-site sigmoidal dose-response curve with
a variable slope. The pharmacological activity of test compounds was reported
as the concentration of drugs that reduces the effect of 1 μM epibatidine by 50 %
(IC\textsubscript{50} values). The concentrations of DMSO used to dissolve test compounds
were 1% or less. At these concentrations, DMSO was shown to have no effects
on basal or agonist-induced increase in fluorescence signal. Due to solubility
problems, concentration-response studies were conducted to concentration of up
to 100 μM. Pharmacological activity of compounds that showed no inhibition up
to the highest concentrations was described as ‘no activity up to 100 μM’.
2.1.4. [³H]-Epibatidine Binding to Human Recombinant Nicotinic Receptors.

In order to evaluate the effects of NAMs on the affinity of the orthosteric ligand, binding assays with [³H]-Epibatidine were performed, using a previously reported procedure with minor modifications (González-Cestari et al. 2009). In particular, homologous and heterogeneous competition binding assays were performed with membrane preparations of HEK tsA201 stably expressing Ha4β2 nAChRs. To start the binding reaction, the cell suspension was incubated with 500 μl of binding buffer containing 200 pM of [³H]epibatidine (120 mM NaCl, 5 mM KCl, 8 mM Na₂HPO₄, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide, 2 mM EDTA, 2 mM EGTA, and 5 mM HEPES, pH 7.4) (1 hour, room temperature). For homologous binding assays, the 1 hour incubation with [³H]epibatidine proceeded with increasing concentrations of epibatidine in the absence or presence of nAChR NAMs. For heterologous binding assays, the 1 hour incubation with [³H]epibatidine proceeded with increasing concentrations of nAChR NAMs. After the incubation, the cell suspension in the assay buffer was passed through a Whatman filter presoaked with 5% polyethyleneimine (PEI) using a cell harvester. The filter was rapidly washed three times with the assay buffer and transferred to a counting vial with 4.5 mL of scintillation cocktail. The vial was shaken vigorously and incubated for 20-24 hours. After the incubation, the radioactivity was counted with a Beckman 6800 liquid scintillation counter (Beckman, Irvine, CA). Non-specific binding was determined in the presence of 300 μM nicotine and typically represented less than 3% of total binding. Specific
binding was calculated by subtracting non-specific binding from total binding.

Inhibition curves were determined from the sigmoidal-varied slope curves using Prism (Graphpad, Sand Diego, CA). NAMs were dissolved in DMSO, resulting in a final concentration of < 0.1%. At these concentrations, DMSO had no effects on \[^3\text{H}\]-epibatidine binding.

2.1.5. Pharmacophore Generation.

In order to define structural and chemical features shown in some promising NAMs, pharmacophore models were generated using a previously reported procedure (Henderson et al. 2010; McKay et al. 2007). Briefly, compounds with promising properties (i.e. potency and/or selectivity) were aligned using GASP (Genetic Algorithm Similarity Program, SYBYL 7.1). Orientation of molecules was optimized using similarity constraints (population size, 125; the allele mutate weight, 96; the fitness increment, 0.02). For each set of molecules, pharmacophore alignment was repeated for 10 runs. An optimal pharmacophore model was selected based on visual inspection.

2.1.6. Calculations and Statistics.

Data were calculated from the number of observations (n) performed in duplicate or triplicate. IC\textsubscript{50} values and Hill coefficients were obtained by averaging values that were generated from each individual concentration-
response curve. Results were expressed as geometric means (95% confidence limits) for IC\textsubscript{50} values and arithmetic means for Hill coefficients.
2.2. *In vivo* studies.

2.2.1. Drugs.

Cocaine hydrochloride was provided by the National Institute on Drug Abuse drug supply program. Mecamylamine hydrochloride was purchased from Sigma. Methyllycaconitine citrate (MLA) and dihybro-β-erythroidine hydrobromide (DHβE) were purchased from Tocris. KAB-18 was synthesized and supplied by Dr. Stephen C. Bergmeier in collaboration with our laboratory (Ohio University, Athens, Ohio). Mecamylamine and KAB-18 were tested as racemates. All drugs were dissolved with isotonic saline solution (0.9% NaCl). All drug solutions were made fresh on the test days and administered intraperitoneally (i.p.) in a volume of 0.1 ml/10g of body weight. The doses of the nAChR antagonists were chosen based on previous studies with mice in which they showed nAChR-mediated effects (Gommans et al. 2000; Grabus et al. 2006; Stolerman et al. 1999; Zachariou et al. 2001; Zanetti et al. 2006).

2.2.2. Animals.

Adult male C57Bl/6J mice (8-10 weeks) were purchased from Jackson Laboratories (Bar Harbor, ME) and used for all *in vivo* experiments. Upon arrival, mice were housed in groups of four with food and water available *ad libitum* and acclimated to the animal facility for at least 7 days. Mice were maintained under a 12:12h dark-light cycles (light on at 6:00h) and all behavioral tests were performed during the light phase of the light-dark cycle. In order to reduce the
non-specific behavioral effects associated with experimental procedures, all mice were transported to the testing room and handled for 5 min each for 3 consecutive days prior to the behavioral tests. On the test days, the animals were transferred to the behavior testing room and allowed to acclimate to the experimental environments for 1 hour before the start of the test. All mice were experimentally naïve at the beginning of the test and used only once. All mice studies were conducted in accordance with an animal protocol approved by The Ohio State University Institutional Animal Care and Use Committee (IACUC).

2.2.3. Apparatus.

The conditioning apparatus is an acrylic box (12.5 cm x 42.5 cm) subdivided into three compartments: a middle compartment (12.5 cm x 7.5 cm) and two side compartments (12.5 cm x 17.5 cm) (Figure 2.1). The conditioned place preference (CPP) procedure consists of three phases: a pre-conditioning preference test, a conditioning phase, and a post-conditioning preference test (Figure 2.1). On the pre- and post-conditioning preference test days, the conditioning apparatus was constructed with three compartments that have different sets of tactile and visual cues (Figure 2.1). During the conditioning phase, the entire apparatus was configured with a single set of tactile and visual cues. Throughout the trial, animal behaviors were recorded using overhead cameras and analyzed by the ANYMAZE software v4.81 (Stoelting Co.; Wood Dale, IL, USA).
Figure 2.1. CPP paradigm and conditioning apparatus. (A) The CPP paradigm consisted of a pre-conditioning preference test, a conditioning phase, and a post-conditioning preference test. (B) The conditioning apparatus was comprised of three compartments. On the pre- and post-conditioning preference test days, the three compartments of the conditioning apparatus have different sets of tactile and visual cues in order to determine animals’ preference for the different environmental cues. During the conditioning phase, the entire apparatus was configured with a single set of tactile and visual cues. Each mouse was administered with cocaine (C) or saline (S) injections and placed into the apparatus configured with cocaine- or saline-paired environmental cues. There were 4 cocaine pairing sessions alternating with 4 saline pairing sessions over the 8 consecutive days.
2.2.4. Conditioned Place Preference Test.

The CPP paradigm is one of the most widely used behavioral models to assess the addictive properties of drugs (Bardo and Bevins 2000). In this paradigm, animals are repeatedly treated with drugs in a particular environment. As the environment becomes associated with the rewarding effects of drugs, animal are more likely approach to the drug-associated environment.

In this study, the CPP procedure consists of a pre-conditioning preference test, a conditioning phase, and a post-conditioning preference test. On the pre-conditioning preference test day, the animal’s pre-existing preference was assessed by placing mice into the middle, neutral compartment and allowing them to freely explore three environmentally distinct compartments (30 min). The time spent in each compartment was measured and used to determine the animal’s pre-existing preference. Following the pre-conditioning preference test, mice were assigned to receive cocaine in one set of environmental cues in a counterbalanced manner; half of the mice received cocaine in the initially preferred environment and the other half received cocaine in the initially non-preferred environment so that the total group bias can be minimized. The conditioning phases consisted of four drug pairing sessions over 8 consecutive days. During this phase, mice were treated with alternative injections of cocaine (days 1,3,5, and 7) and saline (days 2,4,6, and 8). Immediately after cocaine (10 mg/kg, i.p.) or saline administration, mice were placed into the apparatus and exposed to the corresponding environmental cues for 30 min. As control, one
group of mice received saline throughout the 8 days of conditioning sessions. The post-conditioning preference test was performed under drug-free conditions 24 h after the last conditioning session. As in the pre-conditioning preference test, the mice were placed into the apparatus and allowed to explore three distinct compartments for 30 min. The time spent in each compartment was recorded and used to determine the CPP score. The CPP score was defined as the difference in time spent in the compartment with cocaine-designated cues during the pre- and post-conditioning preference tests. A positive value for the CPP score indicates that mice show preference toward the cocaine-paired cues and this is believed to be due to the rewarding effects of cocaine. Effects of nAChR antagonists on the cocaine-induced CPP response were assessed by administering drugs to the mice 30 min prior to saline or cocaine injection. For this study, the following nAChR antagonists were tested: mecamylamine (3 mg/kg, i.p), methyllycaconitine citrate (MLA, 5 mg/kg), dihydro-β-erythroidine hydrobromide (DHβE; 2.5 mg/kg), and KAB-18 (0.3 mg/kg, 1 mg/kg, and 3 mg/kg). Since many drugs of abuse are known to stimulate locomotor activity through dopamine-dependent mechanisms, locomotor data were also recorded during the trial. The collected locomotor data were analyzed to assess the effects of nAChRs on locomotor response to acute and repeated cocaine administration.
2.2.5. Statistical Analysis.

Data were analyzed by one-way ANOVA or two-way repeated measures ANOVA for comparison between treatments or time (Prism 4.0, Graphpad, San Diego, CA). Individual treatments were compared by Dunnett’s post-hoc analyses. All differences were considered significant at p < 0.05. Data were represented as mean ± SEM.
Chapter 3: SAR and Mechanisms of action of DCNR-I-235

3.1. Introduction to Study.

Structure-based design is emerging as an increasingly useful tool for drug discovery (Marrone et al. 1997; Patny et al. 2006). With the advancement in structural biology, structural information on drug targets (e.g. receptors, enzymes, and transporters) has become available and provides valuable insight for rational drug design. Introduction of ~ 50 compounds into clinical trials and numerous drug approvals demonstrate an important role for structure-based approach in the drug discovery process (Böhm and Stahl 2000; Jorgensen 2004). In the field of nAChR research, tremendous efforts have been made to gain insight into the structure of the integral membrane-bound nAChR. Specifically, a variety of approaches (e.g. X-ray crystallography, cryo-electron microscopic analysis, NMR studies, and computational modeling) have been utilized to understand the structure of nAChRs (Ryan and Baenziger 1999). As a result, substantial progress has been made in obtaining highly informative and well-defined view of functional pentameric forms of nAChRs. The comprehensive structure of the *Torpedo marmorata* muscle-type of nAChR at 4 Å resolution was obtained from electron diffraction studies (Unwin, 2005). More recently, an α1 extracellular domain of the mouse nAChR was characterized to a resolution of
1.94 Å. (Dellisanti et al., 2007). This first crystal structure of a mammalian nAChR provides important findings such as a main immunogenic region (MIR; an important structure for autoantibodies in myasthenia gravis) and a Cys loop. The identification of the acetylcholine binding protein (AChBP), a pentameric water-soluble protein found in the synapses of snails, also yielded important information on the structure and ligand binding properties of the nAChRs (Smit et al., 2001). As a glia-derived protein that binds to ACh in the synapse and modulates the cholinergic synaptic transmission, AChBP shares structural features with the extracellular ligand binding domain of nAChRs (Bourne et al. 2010). Furthermore, AChBP possesses all the amino acid residues required for classical nAChR ligand binding and displays pharmacological profiles close to those of nAChRs. For example, AChBPs bind classic nAChR agonists and antagonists such as ACh, nicotine, epibatidine, (+)-tubocurarine and α-bungarotoxin with a spectrum of affinities similar to that of homomeric α7 nAChRs (Karlin, 2002, Celie et al., 2004; Celie et al., 2005). Therefore, AChBP has been widely used as structural surrogates for the extracellular ligand binding domain of nAChRs. In particular, the x-ray crystal structures of AChBP identified in three different species (Lymnaea stagnalis, Bulinus truncates, and Aplysia californica) have been utilized as templates for nAChR homology models (Smit et al., 2001; Celie et al., 2005; Schaus et al., 2002).
AChBP-based nAChR homology models are used to address a number of questions regarding pharmacological and functional properties of nAChRs (e.g. gating dynamics, agonist binding, and agonist selectivity) (Le Novere et al., 2002; González-Cestari et al., 2009; Henderson et al., 2010; Pavlovicz et al., 2011; Parthiban et al. 2009). In addition to their role in elucidating basic principles of nAChR function, homology models developed based on AChBP structures have served as powerful tools in the drug discovery process. For example, nAChR homology models have been applied for structure-based virtual screening (SBVS) and led to the identification of novel chemical classes of nAChR ligands (Babakhani et al. 2009; Ulens et al. 2009; Mahasenan et al., 2011). In addition to the early stage of the drug discovery process, the late lead optimization stage has also benefited from studies with the nAChR homology models. Traditionally, lead optimization has been a lengthy process that involves modifications of the chemical structure of a known lead compound in an iterative manner to improve pharmacological profiles of ligands (Cavasotto and Phatak 2009). With nAChR homology models, however, information important for understanding receptor-ligand interactions and designing drugs with improved pharmacological properties can be obtained (Kombo et al. 2011; Slavov et al. 2010; Yamauchi et al. 2012). That is, various studies performed with nAChR homology models (e.g. ligand docking studies, binding energy calculations, and molecular dynamics simulations) can provide the rationale to make structural
modifications to the lead compound. Thus, homology models can support and expedite the challenging lead optimization process.

Previously, our group developed a human nAChR homology model (Mahasenan et al. 2011; Pavlovicz et al. 2011). In contrast to conventional nAChR homology models that were generated based on a single template, our model was constructed by using four crystallographic templates: the mouse α1 monomer and three molluskan species of AChBPs (Celie et al. 2004; Celie et al. 2005; Dellisanti et al. 2007; Hansen et al. 2005; Pavlovicz et al. 2011). Upon validation of the model through the use of nAChR ligands with known binding modes, the nAChR homology model has been utilized to aid our rational and structure-based drug design efforts. One of the most important findings of our modeling studies is the discovery of the novel allosteric binding of nAChRs (i.e. ‘β subunit site’) (Henderson et al. 2010; Henderson et al. 2012; Pavlovicz et al. 2011). As mentioned in the introduction, the ‘β subunit site’ shows structural and sequence diversity across nAChR subtypes and represents a promising molecular target to develop subtype-selective nAChR drugs. Thus, with the aim of utilizing its unique properties as a non-conserved allosteric binding site, the ‘β subunit site’ was used for structure-based virtual screening (SBVS) and led to the identification of several chemical classes of nAChR NAMs (Henderson et al. 2011; Mahasenan et al. 2011). The present study describes a novel chemical class of nAChR NAMs identified through the SBVS with the ‘β subunit site’.
Pharmacological activities of analogs bearing diverse substituents at different positions were characterized. Chemical/structural properties of these analogs were then correlated to their activity on nAChRs through the SAR analyses (Figure 3.1). In particular, effects of the analogs were evaluated on two nAChR subtypes that are abundantly expressed in the body: hα4β2 nAChRs and hα3β4 nAChRs. Furthermore, a series of functional and binding studies using the radio-labeled orthosteric ligand was performed with one of the reported analogs. These studies contributed to our understanding of the mechanisms by which the nAChR NAMs produce inhibitory effects on nAChR functions. Analogs reported in this chapter were synthesized by Dr. Dan Carper in the Coleman laboratory.

