STRUCTURE ACTIVITY RELATIONSHIPS OF NICOTINE ANALOGS AND *Erythrina* ALKALOIDS ON THE ALPHA 4 BETA 2 NICOTINIC ACETYLCHOLINE RECEPTOR

By

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To those I hold dearest: my parents

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Neuronal α4β2 nicotinic acetylcholine receptors (nAChRs) are the major high affinity nAChRs in the mammalian brain and have been implicated in mediating the dopamine-releasing and cognition-enhancing effects of nicotine. Nicotine, an alkaloid from the plant genus *Nicotiana*, is an agonist with a high affinity for the α4β2 receptor. Another high-affinity α4β2-nAChR ligand is dihydro-β-erythroidine (DHβE), which is a competitive antagonist from the plant genus *Erythrina*. The goal of this study was to assess structural activity relationships of these prototypic plant alkaloids for α4β2 through characterization of the receptor binding and functional properties of several natural, semi-synthetic and synthetic analogs.

Substituents were added at four different positions on nicotine: the 5'-, 3'- and 1'-N-positions on the pyrrolidine ring, and position 5 on the pyridine ring. We hypothesized that substituents at these positions would permit interaction with the α4β2 receptor yet

influence the functional properties of these compounds. Modifications at any of these positions resulted in a decreased affinity for α4β2 relative to nicotine. Six of eleven analogs displayed partial agonist activity, three were antagonists and the remaining two were inactive on human α4β2. Increasing substituent size at the 1'-N-pyrrolidine position greatly reduced affinity but had less influence on the maximum effects measured. The 5'-trans-methyl-(S)-nicotine had over 90-fold greater affinity than the cis-isomer and was an antagonist. Separated enantiomer fractions of 1'-ethyl-(S,R)-nornicotine and 5-phenyl-(S,R)-nicotine bound with 10-fold or greater difference in affinity to rat α4β2.

Two groups of *Erythrina* compounds were studied: the D-ring lactones (β-erythroidines) and the D-ring aromatic compounds. All alkaloids that had measurable affinity for the receptor also retained their full competitive antagonism. The aromatic compounds were the most active; erysovine displayed a higher affinity than its isomer erysodine. Opening the D-ring of β-erythroidine did not result in loss of activity; major determinants for binding to α4β2 must also exist within the other three rings. Finally, dihydro-β-erythroidine was more active than either β-erythroidine or tetrahydro-β-erythroidine.

CHAPTER 1
INTRODUCTION

> The brain is a monstrous, beautiful mess. Its billions of nerve cells—called neurons—lie in a tangled web that displays cognitive powers far exceeding any of the silicon machines we have built to mimic it. -Allman, 1989 page 3

The brain remains a mystery of intricate circuits that allow for acquisition, integration, and retrieval of information. This "tangled web" of neurons forms synapses that allow for information transfer through specialized methods of signaling, both chemical and electrical. Neurotransmitters are chemical signals that, upon binding to specific target sites on neurons (known as receptors) can change the electrical properties of the target cell. Receptors, along with neurotransmitters, are critical for communication between neurons.

The theory of receptors first originated in 1878, when the British physiologist John N. Langley was studying the actions of atropine and pilocarpine on salivary secretion of cats, and found that the inhibitory action of atropine could be overcome by increasing the concentration of pilocarpine. In 1905, Langley was studying the effects of nicotine on striated muscle in fowl when he first used the term "receptive substance" which (we now know) referred to the nicotinic acetylcholine receptor (nAChR).

Superfamily of Ligand-Gated Ion Channels

Nicotinic acetylcholine receptors belong to a superfamily of ligand-gated ion channels. The superfamily also includes 5-HT3, glycine and A and C type GABA (γ-amino butyric acid) receptors. Members of this superfamily of receptors all have a conserved sequence consisting of a pair of cysteines separated by 13 amino acids and

linked by a disulfide bridge; thus they are also known as the Cys-loop receptors (Kao and Karlin, 1986). Each of these receptors has a central ion channel, around which are organized five homologous subunits, to which endogenous neurotransmitter may bind and cause a conformational change: allowing ions (including sodium, potassium, calcium and/or chloride) to flow through the channel. The vertebrate nAChRs are cationic ligand-gated ion channels that allow for the passage of positively charged ions (cations) through the channel (Takeuchi and Takeuchi, 1960), whereas glycine and GABA receptors permit the flow of anions.

Characterization of Nicotinic Acetylcholine Receptors (nAChRs)

The nicotinic acetylcholine receptor is the best-studied of the ligand-gated ion channels. Its name arises from the fact that nicotine, a plant alkaloid extracted from tobacco, binds to these receptors, as does the endogenous neurotransmitter acetylcholine. A primary reason that nAChRs are well characterized is due to the use of tissues derived from the electric organs of certain fishes, including the *Torpedo*, which are a rich source of nAChRs (Feldberg *et al*. 1940). The receptors in these electric organs were found to have a high degree of homology to those of the vertebrate embryonic muscle type nAChR.

Studies of the Muscle-type nAChR

Receptors from the *Torpedo* were first affinity-purified to determine the number, stoichiometry, and subunits involved (Olsen *et al*. 1972). Each receptor was determined to be a 250-kilo-Dalton protein consisting of α, β, δ, and γ-subunits (Reynolds and Karlin, 1978; Lindstrom *et al*. 1979). When covalently labeling the subunits, the α-subunit was found to be twice as abundant as the others (Reiter *et al.* 1972; Weiland *et*

al. 1979). This led to the theory that the receptor consisted of two α-subunits in the formation of the pentameric receptor.

Acetylcholine (ACh) produces its effects on a cell by binding to the nAChR. The location of and number of binding sites on the *Torpedo* (muscle-type) receptor were determined by using snake venom peptides (α-toxins) that served as high-affinity labels for the ACh binding sites (Lee and Chang, 1966). The ability of ligands to bind to the receptor could now be studied using radiolabeled α-toxins that would compete with unlabeled agonists and antagonists for the ACh-binding site on the receptor (Weber and Changeux, 1974). One of the snake α-toxins, α-bungarotoxin (α-btx), bound to two sites per receptor oligomer, while the ratio of ACh to α-btx binding was close to one (Neubig and Cohen, 1979). These data indicated that there were two ACh binding sites for the *Torpedo* receptor. It was initially presumed that ACh was binding to some extracellular portion of the receptor because the positive charge of the quaternary ammonium of ACh prevents it from crossing the cell membrane. More specifically, Silman and Karlin (1969) showed that the ACh binding sites were located near a disulfide bond by using bromoacetylcholine to form a covalent bond with the receptor after reduction of exposed cysteines.

Muscle-type nAChR Stoichiometry

The stoichiometry of the *Torpedo* receptor subunits was confirmed as $(\alpha 1)_2(\beta 1)\gamma\delta$ in the early 1980s by Raftery *et al.* (1980) who performed N-terminal microsequencing of the subunits. These N-terminal sequences allowed for creation of degenerate oligonucleotides to probe the *Torpedo* cDNA library (Ballivet *et al.* 1982; Claudio *et al.* 1983; Noda *et al.* 1982, 1983). The sequences showed homology among the subunits;

and based on these sequences, hydrophobicity plots were created (Claudio *et al.* 1983) that led to models of how each of the subunits folds in the cell membrane. Hydrophobicity plots suggested that each subunit contains a large extracellular domain, followed by four hydrophobic transmembrane domains (known as M1 to M4): with a large cytoplasmic domain between M3 and M4 (Schofield *et al.* 1987; Unwin, 1993). The receptor is about 80 Å in diameter and extends 65 Å above the membrane, 35 Å below the membrane, and 40 Å across it. The central channel is about 30 Å in diameter, while the ACh-binding sites are located about 30 Å above the membrane (Unwin, 1993).

Availability of cDNAs allowed the development of expression systems. Expression systems allowed for manipulation of subunits by omissions and mutations that permitted further exploration of the ligand-binding domain. The difference between the α-δ and α-γ pairs of subunits accounted for the difference in affinity of curare (a competitive antagonist) binding to the wild type receptor (Blount and Merlie, 1989). The binding sites in the case of vertebrate adult muscle exist between α-δ and α-ε subunits, because the γ-subunit is replaced by an ε-subunit in adults to give a stoichiometry of $(\alpha 1)_2(\beta 1)\varepsilon\delta$ (Mishina *et al.* 1986). It has been proposed, based on DNA sequences, that the subunits that compose the muscle-type nAChR are the most recent evolutionary type of nAChR (estimated to have developed 490-520 million years ago); and that the most ancient form of nicotinic receptor is the homomeric receptor (Le Novere and Changeux, 1995; Ortells and Lunt, 1995).

Using Electron Microscopy to Study the Two-Dimensional Structure

Through the use of cryoelectron microscopy, the two-dimensional structure of nAChRs began to take shape. Unwin and colleagues (1988) established conditions for

forming ice crystals of nAChRs from *Torpedo* that showed the receptor to consist of five "barrel staves" in a circular arrangement surrounding a central channel at a resolution of 17 and 18 Å (Toyoshima and Unwin, 1988). As previously mentioned, each subunit is made up of four transmembrane domains known as M1 to M4. At a higher 9 Å resolution, it was possible for Unwin to study the electron densities of each subunit. Initial evidence (Wilson and Karlin, 1998) indicated that the M2 transmembrane domain of each subunit lined the central channel. It was also postulated that the four transmembrane domains were composed of α-helices based on their amino-acid sequence (Claudio *et al.* 1983; Devillers-Thiery *et al.* 1983; Noda *et al.* 1983; Unwin, 1993).

Unwin provided valuable data on the motion of nAChR activation by collecting images of the nAChR from *Torpedo* before and after a brief exposure to ACh at 9 Å. Results showed that upon activation, the subunits rotate on an axis parallel to the central channel. The data also corroborated the proposed location of channel gate as determined by Wilson and Karlin (1998) at a position halfway in the membrane postulated to be formed by leucine residues from each subunit (Unwin, 1995). At a resolution of 4.6 Å, the extracellular structures of the subunits appeared to be composed of β-sheets. These higher-resolution images also showed that these β-sheets form the extracellular entrance to the channel (which is lined by α-helices) by connecting it to the ACh-binding pockets and that a central channel exists with lateral openings below the membrane that serve as a filter for ions (Miyazawa *et al.* 1999).

Studies of Neuronal nAChRs

In the early 1990s the α7 gene was cloned from both chick and rat brain (Couturier *et al.* 1990; Schoepfer *et al.* 1988; Seguela *et al.* 1993). These receptors were shown to

have a very high affinity for α-btx. They were determined to be homomeric, or consist of five identical subunits. There also existed a group of nAChRs that did not bind α-btx with high affinity, but rather had a high affinity for nicotine or ACh and had a distinctly different labeling pattern from that of α7 (Clarke *et al.* 1985). This group of receptors was found to be composed of both α- and non-α subunits. Cloned subunits were classified as α subunits if they contained vicinal cysteines in their N-terminal domain, homologous to positions 192 and 193 in the *Torpedo*. The non-α subunits that lacked these vicinal cysteines were designated as β-subunits.

There are currently 12 known neuronal nAChR subunits, α2 to α10 and β2 to β4. The α7-, α8-, and α9-subunits can form homomeric channels. The most commonly expressed homomeric nAChR in mammals is the α7-receptor. While α9 and α10 are also expressed in mammals, α8 nAChR expression has only been found in chickens (Gerzanich *et al.* 1993; Keyser *et al.* 1993). Heteromeric receptors contain combinations of α- and β-subunits. The stoichiometry ratio of α- to β-subunits was shown to depend on the expression system of the receptors and other factors, including temperature and pharmacological treatment (Nelson *et al.* 2003). The assembly composition of these heteromeric receptors is what gives rise to the diversity of nAChR properties, both binding and function.

The location at which the nicotinic agonist ACh binds is known as the ligand-binding domain (LBD). The LBD is located between the interface of the α-subunit and its adjacent subunit on the N-terminal domain. The N-terminal domain is about 210 amino-acid residues long. Heteromeric receptors contain two ACh-binding sites, while homomeric receptors might contain up to five ACh-binding sites. The LBDs

are composed of six loops, three from the contributing α-subunit and three from the non-α-subunit. In the case of homomeric receptors the adjacent α-subunits each contribute three loops. The loops from the α-subunits are considered the primary loops and are known as A, B and C (Galzi *et al.* 1991) while the loops from the non-α-subunits are the complementary loops D, E and F (Corringer *et al.* 1995). These loops and their residues were determined through numerous photoaffinity labeling, mutatgenesis, radioligand binding and functional assays.