**Figure 3.1. The locations of the chemical modifications for SAR study.**
3.2. Results

3.2.1. SAR studies on Series 1 Analogs.

The series 1 SAR studies focused on investigating the effects of various aryl or alkyl groups at position $R_1$ and nitro vs. amine groups at position $X_1$ (Figure 3.1). Thirteen analogs incorporating different functional groups were synthesized and tested with regard to their functional activities as nAChR antagonists (Table 3.1). Among analogs with nitro substitution at the position $X_1$, an analog containing a 2-pyridyl group at $R_1$ (DCNR-I-136) inhibited the nAChR activity with IC$_{50}$ values of 18.5 μM and 18.2 μM on h$\alpha$4$\beta$2 and h$\alpha$3$\beta$4 nAChRs, respectively. Replacing the 2-pyridyl ring with a 6-trifluoromethyl-2-pyridyl group, an o-fluorophenyl substituent, or a p-fluorophenyl substituent (DCNR-I-136 vs. DCNR-I-203, DCNR-I-134, and DCNR-I-211) resulted in significant decreases in potency on both h$\alpha$4$\beta$2 and h$\alpha$3$\beta$4 nAChRs (IC$_{50}$ values > 50 μM, p<0.05). An analogous amide with a p-methoxyphenyl group (DCNR-I-163) was ~ 2 fold less potent than the 2-pyridyl containing analog (DCNR-I-136) on both subtypes. It showed nAChR antagonist activity with IC$_{50}$ values of 32.2 μM and 39.4 μM on h$\alpha$4$\beta$2 and h$\alpha$3$\beta$4 nAChRs, respectively. In contrast to other analogs that are equipotent on two subtypes, an indazole-substituted analog (DCNR-I-164) showed ~ 2 fold preference for h$\alpha$3$\beta$4 nAChRs against h$\alpha$4$\beta$2 nAChRs. It inhibited nAChR activity with IC$_{50}$ values of 47.8 μM and 22.4 μM on h$\alpha$4$\beta$2 and h$\alpha$3$\beta$4 nAChRs, respectively. An analog containing a cyclohexyl group at $R_1$ (DCNR-I-234) showed higher inhibitory potency than other analogs with aryl
groups at this position in this series. DCNR-I-234 inhibited nAChR function with IC$_{50}$ values of 9.1 μM and 7.2 μM on hα4β2 and hα3β4 nAChRs, respectively. An analog bearing 2-fluorophenyl-sulfonyl piperazine group at position R$_1$ (DCNR-I-151) showed nAChR antagonist activity with potency similar to that of DCNR-I-234. DCNR-I-151 inhibited the activity of nAChRs with IC$_{50}$ values of 6.5 μM and 7.8 μM on hα4β2 and hα3β4 nAChRs, respectively. Generally, reduction of the nitro group to the amine at position X$_1$ did not affect the compounds’ potency or selectivity. Among analogs with an amine substitution at position X$_1$, compounds containing o-fluorophenyl, p-methoxyphenyl, or a cyclohexyl moiety at R$_1$ (DCNR-I-175, DCNR-I-173, and DCNR-I-235) displayed equipotent nAChR antagonist activity compared to their nitro group containing counterparts. One exception for this trend is a set of analogs that are substituted with a p-fluorophenyl moiety at position R$_1$: For these analogs, reduction of the nitro group to the amine led to increases in potency on both hα4β2 and hα3β4 nAChRs (DCNR-I-211 vs. DCNR-I-228). The nitro containing analog (DCNR-I-211) showed no activity on both subtypes of nAChRs up to 100 μM. On the other hand, the amine containing analog (DCNR-I-228) inhibited the activity of nAChRs with IC$_{50}$ values of 43.0 μM and 58.4 μM on hα4β2 and hα3β4 nAChRs, respectively. DCNR-I-174 is the only analog substituted with the furan-2-ylmethanamine moiety at position X$_1$. Compared to DCNR-I-173 that contains the same functional group at the position R$_1$, DCNR-I-174 showed no inhibitory
activity on both subtypes. This indicates that the introduction of the furan-2-ylmethanamine moiety into position $X_1$ led to the loss of activity.
### Table 3.1. Series 1 SAR Studies

<table>
<thead>
<tr>
<th></th>
<th>hα4β2 nAChRs</th>
<th>hα3β4 nAChRs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\textsubscript{50} Value (μM)\textsuperscript{a}</td>
<td>n\textsubscript{h}\textsuperscript{b}</td>
</tr>
<tr>
<td>DCNR-I-136</td>
<td>18.5 (14.3-24.0)</td>
<td>-1.3</td>
</tr>
<tr>
<td>DCNR-I-203</td>
<td>&gt; 100\textsuperscript{c}</td>
<td>~\textsuperscript{d}</td>
</tr>
<tr>
<td>DCNR-I-134</td>
<td>73.2 (62.6-85.5)</td>
<td>-0.7</td>
</tr>
<tr>
<td>DCNR-I-211</td>
<td>&gt; 100\textsuperscript{c}</td>
<td>~\textsuperscript{d}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values represent geometric means (confidence limits), n = 4-10.

\textsuperscript{b}n\textsubscript{h}, Hill coefficient.

\textsuperscript{c}No activity up to 100 μM.

\textsuperscript{d}Could not be determined.

Continued
<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ Value (μM)$^a$</th>
<th>$n_h^b$</th>
<th>IC$_{50}$ Value (μM)$^a$</th>
<th>$n_h^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCNR-I-163</td>
<td>32.2 (15.8-65.4)</td>
<td>-0.7</td>
<td>39.4 (28.0-55.4)</td>
<td>-0.9</td>
</tr>
<tr>
<td>DCNR-I-164</td>
<td>47.8 (33.8-67.6)</td>
<td>-1.0</td>
<td>22.4 (18.1-27.7)</td>
<td>-0.8</td>
</tr>
<tr>
<td>DCNR-I-234</td>
<td>9.1 (5.5-14.9)</td>
<td>-1.1</td>
<td>7.2 (5.4-9.6)</td>
<td>-1.0</td>
</tr>
<tr>
<td>DCNR-I-151</td>
<td>6.5 (4.6-9.1)</td>
<td>-1.1</td>
<td>7.8 (5.2-11.8)</td>
<td>-0.7</td>
</tr>
</tbody>
</table>

$^a$Values represent geometric means (confidence limits), $n = 4$-$10$.

$^b$n$_h$, Hill coefficient.

$^c$No activity up to 100 μM.

$^d$Could not be determined.
Table 3.1. Continued

<table>
<thead>
<tr>
<th>Compound</th>
<th>hα4β2 nAChRs</th>
<th>hα3β4 nAChRs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; Value (μM)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>n&lt;sub&gt;h&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DCNR-I-175</td>
<td>&gt; 100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>~&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>DCNR-I-228</td>
<td>43.0 (34.3-53.8)</td>
<td>-1.2</td>
</tr>
<tr>
<td>DCNR-I-173</td>
<td>21.0 (17.4-25.5)</td>
<td>-1.3</td>
</tr>
<tr>
<td>DCNR-I-235</td>
<td>8.8 (7.1-10.9)</td>
<td>-1.4</td>
</tr>
<tr>
<td>DCNR-I-174</td>
<td>&gt; 100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>~&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values represent geometric means (confidence limits), n = 4-10.

<sup>b</sup> n<sub>h</sub>, Hill coefficient.

<sup>c</sup> No activity up to 100 μM.

<sup>d</sup> Could not be determined.
3.2.2. SAR Studies on Series 2 Analogs.

The series 2 SAR studies focused on the structural modifications on the core benzene ring as well as the nitro vs amine substitution at position X₁ (Figure 3.1). SAR studies were conducted on ten analogs substituted with different functional groups at positions X₁ and R₁ to determine how these structural features are related to their activity on nAChRs (Table 3.2). Among analogs with a nitro substituent at position X₁, analogs containing aliphatic groups (DCNR-I-187, DCNR-I-237, and DCNR-I-169) at position R₁ showed higher potency on both hα4β2 and hα3β4 nAChRs compared to aromatic-substituted analogs (DCNR-I-206 and DCNR-I-172). In addition, acylation of the piperazine ring (DCNR-I-187 vs. DCNR-I-237) or replacement of piperazine with a morpholine (DCNR-I-187 vs. DCNR-I-169) resulted in 2-3 fold increases in potency for both hα4β2 and hα3β4 nAChRs. Similar to the series 1 SAR studies, reduction of the nitro group to the corresponding amine generally did not affect potency or selectivity. As observed with the nitro containing counterparts, aliphatic-substituted compounds (DCNR-I-190, DCNR-I-238, and DCNR-I-244) were more potent against both hα4β2 and hα3β4 nAChRs compared to the aromatic-substituted compounds (DCNR-I-213 and DCNR-I-246). Acylation of the piperazine ring (DCNR-I-190 vs. DCNR-I-238) or replacement of piperazine with morpholine (DCNR-I-190 vs. DCNR-I-244) also led to 2-3 fold increases in inhibitory potency for both hα4β2 and hα3β4 nAChRs. The only analogs in this series that showed different potency depending on the substituent at position X₁
are those substituted with the acylated piperazine ring (DCNR-I-237 and DCNR-I-238). For these analogs, amine substitution was favored at position X₁ for inhibitory effects on nAChR function compared to the nitro substitution. The amine containing analog DCNR-I-238 was slightly more potent on both hα4β2 and hα3β4 nAChRs compared to the nitro containing derivative DCNR-I-237.
| Compound  |  
|----------|----------|  
|          | hα4β2 nAChRs | hα3β4 nAChRs |
|          | IC₅₀ Value (μM)ᵃ | nₕᵇ | IC₅₀ Value (μM)ᵃ | nₕᵇ |
| DCNR-I-187 | 88.0 (78.2-99.0) | -1.3 | 94.8 (68.3-132.0) | -1.3 |
| DCNR-I-237 | 38.8 (28.5-52.9) | -0.9 | 44.9 (37.7-53.4) | -0.9 |
| DCNR-I-169 | 24.4 (17.8-33.4) | -1.1 | 25.6 (22.6-29.0) | -1.0 |
| DCNR-I-206 | > 100ᶜ | ~ᵈ | > 100ᶜ | ~ᵈ |
| DCNR-I-172 | > 100ᶜ | ~ᵈ | > 100ᶜ | ~ᵈ |

ᵃValues represent geometric means (confidence limits), n = 4-10.
ᵇnₕ, Hill coefficient.
ᶜNo activity up to 100 μM.
ᵈCould not be determined.
<table>
<thead>
<tr>
<th>Compound</th>
<th>hα4β2 nAChRs IC₅₀ Value (μM)ᵃ</th>
<th>nₜᵇ</th>
<th>hα3β4 nAChRs IC₅₀ Value (μM)ᵃ</th>
<th>nₜᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCNR-I-190</td>
<td>77.7 (60.7-99.5)</td>
<td>-1.0</td>
<td>63.7 (45.1-90.9)</td>
<td>-1.1</td>
</tr>
<tr>
<td>DCNR-I-238</td>
<td>22.4 (17.0-29.5)</td>
<td>-0.9</td>
<td>22.0 (16.5-29.5)</td>
<td>-1.1</td>
</tr>
<tr>
<td>DCNR-I-244</td>
<td>23.6 (13.1-42.6)</td>
<td>-0.8</td>
<td>10.9 (7.3-16.3)</td>
<td>-0.8</td>
</tr>
<tr>
<td>DCNR-I-213</td>
<td>&gt; 100ᶜ</td>
<td>~ᵈ</td>
<td>&gt; 100ᶜ</td>
<td>~ᵈ</td>
</tr>
<tr>
<td>DCNR-I-246</td>
<td>&gt; 100ᶜ</td>
<td>~ᵈ</td>
<td>&gt; 100ᶜ</td>
<td>~ᵈ</td>
</tr>
</tbody>
</table>

ᵃValues represent geometric means (confidence limits), n = 4-10.

ᵇnₜ, Hill coefficient.

ᶜNo activity up to 100 μM.

ᵈCould not be determined.
3.2.3. SAR Studies on Series 3 Analogs.

In the series 3 SAR studies, structural modifications were made at the 3 and 5 positions of the aryl ring and at the amide linkage (Figure 1, positions X_1, X_2, and X_3). An analog substituted with a nitro moiety at the positions X_1 and X_2 (DCNR-I-222A) inhibited receptor functions with IC_{50} values of 14.2 μM and 22.0 μM on hα4β2 and hα3β4 nAChRs, respectively. On the other hand, an analog containing an amino group at positions X_1 and X_2 (DCNR-I-223) was 2~3 fold less potent than the nitro containing analog. DCNR-I-223 showed antagonist activity on hα4β2 and hα3β4 nAChRs with IC_{50} values of 44.0 μM and 50.8 μM, respectively. DCNR-I-231A and DCNR-I-231B are the only analogs that lack amide linkage at the position X_3. An analog with a nitro group at position X_1 (DCNR-I-231A) displayed no activity on either hα4β2 or hα3β4 nAChRs, whereas an analog with an amino group at position X_1 (DCNR-I-231B) inhibited receptor function with IC_{50} values of 37.1 μM and 57.4 μM on hα4β2 and hα3β4 nAChRs, respectively.
<table>
<thead>
<tr>
<th>Compound</th>
<th>hα4β2 nAChRs IC₅₀ Value (μM)ᵃ</th>
<th>hα4β2 nAChRs nₜᵇ</th>
<th>hα3β4 nAChRs IC₅₀ Value (μM)ᵃ</th>
<th>hα3β4 nAChRs nₜᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCNR-I-222A</td>
<td>14.2 (11.9-16.9)</td>
<td>-0.8</td>
<td>22.0 (15.4-31.4)</td>
<td>-0.9</td>
</tr>
<tr>
<td>DCNR-I-223</td>
<td>44.0 (35.9-54.0)</td>
<td>-1.2</td>
<td>50.8 (42.1-61.3)</td>
<td>-1.3</td>
</tr>
<tr>
<td>DCNR-I-231A</td>
<td>&gt; 100ᶜ</td>
<td>~ᵈ</td>
<td>&gt; 100ᶜ</td>
<td>~ᵈ</td>
</tr>
<tr>
<td>DCNR-I-231B</td>
<td>37.1 (27.4-50.4)</td>
<td>-0.8</td>
<td>57.4 (46.5-71.0)</td>
<td>-0.8</td>
</tr>
</tbody>
</table>

ᵃValues represent geometric means (confidence limits), n = 4-10.
ᵇnₜ, Hill coefficient.
ᶜNo activity up to 100 μM.
ᵈCould not be determined.
3.2.4. Mechanisms of Action.

DCNR-I-235 is one of the most potent compounds among the 27 analogs reported in this chapter (Table 3.1). In functional studies, DCNR-I-235 inhibited hα4β2 nAChR activity with an IC$_{50}$ value of 8.8 μM (7.1-10.9 μM) (Figure 3.2A). The inhibitory effects of DCNR-I-235 (10 μM) on hα4β2 nAChR functions were not surmountable with increasing concentrations of the orthosteric agonist epibatidine (Figure 3.2 B). This result suggests that DCNR-I-235 inhibited nAChR function through a non-competitive mechanism. In order to assess the effects of DCNR-I-235 on the apparent affinity of the orthosteric ligand, we also performed binding assays with the $[^3]$H]epibatidine. First, in the homologous competition binding assays, $[^3]$H]epibatidine was incubated with increasing concentrations of unlabeled epibatidine in the absence or presence of DCNR-I-235 (10 μM). As seen in figure 3.3 A, DCNR-I-235 did not affect the overall shapes, IC$_{50}$ values, and Hill coefficients of the homologous competitive inhibition curves. This indicates that DCNR-I-235 did not influence the apparent affinity of the orthosteric ligand at the concentration tested. Heterogeneous competition binding assays were also performed to investigate the effects of various concentrations of DCNR-I-235 on the binding of epibatidine to nAChRs. In this assay, the binding of $[^3]$H]epibatidine to the hα4β2 nAChRs was measured in the presence of six different concentrations of DCNR-I-235 (1-100 μM). As seen in figure 3.3.B, DCNR-I-235 decreased $[^3]$H]epibatidine binding in a concentration-dependent manner. However, DCNR-I-235 did not produce complete inhibition of
[^3]H]epibatidine binding and achieved maximum inhibition of 39.0 ± 0.7 % at 100 μM.

Figure 3.2. Effects of DCNR-I-235 on hα4β2 nAChR function.
The effect of DCNR-I-235 on nAChR function was investigated using HEK tsA201 cells expressing hα4β2 nAChRs as described in Chapter 2.
Concentration-response effects of DCNR-I-235 on hα4β2 nAChRs (A) and concentration-response effects of epibatidine on hα4β2 nAChRs in the absence (■) or presence (□) of DCNR-I-235 (10 μM) (B) were expressed as a percentage of peak fluorescence responses for 1 μM epibatidine. Data points represent means ± SEMs (n=4-8).
Figure 3.3. Effects of DCNR-I-235 on $[^3]H$epibatidine binding to hα4β2 nAChRs. Binding assays with $[^3]H$epibatidine were performed as described in the Experimental Section using HEK tsA201 cells expressing hα4β2 nAChRs. Inhibition of $[^3]H$epibatidine binding to hα4β2 nAChRs by unlabeled epibatidine in the absence (■) or presence (□) of DCNR-I-235 (10 μM) (A) and inhibition of $[^3]H$epibatidine binding to hα4β2 nAChRs by various concentrations of DCNR-I-235 (B) were expressed as a percentage of control specific binding. Values shown are means ± SEMs (n=5-6).
3.2.5. Discovery of Novel Hits through the Ligand-based Approach.

Among the benzamide analogs reported in this chapter, DCNR-I-235 is one of the three most potent compounds that show nAChR antagonist activity with IC\textsubscript{50} values less than 10 μM. In addition, a series of functional and binding studies suggest that DCNR-I-235 inhibits nAChR function through a non-competitive and allosteric mechanism. Based on its potency as a nAChR antagonist and well-characterized mechanism as an allosteric modulator, DCNR-I-235 was further used to identify new nAChR NAMs. In particular, based on the hypothesis that structurally similar molecules would have related biological activities, a similarity search with the Chembridge database (~ 900,000 compounds) was performed using DCNR-I-235 as a reference compound (Figure 3.4). The 2D/3D similarity search was performed by the Chembridge’s search engine with a threshold of 70% of similarity (www.hit2lead.com). An initial similarity search using DCNR-I-235 identified two compounds as hit molecules (i.e. Hit 1, 7933751; Hit 2, 7931643). Among these two hits, 79933751 (Hit 1) is identical to DCNR-I-234 from the McKay NAM library. Subsequently, these two hits from the initial similarity search were applied for the second round of similarity search and yielded a total of 6 additional hits (i.e. Hit 3, 7343236; Hit 4, 7939257; Hit5, 7932484; Hit 6, 7321388; Hit 7, 7335081; Hit 8, 7341260). Seven hits (hits 2-8) from the similarity searches were then purchased and tested for their activity on nAChR using HEK tsA201 cells stably expressing either hα4β2 and hα3β4 nAChRs. Among seven newly identified compounds, five were
shown to be active as antagonists against both hα4β2 and hα3β4 nAChRs: Hits 2-3 and Hits 5-7 showed IC$_{50}$ values in the low micromolar range for both subtypes (Table 3.4). As expected from a lack of subtype-selectivity of DCNR-I-235, none of the selected molecules displayed preferential activity for hα4β2 or hα3β4 nAChRs. Despite their structural similarity to DCNR-I-235, Hits 4 and 8 exhibited no activity on both subtypes up to 100 μM (Table 3.4). None of the hit compounds identified through the similarity search showed agonist or positive allosteric activity on nAChRs.
Figure 3.4. Hit identification through a ligand-based similarity search. The structures of compounds selected from the similarity search are shown. Among the seven newly identified compounds, five compounds (i.e. Hits 2-3 and Hits 5-7) showed antagonist activity on both α4β2 nAChRs and α3β4 nAChRs.
<table>
<thead>
<tr>
<th>ID # (Hit #)</th>
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<tbody>
<tr>
<td></td>
<td>hα4β2 nAChRs</td>
<td>hα3β4 nAChRs</td>
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<td>n$_h$$^b$</td>
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<td>~$^d$</td>
<td>&gt; 100$^c$</td>
<td>~$^d$</td>
</tr>
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<td>5.1 (4.2-6.3)</td>
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<td>&gt; 100$^c$</td>
<td>~$^d$</td>
</tr>
</tbody>
</table>

$^a$Values represent geometric means (confidence limits), n = 4-10.

$^b$n$_h$, Hill coefficient.

$^c$No activity up to 100 μM.

$^d$Could not be determined.

$^*$7933751 is identical to DCNR-I-234 from the McKay NAM library.
3.3. Discussion.

Driven by the potential therapeutic advantages of allosteric modulators, efforts in our laboratory have been focused on the discovery and development of nAChR allosteric modulators. As a result of 10 years of our drug discovery efforts, we generated a small focused chemical library of ~ 250 molecules comprising four different chemical classes of nAChR NAMs (i.e. the McKay NAM library) (González-Cestari et al. 2009; Henderson et al. 2010; Henderson et al. 2011). In addition, we also identified and characterized the allosteric ‘β subunit site’ that is located at the interface between α and β subunits approximately 10 Å from the orthosteric binding site (Henderson et al. 2010; Henderson et al. 2012; Pavlovicz et al. 2011). As a part of our ongoing effort to identify and develop nAChR NAMs, the present study reported SAR studies on the novel chemical class of nAChR NAMs. The benzamide analogs reported in this study were discovered through the SBVS with the previously identified and characterized ‘β subunit site’ (Mahasenan et al. 2011). For evaluation of pharmacological activity of the analogs on nAChRs, HEK tsA201 cells stably expressing hα4β2 or hα3β4 nAChRs were used. Previously, it has been shown that pharmacological properties of classical agonists (epibatidine and nicotine) and antagonists (mecamylamine and d-tubocurarine) determined using these cells lines are consistent with what has been determined using other cell lines stably expressing hα4β2 or hα3β4 nAChRs (Henderson et al. 2010). Among the 27 synthesized analogs, DCNR-I-151, DCNR-I-234, and DCNR-I-235 showed
potent antagonist activity on nAChRs. They inhibited the activity of $\alpha_4\beta_2$ and $\alpha_3\beta_4$ nAChRs with $IC_{50}$ in the low micromolar range. For comparison, $IC_{50}$ values of the non-competitive antagonist mecamylamine were 0.6 μM and 0.7 μM on $\alpha_4\beta_2$ nAChRs and $\alpha_3\beta_4$ nAChRs, respectively. The $IC_{50}$ values of the competitive antagonist d-tubocurarine were 9.2 μM and 6.8 μM on $\alpha_4\beta_2$ nAChRs and $\alpha_3\beta_4$ nAChRs, respectively. The most significant finding among series 1 SAR studies is that compounds with aliphatic substituents on the amide nitrogen (DCNR-I-234 and DCNR-I-235) showed higher potency compared to compounds substituted with an aromatic ring at that position (Table 3.1). Series 2 SAR studies revealed that 2-aminoaryl substituents on the benzene core are not desired for nAChR antagonist activity (Table 3.2). Series 3 SAR studies showed that the introduction of the electron-withdrawing 3-nitro group or the electron-donating 3-amino group to the phenyl core (DCNR-I-134 vs. DCNR-I-222A; DCNR-I-175 vs. DCNR-I-223) resulted in significant improvement in potency on both $\alpha_4\beta_2$ and $\alpha_3\beta_4$ nAChRs. Previously, we have addressed the importance of the carbonyl linkage for subtype selectivity toward $\alpha_4\beta_2$ nAChRs over $\alpha_3\beta_4$ nAChRs in other chemical classes of NAMs (Henderson et al. 2010; Henderson et al. 2012). However, the association between the carbonyl moiety and subtype-selectivity was not observed in this class of nAChR NAMs. The reduction of the amide linkage to the corresponding amine did not affect the analog’s subtype-selectivity (DCNR-I-134 vs. DCNR-I-231A; DCNR-I-175 vs. DCNR-I-231B). However, the reduction of the carbonyl moiety caused significant changes in
potency on a particular set of analogs. The reduction of the amide linkage to an amine on analogs bearing a 5-amino substituent at the benzene core (DCNR-I-175 vs. DCNR-I-231B) led to significant increases in potency on both subtypes. On the other hand, the same structural change for analogs containing a 5-nitro substituent at the phenyl core (DCNR-I-134 vs. DCNR-I-231A) did not induce changes in potency. The differential effects of the carbonyl linkage on compounds’ activity might be due to difference in binding modes of these two NAMs. Although they are hypothesized to act on the same allosteric binding site, it is possible that their molecular interactions within the site are different.

It is important to note that 10 of the 27 analogs showed no activity on both hα4β2 nAChRs and hα3β4 nAChRs up to 100 μM. For this observation, several explanations are possible. For example, steric interactions might be responsible for the loss of activity. The introduction of structurally bulky groups to the 2 position of the phenyl ring (DCNR-I-187 vs. DCNR-I-206, DCNR-I-172; DCNR-I-190 vs. DCNR-I-213, DCNR-I-246) may result in steric hindrance that could prevent the analogs from interacting with the receptors. Complex allosteric modulation may also account for the observation of significantly different pharmacological activities among these closely related analogs (e.g. DCNR-I-136 vs. DCNR-I-203; DCNR-I-175 vs. DCNR-I-228). Binding of allosteric modulators to their receptors can induce global conformational changes both on their own and in the presence of bound orthosteric ligand, which can affect multiple factors.
related to receptor activation (e.g. agonist affinity, agonist efficacy, and receptor activation/desensitization kinetics) (Christopoulos and Kenakin 2002; Gregory et al. 2007). Importantly, individual parameters can be altered independently. Thus, it is possible that structural modifications on allosteric modulators can have effects on each parameter in various degree and direction of modulation. For this reason, SAR studies on allosteric modulators have proven to be challenging, with minimal structural changes in a given chemotype leading to dramatic changes in biological activity (e.g. a complete loss of activity, changes from agonist to antagonist or vice versa) (Williams et al. 2009; Zhao et al. 2007). The complexity of allosteric modulation is further discussed below.

nAChR NAMs can produce inhibition of receptor function through multiple mechanisms. One possible mechanism is to decrease intrinsic efficacy of an agonist by perturbing agonist-induced biophysical processes that link the agonist binding to receptor activation. One of the NAMs previously discovered by our laboratory (i.e. KAB-18) is suggested to inhibit nAChR activity through this mechanism (Henderson et al. 2010; Henderson et al. 2012; Pavlovicz et al. 2011). In molecular dynamics simulation studies with the nAChR homology model, KAB-18 was shown to interfere with agonist binding-induced C-loop closure, a process considered to be important for channel gating (Pavlovicz et al. 2011). Another possible mechanism by which nAChR NAMs block or diminish receptor activity is through their effects on agonist binding affinity (Christopoulos and Kenakin
Although they do not directly compete with the orthosteric agonist, nAChR NAMs can induce changes in “geometry” of the orthosteric binding site and indirectly inhibit agonist binding to nAChRs. Reduced agonist binding to nAChRs subsequently results in decreases in receptor activity. When competitive binding assays are performed with radio-labeled orthosteric ligands and allosteric modulators, effects of NAMs acting through this mechanism are reflected as decreases in the orthosteric ligand binding. However, the effects of NAMs on the orthosteric ligand binding affinity can be more diverse in terms of the magnitude of modulation as opposed to orthosteric competitive antagonists. More specifically, orthosteric antagonists cause concentration-dependent decreases in the radio-labeled orthosteric ligand binding through simple competition. Thus, with increasing concentrations, competitive antagonists can always produce complete inhibition of the orthosteric ligand binding (Christopoulos 2002). In contrast, NAMs produce variable degrees of inhibition depending on the strength of modulatory effects that they have on the orthosteric binding sites. NAMs with weak modulatory effects can produce concentration-dependent decreases in orthosteric ligand binding but fail to cause complete inhibition (Gregory et al. 2007; May et al. 2007). On the other hand, NAMs that have strong modulatory effects can achieve complete inhibition of the orthosteric ligand binding. In this case, effects of NAMs on orthosteric ligand binding cannot be discriminated from those of competitive antagonists by simple competition binding assays. Thus, in order to validate the allosteric mechanisms,
different types of binding assays should be used. For example, allosteric mechanisms can be detected using dissociation kinetic assays with the radio-labeled orthosteric ligand. The dissociation kinetic assays exploit the fact that allosteric modulators can bind to the receptor simultaneously with the orthosteric ligand and form a ternary complex with the radio-labeled orthosteric ligand and the receptor (Christopoulos and Kenakin 2002; May et al. 2007). As allosteric modulators cause changes in the ternary complex prior to dissociation of the orthosteric ligand, they can alter the dissociation constant of orthosteric ligands, which can be detected with dissociation kinetic assays. Lastly, it is also possible that nAChR NAMs decrease receptor functions through their effects on receptor activation/desensitization kinetics. For example, NAMs can inhibit nAChR-mediated signaling by enhancing receptor desensitization. That is, NAMs can shift a portion of the nAChR population from a readily activable state to a desensitized state and thus decrease the functional consequence of receptor activation. Since desensitized nAChRs exhibit substantially higher affinity for orthosteric ligands than those in activable state, NAMs with this mode of action will lead to an ironical increase in the orthosteric ligand binding. Indeed, it has been shown that some NAMs previously identified by our laboratories (e.g. APB-8, KAB-24, and KAB-25) increase the total binding of $[^3]$H]epibatidine to native nAChRs, indicating that they act by enhancing receptor desensitization (González-Cestari et al. 2009). In addition to nAChR NAMs reported from our laboratory, many non-competitive antagonists of nAChRs (e.g. chlorpromazine,
vinblastine, dibucaine, and histrionicotoxin) have also been known to inhibit receptor-mediated response through their effects on receptor desensitization (Carp et al. 1983;Kato and Changeux 1976;McKay et al. 1985;Sine and Taylor 1982). Importantly, the above mentioned mechanisms are not necessarily mutually exclusive and allosteric modulators can change receptor functions through their independent effects on the above mentioned parameters (i.e. agonist efficacy, agonist affinity, and desensitization/sensitization kinetics). Overall effects of allosteric modulator on receptor functions would be determined by combinations of their effects on these parameters (Conn et al. 2009;May et al. 2007). Thus, mechanisms of allosteric modulators to change receptor activity can be quite complex. On the one hand, the complex nature of the allosteric modulation renders unpredictability in SAR effort and thereby complicates the rational design of allosteric modulators (Sharma et al. 2008). On the other hand, the intricate mechanisms of allosteric modulators allow them to produce to a larger repertoire of receptor activity and provide them additional benefits as therapeutic compounds (Conn et al. 2009;Gregory et al. 2007).

The series of molecules reported here are analogs of a hit from the SBVS with the allosteric ‘β subunit site’ (Henderson et al. 2010;Mahasenan et al. 2011). Therefore, these compounds are hypothesized to be allosteric modulators that interact with the ‘β subunit site’. In order to gain insight into the mechanisms that these analogs produce inhibitory effects on nAChR functions, a series of
functional and binding studies were performed. Based on its potent antagonist activity, DCNR-I-235 was selected and used for the mechanism studies. In the functional assay, DCNR-I-235 produced insurmountable inhibition of the maximal responses to epibatidine, while leaving the agonist’s EC₅₀ value unaffected (Figure 3.2 B). This data suggest that DCNR-I-235 is a non-competitive antagonist. Effects of DCNR-I-235 on orthosteric agonist binding affinity were also assessed through binding studies with [³H]epibatidine. In the homologous competition binding studies, DCNR-I-235 (10 μM) did not change overall shape, Hill coefficients, or IC₅₀ values of the homologous displacement curves. This indicates that the binding of epibatidine to nAChRs was not affected by DCNR-I-235 at the tested concentration, which is close to the compound’s IC₅₀ value obtained from the functional assay: DCNR-I-235 inhibited hα4β2 nAChR functions with IC₅₀ value of 8.8 μM. It would be worth mentioning that a saturation binding study could provide more direct measures on antagonists’ effects on the radio-ligand binding affinity (Mathiesen et al. 2006). However, it requires a large amount of the radiolabeled ligand. In order to circumvent this limitation, homologous competition binding assays were employed as an alternative to the saturation binding assays and allowed the indirect assessment of the effects that DCNR-I-235 has on the orthosteric ligand binding. In addition to the homologous competition binding assays, heterogeneous competition binding assays were also performed to determine the effects of various concentrations of DCNR-I-235 on the apparent affinity of epibatidine. The results of heterogeneous competition
binding assays revealed that DCNR-I-235 decreased \[^3\text{H}\]epibatidine binding in a concentration-dependent manner (Figure 3.3.B). This indicates that DCNR-I-235 binding causes a conformational change in nAChRs that compromises their interaction with the orthosteric agonist epibatidine. However, the inhibition of \[^3\text{H}\]epibatidine binding by DCNR-I-235 was incomplete and produced maximum of 39.0 ± 0.7 % inhibition at 100 μM. This indicates that \[^3\text{H}\]epibatidine still can occupy the orthosteric binding site in the presence of DCNR-I-235 and thus suggests that DCNR-I-235 is an allosteric modulator of nAChRs.

In conclusion, the present study described the SAR studies of benzamide analogs on nAChRs. Among 27 benzamide analogs reported here, three compounds (DCNR-I-151, DCNR-I-234, and DCNR-I-235) showed potent antagonist activity on nAChRs with IC\(_{50}\) values lower than 10 μM. A similarity search of the Chembridge database with DCNR-I-235 further led to the identification of novel compounds that inhibit the activity of nAChRs with IC\(_{50}\) values in low micromolar range. A series of functional and binding studies performed with DCNR-I-235 suggested that it inhibited nAChR function through non-competitive and allosteric mechanisms. Considering that these analogs were identified by the SBVS with the allosteric binding site (i.e. β subunit site), the characterization of DCNR-I-235 as a nAChR NAM documents the successful utilization of the structural information for the identification of novel drug scaffolds. However, it would be important to realize that none of the reported
analogs showed preferential activity for either hα4β2 or hα3β4 nAChRs. Further studies aimed to address this issue of subtype-selectivity are warranted.
4.1. Introduction to Study.

The ongoing research efforts of our laboratory have been in the discovery of nAChR ligands that have high subtype-specificity. Our overall strategy for designing and identifying subtype-selective nAChR ligands is to target allosteric binding sites. Compared to the highly conserved orthosteric binding sites, allosteric binding sites typically display higher divergence across receptor subtypes (Christopoulos 2002; Taly et al. 2009). Therefore, allosteric modulators have potential to attain a high degree of subtype-selectivity by interacting with non-conserved amino acids within the allosteric binding sites. This chapter describes a proof-of-concept study to document the utilization of allosteric binding sites to discover drugs that have specificity for certain nAChR subtypes. In particular, the present study focused on the subtype-selectivity for hα4β2 nAChRs over hα3β4 nAChRs. As one of the main subtypes expressed in the central nervous system (CNS), hα4β2 nAChRs have been implicated in various brain diseases and disorders (e.g. nicotine addiction, anxiety, and depression). On the other hand, hα3β4 nAChR is largely expressed in the peripheral autonomic ganglia where it modulates the release of multiple neurotransmitters.
Due to the roles of hα3β4 nAChR in the autonomic ganglia, the activity of nicotinic drugs on this subtype has been postulated to mediate a wide range of autonomic side effects (e.g. constipation, urinary retention, dilation of the pupils, and postural hypotension) (Dani and Bertrand 2007; LLoyd et al. 1998; Taly et al. 2009). Therefore, with an interest in developing safer drugs for CNS applications, subtype-selectivity was pursued for the hα4β2 nAChRs over the hα3β4 nAChRs.

Previously, our laboratory has identified several chemical classes of nAChR NAMs (González-Cestari et al. 2009; Henderson et al. 2010; Henderson et al. 2011). These NAMs produced insurmountable antagonism, indicating that they inhibited the nAChR functions through non-competitive mechanisms. Importantly, some of these nAChR NAMs showed selectivity for hα4β2 nAChRs over hα3β4 nAChRs. An allosteric binding site for these NAMs was also identified through a combination of homology modeling, blind docking, and site-directed mutagenesis studies (Henderson et al. 2010; Henderson et al. 2012; Pavlovicz et al. 2011). As mentioned in Chapter 1, this site is named as the ‘β subunit site’ due to the fact that major amino acid residues comprising this allosteric site are located at the β subunit. The ‘β subunit site’ is sequentially and structurally diverse among subtypes, which provides an explanation for the relative subtype-selectivity shown with some of the NAMs (Henderson et al. 2010; Henderson et al. 2011). The diversity within the ‘β subunit site’ and the identification of subtype-selective NAMs acting on this site provide support for
our approach of targeting allosteric binding sites to develop subtype-selective nAChR antagonists.