Structure of nAChRs

Site-Directed Mutagenesis of the Receptor and Substituted Cysteine Accessibility Method

Site-directed mutagenesis has been a valuable tool for investigating residues important in various aspects of the receptor. The receptor channel incorporates negatively charged amino acids that promote the passage of cations (Pascual and Karlin, 1998). Mutating these residues to uncharged amino-acids decreases the cationic conductance of the receptor (Imoto *et al.* 1998). Interestingly the cationic selectivity of the receptor can be converted to an anionic selectivity by mutating only three residues in the M2 domain and the M1-M2 loop, as was done with the chick α7 receptor (Galzi *et al.* 1992). Mutagenesis studies have determined that a threonine residue at position 59 within the β2-subunit is important for the interaction of the competitive antagonist dihydro-β-erythroidine with α3β2 receptors (Harvey and Luetje, 1996).

The substituted cysteine accessibility method (SCAM) proved to be a valuable tool for initial identification of all the residues lining the M2 domains of the muscle type nAChR (Akabas *et al.* 1994; Zhang and Karlin, 1998); which (as previously determined) is the domain that lines the channel of the receptor and allows conduction of cations.

SCAM has identified four important residues that exist in the middle of the M2 domain and are part of the channel gate for the active state of the receptor (Wilson and Karlin, 1998).

The ACh-Binding Protein

In 2001 there was a breakthrough in the structural analysis of the nAChR. A group in the Netherlands discovered a soluble protein from the fresh water snail *Lymnaea stagnalis* (Brejc *et al.* 2001; Smit *et al.* 2001). The protein had a sequence identity similar to subunits from the nAChR, as well as the other cys-loop ligand-gated ion channels. The discovery came about when experiments on pre- and postsynaptic cholinergic neurons from *Lymnaea* determined that excitatory postsynaptic potentials (EPSP) occurred only in the absence of glia cells. Further exploration found a protein secreted from the glia that bound the ACh released in the synapse. Thus it was named the acetylcholine-binding protein (AChBP). It has been proposed that *Lymnaea* can use the AChBP as a buffer to directly modulate cholinergic synaptic transmission (Smit *et al.* 2003).

Purification and molecular cloning of the protein determined that it is made up of five subunits. Each subunit consists of 210 residues forming a homopentamer (Brejc *et al.* 2001). Each subunit has a ligand binding-domain between the N-terminals of adjacent subunits but lacks the pore-forming transmembrane domains. The majority of the residues that are conserved among the nAChRs are also conserved in the AChBP, which makes it a good model for the N-terminal domain of the nAChR including the LBD. The AChBP has the highest sequence homology to the nAChR α-subunit extracellular domains with a 23.9% similarity to the α7 nAChR (Smit *et al.* 2001). Using radiolabeled

α-btx the IC_{50} values were determined for several nicotinic agonists and antagonists including α-btx (2.6 nM), nicotine (98 nM) and ACh (4.2 μM), these values are more comparable to the pharmacology of homomeric receptors, specifically α7 and α9, over the high affinity heteromeric nAChRs (Smit *et al.* 2001).

Brejc *et al*. (2001) crystalized this protein at 2.7 Å. The measurements of the AChBP are 62 Å in height, a diameter of 80 Å and a pore size of 18 Å, these measurements parallel Unwin's data on the *Torpedo* nAChR. The residues important in the LBD were previously determined by biochemical and mutagenesis data. The crystal structure confirmed the involvement of these residues as well as elucidated how these residues are positioned in the binding domain. The principal side of the LBD consists of loops from the α-subunit homolog known as loops A, B and C while the complementary side, the β-subunit homolog in heteromeric receptors and α-subunits in homopentameric receptors, consists of loops D, E and F made up of β-sheets. The residues in the binding site (as crystallized with a HEPES molecule), which are primarily aromatic and hydrophobic, are as follows: loop A, tyrosine 89; loop B, tryptophan 143 and 145; loop C, tyrosine 185 and 192, and cysteines 187 and 188. Important LBD residues from the complementary side are: loop D, tryptophan 53 and glycine 55; loop E, arginine 104, valine 106, leucine 112, and methionine 114; loop F tyrosine 164 (Brejc *et al.* 2001; Corringer *et al.* 2000; Dougherty and Lester, 2001). AChBP has yet to be crystallized with an empty binding pocket. The original crystals contained a HEPES molecule in the ACh binding site.

Recently nicotine and carbamylcholine, both nicotinic agonists have been crystallized in the AChBP (Celie *et al.* 2004). The data reaffirmed the proposed aromatic

and hydrophobic residue contacts between these nicotinic agonists and the ACh binding site. The study by Celie *et al*. (2004) determined that hydrogen bonds exist between the receptor and the nitrogens on the pyridine and pyrrolidine rings of nicotine. Also, the carbonyl oxygen of W143 interacts with the positively charged nitrogens of nicotine and carbamylcholine. Interestingly they found that both ligands bind with their nitrogen atoms at nearly the same position in the binding pocket. A comparison between the LBD of the α4β2 nAChR and the AChBP reveal three residue substitutions, R104 (AChBP) is replaced by V109 (α4β2), L112 by F117 and M114 by L119 (Celie *et al.* 2004).

The utilization of the AChBP for molecular modeling has its limitations. One of the major limitations being that the AChBP is not a functioning channel so modeling ligand binding may not reflect molecular changes in movement that the nAChR undergo upon activation and desensitization. Unwin (2005) has helped to shed some light on the conformation of the receptor in the unliganded, or closed state. He has determined at a 4Å resolution of the *Torpedo* nAChR in a closed conformation that the C loop of the α subunits projects away from the receptor and that upon agonist binding the C loop moves in toward the receptor's ACh binding site, which along with the movement of the B loop allow for a conformational change that would permit receptor activation and channel opening. Unwin (2005) terms this conformational state of the B and C loops of the α subunits as "distorted" and that upon ligand binding both a salt bridge and hydrophobic interactions are broken, which allow for the loops to change to a conformationally "relaxed" state of the receptor.

Pharmacology and Function of nAChRs

The majority of the initial information about nAChRs came from ligand binding, photoaffinity-labeling, electrophysiological and mutagenesis experiments. Although the initial properties were determined by electrophysiological methods in the 1940s, it was only after the cloning of genes coding for nAChRs that electrophysiological experiments could be put to use for studying the physiology of specific subunit combinations. Much of the data up to that point relied upon radioligand-binding studies to elucidate the pharmacology of nAChRs expressed in various tissues and regions throughout the brain.

Pharmacology of nAChRs

The initial pharmacology of AChRs was largely based on the study of natural products and their ability to bind and affect nAChRs. Plant compounds including nicotine, muscarine, atropine, curare and physostigmine were all utilized due to the finding that they produced either activation or inhibition, depending upon concentrations used, of cholinergic (either nicotinic or muscarinic) transmission. The ability to radiolabel these compounds provided the evidence for the location of nAChRs in various tissues as well as provided the capability to measure the tightness with which these radiolabeled compounds bound to the receptor, known as affinity. A ligand that reversibly binds directly to the ACh binding site is called a competitive ligand. It will compete with ACh for the same binding site on the nAChR and with increasing concentrations of ligand it will displace ACh from the binding pocket. A ligand that does not bind to the ACh binding site is termed non-competitive and will not displace ACh with increasing concentrations. Non-competitive ligands bind to other sites on the receptor known as allosteric sites. Local anesthetics are ligands that bind to nAChRs at sites other than the ACh binding site (Papke and Oswald, 1989; Sine and Taylor, 1982).

A group of alkaloids from the venom of *Tetraponera* ants have also been initially characterized as noncompetitive antagonists at several nAChR subtypes and are likely binding at a site within the channel (Kem *et al*. 2004b).

There are several considerations when radiolabeling a compound that is to be used as a ligand in studying nAChRs. One concern is that the radiolabel must have a high enough specific radioactivity that binding of nanomolar (nM) concentrations of ligand can be reliably measured. It is also a requirement that the radiolabel not interfere with binding of the ligand to the receptor. Also, it is important that the label is not metabolized or exchanged during the experiment. Non-saturating sites on the membrane to which a radioligand binds are known as non-specific binding sites. It is important that the radioligand can be used to differentiate between non-specific binding and binding to only the receptors, known as specific binding. Obtaining specific binding is usually performed by adding a high concentration of a compound, such as nicotine, that binds specifically to the receptor sites in order to knock off the radioligand from the receptors, thus the radioligand is only bound to the non-specific sites. The counts due to non-specific binding (these are proportional to ligand concentration) can be subtracted from the total binding counts of radioligand bound to the cell membranes in order to determine specific binding. Receptor binding should be saturable, reversible, and over a period of time reach equilibrium binding. It is also ideal if the ligand is selective so that is binds with a high specificity to a specific subtype of receptor, or in the case of early development of nAChR radioligands specifically to nAChRs.

One such compound that was initially employed for utilization in studying nAChRs was radiolabled muscarine (Waser, 1961). The problem with using muscarine, a toxin

extracted from mushrooms, to study nAChRs is that it labeled two types of acetylcholine receptors, the ligand-gated nicotinic as well as the G-protein coupled muscarinic receptors. Acetylcholine also labels both types of ACh receptors and is readily hydrolyzed by acetylcholinesterase into acetate and choline, thus it is usually preferred to work with a more stable and selective ligand for nAChRs although the binding of radiolabeled ACh has been characterized in the presence of atropine, to prevent binding to muscarinic receptors (Schwartz *et al.* 1982). Several such ligands discussed earlier were the snake neurotoxins. α-Bungarotoxin has been shown to be specific for certain nAChRs and has a low nanomolar affinity (Barrantes *et al.* 1995; Gopalakrishnan *et al.* 1995). The equilibrium dissociation constant K_d (or K_i) is an inverse measure of this affinity. Several studies have compared the binding of ACh to nicotine and α-btx in the rat and mouse brain (Clarke *et al.* 1985; Marks *et al.* 1986). These studies indicated that radiolabeled ACh and nicotine were binding to the same sites with high affinity while α-btx was labeling different sites. Acetylcholine and nicotine were labeling sites in the cortex, cerebellum, ventral tegmental area and the thalamus while α-btx was binding at higher densities in the thalamus and hippocampus. It was soon determined that the nAChR in the central nervous system to which α-btx was binding was that of a homomeric α7 receptor (Couturier *et al.* 1990). Since its first utilization radiolabeled α-btx has been a commonly used label for α7.

A few years later a relatively selective label, as compared to nicotine or ACh, for the heteromeric α4β2 nAChR was discovered. Cytisine, a plant alkaloid from *Laburnum anagyroides*, is an autonomic ganglionic agonist. Exploration of its properties on neuronal cell membranes revealed that cytisine had a very high affinity for those sites

labeled by radioactive nicotine and ACh. It had a measured K_d value of less than 1 nM, with high levels of binding in the cortex, thalamus and striatum and represented 60-90% of total binding at the various concentrations examined (Pabreza *et al.* 1991). Flores *et al.* (1992) later determined that cytisine was primarily labeling the heteromeric α4β2 nAChR. Several ligands that also possess a high affinity for these receptors have been radiolabeled and studied since the development of cytisine. The compound known as ABT-418 is a relatively high affinity ligand for α4β2 that functions as an agonist, and like nicotine may function as a concentration dependent noncompetitive antagonist (Papke *et al.* 1997). Anderson *et al*. (1995) radiolabeled ABT-418 and measured a K_d value of 2.9 nM for rat brain. Another ligand that has been radiolabeled for the study of α4β2 receptors is a semi-synthetic compound derived from the plant genus *Erythrina*. The alkaloid known as dihydro-β-erythroidine is commonly used in functional assays as a competitive nicotinic antagonist. Williams and Robinson (1984) investigated a tritiated this alkaloid and measured high and low affinity binding sites in rat brain membranes with respective K_d values of 4 and 22 nM. They found that its distribution of binding in rat brain was highest in the thalamus and that cytisine was a more potent inhibitor than nicotine in displacing the radiolabeled alkaloid. Also, mecamylamine did not displace the radiolabeled dihydro-β-erythroidine at concentrations up to 100 μM. Their data indicated that the tritiated alkaloid was preferentially interacting with α4β2 over the other neuronal subtypes of nAChRs.