In this chapter, the discovery of a novel chemical class of nAChR antagonists that allosterically modulate receptor activity is described. In particular, these nAChR NAMs were identified based on the hypothesis that nAChR drugs with high subtype-specificity can be identified through a rational drug design strategy. To this end, knowledge gained from our previous studies was utilized by incorporating multiple approaches encompassing ligand-based modeling, structure-based modeling, pharmacology, and medicinal chemistry. Four NAMs (i.e. KAB-18, DDR-5, DDR-13, and DDR-18) selectively targeting hα4β2 nAChRs provided information on physicochemical properties that might be important for subtype-specificity (Henderson et al. 2010). With its biochemical characteristics, the sequentially and structurally diverse allosteric binding site (i.e. the ‘β subunit site’) also contributed to lead identification. A lead compound of this study (9012620, (4-(allyloxy)-N-(6-methylpyridin-2-yl)benzamide), which was identified by using our knowledge of the subtype-selective nAChR NAMs and their allosteric binding site, shows a ~5-fold relative selectivity for hα4β2 nAChRs against hα3β4 nAChRs. Upon the discovery of 9012620, 26 analogs based on the lead compound were synthesized, permitting the development of a SAR for allosteric modulation of nAChRs based on the 9012620 scaffold. Analogs
reported in this chapter were synthesized by Dr. Sihui Long in the Werbovetz laboratory.

4.2. Results.

4.2.1. Pharmacophore and Ligand-Based Virtual Screening.

In order to identify novel chemical entities that exhibit subtype-selectivity, information obtained from our previous studies was utilized (González-Cestari et al. 2009; Henderson et al. 2010; Mahasenan et al. 2011; Pavlovicz et al. 2011). Initially, four NAMs (i.e. KAB-18, DDR-5, DDR-13, and DDR-18) previously identified by our laboratory were selected based on their preference for hα4β2 nAChRs against hα3β4 nAChRs (Henderson et al. 2010) (Figure 4.1). These NAMs were then used to generate an initial pharmacophore (Data not shown). In parallel, structure-based virtual screening (SBVS) using the allosteric binding sites for these four NAMs (i.e. β subunit site) was performed and led to the identification of four novel scaffolds that inhibit the activity of nAChRs with relative subtype-selectivity for hα4β2 nAChRs (Mahasenan et al. 2011). Among the top hits from this SBVS, Hit 2 ((5-amino-N-(6-methylpyridin-2-yl)-2-(piperidin-1-yl)benzamide) shares structural similarities with the four NAMs used to generate the initial pharmacophore (Figure 4.1). In addition, Hit 2 has a lower molecular weight; lacking the substitutions linked to the piperidine ring and thereby possessing potential to exhibit improved bioavailability (Figure 4.1). As Hit 2 shows a preference for hα4β2 nAChRs, shares structural similarity with the
four NAMs used for initial pharmacophore development, and possesses more desirable drug properties with regard to in vivo bioavailability, the initial pharmacophore was refined using Hit 2 from the SBVS. The refined pharmacophore reported here features three hydrophobic regions (HYD1, HYD2, and HYD3) and one hydrogen bond acceptor (HBA) (Figure 4. 2). This refined pharmacophore model was then applied for ligand-based virtual screening (LBVS) as described below.
Figure 4.1. Structures of the compounds used for pharmacophore generation. Asterisk (*) indicates chiral centers.
Figure 4.2. Proposed pharmacophore describing the features of subtype selective NAMs of nAChRs. The features of the pharmacophore model generated using GASP are illustrated. Three hydrophobic areas (HYD1, HYD2, and HYD3) and a hydrogen bond acceptor (HBA1) are marked. This pharmacophore was developed as described in the experimental section by Dr. Brandon Henderson.
4.2.2. Lead Compounds.

Chembridge’s CNS diversity set of small molecules (~10,000) were virtually screened using the refined pharmacophore (Figure 4.2) with 220 hits being identified. The hits were then ranked based on a query fit (QFIT) score and the pharmacological activity of the top 10 hits was evaluated as described in chapter 2 using HEK tsA201 cells stably expressing either hα4β2 nAChRs or hα3β4 nAChRs (data not shown). One of the top ranked hits from this LBVS is the focus of this study: 9012620, 4-(allyloxy)-N-(6-methylpyridin-2-yl)benzamide. As a lead molecule, 9012620 inhibited the activity of hα4β2 nAChRs with an IC_{50} value of 6.0 (3.4-10.6) μM with ~5-fold preference against hα3β4 nAChRs (Figure 4.3 A and 4.3 B; Table 4.1). On the other hand, SL-2-001 (4-(6-methylpyridin-2-yl)-4-propoxybenzamide), in which the propene moiety in the alkoxy portion of 9012620 was replaced with the propane moiety, showed no preference for either subtype; SL-2-001 produced antagonistic activity on both hα4β2 and hα3β4 nAChRs with IC_{50} values of 9.5 (3.7-24.1) μM and 11.1 (8.2-15.2) μM, respectively (Figure 4.3 A and 4.3 C; Table 4.1). Based on their potency and/or selectivity, 9012620 and SL-2-001 were selected as lead molecules and structural modifications were made at the pyridyl or alkoxy portions of these compounds as described in Figure 4.3 A. Analyses of SAR studies with 25 analogs of these lead compounds were then performed to determine how modifications are related to the compounds’ activity on nAChRs. With regard to their mechanisms of action, the effects of 9012620 and SL-2-001 were not
surmountable with increasing concentrations of epibatidine but led to a decrease in the maximum effect of epibatidine (Figure 4.4). This suggested that 9012620 and SL-2-001 do not compete with epibatidine for the orthosteric binding site but instead act as non-competitive antagonists of nAChRs.
Figure 4.3. Concentration-response effects of 9012620 and SL-2-001 on hα4β2 nAChRs and hα3β4 nAChRs. (A) Structure of the lead compounds with the location of the primary substitutions for SAR studies. The concentration-response effects of 9012620 (B) and SL-2-001 (C) were investigated on hα4β2 nAChRs (■) and hα3β4 nAChRs (□) as described in the experimental section. Values represent means ± SEMs (n = 4–7). Data are reported as a percentage of peak fluorescence responses for 1 μM (±)-epibatidine. IC<sub>50</sub> values of 9012620 and SL-2-001 are reported in Table 4.1.
Figure 4.4. Concentration-response effects of epibatidine in the absence and presence of lead compounds. The concentration-response effects of epibatidine were investigated in the absence (□) and the presence (■) of 9012620 (10 μM) (A) and SL-2-001 (10 μM) (B) on human α4β2 nAChRs, using calcium accumulation assays described in the experimental section. Values represent means ± SEM (n = 4).
4.2.3. SAR Studies on Analogs of 9012620.

In the first series of SAR studies, chemical modifications were made primarily to the alkoxy portion of the lead molecule, 9012620, to determine functional effects of those structural changes (Table 4.1). Concerning modifications in the alkoxy portion of 9012620, replacement of the propene group with a propane moiety (SL-2-001) resulted in an increase in potency against hα3β4 nAChRs with no change in potency on hα4β2 nAChRs; thus, this modification led to the loss of relative selectivity for hα4β2 nAChRs. Introduction of a methyl moiety (9059652) and replacement of the propene group with a propyne group (9061705) induced changes in functional activity on nAChR in a subtype-specific manner. While those changes led to decreases in potency against hα4β2 nAChRs, the same changes resulted in a trend toward increased potency against hα3β4 nAChRs. However, the increases in potency were not statistically significant (9012620 vs SL-2-001, p = 0.261; 9012620 vs 9061705, p = 0.143). 5561105 had structural modifications on both the pyridyl and alkoxy portions of 9012620; a methyl group was introduced to the methylpyridine in the pyridyl portion and the propene moiety in the alkoxy portion was replaced by an ethyl residue. These modifications led to a decrease in potency against hα4β2 nAChRs, whereas they caused an increase in potency against hα3β4 nAChRs. Overall, these structural changes decreased the selectivity ratio and 5561105 inhibited the activity of hα4β2 and hα3β4 nAChRs with comparable potency. The pyridyl portion of 9012620 was also modified using several different substitutions.
and functional effects of those changes were investigated (Table 4.2). Replacement of 2-methylpyridine with pyridine (6114880) caused decreases in potency for both hα4β2 and hα3β4 nAChRs. 6114880 still maintained relative selectivity for hα4β2, although the selectivity ratio decreased to ~3-fold. The introduction of halogen atoms (e.g. chlorine and bromine) led to decreases in potency on both hα4β2 and hα3β4 nAChRs. Bromine introduction (SL-2-062) resulted in ~11-fold and ~2-fold decreases in potency on hα4β2 and hα3β4 nAChRs, respectively, while the chlorinated analog (SL-2-057) showed no activity up to 100 μM on both subtypes. Replacement of 2-methylpyridine with 2-ethylpyridine (SL-2-054) led to a decrease in potency against hα4β2 nAChRs. However, potency against hα3β4 nAChRs was not affected by this substitution, leading to a decrease in the selectivity ratio. On the other hand, replacement of 2-methylpyridine with N-propylpyridin-2-amine (SL-2-070) or N-butylpyridin-2-amine (SL-2-067) resulted in decreases in potency on hα4β2 nAChRs and slight increases in potency on hα3β4 nAChRs.
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<td>1.3</td>
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<tr>
<td>9061705</td>
<td>19.4 (15.7-24.0)</td>
<td>-1.1</td>
<td>21.7 (15.3-31.0)</td>
<td>-1.6</td>
<td>1.1</td>
</tr>
<tr>
<td>5561105</td>
<td>17.1 (11.2-26.0)</td>
<td>-0.9</td>
<td>17.4 (14.9-20.4)</td>
<td>-0.8</td>
<td>1.0</td>
</tr>
</tbody>
</table>

$^a$Values represent geometric means (confidence limits), n = 5-10;

$^b$n$_h$, Hill coefficient;

$^c$Selectivity; Fold ratio of IC$_{50}$-hα3α4β nAChRs/IC$_{50}$- hα4β2 nAChRs.
### Table 4.2. SAR studies on the pyridyl portion of 9012620

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ Value (μM)$^a$</th>
<th>n$_h$$^b$</th>
<th>IC$_{50}$ Value (μM)$^a$</th>
<th>n$_h$$^b$</th>
<th>Selectivity$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>9012620</td>
<td>6.0 (3.4-10.6)</td>
<td>-0.7</td>
<td>31.7 (22.8-44.0)</td>
<td>-1.1</td>
<td>5.3</td>
</tr>
<tr>
<td>6114880</td>
<td>19.3 (14.5-25.7)</td>
<td>-1.0</td>
<td>61.4 (40.6-92.8)</td>
<td>-1.3</td>
<td>3.2</td>
</tr>
<tr>
<td>SL-2-062</td>
<td>70.1 (53.9-91.2)</td>
<td>-1.1</td>
<td>65.5 (54.4-78.9)</td>
<td>-0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>SL-2-057</td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>~$^e$</td>
</tr>
<tr>
<td>SL-2-054</td>
<td>20.5 (15.5-27.3)</td>
<td>-1.3</td>
<td>27.4 (14.6-51.2)</td>
<td>-0.9</td>
<td>1.3</td>
</tr>
<tr>
<td>SL-2-070</td>
<td>14.8 (10.6-20.8)</td>
<td>-2.0</td>
<td>11.7 (8.2-16.8)</td>
<td>-2.1</td>
<td>0.8</td>
</tr>
<tr>
<td>SL-2-067</td>
<td>22.4 (12.4-40.4)</td>
<td>-1.6</td>
<td>19.2 (13.7-26.9)</td>
<td>-1.7</td>
<td>0.9</td>
</tr>
</tbody>
</table>

$^a$Values represent geometric means (confidence limits), n = 5-10;

$^b$n$_h$, Hill coefficient;

$^c$Selectivity; Fold ratio of IC$_{50}$-h$_{3α4β}$ nAChRs/IC$_{50}$- h$_{α4β2}$ nAChRs.

$^d$No activity up to 100 μM;

$^e$Could not be determined.
4.2.4. SAR Studies on Analogs of SL-2-001.

In the second series of SAR studies, SL-2-001 was used as the basis of comparison and functional effects of different substitutions on the pyridyl portion of SL-2-001 were explored (Table 4.3). Similar to the SAR studies on 9012620, introduction of halogen atoms to the pyridyl portion of SL-2-001 produced significant decreases in potency for both subtypes. Analogs with bromine or chlorine substitutions (SL-1-065 and SL-I-021) either showed weak inhibitory activity (IC$_{50}$ > 50 μM) or no effects up to 100 μM on both subtypes. Replacement of 2-methylpyridine with pyrazine (SL-2-063) resulted in a ~3-fold decrease in potency on hα4β2 nAChRs and a ~2-fold decrease in potency on hα3β4 nAChRs. Substitution of 2-ethylpyridine at the pyridyl portion of SL-2-001 (SL-2-038B) led to ~5-fold and ~2-fold decreases in potency on hα4β2 and hα3β4 nAChRs, respectively. Four analogs that have different alkylamine substitutions at the pyridine ring provided insight into the functional effects of chain length on the pyridyl portion of SL-2-001. Analogs incorporating longer alkylamine side chains showed lower potency on both hα4β2 and hα3β4 nAChRs, suggesting an inverse correlation between chain length and potency. Introduction of structurally bulky groups to the pyridine ring led to loss of activity against both hα4β2 and hα3β4 nAChRs (SL-1-083, SL-1-095, and SL-1-096, SL-1-089, SL-1-085, SL-1-75, SL-1-087, and SL-I-093).
<table>
<thead>
<tr>
<th>Compound</th>
<th>IC(_{50}) Value (μM)(^{a})</th>
<th>(n_h) (^{b})</th>
<th>IC(_{50}) Value (μM)(^{a})</th>
<th>(n_h) (^{b})</th>
<th>Selectivity(^{c})</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL-2-001</td>
<td>7.5 (3.6-15.5)</td>
<td>-1.3</td>
<td>11.5 (8.0-15.2)</td>
<td>-0.8</td>
<td>1.5</td>
</tr>
<tr>
<td>SL-1-065</td>
<td>80.7 (49.3-132.0)</td>
<td>-1.0</td>
<td>&gt;100(^{d})</td>
<td>~(^{e})</td>
<td>~(^{e})</td>
</tr>
<tr>
<td>SL-1-021</td>
<td>&gt;100(^{d})</td>
<td>~(^{e})</td>
<td>&gt;100(^{d})</td>
<td>~(^{e})</td>
<td>~(^{e})</td>
</tr>
<tr>
<td>SL-2-063</td>
<td>23.2 (16.4-32.6)</td>
<td>-1.2</td>
<td>22.5 (12.0-42.0)</td>
<td>-0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>SL-2-038B</td>
<td>37.6 (22.6-62.7)</td>
<td>-1.7</td>
<td>29.2 (27.1-31.5)</td>
<td>-1.6</td>
<td>0.8</td>
</tr>
<tr>
<td>SL-2-017</td>
<td>15.4 (10.5-22.6)</td>
<td>-1.4</td>
<td>12.6 (6.4-24.6)</td>
<td>-1.1</td>
<td>0.8</td>
</tr>
</tbody>
</table>

\(^{a}\)Values represent geometric means (confidence limits), \(n = 5-10\);  
\(^{b}\)\(n_h\), Hill coefficient;  
\(^{c}\)Selectivity; Fold ratio of IC\(_{50}\)-h\(\alpha 4\beta 2\) nAChRs/IC\(_{50}\)-h\(\alpha 3\beta 4\) nAChRs.  
\(^{d}\)No activity up to 100 μM;  
\(^{e}\)Could not be determined.  

Continued
### Table 4.3. Continued

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ Value (μM)$^a$</th>
<th>$n_H^b$</th>
<th>IC$_{50}$ Value (μM)$^a$</th>
<th>$n_H^b$</th>
<th>Selectivity$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="SL-2-014" /></td>
<td>54.1 (24.7-118.0)</td>
<td>-0.8</td>
<td>77.8 (42.2-143.0)</td>
<td>-0.7</td>
<td>1.4</td>
</tr>
<tr>
<td><img src="image" alt="SL-2-010" /></td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>&gt;100$^d$</td>
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<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>~$^e$</td>
</tr>
<tr>
<td><img src="image" alt="SL-1-083" /></td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>~$^e$</td>
</tr>
<tr>
<td><img src="image" alt="SL-1-095" /></td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>~$^e$</td>
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<tr>
<td><img src="image" alt="SL-1-096" /></td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>~$^e$</td>
</tr>
</tbody>
</table>

$^a$Values represent geometric means (confidence limits), $n = 5-10$;

$^b$n$_H$, Hill coefficient;

$^c$Selectivity; Fold ratio of IC$_{50}$-h3α4β nAChRs/IC$_{50}$- h4β2 nAChRs.