The α4β2-subtype has a high affinity for the agonist nicotine that induces a conformational change (desensitization), which displays this high affinity state (Changeux *et al.* 1984; Higgins and Berg, 1988; Buisson and Bertrand, 1998). Agonists

for the α7 nAChR have not been found to induce such a high affinity state of this particular receptor. Thus, the α4β2-subtype is referred to as the high affinity nAChR, while the α7-subtype is known as a low affinity nicotine-binding receptor. Although there are numerous combinations of nAChR subtypes that form neuronal receptors, the α4β2 and α7 nAChRs are the two major subtypes in the mammalian central nervous system (CNS) (Clarke *et al*. 1985; Hogg *et al*. 2003; Pauly *et al*. 1989; Seguela *et al*. 1993; Wada *et al*. 1989; Whiting *et al*. 1991).

Distribution and Physiology of nAChRs in the Mammalian CNS

Nicotinic receptors in the CNS are known to facilitate synaptic transmission. There are several studies that indicate the existence of postsynaptic nAChRs in the mammalian CNS (Alkondon *et al.* 1998; Clarke, 1993; de la Garza *et al.* 1987; Frazier *et al.* 1998; Schroder *et al.* 1989) but the lack of evidence for the involvement of these receptors in EPSPs has lead to the theory that the primary location of nAChRs is presynaptic. Through autoradiography, immunolabeling, and co-localization with presynatpic markers, the existence of nAChRs on presynaptic neurons has been firmly established (Clarke and Pert, 1985; Lubin *et al.* 1999; Jones *et al.* 2001). The existence of these presynaptic nAChRs allows for the regulation of neurotransmitter release. Activation of these receptors has been shown to result in release of not only ACh (Wilkie *et al.* 1993), but also in dopamine (Rapier *et al.* 1990, Grady *et al.* 1992), glutamate (Alkondon *et al.* 1997; McGehee and Role, 1995) and GABA (Alkondon *et al.* 1999; Radcliffe *et al.* 1999; Yang *et al.* 1996). The release of neurotransmitter through activation of nAChRs on presynaptic terminals is due to the entry of calcium both through the nAChR channel and through voltage-gated calcium channels, which are activated upon depolarization of the

cell (Soliakov *et al.* 1995). All nAChRs are permeable to calcium but the neuronal receptors have a higher permeability ratio as compared to the muscle subtype. The α7-homomeric receptors have the highest permeability to calcium, with a permeability ratio of about 6.6 or higher (P_{Ca}/P_{Na}) (Bertrand *et al*. 1993; Fucile, 2004; Sands *et al*. 1993; Sequela *et al*. 1993). The activation of nAChRs and their subsequent ion permeability of is not the result of a static entity but rather a fluid movement in the conformation of the receptor.

Function of nAChRs

The prototypical site for the study of the mammalian nAChR is the neuromuscular junction. Postsynaptic cells of the muscle form large synapses, which contain millions of nAChRs. Large amounts of ACh are released from the presynaptic membranes directly onto the postsynaptic receptors in order to ensure that an action potential occurs in order to facilitate the movement of muscle. In the early 1950s, there were rapid developments in the understanding of synaptic transmission thanks in a large part to the study of the neuromuscular junction. In 1952 Hodgkin and Huxley revealed the mechanism of the action potential involved in electrical signaling in the giant squid axon. Shortly after Fatt and Katz (1950, 1951, 1952) provided a better understanding of neurotransmitter release and the resultant change in ion permeability by ACh. Hodgkin and Huxley employed the voltage clamp technique to study the permeability changes underlying the action potential in the giant squid axon. Through the use of a recording and current passing electrode, voltage clamp allows for the membrane potential of a cell to be held constant while measuring the change in ionic current flow required to keep the membrane potential at a constant voltage. This technique allowed for macroscopic measurements of receptor

activation and inhibition. The voltage clamp technique known as patch clamp recording allowed for microscopic measurements and kinetic analysis of single receptors (Hamill *et al.* 1981; Neher and Sakmann, 1976). These techniques were major breakthroughs in the study of all ligand gated ion channels.

In the past few years other methods of measuring functionality have been developed. These include both calcium and membrane potential dye measurements. Fitch *et al*. (2003) determined the potency and efficacy of nicotinic agonists on various cell lines expressing several subtypes of nAChRs using either a membrane potential or calcium fluorescent dye. Both dyes fluoresce due to either changes in membrane potential or increases in intracellular calcium reflecting activation of the expressed receptors. Changes in fluorescence can be calculated and graphed to determine either activation or inhibition concentration constants. They determined that the membrane potential dye was more sensitive than the calcium dye but that the overall results were comparable to those of the calcium dye as well as previously published radiolabeled rubidium efflux assays. The membrane potential dye was also useful in cell lines that have a low calcium conductance. One disadvantage to using these dyes is the inability to wash away compound after application. Compounds that function as agonists may produce a time dependent desensitization of the receptors. Another disadvantage in using the membrane potential dye is that agonists may appear more potent and antagonists less potent, as compared to electrophysiological data. This may be due to the phenomenon of spare receptors (fraction of receptors occupied that produces a maximum response).

As discussed earlier, the ability and strength with which a ligand binds to the nAChR is known as affinity. It describes how a ligand binds but it does not characterize

its capability to functionally activate the receptor. The term efficacy was coined to describe the ability of a ligand to activate a receptor (Colquhoun and Sakmann, 1998; Stephenson, 1956).

A ligand can have varying degrees of efficacy based upon the subtype of receptor and concentration of ligand. The term agonist describes a ligand that activates a receptor. The endogenous neurotransmitter ACh is a full agonist for nAChRs. A ligand's efficacy is often compared to that of a known full agonist, such as ACh, for the subtype of nAChR being studied. If a ligand activates a receptor with less than maximum activation as compared to ACh it is termed a partial agonist. For example, cytisine is a partial agonist for $\alpha4\beta2$. It has high affinity for the receptor, a K_d less than one nanomolar (Pabreza *et al.* 1991), but it causes only 15% of the maximum response at 1 mM as compared to ACh (Papke and Heinemann, 1994). Thus, cytisine is less efficacious for the $\alpha4\beta2$ receptor than ACh.

Ligands that bind to nAChRs but produce no activation are called antagonists. Antagonists exist as competitive or non-competitive ligands. Competitive antagonists bind to the ACh binding site and alone cause no change in the activity of the receptor. However, when present with an agonist that binds to the same site, the competitive antagonist will inhibit the receptor from activation at specific concentrations. α-Bungarotoxin, as previously described, is a competitive antagonist for $\alpha7$ receptors. It binds to the $\alpha7$ ACh binding site with a K_d of 0.16 nanomolar (Marks *et al.* 1986) and inhibits activation at higher nanomolar concentrations, such as 100nM (Frazier *et al.* 1998). Non-competitive antagonists can produce the same inhibition as competitive antagonists, but they do not bind to the ACh binding site. A non-competitive antagonist,

such as the local anesthetics dibucaine or tetracaine, prevents activation of the muscle type nAChR by binding to one or possibly more allosteric sites on the receptor (Papke and Oswald, 1989; Sine and Taylor, 1982). Non-competitive antagonists may also inhibit activation without binding to an allosteric site but rather through blocking passage of ions through the channel, termed channel-block. Competitive agonists, such as nicotine, may become non-competitive antagonists at high concentrations due to the ability of nicotine to cause channel block. The capability of a compound to act as an agonist or antagonist depends upon the stoichiometry of the nAChR and the stoichiometry of the receptor determines the kinetics and ion permeability.

Functional states of the nAChR

The ability of nAChRs to successfully facilitate neurotransmission involves several different conformational states of the receptor. The transition from one state to another is dependent upon several factors including agonist concentration, time of exposure, and association and dissociation rate constants for binding. There exist at least three conformational states for nAChRs, closed, active and desensitized. Monod *et al*. (1965) produced their model of allosteric interactions to help explain the transition from one state to the next. The closed state is an unbound resting state of the receptor such that a net flow of ions is not passing through the channel. In the absence of agonist nAChRs exist in an equilibrium that greatly favors the closed state. Upon exposure to an agonist the receptors no longer exist in that resting equilibrium but rather have become active, opening their channels and allowing ions to flow through and thus producing a current across the membrane. The active state requires bound ligand, with at least two agonist molecules bound to the receptor. With two agonist molecules bound the receptor has a higher probability of opening than monoliganded receptors (Sine and Taylor, 1980,

1981). The third state that exists is the desensitized state of the receptor. This state, which was originally studied at the motor end-plate by Katz and Thesleff (1957), is a nonconducting state that does not respond to additional application of agonist and must recover before subsequent activation or before returning to the resting state. Although this is a nonconducting state the conformation of the desensitized receptor is distinctly different from that of the resting state (Wilson and Karlin, 2001; Unwin, 1995). The properties of desensitization, such as onset and recovery, are dependent upon receptor stoichiometry. Desensitization can be induced by properties ranging from agonist concentration to disruption of the tissue (Whiteaker *et al.* 1998; Fenster *et al.* 1999), such as occurs during homogenization. The neuronal α7 nAChR are the most rapidly desensitizing of the nAChRs. The rapid desensitization of α7, by high concentrations of agonist (ACh), is so quick that it complicates data analysis due to the fact that by the time the agonist solution reaches its full concentration the peak response of the receptor has already occurred (Papke and Porter Papke, 2002). The fast desensitization of α7 is in part due to a leucine residue at position 247 in the second transmembrane domain. Through substitution of a threonine residue for the leucine (L247T) the rate of desensitization is slowed, but the pharmacology of the receptor is dramatically different from that of the wild type receptor (Revah *et al.* 1991; Bertrand *et al.* 1992). Placzek *et al*. (2005) created an α7 gain of function mutant that contained a serine residue in place of a threonine (as found in wild-type α7) in the second transmembrane domain. This mutant receptor has longer open times as compared to wild-type α7 with its pharmacology (for agonists and antagonists) similar to that of the wild-type receptor, unlike the confused pharmacology of the L247T mutant.

Neuronal nAChR Involvement in Disease

Since nAChRs do facilitate neurotransmission, it is not surprising that they are also involved in neuronal dysfunction of several disease states. A downregulation of nAChRs has been found in brains of persons diagnosed with Alzheimer's disease (AD) (Norberg and Winblad, 1986; Schroder *et al.* 1991; Court *et al.* 2000). The primary target for downregulation is the α4β2-subtype (Warpman and Nordberg, 1995) although α7 may also be affected (Perry *et al.* 2000). The exact involvement of nAChRs in the impairment of memory and cognition in AD patients is not known, but treatment with nicotinic receptor agonists and acetylcholine esterase inhibitors have been found to alleviate some of the clinical symptoms of AD in the early stages of the disease.

Nicotinic acetylcholine receptors have also been found to be downregulated in Parkinson's disease based upon ^{3}H-nicotine binding assays (Rinne *et al.* 1991; Perry *et al.* 1995; Court *et al.* 2000). The pathology of Parkinson's disease is the loss of dopaminergic neurons in the nigro-striatal and ventral tegmentum area-mesocortical pathways in the brain. Several subtypes of nAChRs have been found to play a role in the release of dopamine from these neurons. Both α4β2 and α6β3β2 nAChR subtypes are located on dopaminergic neurons (Champtiaux *et al.* 2002, 2003; Cui *et al.* 2003; Zoli *et al.* 2002).

There exist at least two neuronal disorders that involve mutations of the gene encoding for specific nAChR subunits, schizophrenia and autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). The chromosome encoding the α7 gene has been found to be partially duplicated in schizophrenic families (Chini *et al.* 1994; Leonard *et al.* 1996; Freedman *et al.* 2001; Xu *et al.* 2001). Interestingly, 90% of schizophrenic

patients self-administer some form of nicotine (Lohr and Flynn, 1992; Poirier *et al.* 2002). Mutations on genes that encode both the α4 and β2 nAChR subunits are found in ADNFLE patients. The manifestation of these mutations can be traced to residue mutations of both the α4 and β2 membrane-spanning domains M2 (Phillips *et al.* 1995, 2001; De Fusco *et al.* 2000; Hirose *et al.* 1999; Steinlein *et al.* 1995, 1997). The culmination of these mutations is an increased sensitivity of the receptors to ACh, which has been proposed to generate seizures resulting from a synchronization of spontaneous oscillations in the thalamo-cortical circuits (Raggenbass and Bertrand, 2002).

It has been established that nicotinic receptors are involved in the reinforcing properties of nicotine, in part due to activation of nAChRs on dopaminergic neurons in the nucleus accumbens and ventral tegmental area (Corrigall *et al*. 1994; Picciotto and Corrigall, 2002). Nicotine dependence is often established and sustained through smoking cigarettes. The chronic and prolonged use of tobacco products often results in negative health effects including cancer and cardiovascular disease. Although the detrimental effects of tobacco dependence are produced not through nicotine but the many other ingredients in cigarettes, it is the nicotine that has been determined to produce the reinforcement. Nicotine itself has been found to produce the enhancement of cognition and memory (Levin, 1992; Arendash *et al.* 1995a). It also has the ability to both activate and inhibit nAChRs based upon concentration and length of exposure.

The development of knock out mice in which a gene for a specific nAChR subunit has been mutated to prevent expression have allowed for investigation of subunits involved in nicotine reinforcement. Picciotto *et al*. (1995) were the first to develop a β2-knock out mouse. They showed that these knock out mice lacked the majority of high

affinity nAChRs while the levels of α-btx binding remained the same as in wild-type mice. Most importantly, these mice were shown to have decreased dopamine release in response to nicotine exposure and do not self-administer nicotine (Picciotto *et al*. 1998). Although it has recently been shown that α6-subunits associate with the β2-subunit in the CNS, the majority of β2 are associated with α4 (Champtiaux *et al*. 2003).

The involvement of α4β2 nAChRs in addiction has lead these receptors to become targets for the design of pharmacological therapeutics. Over the years several approaches and therapeutic agents have been tested for their ability to aid in smoking cessation. In the 1960's cytisine, a partial agonist for α4β2, was examined but lacked the ability to readily cross the blood brain barrier. The combination of mecamylamine, a nAChR antagonist, and nicotine administered through a nicotine skin patch has been shown to be somewhat beneficial in aiding cessation (Rose *et al*. 1994). Bupropion, a compound used to treat depression, has also been determined to aid smoking cessation. The precise mechanism of action is unknown but it is though to prevent the reinforcing properties of nicotine (Cryan *et al*. 2003). More recently a compound known as varenicline, a partial α4β2 agonist, has begun clinical trials for smoking cessation (Coe *et al*. 2005). Based on these studies it may be expected that a partial agonist would be a useful smoking cessation agent due to its ability to result in a release of dopamine, yet block additional dopamine release upon stimulation with a full agonist such as nicotine. The competitive antagonist, DHβE, has been shown to prevent nicotine self-administration when applied directly to the ventral tegmental area (Corrigall *et al*. 1994). Thus, it is also possible that an antagonist may also function as a smoking cessation agent.

The development of either partial agonists or antagonists may be useful not only for smoking cessation, but also as *in vivo* probes for the involvement of α4β2 in neuronal processes. Although knock out mice have been created, the involvement of α4β2 receptors in cognition and nicotine addiction may be masked by compensatory mechanisms from other nAChR subunits. In order to develop ligands that display partial agonist or antagonist activity on the α4β2 receptor, it is necessary to better understand the interaction of a compound with the receptor. Specifically, what features of the compound confer affinity and influence functional properties. Structure activity relationship studies involve analysis of a group of similar compounds, such as nicotine analogs, where slight to large modification are made to the structure of the compound. The ability of these modified compounds to bind and activate or inhibit the receptors provides information useful in the design of α4β2 specific ligands.

Structure Activity Relationships of Neuronal nAChRs

Nature has provided various molecules, including cytisine and α-btx, which have allowed for elucidation of structure, location and function of nAChRs expressed in various tissues. With increased understanding of these receptors and their involvement in several diseases the possibility of alleviating symptoms or correcting deficiencies often leads to the re-visitation of natural products. The design of pharmacological agents often starts with a lead compound, one that has been found to interact with the receptor of interest. One lead compound that has proved useful in the development of clinically based therapeutics is a natural product from a marine worm, the toxin known as anabaseine. Anabaseine itself stimulates all nAChRs (Kem, 1971; Kem *et al.* 1997) but is most potent on muscle-type and neuronal α7 receptors (Kem *et al.* 1997). Through the

addition of a substituted benzylidene moiety, anabaseine becomes a selective partial agonist for α7 and an antagonist for α4β2. This compound known as DMXBA (GTS-21), whose chemical name is 3-(2,4-benzylidene)-anabaseine, has been shown to be neuroprotective in a PC12 neuronal growth factor deprived model of cytotoxicity (Li *et al.* 1999). It also has been found to enhance cognition in various behavioral tasks performed by aged rats (Arendash *et al.* 1995b), and therefore may prove a useful therapeutic for Alzheimer's disease (Kem, 2000). DMXBA is currently in clinical trials for correcting the auditory gating deficiency experienced by schizophrenic patients (Stevens *et al.* 1998; Simosky *et al.* 2001).

Similar to the development of DMXBA from the natural product anabaseine, nicotine may serve as a good lead compound for the design of smoking cessation drugs. Several previous studies have determined structure activity relationships of nicotine for the α4β2 nAChR. Substituents at various positions on both the pyridine and pyrrolidine rings of nicotine have been determined to affect affinity. Fewer studies have addressed the functional consequences of these substitutions on the α4β2 receptor. Including Beers and Reich's (1957) well-known structure activity relationship for agonists and antagonists with cholinergic binding sites, other findings about the interaction of ligands with nicotinic receptors have since been discussed. One theory proposed by several groups is that a cation-π interaction exists between Trp149, of the α-subunit of the *Torpedo* receptor as well as the AChBP, with the nitrogen atom in an interacting ligand (such as nicotine) (Beene *et al*. 2002; Tønder and Olesen, 2001). Structure activity relationships also exist for other ligands and the α4β2 nAChR. Epibatidine, the most potent nAChR agonist, has 7-azabicyclo[2.2.1]heptane ring attached to an

exo-5-(2'-chloropyridinyl) substituent. Although it is exceptionally potent, epibatidine is not selective for particular nAChRs. Carroll *et al*. (2001) reported that a phenyl substituent at the 3'-position of epibatidine changed it into an α4β2 antagonistic *in vitro*.

Nicotine is a tertiary amine with a chiral carbon at the 2'-pyrrolidine position. In 1957 Beers and Reich proposed that the ability of the naturally occurring (S)-nicotine to interact with the nAChR existed due a hydrogen bond formed between the nitrogen on the pyridine ring and through electrostatic interactions by the nitrogen on the pyrrolidine ring. They measured a minimum distance of 5.9 Å from the nitrogen group to the hydrogen bond. They found this measurement existed for both nicotinic agonists (ACh, cytisine, nicotine) and nicotinic antagonists (strychnine, β-erythroidine). This initial analysis of nicotinic ligands was performed in order to determine structural basis for the interaction with nAChRs.

Although nicotine has a higher affinity for neuronal α4β2 receptors over α7, it is not a selective molecule. Thus, the structure of nicotine may be used as a template for the design of other compounds. This method of study, structure activity relationship, involves determining the affinity (K_i value) and EC_{50} or IC_{50} values of a group of compounds. This dissertation characterizes the SAR relationship for two groups of plant alkaloids on the high affinity neuronal α4β2 nAChR. The first group of compounds are nicotine analogs, which as we have determined act as weak partial agonists and antagonists on the α4β2 receptor.

The other group of compounds are *Erythrina* alkaloids, which like the commonly used dihydro-beta erythroidine, also act as antagonists on the neuronal α4β2 receptor. Like nicotine they too are tertiary amines. In Beers and Reich's classic study from 1970

they examined the structure of β-erythroidine as compared to ACh in order to determine structural elements required for binding to the ACh receptor. β-Erythroidine with its fused ring system is a more rigid molecule than nicotine. Beers and Reich (1970) determined that the oxygen atoms on rings D and A can both form hydrogen bonds that measure 5.9 Å from the nitrogen between rings C and B. They proposed that although β-erythroidine is a rigid molecule its ability to bind and inhibit receptor function might be due to dual hydrogen binding sites.

The goal of this dissertation was to create structure activity relationships for each of these two groups of plant alkaloids that may be useful in designing partial agonists and antagonists for the possible treatment of nicotine dependence. These structure activity relationships may also be useful for designing *in vitro* and *in vivo* probes for studying the function of α4β2 nAChRs.

CHAPTER 2
MATERIALS AND METHODS

Radioligand Binding

Compound Syntheses

Dr. Ferenc Soti synthesized all nicotine analogs; purity was determined by reversed phase HPLC and NMR confirmed compound structure. The nicotine analogs were free bases. All were suspended in high performance liquid chromatography (HPLC) grade methanol to create stock solutions of 10 to 100 mM. These stock solutions were further diluted in 50 mM Tris binding saline containing 2 mg/ml of bovine serum albumin (Sigma, St. Louis, MO) to create desired concentrations used in binding or functional assays. The extraction and semi-synthetic preparation of the *Erythrina* alkaloids were also performed by Dr. Ferenc Soti according to traditional methods. Following extraction of several natural aromatic *Erythrina* compounds, separation was performed by high performance liquid chromatography (HPLC) using a C-18 column (Beckman Coulter, Fullerton, CA) as well as by silica gel chromatography. Erysotrine was the only *Erythrina* alkaloid that was isolated as a free base. All the other alkaloids were acetate or hydrochloride salts. The *Erythrina* alkaloids also were suspended in HPLC grade methanol to create stock solutions and then further diluted in 50 mM Tris binding saline containing 2 mg/ml bovine serum albumin.

The (R)-form of nicotine was obtained from Research Biochemicals Inc. (Natick, MA) as a di-p-toluoyl tartrate salt. The (S)-form of nicotine was obtained from Sigma (St. Louis, MO) as a tartrate salt. The (R)- and (S)-nornicotines were obtained from

Peyton Jacobs III (San Francisco General Hospital, San Francisco, CA) and both existed as dicamsylate and camsylate salts, respectively.

Rat Brain Membranes

Whole male Sprague-Dawley rat brains obtained from Pel-Freeze Biologicals (Rogers, AZ) were prepared according to Marks and Collins (1982). Whole rat brains were homogenized with a 30 ml Wheaton (location) glass homogenizing tube and pestle attached to a motor source. The tissue was homogenized in Tris binding saline consisting of 120 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2 and 50 mM Trizma HCl Buffer pH=7.4. After homogenization the tissue was centrifuged at 11,000 rpm for a ten-minute period. After centrifugation the pellet was resuspended in fresh binding saline and again homogenized. A BCA protein assay (Pierce, Rockford, IL) was then performed to obtain the protein concentration of the rat brain membranes. Homogenized tissue was stored at -80°C until use. Rat brain membranes at a concentration of 200 μg protein were used per tube for radioligand binding.

Human Embryonic Kidney Subclone Cell (tsA201) Membranes

A subclone of the human embryonic kidney cell line (tsA201 cell line) was graciously provided by John Lindstrom (University of Pennsylvania, Philadelphia, PA). These cells stably express human α4β2 nAChRs on their membranes. These cells were prepared according to Xiao and Kellar (2004). Cells at about 80 to 90% confluence were collected after removing the culturing media from the flask (75 cm^2) and adding 6-10 mls of ice-cold Tris binding saline (pH = 7.4) with a disposable cell scraper. The dislodged cells were then collected in centrifuge tubes and spun down at 1000 rpm for five minutes. The loose pellet was then collected and homogenized in the same method as the rat brain

membranes. After performing a protein assay the homogenized membranes were stored at -80°C until use. Homogenized tsA201 cell membranes at concentrations of 50 to 125 μg were used per tube for radioligand binding.