$^d$No activity up to 100 μM;

$^e$Could not be determined.
Table 4.3. Continued

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ Value ($\mu$M)$^a$</th>
<th>$n_h^b$</th>
<th>IC$_{50}$ Value ($\mu$M)$^a$</th>
<th>$n_h^b$</th>
<th>Selectivity$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="SL-1-089" /></td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>~$^e$</td>
</tr>
<tr>
<td><img src="image" alt="SL-1-085" /></td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>~$^e$</td>
</tr>
<tr>
<td><img src="image" alt="SL-1-075" /></td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
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<tr>
<td><img src="image" alt="SL-1-087" /></td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>~$^e$</td>
</tr>
<tr>
<td><img src="image" alt="SL-1-093" /></td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>&gt;100$^d$</td>
<td>~$^e$</td>
<td>~$^e$</td>
</tr>
</tbody>
</table>

$^a$Values represent geometric means (confidence limits), n = 5-10;

$^b$n$_h$, Hill coefficient;

$^c$Selectivity; Fold ratio of IC$_{50}$-h$\alpha$3$\beta$4 nAChRs/IC$_{50}$- h$\alpha$4$\beta$2 nAChRs.

$^d$No activity up to 100 $\mu$M;

$^e$Could not be determined.
4.3. Discussion.

Computer aided drug discovery (CADD) has become an increasingly useful tool to facilitate many steps of the drug discovery process such as identifying hits, enabling de novo design of ligands, and modeling ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties (Jorgensen 2004; Shoichet et al. 2002; Yang and Huang 2006). In particular, virtual screening (VS) has been widely used to guide lead identification by employing various statistical analyses and algorithms designed to search large chemical libraries in silico (Walters et al. 1998). Ligand-based virtual screening (LBVS) takes advantage of available information for known ligands such as structures, the shape of individual fragments, and electrostatic properties; whereas SBVS exploits the knowledge of the structure of the target protein (Floriano et al. 2004; Jain 2004). In this study, a novel scaffold (9012620) was identified through a virtual screening, using ligand-based and structure-based approaches.

Previously, our laboratory identified NAMs (i.e. KAB-18, DDR-5, DDR-13, and DDR-18) that showed relative selectivity for hα4β2 nAChRs against hα3β4 nAChRs (Figure 4.1) (Henderson et al. 2010). Furthermore, a combination of homology modeling, blind docking, and site-directed mutagenesis identified the ‘β subunit site’, a novel allosteric binding site on the receptor where these NAMs interact (Henderson et al. 2010; Henderson et al. 2012; Pavlovicz et al. 2011). In
contrast to the orthosteric binding site, the ‘β subunit site’ showed reduced sequence conservation. The ‘β subunit site’ was then used for SBVS with the hypothesis that its sequence and structural diversity could provide molecular foundations to develop selective nAChR drugs. Supporting this hypothesis, hits from this SBVS showed preference for hα4β2 nAChRs (Mahasenan et al. 2011). Interestingly, one of the hits from the SBVS, (5-amino-N-(6-methylpyridin-2-yl)-2-(piperidin-1-yl)benzamide; Hit 2) shares the following structural features with the previously identified four subtype-selective NAMs (Figure 4.1): 1) The carbonyl group is attached to the aryl ring and 2) the aryl ring has a six-membered ring at the ortho position relative to the carbonyl substitution. In order to identify a novel chemical scaffold with relative selectivity for hα4β2 nAChRs, the above mentioned information obtained from the previous studies was utilized. Initially, chemical and structural features of four subtype-selective NAMs (i.e. KAB-18, DDR-5, DDR-13, and DDR-18) were used to generate a pharmacophore. This initial pharmacophore was then refined using the structure of Hit 2, one of the hits from SBVS (Mahasenan et al. 2011), as it 1) shows relative selectivity for hα4β2 nAChRs over hα3β4 nAChRs, 2) displays structural similarity to the four NAMs used to develop the initial pharmacophore, and 3) has physicochemical properties associated with more desirable ADMET properties. The refined pharmacophore was subsequently applied to the LBVS and led to the identification of 9012620, a lead molecule of this study. In particular, with an aim of developing CNS active drugs, we performed the LBVS using Chembridge’s
CNS diversity set. Molecules contained in this library generally possess favorable physicochemical properties with regard to CNS bioavailability due to multiple strict sets of property filters. However, the strict criteria for drug-like properties result in the small-sized library with decreased molecular diversity. This might have limited the potential of identifying more potent and/or selective drugs, as well as the chemical diversity of hit molecules.

The lead molecule (9012620) exhibited hα4β2 nAChR-selective antagonism. 9012620 showed antagonist activity with an IC_{50} value of 6.0 (3.4-10.6) μM on hα4β2 nAChRs with ~5-fold preference against hα3β4 nAChRs (Table 4.1). Its non-competitive mechanism of action was demonstrated by a reduction in the maximum effects of the orthosteric ligand epibatidine (Figure 4.4A). Unlike the lead compound 9012620, the non-competitive nAChR antagonist mecamylamine showed non-selective actions on hα4β2 nAChRs and hα3β4 nAChRs (IC_{50} values of 0.6 μM and 0.7 μM on hα4β2 nAChRs and hα3β4 nAChRs, respectively). Similarly, the competitive antagonist d-tubocurarine displayed equipotent antagonist activity on the two subtypes with IC_{50} values of 9.2 μM and 6.8 μM on hα4β2 nAChRs and hα3β4 nAChRs, respectively.

In order to determine effects of structural modifications to the pyridyl or alkoxy portions of the molecules with regard to their functional activities, analyses of the SAR studies on analogs of 9012620 and SL-2-001 were performed. The
most significant finding from the SAR studies of analogs of 9012620 is that modification of propene to propane (9012620 vs. SL-2-001) led to loss of preference for α4β2, suggesting the importance of a double bond in the alkoxy portion of the molecules with regard to subtype-selectivity. Another analog containing the propene moiety (6114880) also exhibited ~ 3-fold preference for α4β2 nAChRs against α3β4 nAChRs. However, relative selectivity for α4β2 nAChRs was not observed for other analogs that also have the propene moiety (SL-2-062, SL-2-057, SL-2-05, SL-2-070, and SL-2-067). Introduction of halogen atoms, an ethyl moiety, and alkyl amines (SL-2-062, SL-2-057, SL-2-05, SL-2-070, and SL-2-067) to the pyridyl portion of the molecules abolished the subtype-selectivity. Comparison with their counterparts containing the propane moiety (SL-1-065, SL-1-021, and SL-2-038B) suggested that functional activities were mainly determined by the pyridyl portion of the molecules for this series of compounds and modification of propene to propane did not affect either potency or subtype-selectivity. Structural modifications to both the pyridyl and alkoxy portions of the molecules led to an analog (5561105) that has comparable potency on α4β2 and α3β4 nAChRs. Collectively, these data suggest that both portions of the molecules contribute to the selectivity for α4β2 nAChRs.

The SAR studies on analogs of SL-2-001 also provide insight into the functional effects of chemical modifications on the pyridyl portion of the molecules. With the aim of exploring the appropriate chain length linked to the
pyridine ring, a series of compounds (SL-2-001, SL-2-107, SL-2-014, SL-2-010, and SL-2-005) were synthesized. SAR studies on these compounds suggested that steric hindrance exists at the portion of the receptor-binding pocket where the pyridine moiety interacts. Increasing the chain length resulted in decreases in potency for both subtypes. Similarly, substitutions with structurally bulky moieties to the pyridine ring (SL-1-083, SL-1-095, SL-1-096, SL-1-089, SL-1-085, SL-1-075, SL-1-087, and SL-1-093) led to loss of activity on both subtypes.

In summary, this chapter describes the identification of a novel chemical class of NAMs of nAChRs through the application of multiple approaches. Iterative cycles of virtual screening using ligand-based and structure-based approaches led to the identification of the lead molecule, 9012620 (4-(allyloxy)-N-(6-methylpyridin-2-yl)benzamide). As the lead, 9012620 inhibits the activity of hα4β2 nAChRs with ~5-fold preference against hα3β4 nAChRs. To gain insight into the chemical/structural properties of the molecules pertaining to antagonistic activity on nAChRs, we have obtained 27 benzamide analogs and performed SAR studies on these analogs. It is notable that among the 27 analogs, two analogs (i.e. 9012620 and 6114880) exhibit preference for hα4β2 nAChRs, although the selectivity ratios remain modest. The study described here documents the successful utilization of a multifaceted approach for rational drug discovery employing computational modeling, pharmacology, and medicinal
chemistry, which has led to the identification of a novel chemical class of nAChR NAMs including molecules that show relative selectivity for hα4β2 nAChRs.

5.1. Introduction to Study.

Cocaine is a powerfully addictive psychostimulant. Due to its strong modulatory effects on the brain reward system, cocaine produces rapid and intense feeling of euphoria (Wise 2002; Xu et al. 2010). When repeatedly used, cocaine also induces chronic neuronal adaptations in the brain reward system that lead to abstinence symptoms such as craving, depression, and irritability (Goldstein and Volkow 2002). As a result, it remains to be one of the most problematic drugs of abuse and causes devastating social, medical, and economic consequences in the United States as well as globally (Degenhardt and Hall 2012). In addition to its effects on the brain reward system, cocaine also has profound impacts on multiple physiological systems (e.g. cardiovascular, respiratory, gastrointestinal, reproductive, and renal systems) (Karila et al. 2012). Thus, a number of medical complications including arrhythmia, respiratory arrest, pneumothorax and hyperthermia often arise from cocaine use (Benowitz 1993). In particular, toxic effects of cocaine on the cardiovascular system are frequent causes of drug-related emergency department visits and significantly contribute to the cocaine-related fatalities (Pilgrim et al. 2013). Unfortunately, no
pharmacotherapy is available for the treatment of cocaine addiction and the prevalence of cocaine use remains to be high. Indeed, cocaine ranks as the second most widely used illegal drug following cannabis with 14 million to 21 million users around the world (Degenhardt and Hall 2012). This emphasizes an urgent need for an effective therapeutic agent that can be used to help cocaine addicts quit the pathological drug-seeking behavior.

Accumulating data suggest that nAChRs are involved in various aspects of cocaine addiction. In preclinical studies, it has been shown that addiction-relevant behavioral responses to cocaine were influenced by manipulating nAChR activity with nicotinic agonists and antagonists. For example, pre-exposure to the nAChR agonist nicotine led to an increase in self-administration of cocaine under a fixed-ratio schedule and induced higher breakpoints under a progressive ratio schedule (Bechtholt and Mark, 2002). In addition, nicotine pretreatment enhanced cocaine-induced locomotor stimulation, a behavior mediated by dopaminergic signaling in the addiction-relevant brain area (Collins and Izenwasser, 2004). On the other hand, administration of the nAChR antagonist mecamylamine or genetic deletion of the β2 containing nAChRs decreased cocaine conditioned place preference (CPP) (Zachariou et al., 2001; Levin et al., 2000; Blokhina et al., 2005). Inactivation of nAChRs prevented escalation of drug self-administration with extended daily access to cocaine, a characteristic of pathological drug use (Hansen and Mark, 2007). Human studies
further suggested the roles that nAChRs play in cocaine addiction. 
Epidemiological studies indicated that nicotine and cocaine are often co-abused 
and concurrent use of nicotine is associated with increases in cocaine intake 
(Roll et al., 1996; Budney et al., 1993). Similarly, it has been shown that nicotine 
enhanced cue-induced craving of cocaine in human subjects, whereas 
mecamylamine attenuated it (Reid et al., 1999; Reid et al., 1998). Finally, in a 
recent small preliminary clinical trial, the nAChR partial agonist varenicline 
(Chantix) has been shown to have a small to moderate effect in decreasing 
cocaine use and reward (Plebani et al. 2012). Collectively, these studies suggest 
that nAChRs may play an important role in processing the rewarding effects of 
cocaine. Therefore, modulation of nAChR signaling may prove valuable as a 
therapeutic approach for the treatment of cocaine addiction.

As described in Chapter 1, the pentameric nAChRs exist as multiple 
subtypes that have different subunit compositions (combinations of α2-α10 and 
β2-β4) (Gotti et al. 2006b). Importantly, each subtype displays distinct functional 
properties and expression patterns, suggesting that each subtype may play 
different roles in certain physiological or pathological states (Dani 2001). Given 
this potential relevance of nAChR diversity, subtype-selective nAChR drugs may 
hold considerable potential as therapeutic agents. By selectively targeting certain 
nAChR subtypes involved in a disease of interest, they would modify impaired 
functions without perturbing other aspects of cholinergic neurotransmission (Taly
et al. 2009). Therefore, it would not be surprising that a great deal of effort has been directed to the development of drugs that can selectively target certain nAChR subtypes (Williams et al. 2011). A promising approach to design and develop subtype-selective nicotinic drugs would be to target allosteric binding sites. These sites are spatially distinct from the highly conserved endogenous ligand binding site and often exhibit sequence divergence among subtypes (Hogg et al. 2005; Taly et al. 2009). Due to this difference in the allosteric binding sites among subtypes, allosteric modulators can potentially exhibit a high degree of subtype-selectivity (Williams et al. 2011). In addition to the improved subtype-selectivity, allosteric modulators can also exhibit unique properties that will lead to a significant therapeutic benefit. For example, they are quiescent in the absence of endogenous ligand, which will lead to the preservation of normal spatial and temporal patterns of neurotransmission (Huang et al. 2011). Since they impart diverse effects on receptor behavior, 'fine-tuning' of the receptor activity can also be possible with allosteric modulators (Christopoulos 2002). Thus, the discovery and development of nAChR allosteric modulators is a promising field of research.

Previously, our laboratory has identified and reported phenylpropylpiperidine analogs as a novel chemical class of nAChR NAMs (Henderson et al. 2010). Importantly, some NAMs (i.e. KAB-18, DDR-5, DDR-13, and DDR-18) in this series displayed preferential activity for hα4β2 nACHRs against hα3β4
nAChRs: In *in vitro* studies using the cell lines stably expressing hα4β2 nAChRs or hα3β4 nAChRs, they were shown to inhibit the functions of the hα4β2 nAChRs with an IC₅₀ values in low micromolar range without affecting the hα3β4 nAChR activity up to 100 μM (Henderson et al. 2010; Henderson et al. 2012). In the site-directed mutagenesis studies, it has been shown that these subtype-selective NAMs interact with non-conserved amino acids within the ‘β subunit site’, an allosteric binding site identified and characterized by our laboratory (Henderson et al. 2010; Henderson et al. 2012; Pavlovicz et al. 2011). Thus, it was concluded that these NAMs attain relative selectivity for hα4β2 nAChRs by exploiting the structural divergence within the allosteric binding site. As one of the most prominent subtypes found in the CNS, α4β2 nAChRs have been implicated in several neuropathologies including addiction, anxiety, and depression (Gotti et al. 2006a; Picciotto et al. 1997; Picciotto et al. 2002). On the other hand, the α3β4 nAChR is a primary subtype responsible for synaptic transmission at autonomic ganglia and activities of nicotinic drugs on the hα3β4 nAChR have been associated with a number of autonomic side effects (Williams et al. 2011; Young et al. 2001). Thus, subtype-selectivity for hα4β2 nAChRs against hα3β4 nAChRs would be desired properties for drug candidates being developed for CNS indications.

The present study was carried out to evaluate the therapeutic potential of KAB-18, one of the subtype-selective nAChR NAMs (Figure 5.1). In particular, its
potential value in the treatment of cocaine addiction was examined with a hypothesis that KAB-18 would decrease the addictive properties of cocaine by blocking nAChR-mediated signaling. This hypothesis was suggested by the previous findings that the non-selective nAChR antagonist mecamylamine and the genetic deletion of β2 subunit of the nAChR block or diminish addiction-relevant biochemical and behavioral responses to cocaine (Blokhina et al. 2005; Hanson and Markou 2007; Zachariou et al. 2001; Zanetti et al. 2006). The effects of KAB-18 on the rewarding properties of cocaine were assessed in a CPP paradigm. Based on the assumption that drugs of abuse would produce positive emotional effects that can be associated with the environment in which the drug is administered, drug-induced CPP response has been wildly used as a behavioral measure of the rewarding value of drugs (Tzschtenteke 1998). Indeed, all drugs abused by humans produce CPP responses in rodents in that animals acquire preference for a place paired with the addictive drugs after two or three pairings (Huston et al. 2013). Thus, modulatory effects of KAB-18 on cocaine-induced CPP would suggest its potential utility in the treatment of cocaine addiction. Additional behavioral measures for the rewarding properties of cocaine used in this study are cocaine-induced hyperlocomotion and behavioral sensitization. Since all addictive drugs produce robust locomotor stimulating effects that are often sensitized with repeated drug exposures, locomotor responses to addictive drugs have been used as addiction-relevant behavioral indices (Weinshenker and Schroeder 2007). Thus, effects of KAB-18 on the
locomotor response to cocaine could provide insight into its therapeutic potential in the treatment of cocaine dependence. The effects of classical nAChR antagonists on the addictive properties of cocaine were also determined and compared with those of KAB-18. The three classical antagonists used in this study are mecamylamine, dihydro-β-erythroidine hydrobromide (DHβE), and methylycacaonitine (MLA) (Figure 5.1). To our knowledge, this is the first study where the in vivo effects of a nAChR NAM were evaluated in an animal model of cocaine addiction.

Figure 5.1. Chemical structures of nAChR antagonists used in the study.

Asterisk (*) indicates chiral centers.
5.2. Results.