Binding Assay Protocol

The radioligands used in the competition and saturation binding assays were obtained from Perkin Elmer Life and Analytical Sciences (Boston, MA). ^{3}H-Cytisine (34 Ci/mmol) experiments were performed according to Flores *et al*. (1992) with a few minor alterations, specifically that the incubation time was increased to four hours at 4°C to allow for the binding to reach equilibrium. The ^{125}I-α-bungarotoxin (α-btx) (136 Ci/mmol) experiments were incubated at 37°C for three hours. Both radioligands were used at a final concentration of 1 nM in competition binding assays. Homogenized tissue at the above mentioned concentrations were suspended in 50 mM Tris binding saline containing 2 mg/ml of bovine serum albumin (Sigma, St. Louis, MO). The tissue along with the radioligand and compound of interest were incubated in 13x100 mm disposable glass culture tubes at a final volume of 0.5 ml. For each radioligand nonspecific binding was measured in the presence of a final concentration of 1 mM (S)-nicotine hydrogen tartrate salt (Sigma, St. Louis, MO). After incubation radioligand bound membranes were collected by vacuum filtration with a Brandel cell harvester (Gaithersburg, MD) onto Whatman GF/C glass fiber filters that had been pre-soaked in 0.5% polyethylenimine for 45 minutes. The radiolabeled membranes were washed three times with 3 mls of ice-cold 50 mM Tris binding saline. Filters containing ^{3}H-cytisine bound membranes were collected in 20 ml scintillation tubes and suspended in 8 mls of 30% Scintisafe scintillation fluid (Fisher), then counted in a Beckman LS-6500 liquid

scintillation counter (Fullerton, CA). Filters containing ^{125}I-α-btx bound membranes were placed in 4 ml scintillation vials and counted in a Beckman 5500B gamma counter (Fullerton, CA). Whereas each concentration used for saturation and competition binding assays with rat brain membranes was performed in quadruplicate, each concentration for assays involving tsA201 membranes was performed in triplicate.

Binding Assay Data Analysis

Binding assay data were analyzed using GraphPad Prism software (San Diego, CA). The count per minute values for each concentration were averaged and normalized to the specific binding value obtained within each experiment. Saturation assay data were analyzed by fitting the data to a one site binding hyperbola model ($Y=B_{max}*X/(K_d+X)$) in order to determine the K_d and Bmax value for the respective radioligand and tissue. The data could be transformed into a Scatchard plot for visualization of the respective slopes and X-axis intercepts using the Prism software. Competition assay data were analyzed using a sigmoidal dose response with variable slope ($Y=Bottom+((Top-Bottom)/(1+10^{((LogIC50-X)*Hillslope)}))$) from which a hillslope and IC_{50} value were determined; Top = Y value at the top plateau of the curve; Bottom = Y value at the bottom plateau of the curve. The IC_{50} value along with the pre-determined K_d value for the radioligand and nAChR-containing membrane of interest were then used in the Cheng Prusoff equation ($K_i=IC_{50}/(1+(Ligand)/K_d)$) to calculate the K_i value. Significant differences were determined using an unpaired, two-tailed T-test in GraphPad Prism.

Functional Measurements

Cell Culture

Cells were cultured essentially according to Nelson et al. (2003). The tsA201 cells expressing α4β2 nAChRs were maintained in a culture medium consisting of Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA) supplemented with 10% FBS (MediaTech Inc., Herdon, VA), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Irvine Scientific, Santa Ana, CA), 0.5 mg/ml Zeocin (Invitrogen, Carlsbad, CA), and 0.6 mg/ml G-418. Cells were grown in 75 cm^2 culture flasks which were housed in a humidified incubator at 37°C in an atmosphere of 5% CO_2. Cells were grown to around 80-90% confluence before being split with 0.25% Trypsin (Gibco, Carlsbad, CA) at a subcultivation ratio of between 1:6 and 1:10 weekly.

Membrane Potential Dye, Cell Loading and Compound Plate Preperation

Cells were seeded at a density of roughly 5×10^4 cells/well to 10×10^4 cells/well onto 96-well flat-bottom black-wall culture plates that had been coated with 50 μl per well of 50 μg/ml poly-D-lysine (70-150kDa). Cells were then grown overnight in 100 μl of culture medium in order to achieve a single layer of cells on the bottom of each well. The membrane potential dye, obtained from Molecular Devices, was prepared by dissolving one bottle of the dye into 30 mls of 1X Hanks' balanced salt solution (HBSS) supplemented with 20 mM Hepes (pH=7.4). The dye was then added (100 μl to each well) on top of the existing 100 μl of media. Finally, the cells were incubated in the dark with the dye at 37°C for 30 minutes before reading. Serial dilutions of a compound were prepared in a separate clear-walled 96-well V-bottom plate by evaporation of the correct

volume of a methanol stock solution. The evaporated compounds were then reconstituted in 250 μl of HBSS/Hepes (pH=7.4) containing 1 μM atropine.

Membrane Potential Measurements

Flexstation protocol was performed essentially as described by Fitch *et al*. (2003) with several modifications. Fluid transfer and readings were performed by a Flexstation fluorometer (Molecular Devices, Sunnyvale, CA). The dye (composition withheld for proprietary reasons) is a lipophilic, anionic, bis-oxonol dye. When the cells are depolarized the dye enters and binds to cytosolic proteins, causing an increase in fluorescence signal. During hyperpolarization the dye exits the cells and there is a decrease in fluorescence signal. Excitation and emission wavelengths were set to 530 nm and 565 nm with a cutoff of 550 nm. A reading was taken every 1.44 seconds over about a three minute period for a total of 139 readings per well. The first 17 seconds were used for a basal read. At 18 seconds the addition of 50 μl of a test compound is added (to assess possible agonist activity, EC_{50}), followed by a 25 μl addition of KCl (40 mM final concentration) at 160 seconds to measure the maximum possible signal and to correct for differences in dye loading and cell count (only the KCl response of the drug-naïve cells was used for normalization). Compound applications were done at about 78 μl per second. Compounds that had no measurable intrinsic activation properties upon the tsA201 cells were then examined to determine their IC_{50} values. The compound of interest was applied to the cells as above except that they were simultaneously applied with 5 μM acetylcholine (ACh) in order to measure inhibition of the ACh response. 5 μM ACh was slightly above the measured EC_{50}, but well below the dose required to

produce a maximal response, for ACh upon the α4β2-expressing tsA201 cells as measured with the Flexstation.

Functional Assay Data Analysis

Responses of experimental compounds were measured as the maximum RFU (relative fluorescent unit) subtracted from the averaged basal read for each individual well. Data was collected with SOFTmax Pro software from Molecular Devices (Sunnyvale, CA). These raw compound values were then divided by the average maximum response of cells in control wells to 40 mM KCl (compound naïve cells run in parallel). It was necessary to use values from control wells because it was determined that KCl response larger for cells previously exposed to agonist. The compound values were then graphed using the sigmoidal dose response with variable slope equation in Prism (Y=Bottom+((Top-Bottom)/(1+$10^{((LogEC50-X)*Hillslope)}$))) to calculate either an EC_{50} or IC_{50} value as well as the hillslope for each compound. The EC_{50} responses were normalized to the maximum response of ACh.

High Performance Liquid Chromatography (HPLC) Chiral Separation of Nicotine Analogs

Racemic nicotine analogs were separated on a Daicel chiral OJ-H semi-preparative column (250 x10 mm i.d.) from Chiral Technologies, Inc. (West Chester, PA) using a Beckman System Gold 126 solvent module attached to a System Gold 168 photodiode array detector (Fullerton, CA). The column was eluted with an increasing gradient (0-94% buffer B) over a 35-minute period where buffer A was n-hexane/ethanol/diethylamine/trifluoroacetic acid (97:3:0.1:0.1 v/v/v/v) and buffer B consisted of the same solvents at a volume ratio of 80:20:0.1:0.1 v/v/v/v for 1'-ethyl-nornicotine-(S,R)-nicotine. The pyridyl and phenyl nicotine compounds were

separated using an isocratic method (90:10:0.1:0.1 v/v/v/v for pyridyl nicotine and 94:6:0.1:0.1 for phenyl nicotine v/v/v/v). The separated compounds were collected with a Foxy Jr. Fraction collector using PeakTrak software (Isco Inc., Lincoln, NE). The amount of collected compound was determined by absorbance spectrum measurements in 95% ethanol using a Beckman DU 650 spectrophotometer (Fullerton, CA) and published molecular extinction coefficients (in 95% ethanol) for unionized forms of nicotine and nornicotine (Swain et al., 1949).

CHAPTER 3
RESULTS

Nicotine Analogs

The involvement of nicotinic receptors in nicotine addiction has led to the synthesis of a variety of nicotine analogs in order to determine relationships between structure and function. Numerous studies have characterized the binding of analogs with substituents at various positions on either the pyridine or pyrrolidine ring of nicotine. However, most of these studies provided binding data for only the high-affinity ($\alpha4\beta2$) receptor and lacked functional data. In order to determine the $\alpha4\beta2$ versus $\alpha7$ selectivity for several previously published as well as novel nicotine analogs, binding assays were performed to measure the ability to displace either ^{3}H-cytisine ($\alpha4\beta2$) or ^{125}I-α-btx ($\alpha7$) in rat brain membranes. The nicotine analogs were then tested in functional assays utilizing tsA201 cells expressing human $\alpha4\beta2$ receptors and a membrane potential dye to measure agonistic activity (EC_{50}) and inhibition of the ACh response (IC_{50}).

(S)-Nicotine has a high affinity for the neuronal $\alpha4\beta2$ nAChR; it binds at low nanomolar (2.3 nM) concentrations (Dukat *et al*. 1996). The naturally occurring (S)-nornicotine, which is also a metabolite of (S)-nicotine, has been reported to have a 16- to 18-fold lower affinity for the $\alpha4\beta2$ receptor (Copeland *et al*. 1991; Glassco *et al*. 1994). The functional effects of (S)-nicotine have also been well characterized. Much of the functional data for $\alpha4\beta2$ has been provided by electrophysiological experiments, radioactive rubidium efflux and radiolabeled-dopamine release assays. (S)-Nicotine acts

as a concentration and time-dependent agonist on α4β2 nAChRs with measured EC_{50} values ranging from 1 to 10 μM. At increasing concentrations and times after application nicotine may behave as an antagonist, primarily due to receptor desensitization. The functional effects of (S)-nornicotine are less well characterized than (S)-nicotine. In dopamine release assays from rat striatal slices, (S)-nornicotine at concentrations up to 100 μM has been found to cause nAChR mediated ^{3}H-dopamine release (Teng *et al*. 1997).

Of the published nicotine analogs, only the 5-pyridyl substituents have been electrophysiologically characterized to obtain IC_{50} values for α4β2. Dukat *et al*. (2002) found that adding smaller substituents at the 5-pyridyl position of nicotine did not greatly reduce the affinity for the α4β2 receptor. They measured the K_i values for 5-bromonicotine and 5-methoxynicotine for displacing ^{3}H-nicotine as 6.9 ± 2.6 nM and 14.3 ± 1.5 nM, respectively, as compared to the K_i value of 2.4 ± 0.4 nM for (S)-nicotine. However, they found that substituents at this position even more significantly influenced their functional properties. Dukat *et al*. (2002) determined that although the K_i values for both compounds were similar to that of nicotine, the 5-bromo compound functioned as a partial agonist on rat α4β2 receptors expressed in *Xenopus* oocytes whereas the 5-methoxy compound had no agonistic activity.

Substituents at the 6-position on the pyridine ring were characterized for binding to α4β2 as well as for *in vivo* effects on mice (Dukat *et al*. 1996). They determined that a chlorine at the 6-position of nicotine produced an analog with 3.8-fold greater affinity than (S)-nicotine for α4β2. Other analogs with small substituents, including 6-bromo and 6-fluoronicotine had affinities that were respectively 1.4- and 1.6-fold lower than that

of (S)-nicotine. However, a larger methoxy substituent at this position decreased affinity 35 times that of nicotine for α4β2. Both the 6-bromo and 6-fluoro analogs were as equipotent as (S)-nicotine in antinociceptive measurements on mice whereas the 6-methoxy analog was much less active. Dukat *et al*. (1996) determined that both lipophilicity and size of the substituent at the 6-pyridyl position influence binding properties as well as *in vivo* effects.