5.2.1. Effects of KAB-18 on Cocaine-induced Conditioned Place Preference.

CPP responses were assessed by comparing the amount of time that mice spent in the cocaine-paired compartment during the post-conditioning preference test as compared to the pre-conditioning preference test. As seen in Figure 5.2, cocaine produced the rewarding effects as measured by CPP. Mice conditioned with cocaine (10 mg/kg, i.p.) spent significantly more time in the cocaine-paired compartment during the post-conditioning preference test than during the baseline pre-conditioning preference test (One-way ANOVA followed by Dunnett’s analysis, p < 0.05, Figures 5.2 and 5.3). The effects of KAB-18 on the rewarding properties of cocaine were evaluated by administrating KAB-18 to the mice during the conditioning phase. In particular, KAB-18 were given prior to cocaine- and saline pairing sessions in order to condition non-specific effects that it might have both to saline- and cocaine-paired compartments. As seen in Figure 5.2, pretreatment with KAB-18 attenuated the cocaine-induced CPP response, reaching at statistical significance at the dose of 1 mg/kg (i.p.) (One-way ANOVA followed by Dunnett’s analysis, p < 0.05). The effects of three classical nAChR antagonists on the rewarding properties of cocaine were also determined. Pretreatment with the non-selective nAChR antagonist mecamylamine (3 mg/kg, i.p.) significantly decreased the cocaine-induced CPP response (One-way ANOVA followed by Dunnett’s analysis, p < 0.05, Figure 5.3). On the other hand, pretreatments with the subtype-selective nAChR antagonists preferentially acting
at β2-containing nAChRs or α7 nAChRs (i.e. DHβE and MLA, respectively) did not modify the cocaine-induced CPP response (Figure 5.3). The pretreatment with KAB-18 and the other three nAChR antagonists alone did not induce CPP or conditioned place aversion (CPA) (Data not shown).
Figure 5.2. Effects of KAB-18 on the cocaine-induced CPP. Mice conditioned with cocaine developed a significant CPP response. Numbers shown in brackets indicate the doses of KAB-18 (mg/kg, i.p.). KAB-18 pretreatment attenuated the cocaine-induced CPP responses, reaching statistical significance at the intraperitoneal dose of 1 mg/kg. CPP score is defined as the time difference that mice spent in the cocaine-paired compartment during post-conditioning preference test versus pre-conditioning preference test. Data point represents the mean ± SEM (n=8-12 per groups). Data were analyzed by one-way ANOVA followed by Dunnett's post hoc analyses. # p < 0.05 compared to saline/saline, * p < 0.05 compared to saline/cocaine.
Figure 5.3. Effects of the nAChR antagonists on the cocaine-induced CPP.

Mice conditioned with cocaine developed a significant CPP response. Pretreatment with mecamylamine (3 mg/kg, i.p.) decreased cocaine-induced CPP responses, while such modulatory effects were not produced by DHβE (2.5 mg/kg, i.p.) or MLA (5 mg/kg, i.p.). CPP score is defined as the time difference that mice spent in the cocaine-paired compartment during post-conditioning test versus pre-conditioning test. Data point represents the mean ± SEM (n=8-12 per groups). Data were analyzed by one-way ANOVA followed by Dunnett’s post hoc analyses. # p < 0.05 compared to saline/saline, * p < 0.05 compared to saline/cocaine.
5.2.2. Effects of KAB-18 on locomotor stimulating effects of Cocaine.

The effects of KAB-18 on the locomotor response to acute cocaine administration were determined by analyzing locomotor data recorded on the first cocaine-pairing session. In this session, KAB-18 was administered as a pretreatment 30 min prior to a cocaine injection. Locomotor activity was then measured for 30 min immediately after cocaine injection while animals were placed in the conditioning apparatus for cocaine-place pairings. Since the present study used mice that were naïve to cocaine, mice were exposed to cocaine for the first time during this session. When administered alone, cocaine at the dose of 10 mg/kg (i.p.) induced a robust locomotor stimulation (One-way ANOVA followed by Dunnett’s analysis, p < 0.05, Figures 5.4 and 5.5). KAB-18 pretreatment attenuated the locomotor stimulating effects of cocaine in a dose-dependent manner (Figure 5.4). Mice pretreated with KAB-18 showed lower locomotor response to cocaine compared to mice that did not receive KAB-18 pretreatment (One-way ANOVA followed by Dunnett’s analysis, p < 0.05 at 1 mg/kg and 3 mg/kg). KAB-18 did not stimulate or suppress spontaneous locomotor activity (Data not shown). The three classical nAChR antagonists were also tested with regard to their effects on cocaine-induced locomotor response. Pretreatment with mecamylamine, DhβE, and MLA produced a trend toward a decrease in cocaine-induced locomotor stimulation (Figure 5.5). However, their effect did not reach a statistical significance. Mecamylamine, DHβE, and MLA did not affect the spontaneous locomotor activity (Data not shown).
**Figure 5.4. Effects of KAB-18 on the cocaine-induced hyperlocomotion.**

Locomotor activity recorded on the first cocaine-pairing session is illustrated. KAB-18 was administered 30 min prior to saline or cocaine injection. Numbers shown in brackets indicate the doses of KAB-18 (mg/kg, i.p.). Locomotor activity was measured for 30 min immediately after saline or cocaine injection while animals were placed in the conditioning apparatus for the cocaine-place pairing. Cocaine produced a robust locomotor stimulation. When given as a pretreatment, KAB-18 attenuated the locomotor stimulating effects of cocaine in a dose dependent manner. Data are expressed as mean ± SEM of the total distance traveled during the 30 min period after saline or cocaine administration (n=8-12 per groups). Data were analyzed by one-way ANOVA followed by Dunnett’s post hoc analyses. # p < 0.05 compared to saline/saline, * p < 0.05 compared to saline/cocaine.
Figure 5.5. Effects of the nAChR antagonists on the cocaine-induced hyperlocomotion. Locomotor activity recorded on the first cocaine-pairing session is illustrated. nAChR antagonists were administered 30 min prior to saline or cocaine injection. Locomotor activity was measured for 30 min immediately after saline or cocaine injection while animals were placed in the conditioning apparatus for cocaine-place pairing. Cocaine produced a robust locomotor stimulation. Pretreatment with mecamylamine (3 mg/kg, i.p.), DhβE (2.5 mg/kg, i.p.), and MLA (5 mg/kg, i.p.) produced non-significant trend toward a decrease in cocaine-induced hyperlocomotion. Data are expressed as mean ± SEM of the total distance traveled during the 30 min period after saline or cocaine administration (n=8-12 per groups). Data were analyzed by one-way ANOVA followed by Dunnett’s post hoc analyses. # p < 0.05 compared to saline/saline, * p < 0.05 compared to saline/cocaine.
5.2.3. Effects of KAB-18 on behavioral sensitization to Cocaine.

In laboratory animals, repeated exposures to drugs of abuse produce behavioral sensitization, a phenomenon characterized by progressive augmentation in drug-induced behavioral responses (Robinson and Berridge 2001; Vanderschuren and Pierce 2009). Once established, the behavioral sensitization is long-lasting and persists for weeks or months after the last drug treatment (Robinson and Kolb 1997; Robinson and Kolb 1999). This phenomenon that occurs with repeated drug exposure is believed to be a reflection of drug-induced neuronal adaptations that play an important role in developing and maintaining the pathological drug-seeking behavior (Robinson and Kolb 1999; Wolf 2010). As such, it has been widely utilized as a prominent animal model to address several aspects of drug addiction (e.g. persistent and intensified drug craving) (Vanderschuren and Pierce 2009). In the present study, cocaine was repeatedly administered to mice during the conditioning phase for the cocaine-place pairings. The repeated administration of cocaine resulted in sensitization in its locomotor stimulating effects, which could be detected through the monitoring of the locomotor behavior during the four-trials of cocaine-conditioning sessions. As seen in Figure 5.6, locomotor response to cocaine was progressively increased upon repeated administrations (Two-way repeated measures ANOVA showed significant effects of drugs ($F_{1,33}=103.95$, $p<0.0001$), time ($F_{4,132}=11.42$, $p<0.0001$), and drug x time ($F_{4,132}=45.75$, $p<0.0001$)). As results, the same dose of cocaine (10 mg/kg, i.p.) produced ~ 1.5 fold greater
locomotor stimulating effects on the last cocaine-pairing session compared to the first cocaine-pairing session (Figure 5.6).

![Figure 5.6. Locomotor activity after injections of saline or cocaine during drug-pairing sessions. Mice were administered with either cocaine (10 mg/kg, i.p., closed circles) or saline (open circles) during the four drug-pairing sessions. Repeated administration of cocaine resulted in sensitization to its locomotor stimulating effects. Data represent mean ± SEM (n=8-12). Two-way repeated measures ANOVA showed significant effects of drugs ($F_{1,33}=103.95$, $p<0.0001$), time ($F_{4,132}=11.42$, $p<0.0001$), and drug x time ($F_{4,132}=45.75$, $p<0.0001$). # $p < 0.05$ compared to saline-administered group, * $p < 0.05$ compared to the first cocaine-pairing session.](image)
During all four cocaine-pairing sessions, KAB-18 pretreatment decreased the locomotor stimulating effects of cocaine (Figure 5.8). KAB-18 led to a statistically significant decrease in the cocaine-induced locomotor response at the dose of 3 mg/kg (i.p.), while it produced only a trend toward a reduction in cocaine’s locomotor stimulating effects at the lower doses. However, KAB-18 pretreatment did not block or attenuate the development of cocaine-induced behavioral sensitization. Mice pretreated with KAB-18 exhibited sensitized locomotor response following repeated administration of cocaine to the same extent as mice that were not pretreated with KAB-18. Pretreatments with mecamylamine, DHβE, and MLA appear to produce inhibitory effects on the cocaine-induced hyperlocomotion (Figure 5.8). However, the inhibitory effects on the behavioral responses to cocaine did not reach a statistical significance. Similar to KAB-18, mecamylamine, DHβE, and MLA also failed to prevent the development of the cocaine-induced behavioral sensitization when they were given as a pretreatment prior to cocaine administration.
Figure 5.7. Effects of KAB-18 on the cocaine-induced behavioral sensitization. Locomotor activity recorded during the four cocaine-pairing sessions is illustrated. KAB-18 was administered 30 min prior to saline or cocaine injections. Numbers shown in brackets indicate the doses of KAB-18 (mg/kg, i.p.). Data are expressed as mean ± SEM of the total distance traveled during the 30 min period after saline or cocaine administration. Data point represents the mean ± SEM (n=12-16 per group). Data were analyzed by two-way repeated measures ANOVA followed by Bonferroni post hoc analyses. # p < 0.05 compared to saline/saline, * p < 0.05 compared to saline/cocaine.
Figure 5.8. Effects of the nAChR antagonists on the cocaine-induced behavioral sensitization. Locomotor activity recorded during the four cocaine-pairing sessions is illustrated. nAChR antagonists were administered 30 min prior to saline or cocaine injection. Numbers shown in brackets indicate the doses of nAChR antagonists (mg/kg, i.p.). Data are expressed as mean ± SEM of the total distance traveled during the 30 min period after saline or cocaine administration. Data point represents the mean ± SEM (n=8-12 per groups). Data were analyzed by two-way repeated measures ANOVA followed by Bonferroni post hoc analyses. # p < 0.05 compared to saline/saline, * p < 0.05 compared to saline/cocaine.
5.3. Discussion.

For the last decade, a number of nAChR genetic mouse models that lack one or more nAChR subunits have been generated. Studies with these mouse models have significantly improved our understanding of physiological and pathological roles of individual nAChR subtypes and provided a rational basis for the development of subtype-selective nicotinic drugs. Ideally, drugs selective for specific nAChR subtypes will produce maximized therapeutic benefits. By selectively targeting nAChR subtypes involved in a disease of interest, they will produce therapeutic effects. At the same time, they are less likely to produce side effects that are caused by the non-selective activation or blockade of all receptor subtypes. One emerging approach to develop subtype-selective nAChR drugs is to target allosteric binding sites, a different site than the highly-conserved endogenous agonist binding site. Due to the sequence and structure differences within allosteric binding sites, allosteric modulators have potential to attain a high degree of subtype-selectivity. Many subtype-selective nAChR PAMs and NAMs discovered over the last decade clearly demonstrate the potential of exploiting allosteric sites to develop highly selective nAChR drugs (Abdrakhmanova et al. 2006; Faghih et al. 2009; Henderson et al. 2010; Henderson et al. 2011; Ng et al. 2007; Pandya and Yakel 2011).

The goal of the present study was to evaluate the therapeutic potential of KAB-18, a nAChR NAM that exhibited subtype-selectivity for hα4β2 nAChRs
against hα3β4 nAChRs. In particular, the potential utility of this subtype-selective nAChR NAM in the treatment of cocaine addiction was assessed based on accumulating evidence that suggest the involvement of nAChR in cocaine reward. Thus, the in vivo effects of KAB-18 on the addiction-relevant behavioral responses to cocaine were determined. The behavioral measures of the reinforcing properties of cocaine used in this study are cocaine-induced CPP response, cocaine-stimulated locomotion, and locomotor sensitization. In addition to KAB-18, three classical nAChR antagonists were also evaluated with regard to their effects on the behavioral response to cocaine. It would be worth mentioning that the three nAChR antagonists tested in this study possess distinct subtype-selectivity profile. Mecamylamine is a non-selective antagonist that blocks all nAChR subtypes (Hurst et al. 2013). DHβE preferentially acts on nAChRs containing β2 subunit (Dawson et al. 2013). MLA is a highly selective and potent antagonist of the α7 nAChR (Ward et al. 1990; Yum et al. 1996). Thus, although not directly designed to address the nAChR subtypes involved in cocaine addiction, the present study provided some insight into the involvement of different nAChR subtypes in mediating the rewarding properties of cocaine.

KAB-18 was shown to be effective in reducing the reinforcing properties of cocaine in a CPP paradigm. When given as a pretreatment, KAB-18 attenuated cocaine-induced CPP response at the dose of 1 mg/kg (i.p.) (Figure 5.2). On the other hand, KAB-18 alone did not induce CPP or CPA. This suggests that the
effects of KAB-18 on cocaine-induced CPP responses cannot be attributed to its intrinsic aversive properties. The non-selective nAChR antagonist mecamylamine also diminished the cocaine-induced CPP response at the dose that did not produce aversive effects. The demonstration of mecamylamine reducing the reinforcing properties of cocaine added to the existing evidence that suggests the therapeutic potential of nAChR antagonists for the treatment of cocaine addiction. In previous studies, it has been shown that mecamylamine decreased the cocaine reinforcement in a CPP paradigm and a self-administration paradigm (Blokhina et al. 2005; Levin et al. 2000; Zachariou et al. 2001). These animal studies are also in good agreement with a human study showing that mecamylamine reduces cue-induced craving for cocaine (Reid et al. 1999). Altogether, these previous data and the present study suggest that endogenous nAChR signaling plays a profound role in mediating the rewarding effects of cocaine. Indeed, nAChRs have well documented roles in various physiological processes involved in addiction (e.g. reward, emotion, motivation, and cognition) (Buccafusco and Terry 2000; Decker and Majchrzak 1992; Levin et al. 2006; Meyer et al. 1998). Furthermore, a number of drugs of abuse including cocaine are known to stimulate the release of ACh in brain regions involved in addiction and reward (e.g. prefrontal cortex, hippocampus and striatum) (Imperato et al. 1993; Mark et al. 1999). Therefore, cocaine-induced release of ACh and subsequent activation of nAChRs may be a critical event for the development of compulsive cocaine use. In particular, nAChRs may affect the addictive qualities
of cocaine by regulating activity of the mesolimbic dopamine system (Changeux 2010; Mathieu-Kia et al. 2002). According to the dopamine hypothesis, drugs of abuse produce motivational and rewarding effects and ultimately lead to addictive behaviors by potentiating dopaminergic neurotransmission in the mesolimbic dopamine system (Fibiger 1978; Wise 1978). This hypothesis is supported by numerous behavioral, pharmacological, neurochemical and biochemical studies demonstrating that all major classes of abused drugs (i.e. psychostimulants, opioids, nicotine, alcohol, and cannabinoids) increase dopaminergic neuron’s activity and/or dopamine release in the mesolimbic dopamine system despite the differences in their primary sites and mechanisms of action (Pierce and Kumaresan 2006). Thus, dopamine within the mesolimbic dopamine system is considered to be a critical player in drug reinforcement (Nisell et al. 1995; Ritz and Kuhar 1993). Importantly, nAChRs are highly expressed in the mesolimbic dopamine system and provide potent regulation to the system’s activity (Mathieu-Kia et al. 2002; Zoli et al. 2002). Activation of nAChRs has been shown to increase the firing rate of dopaminergic neurons and elevate extracellular dopamine levels within the system, while the blockade of nAChRs causes the opposing outcomes. Thus, it is possible that the nAChR antagonist mecamylamine diminishes the reinforcing effects of cocaine by decreasing the dopaminergic transmission within the mesolimbic dopamine system. In particular, if cocaine-induced release of ACh and subsequent activation of nAChRs play an important role in processing cocaine reward,
inactivation of nAChR activity by mecamylamine can lead to a decrease in the cocaine-induced CPP response. However, it would be also important to realize that alternative physiological or behavioral effects of mecamylamine may have contributed to its effects in reducing cocaine-induced CPP responses. One potential confounding effect would be related to the well-established roles that nAChRs have in associative learning and memory. Given the fact that the CPP paradigm involves associated learning dependent on drug-induced effects, the observed decreases in the cocaine-induced CPP responses could have resulted from non-specific deficits in the learning mechanisms caused by blockage of nAChRs. Indeed, mecamylamine has been shown to impair performance in a variety of learning and memory tasks (Marti Barros et al. 2004; Moran 1993; Schildein et al. 2002). However, the doses at which mecamylamine induces learning and memory deficits are higher than the one used in this study (i.e. 3 mg/kg, i.p.). For example, mecamylamine impaired the acquisition of passive avoidance at 8.2 mg/kg but not at 2.4 mg/kg (Hiramatsu et al. 1998). Similarly, administration of mecamylamine led to learning deficits as measured by spatial memory tasks at 10 mg/kg but not at 3 mg/kg (Decker and Majchrzak 1992). Thus, the effects of mecamylamine on cocaine-induced CPP response shown in this study would not probably result from a general learning deficit. Since mecamylamine can also block NMDA receptors at high concentration (> 10 mg/kg), it is also possible that the inhibition of NMDA receptor may have contributed to its inhibitory effects on the cocaine-induced CPP (Snell and
Johnson 1989; Young et al. 2001). However, this seems unlikely because antagonisms of NMDA receptor have been associated with facilitation rather than attenuation of the cocaine’s reinforcing effects. Collectively, the effects of mecamylamine in decreasing the cocaine-induced CPP response are likely due to a selective disruption of the rewarding properties of cocaine rather than due to its own aversive property, its effects on learning and memory impairment, and non-specific effects on NMDA receptors. In contrast to the mecamylamine, the nAChR antagonists with relative selectivity for β2 containing nAChRs and α7 nAChR (i.e. DHβE and MLA, respectively) did not modify the cocaine-induced CPP responses. This suggests that inactivation of DHβE-sensitive subtypes or MLA-sensitive subtype alone was insufficient to reduce the reinforcing effects of cocaine. Given the fact that DHβE and MLA did not modify the cocaine-induced CPP response, the inhibitory effect that KAB-18 has on the cocaine-seeking behavior is worth noting. The differential effects of KAB-18, DHβE, and MLA on the cocaine-induced CPP responses could be due to differences in their relative potency or pharmacodynamic effects on multiple nAChR subtypes.

Unfortunately, pharmacological activity of KAB-18 was characterized only with hα4β2 and hα3β4 nAChR subtypes and data on its comparative potencies on other nAChR subtypes are lacking. Therefore, an important unanswered question would be the relative contribution of nAChR subtypes to the inhibitory effects of KAB-18 on cocaine’s action. The potential subtypes that might be involved are α4α5β2, α4α6β2, α4α6β2β3, or α6β2β3 nAChRs (Quik et al. 2011; Zoli et al.
2002). Being highly expressed within the mesolimbic system, these nAChR subtypes have been shown to play an important role in modulating the dopaminergic transmission within the system. Thus, if activation of these subtypes is necessary for processing the rewarding properties of cocaine, their blockage with KAB-18 would decrease the cocaine-induced CPP responses. More thorough characterization of pharmacological activity of KAB-18 on these nAChR subtypes would provide invaluable information critical for developing therapeutic agents for the treatment of cocaine addiction based on nAChR antagonists.

Nearly all drugs abused by humans are known to produce locomotor stimulation. Since the locomotor stimulating effects appear to be mediated through the enhancement of dopamine transmission within the midbrain, locomotor response to the addictive drugs is often used as a supportive behavior measure for the rewarding properties of drugs. In this study, we recorded locomotor data during the cocaine pairing sessions to assess the modulating effects of KAB-18 on cocaine-induced hyperlocomotion. Similar to what has been shown for the cocaine-induced CPP, KAB-18 diminished the cocaine-stimulated locomotor activity in a dose-dependent manner. KAB-18 itself did not influence basal locomotor activity, suggesting its specificity on cocaine-induced locomotor response. On the other hand, mecamylamine, DHβE, and MLA produced non-significant inhibitory effects on the cocaine-stimulated locomotion. One
interesting observation was that mecamylamine was shown to be less effective in blocking cocaine’s locomotor stimulating effects than it was in blocking cocaine-induced CPP response. While mecamylamine significantly attenuated the cocaine-induced CPP response, it only caused a non-significant trend toward a reduction in the cocaine-induced hyperlocomotion. The differential effects of mecamylamine on cocaine-induced CPP and cocaine-stimulated locomotor activity suggest that two addiction-relevant behavioral indices are not necessarily correlated. Indeed, numerous studies conducted over the last decade provide potential explanation for this observation by refining neuroanatomical framework of the midbrain dopamine system with regard to sub-circuits responsible for drug-induced reward and hyperlocomotion (Figure 5.9) (Janhunen and Ahtee 2007). For example, the hyperlocomotive effects are suggested to be mediated by a dorsal striatum (mesostriatal dopamine pathway) that receives dopaminergic inputs from the substantia nigra pars compacta (SNc). On the other hand, the rewarding effects are believed to be processed by a ventral striatum (mesolimbic dopamine pathway) that receives dopaminergic innervation from the ventral tegmental area (VTA). Because the drug-induced reward and motor response are dissociable, it is also possible that blockage of nAChRs with mecamylamine produced differential effects on the two behavioral measures (Janhunen and Ahtee 2007). These findings also suggest that a caveat needs to be applied in using drug-induced motor response in the context of the addiction-related behavior.
Figure 5.9. nAChR receptors in the mesolimbic and mesostriatal dopamine pathways. The mesolimbic and mesostriatal pathways are two major components of the midbrain dopamine system (A). The mesolimbic pathway that originates from the VTA and terminates in the nucleus accumbens is highly implicated in reward processing. The mesostriatal dopamine pathway that originates from the SNc and projects to the dorsal striatum is involved in the control of locomotor activity. Expression patterns and pharmacological properties of nAChR subtypes differ between mesolimbic and mesostriatal dopamine pathways, suggesting differential contributions of nAChR to the functions of the two pathways (Janhunen and Ahtee 2007) (B). (Figure modified from Calabresi and Di Fillippo, Neuron, 2008). The nAChR subtypes expressed in the two dopamine pathways include but not limited to the one shown in the table. * denotes the potential inclusion of an additional, unspecified subunits.
In the present study, cocaine produced the progressively increased locomotor stimulating effects when it was repeatedly administered during the conditioning phase. This indicates that the behavioral sensitization has developed under the CPP paradigm. As the behavioral sensitization has been associated with several aspects of addiction behavior, modulatory effects of drug candidates on the development or expression of the behavioral sensitization may suggest their therapeutic utility in treating addiction. In the present study, however, none of the nAChR ligands prevented or attenuated the behavioral sensitization. Mice pretreated with one of the nAChR ligands including KAB-18, mecamylamine, DHβE, and MLA exhibited a marked sensitization following repeated cocaine administrations similar to mice that were not pretreated with the nAChR ligands. However, it would be important to mention that the present data on the behavioral sensitization should be interpreted with caution. In behavioral sensitization studies, mice are usually exposed to the addictive drugs on a daily basis and given a drug-free withdrawal phase before being tested for the sensitized response. On the other hand, the present study utilized the locomotor data recorded during the conditioning phase of the CPP paradigm to assess the sensitization effect of the repeated cocaine administration. Thus, mice in this study were administered with cocaine every other day and tested for the sensitized responses without a withdrawal phase. The differences in the sensitizing regimen and experimental procedures may account for the discrepancy with regard to the effects of mecamylamine on the development of
behavioral sensitization. In the previous study where mice were administered with cocaine on a daily basis and tested for the sensitization following the three weeks of withdrawal phase, mecamylamine pretreatment has been shown to prevent the development of cocaine-induced behavioral sensitization (Schoffelmeer et al. 2002). This stands in apparent contrast to the present study where mecamylamine pretreatment failed to modify the development of behavioral sensitization.

In conclusion, the present study is the first to demonstrate the in vivo effects of the nAChR NAM on cocaine-induced behavioral responses that are relevant to the addictive properties of cocaine. When given as a pretreatment, KAB-18 was shown to be effective in reducing cocaine-induced CPP and cocaine-stimulated locomotor activity. Considering a number of therapeutic benefits that can be provided by allosteric modulators, continued development of nAChR NAMs may lead to a novel therapeutic approach for the treatment of cocaine addiction. However, it would be important to realize that the pharmacological activity of KAB-18 was evaluated only on α4β2 nAChR and α3β4 nAChR. Further characterization of pharmacological activity that KAB-18 has on other nAChR subtypes would provide valuable information on nAChR subtypes that are involved in cocaine addiction.
Chapter 6. Summary and Conclusions

As described earlier, this dissertation research was conducted to achieve the following goals: 1) the rational design of nAChR NAMs with desired properties through multidisciplinary and iterative approaches; 2) the characterization of novel chemical classes of nAChR NAMs; 3) gaining an understanding of the molecular mechanisms underlying the inhibitory effects of the nAChR NAMs; 4) the evaluation of therapeutic potential of nAChR NAMs in in vivo model of cocaine addiction. Through multidisciplinary and iterative approaches, two novel chemical classes of nAChR NAMs were discovered. Previously known information on nAChR NAMs as well as their binding sites was successfully utilized through the use of various approaches (e.g. pharmacology, molecular biology, computational modeling, and medicinal chemistry). Each of these approaches contributed unique information and supported the rational drug discovery effort. SAR studies on two sets of analogs determined the chemical/structural properties of each chemotype relevant for antagonistic activity on nAChRs. It is notable that among the total 54 analogs reported here, three analogs displayed preferential activity for hα4β2 nAChRs or hα3β4 nAChRs. DCNR-I-164 (presented in Chapter 3) exhibited ~ 2 fold subtype-selectivity for Hα3β4 nAChRs against Hα4β2 nAChRs. On the other hand,
9012620 and 5561105 (presented in Chapter 4) showed ~ 5 and ~ 3 fold subtype-selectivity for Hα4β2 nAChRs against Hα3β4 nAChRs. With regard to the mechanisms of inhibitory activity of the reported nAChR NAMs, the series of functional and binding assays were performed with DCNR-235. The functional studies with Hα4β2 nAChRs revealed that DCNR-235 produced non-surmountable antagonisms of the responses to the epibatidine. The homologous competition binding assays demonstrated that the orthosteric ligand binding was not affected by DCNR-235 at 10 μM, which is close to its IC₅₀ value from the functional studies. In the heterogeneous competition binding assays, DCNR-235 caused concentration-dependent decreases in orthosteric ligand binding. However, it failed to completely inhibit orthosteric ligand binding and achieved maximum inhibition of 39.1 ± 0.7 % at 100 μM. Collectively, these functional and binding studies suggest that DCNR-I-235 is a nAChR NAM that decreases the receptor function by affecting both the efficacy and affinity of the orthosteric agonist. Evaluation of the in vivo effects of promising drug candidates in preclinical animal models is an important step of the drug discovery and development process. Thus, on the basis of the involvement of nAChRs in addiction to cocaine, therapeutic potential of the nAChR NAM KAB-18 was investigated in the cocaine addiction model. Similar to the classical non-selective nAChR antagonist mecamylamine, KAB-18 was shown to be effective in attenuating addiction-relevant behavioral responses to cocaine: Cocaine-induced CPP responses and hyperlocomotion. Given the fact that KAB-18 shows
subtype-selectivity for Hα4β2 nAChRs over Hα3β4 nAChRs, KAB-18 is expected to produce therapeutic effects in the management of cocaine addiction with reduced risk of side effects as opposed to the non-selective mecamylamine.

Although all of the specific goals were achieved, it would be worth noting some limitations of the work presented in this dissertation. First of all, pharmacological activity of nAChR NAMs was tested on Hα4β2 nAChRs and Hα3β4 nAChRs, which are abundantly expressed in the CNS and PNS, respectively. However, there exist many more nAChR subtypes that have different subunit compositions. Studies on other nAChR subtypes would enable the more thorough characterization of the nAChR NAMs reported in this study with regard to their subtype-selectivity. In particular, improved understanding on subtype-selectivity of KAB-18 would be of great interest. Since KAB-18 was effective in diminishing addictive properties of cocaine, profiling subtype-selectivity of KAB-18 would offer valuable insight for the nAChR subtypes involved in cocaine addiction. Second, among nAChR NAMs reported in this study, a limited number of analogs showed preferential activity for Hα4β2 nAChRs or Hα3β4 nAChRs against the other. Further studies are warranted to identify more subtype-selective nAChR ligands and to improve subtype-selectivity ratio. Finally, since drug addiction is a complex behavioral disorder, employment of other types of cocaine addiction models (e.g. fixed ratio or progressive ratio cocaine self-administration) might enable us to evaluate the in vivo effects of KAB-18 on various aspects of cocaine dependence.
Appendix A. Receptor Pharmacology and The McKay Laboratory


Today, the concept of receptors as physical entities that sense and transduce signals lies at the very heart of pharmacology. Located either in or on cell membranes, receptors bind to a variety of intrinsic and extrinsic signaling molecules (e.g. neurotransmitters, hormones, growth factors, odorant molecules, light, ions, and drugs) (Maehle et al. 2002). Importantly, interactions between receptors and signaling molecules are highly specific. Activation of receptors resulting from bindings of the respective signaling molecules then leads to the initiation of a defined biochemical pathway linked to the responses. Thus, interaction of signaling molecules with the receptor triggers biological responses. Receptor-mediated signaling transduction is the fundamental mechanism by which cells communicate each other. Since living organisms rely on communications within and between cells to maintain biological functions, receptors play critical roles in mediating and regulating a number of physiological processes. Thus, our understanding of biological processes is deeply rooted in this receptor concept. The relevance of receptors in biomedical science is also reflected in the plethora of drugs that use receptors as their molecular targets. However, the fact that the receptor concept is a basic tenet of pharmacology produces the tendency to take the current understanding of receptor for granted. As a result, there is little appreciation of how such an important concept was formulated and elaborated.
Needless to say, this should not be the case and it is necessary to be aware of the historical aspect of the receptor theory. Importantly, the receptor concept emerged from important insights of numerous extraordinary scientists. Early in the 20th century when the idea about receptor was born, the majority of drugs were unknown components of natural products (Rang 2006). Thus, the receptor concept as a theoretical framework to understand drug action was remarkably innovative. More importantly, the receptor concept has been continuously elaborated and refined over the last century based on a number of carefully performed studies. Thus, through the history of the drug receptor theory, one can not only deepen the understanding of present ideas on receptors but also learn the nature of the pharmacological research. Furthermore, learning the history of receptor theory can provide scientists with invaluable opportunities to critically evaluate the current state of the theory and recognize its limitations. An attempt to overcome the limitations would bring novel insights that will advance, devise, and improve our understanding of receptors. Perhaps, looking back and taking lessons from the past might be an important step to move forward in science. As this dissertation concerns receptors as molecular targets for drug discovery in general, and nicotinic acetylcholine receptors (nAhRs) in particular, this appendix section is devoted to the recollections of remarkable findings in history of the emergence and development of the pharmacological receptor concept.

The concept of receptor has a long history. The beginnings of the receptor idea can be traced back to the work of a French chemist, Louis Pasteur (1822-1895). As early as in the 1850s, he observed distinct biological activity of mirror-image crystals of
(-)- and (+)-tartaric acid and postulated specificity in biological responses. Only (+)-tartaric a natural form was metabolized by the microbes. About 30 years later, Emil Fisher also provided notion on the biological specificity. Through his study on sugar utilization by yeasts, he noticed that glycosidases exhibit substrate specificity for different types of sugar. Later, this German chemist attempted to explain the enzyme’s specificity by using a “lock and key” analogy. As specific interactions between drugs and receptors form the basis of the receptor theory, their insight into biological specificity should be acknowledged as a part of the history of receptor theory. The concept of receptor in the context of pharmacology began to be formulated at the end of the 19th century by John Newport Langley (1985-1925) and Paul Ehrlich (1854-1915) (Colquhoun 2006; Rang 2006). Interestingly, these two scientists worked independently with different backgrounds toward different goals. John Newport Langley was a physiologist who was interested in biological actions of naturally occurring alkaloids such as pilocarpine, atropine, curare, and nicotine. In 1905, he claimed the presence of a “receptive substance” at the effector tissues to explain the antagonism between nicotine and curare on skeletal muscle (Bennett 2000; Langley 1901). On the other hand, Paul Ehrlich was an immunologist who developed “side chain theory” based on his research with various toxins and chemical dyes (Strebhardt and Ullrich 2008). In his theory, he proposed that cells have multiple “receptive side chains” where toxins or dyes bind. He believed that the existence of the “receptive side chains” explains the specificity of immunological interactions as well as the selective binding of the dyes. Although his primary interest was not in drug-receptor interactions, his remarkable insight formed the basis for the receptor concept, which also assumes specific
molecular structures of a cell as a cause of biological specificity (Prüll 2003). A few years later in 1900, he replaced the term “receptive side chain” with “receptor” and generalized the receptor concept by suggesting that it could be applied to explain specificity of drugs. The pioneering idea around receptors was then strengthened by scientists who attempted to use mathematical analyses to explain and quantify a drug’s action on cells. Prominent pharmacologists contributing to this effort include A. V. Hill (1886-1977), A.J. Clark (1885–1941), R.F. Furchgott (1916-2009), and H.O. Schild (1906-1984) (Ariens 1954; Furchgott 1954; Furchgott 1964; Rubin 2007; Stephenson 1956). A major conceptual advance in the quantification of drug-receptor interaction was then made by E.J. Ariëns (1918-2002) and R.P. Stephenson (1925-2004) who provided a notion that drugs are not equally effective in producing a response once they bind to receptors. Ariëns introduced the concept of “intrinsic activities” to account for a spectrum of drug-produced functional responses ranging from full agonism to full antagonism (Ariens 1954). Similarly, R.P. Stephenson raised the concept of “spare receptor” and “efficacy” to explain the observation that a maximal response could be reached with submaximal receptor occupancy (Stephenson 1956). Despite significant support for the receptor theory, receptors remained to be viewed as an abstract concept and the existence of receptors were questioned. It was in late 1960 that this skepticism was put to rest when Cheun-Yuan Lee (1915-2001) discovered the snake venoms α-bungarotoxin and β-bungarotoxin that could bind to the nicotinic acetylcholine receptors (nAChRs) with high affinity (Lee and Chang 1966). Importantly, the binding of the toxins to nAChRs is highly specific and irreversible. These properties of the toxins opened the way to isolate nAChRs from the electric organs of the marine ray Torpedo and thus
proving that receptors physically exist. The advances in molecular biology then followed and provided extensive knowledge as to diversity and molecular properties of receptors (Lee 1972). The application of the micro-sequencing techniques makes genetic information for receptors available. Receptor point mutations through site-directed mutagenesis approaches reveal molecular interactions essential for binding of signaling molecules (i.e. ligands) to receptors. Gene-targeting techniques have been used to generate transgenic, knock-out, and knock-in mouse models. The genetically modified mouse models with altered receptor functions then provide in vivo models to assess the physiological role and pathological involvement of the receptors. As briefly summarized above, the concept of receptor was developed through a continuous process and many discoveries were persistently made to support and elaborate the receptor theory (Limbird 2004). With the concept of receptor established, considerable progress has been made in our understanding of biological systems. Improved understanding of the biological systems is then translated into therapeutic benefits by being used in formatting rationales for the development of new therapeutic agents. Therefore, the introduction of receptor as discrete physical entities with unique specificities was a landmark event in the history of pharmacology and many other biomedical science disciplines. However, it is also important to note that the current stage of receptor theory has several limitations as revealed by the advanced techniques and methodologies that enable the detection of previously non-observable phenomenon (e.g. receptor dimerization linked to agonism). As seen in the history of the receptor theory, an attempt to account for these findings is expected to further elaborate our understanding of receptors as well as a number of biological responses mediated by receptors.
A.2. Drugs as Research Tools.