A group of compounds that have been characterized for their ability to inhibit ^{3}H-dopamine release from striatal slices contain substituents (increasing chain length alkyl) at the 1-pyridyl position. Crooks *et al*. (2004) found that adding alkyl substituents with chain lengths ranging from 1 to 4 carbons resulted in low potency antagonists (IC_{50} >10 μM). Interestingly, the most potent compound had a 1-pyridyl-chain length of 12 carbons. This potent antagonist, known as NDDNI, had an IC_{50} value of 0.009 μM and a K_i value for ^{3}H-nicotine displacement of 0.14 μM (Crooks *et al*. 2004).

The binding affinities of substituents at the 3'-, 4'-, and 5'-pyrrolidine positions have been well characterized by Lin *et al*. (1994) in rat brain. They found that increasing substituent size at the 3'-position resulted in decreased affinity, indicating steric effects of the ligand-receptor interaction. A methyl group at the 4'-position had an affinity that was only 3.7-fold lower than that of (S)-nicotine. However, when larger nonpolar or polar substituents were added at the 4'-position, the affinity for α4β2 decreased. Lin *et al*. (1994) noted that electronic along with steric effects may be influencing the binding affinities for the α4β2 receptor. Unlike at the 4'-position, a methyl substituent at the 5'-position was not well tolerated; it decreased affinity over 30-fold as compared to (S)-nicotine. Also, they found that there exists a stereoselectivity for substituents at the

5'-position. The trans-5'-methyl analog had a 34.5-fold greater affinity for the receptor relative to the cis-5'-methyl nicotine. Thus, steric influences as well as stereochemistry produce effects upon binding at the 3'-, 4'- and 5'-positions on (S)-nicotine.

Damaj *et al*. (1996) found that an ethyl at the 1'-N-position reduced affinity for rat α4β2 35-fold as compared to (S)-nicotine. Glassco *et al*. (1994) measured a trend of decreasing affinity for α4β2 in rat brain with increasing 1'-N-substituent size. They found about a 20-fold decrease in affinity (as compared to (S)-nicotine) when the size of the substituent at the 1'-N-position was increased from a methyl to an ethyl, a 369-fold decrease (as compared to (S)-nicotine) when substituting a propyl group and greater than a 7,000-fold decrease when substituting a cyclopropyl group. Like the other pyrrolidine substituents, bulky groups at the 1'-N-position result in steric hindrance for binding with a high affinity to the α4β2 receptor.

Because these previous studies lacked data on efficacy and nAChR selectivity, we have further characterized several of these compounds and additionally, other novel nicotine analogs on α7 as well as α4β2 nAChRs. Our goal was to create a structure activity relationship for nicotine binding to the α4β2 receptor to assist in reaching our ultimate goal of designing a partial agonist for smoking cessation. Based upon hypotheses structured from previously published data (primarily binding) of nicotine analogs and the α4β2 receptor, we created nicotine analogs at four different positions on the nicotine molecule: the 5-pyridyl and 5'-, 1'-N-, and 3'-pyrrolidine positions.

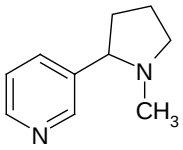
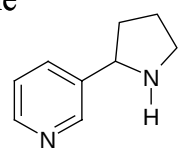
Hypotheses:

1) Relatively bulky substituents at the 5-pyridyl position would permit effective binding but reduce efficacy.
2) A trans-methyl group at the 5'-pyrrolidine position would reduce efficacy with retention of acceptable binding affinity and receptor selectivity.
3) Bulky alkyl groups at the 1'-N-position would decrease the α4β2 affinity but relatively strong partial agonism would be retained.
4) Bulky alkyl groups at the 3'-position would decrease the affinity for rat and human α4β2 but enhance the affinity for α7 (based on previously published DMXBA data).

(S)-Nicotine and (S)-Nornicotine

Using ^{3}H-cytisine as a label for α4β2, nicotine displaced the radiolabel from rat brain with a K_i value of 9.2 nM. As a comparison for selectivity we report selectivity ratios (SR = K_i value for α7/ K_i value for α4β2). The selectivity ratio of (S)-nicotine was 83. (S)-Nornicotine binds with a much lower affinity to rat α4β2 (K_i = 0.11 μM) than (S)-nicotine. It is also less selective for rat α4β2 over rat α7, with a SR of 16 (Table 3-1).

Table 3-1. Inhibition of ^{125}I-α-btx and ^{3}H-cytisine binding to rat brain membranes or inhibition of ^{3}H-cytisine binding to human α4β2 expressing tsA201 cell membranes by (S)-nicotine or (S)-nornicotine.

Compound Name and Structure	K_i (μM)		
	Rat		Human
	^{125}I-α-Btx (α7)	^{3}H-Cytisine (α4β2)	^{3}H-Cytisine (α4β2)
(S)-Nicotine 	0.76 ± 0.11	0.0092 ± 0.0020	0.0089 ± 0.0039
(S)-Nornicotine 	1.7 ± 0.29	0.11 ± 0.029	0.48 ± 0.26

K_i values were calculated according to the Cheng Prusoff equation. The concentration of each radioligand was 1 nM. Each value represents the mean ± SEM of ≥ three separate experiments unless otherwise indicated. Concentrations for each experiment were done in triplicate. The K_d values for ^{125}I-α-btx and ^{3}H-cytisine binding to rat brain membranes were 0.32 nM ± 0.04 and 0.92 ± 0.1 nM respectively. The K_d value for ^{3}H-cytisine binding to tsA201 cell membranes was 0.48 nM ± 0.2.

Because the functional assays were performed using cells (tsA201) that express human α4β2, I also measured the binding properties of the nicotine analogs on homogenized tsA201 cell membranes. Statistical T-tests were performed to determine any significant differences between rat brain α4β2 and human α4β2 K_i values. I also performed a sequence alignment with ClustalW (on the European Bioinformatics website) for human and rat α4 and β2 subunits loops A through F in order to determine any residue differences. The alignment revealed two residue differences between the species as follows:

Loop F	*	Loop E	*
Human β2	EVASLDDF	Human α4	LTKAHLFHDGRVQWT
Rat β2	DVASLDDF	Rat α4	LTKAHLFYDGRVQWT

The F loop of the human β2 subunit contains a glutamic acid at position 190 whereas the rat β2 subunit contains an aspartic acid at position the homologous position (189). The α4 human subunit loop E contains a histidine at position 145 and in its place in the rat α4-receptor subunit is a tyrosine residue at homologous position 147.

Experiments were done to determine the agonist activity of each nicotine analog on α4β2 receptors. These measurements were performed by first adding the nicotine analog alone at various concentrations followed by the addition of KCl (40 mM final concentration), which was used as a calibrant (Figure 3-1). Fitch *et al*. (2003) previously published results using KCl as a calibrant on various cell lines expressing different subtypes of nAChRs, thus we also chose to use KCl. The addition of KCl results in a large depolarization (as measured by changes in fluorescence) that was used to normalize for cell count, dye loading and possible differences in resting membrane potential between passages of cells. After performing a concentration response curve for the tsA201 cells and KCl, it was determined that 40 mM KCl produces about 85% of the maximum KCl response. Measurable changes in fluorescence were relatively rapid, starting within three seconds, after application of agonist. There was no decay of fluorescence after application of (S)-nicotine, thus calibration calculations were performed with the KCl response as measured on agonist naïve cells (those that received only HEPES/HBSS).

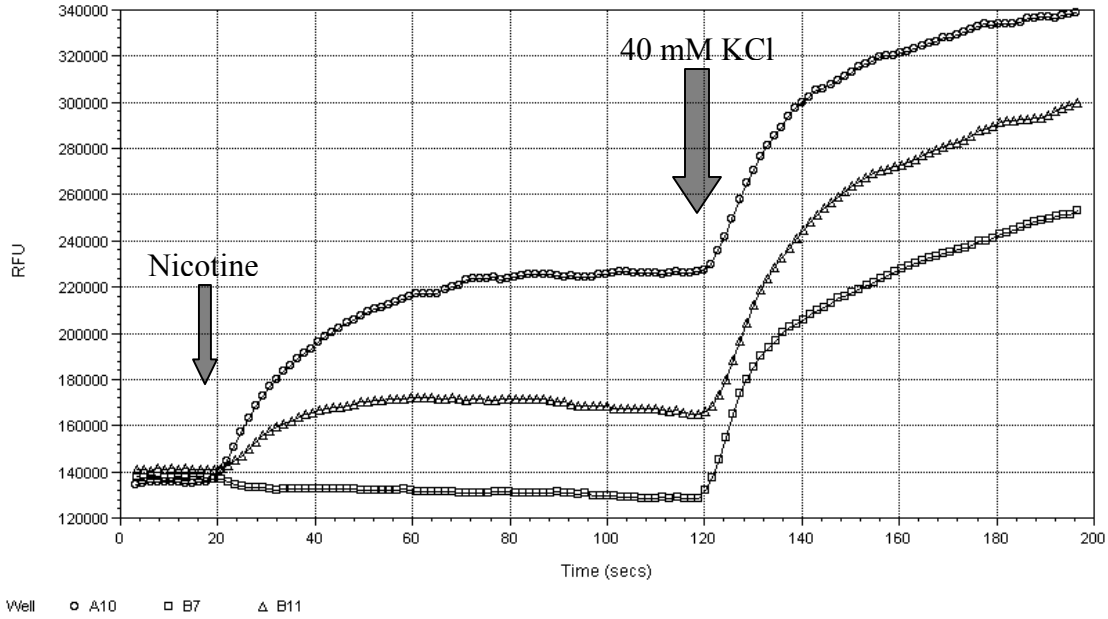


Figure 3-1. Signal acquired for membrane potential fluorescence using tsA201 cells expressing human α4β2 nAChRs. During the first 17 seconds baseline fluorescence was measured for each well. At 18 seconds well A10 (o) received 50 μM nicotine, well B11 (Δ) received 0.16 μM nicotine and well B7 (□) received only 20 mM HEPES/HBSS. All three wells received a final concentration of 40 mM KCl at 120 seconds. A fluorescence reading was taken every 1.44 seconds for a total of 139 reads over a three minute period.

The functional responses of the nicotine analogs as measured with membrane potential dye indicated that several analogs, along with the naturally occurring (S)-nicotine and (S)-nornicotine, had agonistic activity on human α4β2 receptors. The average EC_{50} values for (S)-nicotine and ACh were 1.1 ± 0.15 μM and 3.6 ± 0.9 μM respectively (Figure 3-2). (S)-Nornicotine displayed partial agonist activity: it was half as efficacious as compared to ACh, with an EC_{50} value of 8.9 ± 0.67 μM (Figure 3-2). Several experiments were done to determine the maximum effect of ACh on the human α4β2 expressing tsA201 cells by increasing the concentration of ACh from 2,000 μM to

10,000 μM. The results from those separate experiments (not shown) produced the same EC_{50} and maximum response as the response seen in Figure 3-2.