In the history of pharmacology, drugs have been excellent tools of investigation. First of all, drugs with high specificity for certain receptors have been successfully used to characterize receptors. Indeed, identification and classification of receptors through the use of drugs have been the norm as demonstrated by numerous examples including Sir Henry Dale’s work with muscarine and nicotine, R.P. Ahlquist’s sub-classification of adrenergic receptors, and H.O. Schild’s classification of H₁ and H₂ histamine receptors (Ahlquist 1973; Ash and Schild 1966; Black et al. 1972; Dale 1914). In addition, drugs that alter biological processes mediated by target receptors can advance our knowledge of biological systems. For example, it was nicotine that significantly improved our understanding of the anatomical and functional organization of the autonomic nervous system (ANS). Nicotine’s properties that first activate and then block nerves in the autonomic ganglia were successfully exploited by John Newport Langley, one of the founders of receptor theory, and enables the clarification of many features of ANS. With the ability to alter receptor functions, drugs can also be used as probes to elucidate the physiological roles and pathological involvement of specific receptors. Appreciation of the physiological/pathophysiological roles of receptors then reveals the potential therapeutic utility of receptors and provides a rational basis for the novel strategies to treat diseases. One notable example would be Arvid Carlsson’s research with reserpine that led to the dopamine concept of Parkinson’s disease (PD) (Carlsson 2001a; Carlsson 2001b). He found that the catecholamine-depleting drug reserpine could cause animal to lose movement control, one of the cardinal symptoms of PD (Carlsson et al. 1957). Based on the effects of reserpine in decreasing dopamine levels in the brain, he
suggested that dopamine is an important neurotransmitter that controls movement and depletion of dopamine in the brain is a main cause of PD. His research with reserpine provided a therapeutic basis for the use of the dopamine precursor L-dopa to alleviate symptoms of PD. Today, L-dopa still remains as one of the most widely used therapeutic agents for PD. As exemplified above, drugs that selectively target specific receptors have values as research tools in addition to their therapeutic utility. Thus, discovery of drugs highly specific for receptors would be of great importance both in basic science research and clinical practice. Over the past 25 to 30 years, advancement of molecular biology and cloning techniques has led to the identification and characterization of a host of new receptors and receptor subtypes. However, functional roles of these newly identified receptors and their utility as viable therapeutic targets await discovery. Hence, it is an exciting time for drug discovery research where drugs that possess high specificity to certain receptors can open up a vast new field of research and ultimately be of therapeutic benefit.

**A.3. Drug Discovery in The McKay Laboratory.**

Neuronal nicotinic receptors (nAChRs) are important neurotransmitter receptors that play key roles in regulating neuronal functions. Widely expressed throughout the CNS and peripheral nervous system (PNS), nAChRs influence various aspects of neuronal communications by directly mediating synaptic transmission or indirectly regulating releases of multiple neurotransmitters (e.g. norepinephrine, serotonin, gamma aminobutyric acid (GABA), glutamate, and dopamine) (Role and Berg, 1996). Physiologically, signaling through nAChRs regulates a number of neural functions
including cognition, sleep, arousal, anxiety, food intake and CNS processing of pain (see Gotti et al, 2006). Importantly for human health, nAChRs are also involved in a number of neurological diseases such as schizophrenia, attention deficit hyperactivity disorder, Alzheimer’s disease, Tourette’s syndrome, Parkinson’s disease, autism, some types of epilepsy, and addiction (Gotti et al., 2006b; Lloyd and Williams, 2000). The involvement of nAChRs in a multitude of physiological and pathological processes makes nAChRs attractive targets for drug discovery.

While it is clear that nAChRs represent promising therapeutic targets for various diseases/disorders, the development of drugs that target nAChR has been slow. Major contributing factors that hamper drug discovery efforts in the nAChR arena are, (1) the pentameric nature of nAChRs with twelve genes encoding subunits (α2-α10 and β2-β4), (2) expression of multiple nAChR subtypes based on subunit (α and β) compositions, (3) limited knowledge of subunit composition of naturally expressed nAChRs, and (4) limited understanding of location and role of native nAChRs. These issues can be addressed with molecules that have high specificity for individual nAChR subtypes. Therefore, the availability of potent and subtype-selective nAChR ligands would provide pharmacological tools (1) to investigate the functional role of specific nAChR subtypes in regulating neuronal pathways in the CNS, (2) to clarify questions regarding alterations in the function of specific nAChRs in pathological states linked to nAChRs, (3) to localize the distribution of specific nAChR subtypes under normal and pathophysiological conditions via imaging techniques, and (4) to facilitate the development of new drugs as novel therapeutic interventions for diseases associated with nAChRs.
Over the past 10 years, the focus of The McKay Laboratory has centered on discovery of novel small molecules that modulate nAChR functions. In particular, the major goal of the research focused on the development of a “rational” method to identify molecules that can discriminate certain nAChR subtypes from others. At this point, it should be emphasized that a rational drug-design approach was much needed for nAChR area, where enormous time and effort failed to produce clinically useful drugs. Classical approaches for nAChR drug discovery have focused on binding sites for the endogenous neurotransmitter acetylcholine (i.e. orthosteric binding sites). The basic principle behind this approach is that drugs can mimic the effects of the endogenous agonist by binding to the same regions of the receptor or block agonist-induced nAChR activation by competing with ACh for the same binding site. Unfortunately, however, the orthosteric binding sites are highly conserved among subtypes. This homologous nature of the orthosteric binding sites engenders one of the major challenges in nAChR drug discovery: limited subtype-selectivity of nAChR drugs. Molecules that target the orthosteric binding sites often suffer from poor selectivity among nAChR subtypes and produce multiple side effects. Therefore, The McKay Laboratory’s strategy to develop subtype-selective nAChR drugs is to target binding sites that are distinct from the highly conserved orthosteric binding site (i.e. allosteric binding site). As greater structural diversity exists in allosteric sites than in orthosteric sites, it was hypothesized that molecules interacting with allosteric binding sites (i.e. allosteric modulators) have potential to show a high degree of subtype-selectivity. As a rational drug design approach, The McKay Laboratory utilizes multiple scientific methodologies. For example, various ligand-based approaches were used to exploit structural and chemical
properties of known nAChR allosteric modulators. In addition, The McKay Laboratory had added the nAChR homology model through collaboration with Dr. Chenglong Li’s laboratory by the early 2000s. The availability of a nAChR homology model enables the utilization of structure-based approaches. At an early stage of drug discovery process, the ligand-based and structure-based approaches coupled to virtual screening aid the identification of new lead molecules that possess promising properties. In addition to the lead identification process, The McKay Laboratory also makes use of ligand-based and structure-based computational methods in the lead optimization stage of the drug discovery process. A variety of ligand-based modeling methodologies including genetic algorithm similarity program (GASP), comparative molecular field analysis (CoMFA), comparative molecular similarity indices (CoMSIA), and pharmacophore determine chemical properties that would be favorable for the desired pharmacological activities. These chemical features are then taken into consideration to synthesize compounds that are structurally similar to the lead compound but have more desirable pharmacological activities (e.g. potency and subtype-selectivity). Similarly, various structure-based drug design paradigms including blind docking and molecular dynamics simulation provide insight into the binding pocket of the lead compounds as well as important intermolecular interactions occurring at the pocket. Understanding the biochemical properties of the binding site makes it possible to design analogs of the lead molecules that would have improved pharmacological profiles. Through the knowledge-based strategy, The McKay Laboratory has been able to facilitate the lead optimization process, which is traditionally based on a trial-and-error method that involves repeated cycles of synthesis and biological evaluation. In addition to the
computational methods, structure-activity relationship (SAR) analyses using functional data obtained from bioassays also provide a rationale for chemical modifications to be made on the lead molecule. Importantly, the lead optimization process is performed in an iterative manner rather than a single process. Through multiple iterations of the process, knowledge gained from one set of studies is continuously exploited to design the next set of molecules and ultimately lead to the molecule with optimized properties. For the last decade, the subtype of primary interest to The McKay Laboratory has been the α4β2 nAChR, a subtype highly implicated in nicotine addiction. However, the methodology behind The McKay Laboratory’s approach to develop selective drugs for the α4β2 nAChRs can be applied to any of the subtypes. Therefore, an important implication of The McKay Laboratory’s research is the development of strategy to design subtype-selective nAChR drugs.
Figure A.1. Drug Discovery in The McKay Laboratory.

The figure illustrates the history and milestone of drug discovery research performed in The McKay Laboratory.
A.4. A brief history of nAChR drug discovery in The McKay Laboratory.

In the following section, important findings of nAChR drug discovery research performed in The McKay Laboratory will be reviewed (Figure A.1). The McKay Laboratory’s research on nAChR allosteric modulators started with the methyllycaconitine (MLA). It is a naturally occurring norditerpenoid found in Delphinium species (commonly known as larkspurs) that possess potent antagonist activity at nAChRs. MLA shows high affinity for the homomeric α7 nAChR (Kᵢ value, 5-10 nM) and moderate affinity for α3β4 and α4β2 nAChRs (Kᵢ value, 1.3 μM) (Free et al., 2002a; Free et al., 2002b; Free and McKay, 2003; Free et al., 2003). Despite its potent activity on nAChRs, however, MLA has limited therapeutic utility due to its large molecular weight, high toxicity, and narrow therapeutic index. Therefore, it has been mainly used as a tool of investigation for exploring the heterogeneity of nAChR. In addition, MLA has served as a lead compound for developing novel nAChR antagonists, as the potent activity that MLA has on nAChRs suggests that there may be structural components of MLA that would produce favorable interactions with these nAChRs. Thus, it was hypothesized that isolation of specific regions of MLA would lead to novel compounds that have activity on nAChRs potentially with improved subtype-selectivity. This hypothesis was first tested by simplifying the MLA core structure, which led to the derivatives of MLA containing E ring (Figure A.1) (Bergmeier et al. 1999; Bergmeier et al. 2004; Bryant et al. 2002a; Bryant et al. 2002b). Initially, functional effects of the MLA analogs were evaluated by
neurosecretion assays using cultured bovine adrenal chromaffin cells. In these cells, nAChRs containing α3, α5, and β4 subunits are expressed and mediate adrenal catecholamine secretion. In neurosecretion assays, the E ring analogs of MLA produced inhibition of nAChR-mediated catecholamine release with IC$_{50}$ values in low micromolar ranges (1-11 μM). Their potency was comparable to those of classical nAChR antagonists including d-tubocurarine (IC$_{50}$ values, 2 μM), hexamethonium (IC$_{50}$ values, ~17 μM), and mecamylamine (IC$_{50}$ values, 0.1 μM) (Bryant et al. 2002a). Later investigative studies have demonstrated that the inhibitory effects of these analogs on nAChR function were not surmountable with increasing concentrations of the competitive agonist. On the other hand, these analogs did not show any affinity for the acetylcholine binding sites of either α7, α4β2, or α3β4 nAChRs up to 10 μM. Collectively, the functional and binding studies suggested that the E ring analogs of MLA do not bind to the orthosteric binding sites. Therefore, they were classified as nAChR negative allosteric modulators (NAMs) that non-competitively antagonize the receptor function. Following this initial study, synthetic efforts to define the structural determinants of interactions between MLA and nAChRs has continued, leading to a series of structurally less complex analogs of MLA. Importantly, some of the E ring analogs of MLA showed preferential activity for hα4β2 nAChRs against hα3β4 nAChRs (i.e. KAB-18, DDR-5, DDR-13, and DDR-18) (Henderson et al. 2010). For example, when evaluated with calcium accumulating assay using human recombinant nAChRs, KAB-18 antagonizes α4β2 nAChR function with
IC$_{50}$ value of 13.5 μM. However, it shows no effects on α3β4 nAChR function up to 100 μM. The discovery of drugs that show relative subtype-selectivity is considered to be one of the key findings made in The McKay Laboratory, because few subtype-selective drugs are available. In order to gain insight into the molecular basis for the observed subtype-selectivity, further studies were performed with these subtype-selective nAChR NAMs. Blind docking studies of KAB-18 with homology models of human nAChRs identified a potential binding site for KAB-18. The binding site proposed by the computational studies and potential interactions between KAB-18 and nAChRs occurring at the proposed binding site were then experimentally validated using site-directed mutagenesis studies. Interestingly, a novel binding site for the subtype-selective nAChR NAMs turned out to be located at the interface between α and β subunits in close vicinity to the orthosteric binding site (~10 Å) (Figure A.2). However, this novel binding site differs from the orthosteric binding site. For example, principal ligand-binding elements at the orthosteric binding sites are mainly provided by α subunits. At these sites, β subunits only serve as complementary parts of the binding pocket. In addition, the amino acid residues forming the orthosteric binding sites are highly homologous among nAChR subtypes. In particular, the residues of the principal side (i.e. α subunit) are conserved in all human nAChR subtypes. In contrast to the orthosteric binding sites, major molecular interactions between KAB-18 and nAChRs at the newly identified allosteric binding sites are derived from amino acid residues located at the β subunits (e.g. Thr58, Glu60,
Ser97, Ser133, Ser138, Ser142, Phe118, and Ser137) (Figure A.2). Thus, the novel allosteric binding sites are named ‘β subunit site’.
Figure A.2. The β Subunit site on human α4β2 nAChRs. The interface between α4 (green) and β2 subunits (blue) of nAChR homology model is illustrated. Residues that differ between the β2 and β4 subunits are marked with dark blue. The binding pocket where the orthosteric ligand epibatidine (grey) binds is the orthosteric binding site. Amino acid residues located at the α subunit provide major ligand-binding elements for epibatidine. The binding pocket where KAB-18 (magenta) binds is ‘the β subunit site’. KAB-18 interact nAChRs primarily through interactions with non-conserved amino acid residue in ‘the β subunit site’ (Figure taken with permission from Pavlovecz et al., 2011).
More importantly, the allosteric ‘β subunit site’ show amino acid sequence variation among nAChR subtypes. It is believed that this diversity accounts for the subtype-selectivity achieved by some of the nAChR NAMs. This unique allosteric binding site was then used for a structure-based virtual screening (SBVS) based on a hypothesis that the divergence of the ‘β subunit site’ can be exploited to develop subtype-selective nAChR drugs. The SBVS with the ‘β subunit site’ led to two novel chemical classes of nAChR NAMs; Sulfonylpiperzaine and Benzamide Analogs. Indeed, several compounds belonging to these classes exhibit preferential activity for human α4β2 against human α3β4 nAChRs. This demonstrates the successful utilization of a non-conserved allosteric binding site as a molecular foundation to develop subtype-specific drugs. The last scaffold was obtained from combinations of multifaceted strategy (e.g. pharmacophore modeling, ligand-based virtual screening, and structure-based approaches) using information gained from our previous studies. The scaffold contains a benzamide core structure and has been shown to antagonize nAChR functions through non-competitive mechanisms. In summary, 10 years of drug discovery efforts in The McKay Laboratory has yielded a small focused chemical library containing ~ 250 molecules with four distinct chemical scaffolds. Given that only a few drugs that target nAChRs have been developed, the discovery of novel chemical classes of nAChR drugs would be of great relevance. More importantly, the rational drug design approach of The McKay
Laboratory can be widely applied in the area of nAChR to expedite the drug discovery process.
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