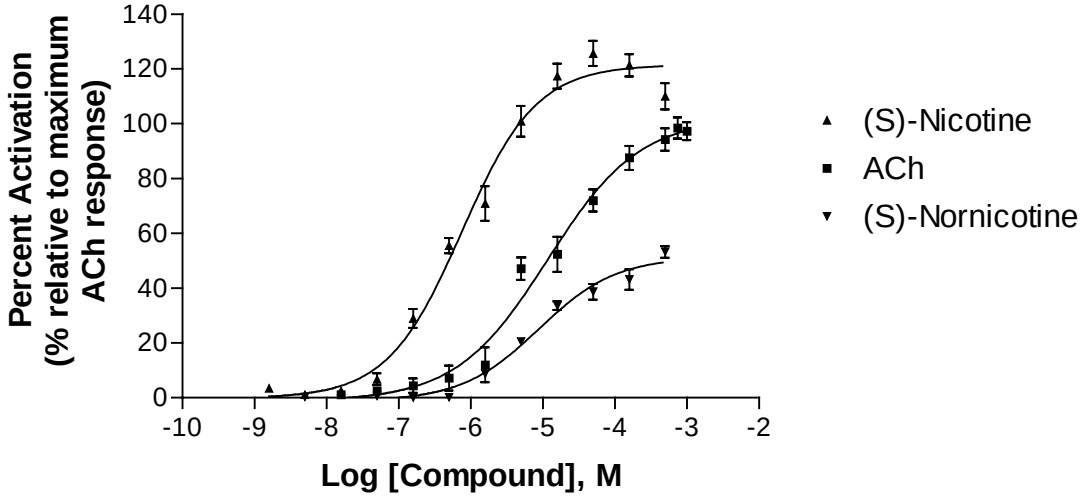
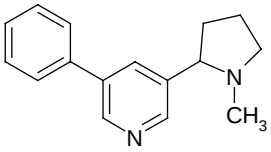
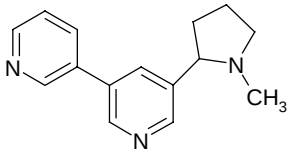


Figure 3-2. Concentration response curves of nicotine, ACh and nornicotine for human α4β2 receptors expressed in tsA201 cells as measured by changes in membrane potential. Responses were normalized to the maximum ACh response. Each curve represents an average of 20 wells for nicotine, 14 wells for ACh and 4 wells for nornicotine (averaged from 2 to 10 separate experiments).

5-Pyridyl Substituted Analogs

The 5-pyridyl substituted analogs, 5-phe-nic and 5-pyr-nic, both bound with low micromolar affinities to rat α4β2: K_is = 0.066 and 0.14 μM, respectively (Table 3-2). Interestingly, these compounds showed no interaction with rat α7 at concentrations up to 20 μM. Thus, although both compounds bound with lower affinities to rat α4β2 than (S)-nicotine, they had higher selectivity ratios (SR): SR of 5-phe-nic was 303 while the SR of 5-pyr-nic was 143. These values are greater than the SR of nicotine, which was 83.

Table 3-2. Inhibition of ^{125}I-α-btx and ^{3}H-cytisine binding to rat brain membranes or inhibition of ^{3}H-cytisine binding to human α4β2 expressing tsA201 cell membranes by 5-pyridyl substituted analogs.

Compound Name and Structure	K_i (μM) Rat ^{125}I-α-Btx (α7)	Rat ^{3}H-Cytisine (α4β2)	Human ^{3}H-Cytisine (α4β2)
5-Phenyl-(S,R)-Nicotine 5-Phe-Nic* 	>20	0.066 ± 0.013 ♦	0.020 ± 0.0037
5-(3-Pyridyl)-(S,R)-Nicotine 5-Pyr-Nic* 	>20	0.14 ± 0.021 ♦	0.053 ± 0.017

Parameters were the same as in Table 3-1. Diamonds indicate a significant difference between the K_i values for rat and human with ^{3}H-cytisine. Abbreviations for the analogs are indicated by an asterisk.

The 5-pyridyl substituted analogs were tested for their ability to activate human α4β2 receptors expressed in tsA201 cells. There was no measurable activation produced by either compound. The 5-pyridyl substituted analogs were then tested for their ability to inhibit the ACh response as measured by changes in membrane potential. ACh was chosen over (S)-nicotine as the standard agonist primarily because the occurrence of channel block by ACh is expected to be less than for (S)-nicotine. A concentration of 5 μM ACh was applied to the cells simultaneously with the nicotine analog of interest. A concentration of 5 μM ACh was chosen because it was slightly above the EC_{50} (3.6 μM) and well below the concentration required to produce a maximum response (500 μM). The 5-phe-nic and 5-pyr-nic compounds had the highest affinities (K_i values = 0.02 μM and 0.053 μM, respectively) for human α4β2 receptors as compared to the other nicotine

analogs. These two compounds produced measurable IC_{50} values of 14.3 (5-phe-nic) and 11.6 μM (5-pyr-nic) for human α4β2 (Table 3-3).

Table 3-3. Inhibition of human α4β2 receptor ACh responses by 5-pyridyl substituted analogs.

Compound Name or Abbreviation	**IC_{50} (μM)**
5-Pyridyl substituted analogs	
5-Phe-Nic	14.3 ± 1.3
5-Pyr-Nic	11.6 ± 0.41

The compounds and ACh (5 μM) were administered simultaneously. Each value represents the average ± SEM of four to six separate wells.

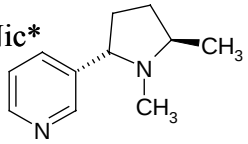
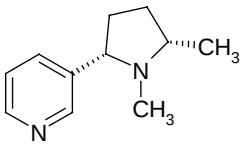
5'-Pyrrolidine Substituted Analogs

The results for the 5'-pyrrolidine substituted analogs indicate that the receptor possesses a strong conformational preference for ligand binding: the 5'-trans-met-nic analog bound with an affinity of 0.150 μM to rat α4β2 whereas 5'-cis-met-nic displayed no interaction with this receptor at concentrations up to 20 μM (Table 3-4). While 5'-trans-met-nic did bind to rat α7 with a K_i of 2.1 μM, 5'-cis-met-nic did not interact with rat α7 at concentrations up to 20 μM. The SR for 5'-trans-met-nic was 14, thus, the methyl substituent at the 5'-trans position dramatically reduced the selectivity for rat α4β2 as compared to nicotine. There was no significant difference between the binding of either 5'-substituted analog to rat α4β2 or human α4β2.

Like the 5-pyridyl substituted analogs, the 5'-pyrrolidine analogs produced no activation of human α4β2 receptors expressed in tsA201 cells. However, the 5'-trans-met-nic analog did inhibit the human α4β2 ACh response (Table 3-5). Full concentration response curves were not obtained at the concentrations tested, therefore

percent of inhibition values were reported. At the highest concentration of 5'-trans-met-nic used (50 μM), the human α4β2 ACh response was inhibited by 50%.

Table 3-4. Inhibition of ^{125}I-α-btx and ^{3}H-cytisine binding to rat brain membranes or inhibition of ^{3}H-cytisine binding to human α4β2 expressing tsA201 cell membranes by 5'-pyrrolidine substituted analogs.

Compound Name and Structure	K_i (μM) Rat ^{125}I-α-Btx (α7)	Rat ^{3}H-Cytisine (α4β2)	Human ^{3}H-Cytisine (α4β2)
5'-Trans-Methyl-(S,R)-Nicotine 5'-Trans-Met-Nic* 	2.1 ± 0.033	0.15 ± 0.013	0.22 ± 0.038
5'-Cis-Methyl-(S,R)-Nicotine 5'-Cis-Met-Nic* 	>20	>20	>20

Parameters were the same as in Figure 3-1. Abbreviations for the analogs are indicated by an asterisk.

Table 3-5. Inhibition of human α4β2 receptor ACh responses by 5'-pyrrolidine substituted analogs.

Compound Name or Abbreviation	Percent Inhibition (I)
5'-Pyrrolidine substituted analogs	
5'-Trans-Nic	50% I (50 μM)
5'-Cis-Nic	0% I (50 μM)

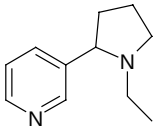
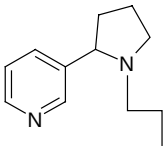
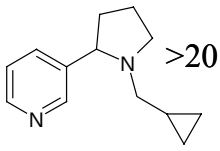
The compounds and ACh (5 μM) were administered simultaneously. The concentrations in parenthesis indicate the highest concentration tested. Each value represents the average ± SEM of four to six separate wells.

1'-N-Pyrrolidine Substituted Analogs

The ability of the 1'-N-pyrrolidine substituted analogs to bind to either rat α4β2 or rat α7 nAChRs decreased as the size of the substituent increased (Table 3-6). The

smallest substituent, an ethyl group, at the 1'-N-position bound with an affinity (K_i = 0.14 μM) similar to that of 5'-trans-met-nic (0.15 μM). As the substituent sizes increased to a propyl and cyclopropyl group, there was no measurable interaction with either rat α4β2 or rat α7 up to 20 μM. The SR of 1'-N-ethyl-nor (SR = 17.9) was less than that of (S)-nicotine (SR = 83). Initial data on the binding of 1'-N-ethyl-nor to human α4β2 indicates a K_i value very similar to that for rat α4β2.

Table 3-6. Inhibition of ^{125}I-α-btx and ^{3}H-cytisine binding to rat brain membranes or inhibition of ^{3}H-cytisine binding to human α4β2 expressing tsA201 cell membranes by 1'-N- pyrrolidine substituted analogs.

	K_i (μM)		
	Rat		Human
Compound Name and Structure	**^{125}I-α-Btx (α7)**	**^{3}H-Cytisine (α4β2)**	**^{3}H-Cytisine (α4β2)**
1'-N-Ethyl-(S)-Nornicotine 1'-N-Ethyl-Nor* 	2.5 ± 0.09	0.14 ± 0.04	0.14 (n=2)
1'-N-Propyl-(S,R)-Nornicotine 1'-N-Propyl-Nor* 	>20	>20	>20
1'-N-Cyclopropylmethyl-(S,R)-Nornicotine 1'-N-Cyclopropyl-Nor* 	>20	>20	>20

Parameters were the same as in Figure 3-1. Abbreviations for the analogs are indicated by an asterisk.

Within the four classes of nicotine analogs, two of them (the 1'-N-pyrrolidine and 3'-pyrrolidine substituted analogs) produced activation. The highest concentrations of analogs utilized in these studies did not produce full concentration response curves

(maximal responses) to allow for EC_{50} values to be determined, therefore only percent activations at these high concentrations are reported (Figure 3-3). The 1'-N-ethyl-nor compound was the only 1'-N-pyrrolidine substituted analog with a measurable affinity for the rat and human receptors and it produced about 35% activation of human α4β2 (at 500 μM) as compared to the maximum ACh response. Both 1'-N-propyl-nor and 1'-N-cyclopropyl-nor activated the human receptor about 40% (at 2,000 μM) and 75% (at 2,000 μM) respectively. Although neither of these compounds had a measurable affinity for the human α4β2 receptor as determined with the radioligand binding assays, which utilized lower concentrations than in the functional assays, the concentrations used for the membrane potential measurements were several-fold higher. Therefore, at concentrations above 20 μM these compounds are binding to the receptor and causing activation.

All three 1'-N-substituted analogs were also found to inhibit the ACh response of human α4β2. The 1'-N-cyclopropyl-nor analog was the most potent at activating human α4β2 (at the concentrations tested) as well as the most potent inhibitor (Table 3-7). The 1'-N-ethyl-nor and 1'-N-cyclopropyl-nor both produced less than 50% inhibition of the human α4β2 ACh response.

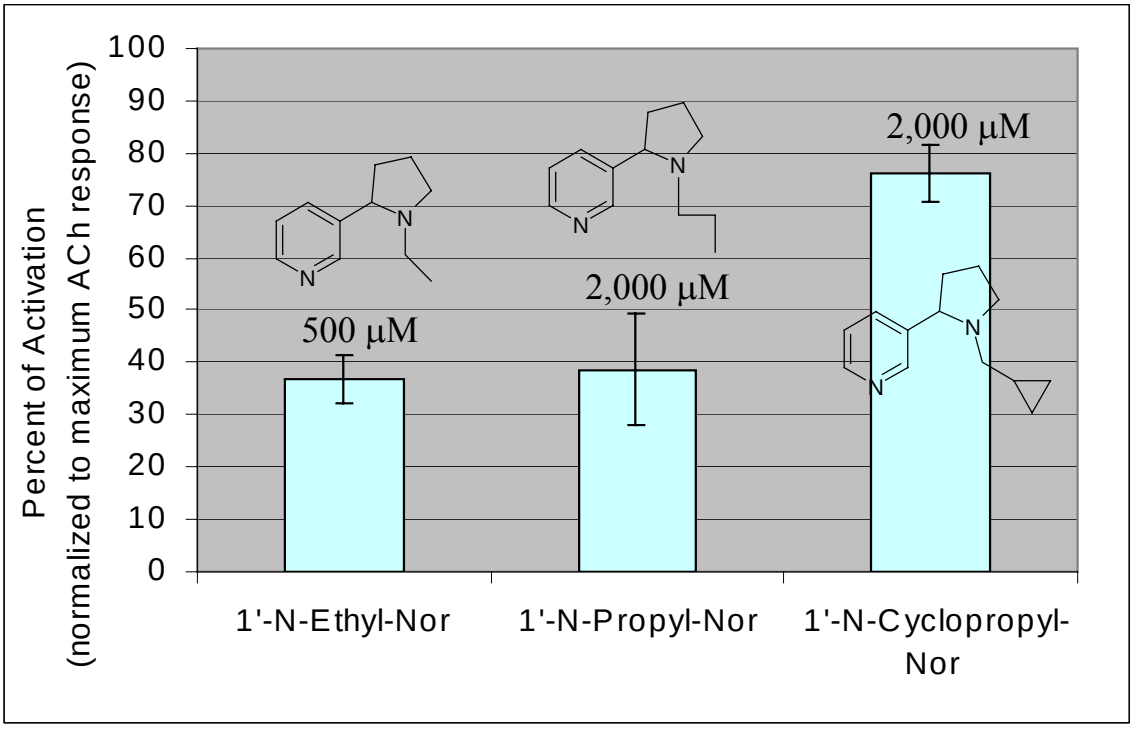


Figure 3-3. Average effects of the 1'-N-pyrrolidine substituted analogs on human α4β2 receptors expressed in tsA201 cells as measured with membrane potential dye. Effects by each analog were produced by the concentration listed above the corresponding bar. Each bar represents 4 separate wells. Responses were normalized to the maximum ACh response (500 μM).

Table 3-7. Inhibition of human α4β2 receptor ACh responses by 1'-N-pyrrolidine substituted analogs.

Compound Name or Abbreviation	**Percent Inhibition (I)**
1'-N-Pyrrolidine substituted analogs	
1'-N-Ethyl-Nor	35% I (500 μM)
1'-N-Propyl-Nor	45% I (500 μM)
1'-N-Cyclopropyl-Nor	80% I (50 μM)

The compounds and ACh (5 μM) were administered simultaneously. The concentrations in parenthesis indicate the highest concentration tested. Each value represents the average ± SEM of four to six separate wells.

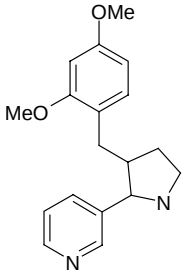
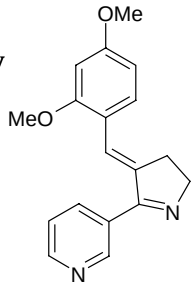
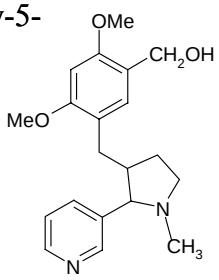
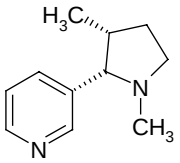
3'-Pyrrolidine Substituted Analogs

The same trend of decreasing affinity with increasing substituent size was observed for the 3'-pyrrolidine substituted analogs (Table 3-8). The 3'-met-nic analog, which has only a methyl group at the 3'-position, bound to rat α4β2 with a decreased affinity

(Ki=0.37 μM) as compared to (S)-nicotine. 3'-Dimethoxybenzyl-nornicotine (DMXBN) has a large substituent, a benzene ring with two methoxy groups attached, yet it still bound to the rat α4β2 receptor with a K_i value of 2.8 μM and had no interaction with rat α7 at up to 20 μM (SR > 7.1). The differences between DMXBM and DMXBN are related to the presence of two additional double bonds in DMXBM, allowing for all these rings of the compound to be electronically conjugated. DMXHMN has an additional hydroxymethyl group attached to the benzyl ring, which is attached at (but not electronically conjugated to) the 3'-pyrrolidine position of nicotine. DMXHMN displayed no interaction with either rat α4β2 or rat α7 at up to 20 μM.

The 3'-pyrrolidine substituted analogs also had very low or no measurable affinity for human α4β2 at concentrations up to 20 μM. They are however activating human α4β2 receptors at higher concentrations (Figure 3-4). DMXBN produced 80% activation (at 500 μM) whereas DMXBM produced only 20% activation (at 500 μM). The conjugated structure of DMXBM makes it more rigid and the ionizable nitrogen less basic. 3'-Met-nic, the 3'-pyrrolidine substituted analog with the smallest substituent, produced 40% activation at 500 μM.

Table 3-8. Inhibition of ^{125}I-α-btx and ^{3}H-cytisine binding to rat brain membranes or inhibition of ^{3}H-cytisine binding to human α4β2 expressing tsA201 cell membranes by 3'-pyrrolidine substituted analogs.

Compound Name and Structure		K_i (μM) Rat ^{125}I-α-Btx (α7)	Rat ^{3}H-Cytisine (α4β2)	Human ^{3}H-Cytisine (α4β2)
3'-Dimethoxy benzyl-(S,R)-Nornicotine DMXBN*		>20	2.8 ± 0.58	Not Determined
3'-(2,4-Dimethoxy benzylidene)-Myosmine DMXBM*		>20	>20	>20
3'-(2,4-Dimethoxy-5-hydroxymethyl) benzyl-(S,R)-Nicotine DMXHMN*		>20	>20	>20
3'-Methyl-(S,R)-Nicotine 3'-Met-Nic*		4.2 n = 2	0.37 ± 0.00015	0.63 ± 0.094

Parameters were the same as in Figure 3-1. Abbreviations for the analogs are indicated by an asterisk.

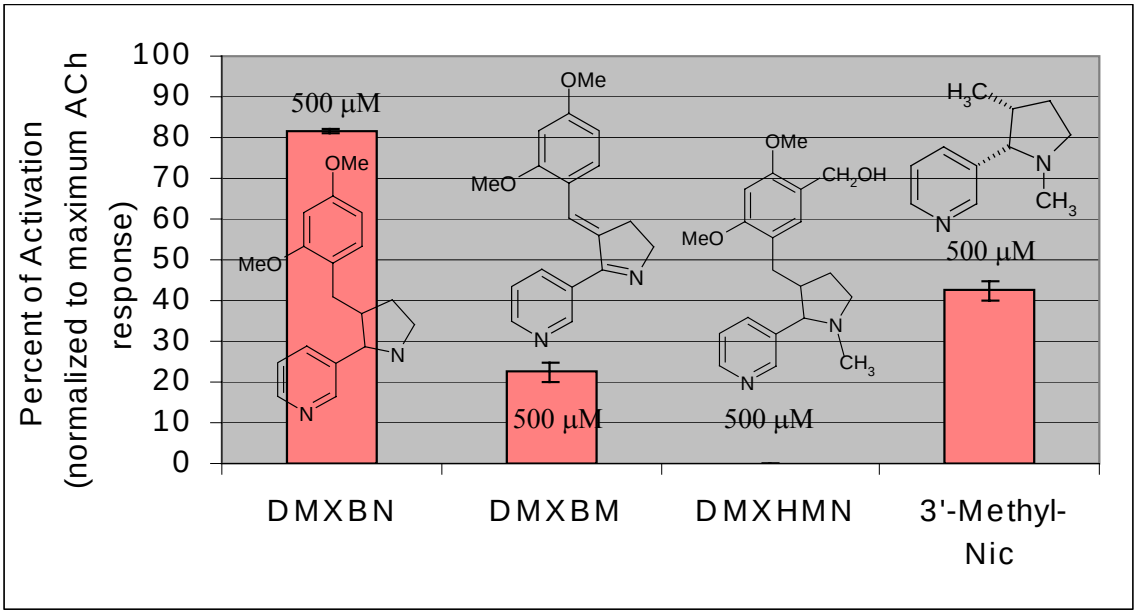


Figure 3-4. Average effects of the 3'-pyrrolidine substituted analogs on human α4β2 nAChRs expressed in tsA201 cells as measured with membrane potential dye. Effects by each analog were produced by the concentration listed within or above the corresponding bar. Each bar represents 4 separate wells. Responses were normalized to the maximum ACh response (500 µM).

Three out of four of the 3'-pyrrolidine substituted analogs produced varying degrees of human α4β2 nAChR activation. They did not however produce much if any inhibition of the ACh response (Table 3-9). The DMXBN, DMXBM and DMXHMN analogs had no inhibitory action at concentrations up to 50 µM (500 µM for DMXBN). 3'-Met-nic did produce a 20% inhibition at 50 µM.

Table 3-9. Inhibition of human α4β2 receptor ACh responses by 3'-pyrrolidine substituted analogs.

Compound Name or Abbreviation	**Percent Inhibition (I)**
DMXBN	0% I (500 µM)
DMXBM	0% I (50 µM)
DMXHMN	0% I (50 µM)
3'-Met-Nic	20% I (50 µM)

The compounds and ACh (5 µM) were administered simultaneously. The concentrations in parenthesis indicate the highest concentration tested. Each value represents the average ± SEM of four to six separate wells.

CHAPTER 4
RESULTS

Separation of Nicotine Analog Enantiomers

Naturally occurring (S)-nicotine (approximately 99% S-form, about 1% R-form as an impurity) has been reported as more potent than its enantiomer in binding to high affinity nicotinic receptors as measured with ^{3}H-nicotine (Copeland *et al*. 1991; Zhang and Nordberg, 1993). Copeland *et al*. (1991) measured a 7-fold difference in the binding of the enantiomers to rat cortex; (S)-nicotine had the higher affinity (K_i = 0.014 μM) relative to (R)-nicotine (K_i = 0.102 μM). Zhang and Nordberg (1993) reported a 3-fold difference in the binding of the stereoisomers to rat cortex but an 11-fold difference in binding to rat cerebellum. Both studies found no significant differences in the binding affinities of the enantiomers of nornicotine. Zhang and Nordberg (1993) determined a 1.6-fold difference in the binding of the nornicotine enantiomers to rat cerebellum whereas Copeland *et al*. (1991) reported a 1.1-fold difference.

The existence of nicotinic receptors on dopaminergic terminals along with evidence of nAChR involvement in nicotine addiction has lead several groups to study the effects of nicotine and nornicotine in dopamine release assays using synaptosomes and rat brain slices. (S)-Nornicotine, like (S)-nicotine is a naturally occurring alkaloid found in tobacco plant (Saitoh *et al*. 1985). It is also an active metabolite of nicotine and has a half-life three times longer than that of (S)-nicotine (Crooks *et al*. 1997; Green *et al*. 2001). Whiteaker *et al*. (1995) measured an EC_{50} of 0.5 μM for (S)-nicotine stimulated ^{3}H-dopamine release from striatal synaptosomes. Based upon the reported decrease in

affinity of (R)-nicotine, it has yet to be characterized in dopamine release assays. The enantiomers of nornicotine however have been investigated. Green *et al*. (2001) determined that (R)-nornicotine (EC_{50} = 0.48 μM) was 6.3 times more potent than (S)-nornicotine (EC_{50} = 3.0 μM) at evoking radiolabeled dopamine release from rat nucleus accumbens slices. It has also been determined that (S)-nornicotine desensitizes nAChRs with 12-fold lower potency than (R)-nornicotine as measured with dopamine release assays from rat striatum (Dwoskin *et al*. 2001).

Several of the nicotine analogs in this dissertation were initially studied as racemic compounds because the enantiomeric species were difficult to synthesize. Given that previous binding data indicates a difference in affinity for the enantiomers of nicotine, it was important to try and separate the (R)- and (S)-forms of the racemic nicotine analogs and determine the affinity and functional properties of these chiral compounds. Our hypothesis was that like (S)-nicotine, the (S)-forms of the racemic analogs would have a higher affinity and functional potency for α4β2.

Three of the racemic nicotine analogs, 5-phe-nic, 5-pyr-nic and 1'N-ethyl-nor, were successfully separated by chiral HPLC (Figure 4-1). The 5-phe-nic and 1'-N-ethyl-nor compounds were tested for their ability to bind to rat α4β2 nAChRs as measured by ^{3}H-cytisine displacement (Table 4-1). The affinities of the nicotine and nornicotine enantiomers were measured for human α4β2 and then further tested for potency and efficacy (Figure 4-2) on the human α4β2 receptor in tsA201 cells

The affinities of the racemic 5-phe-nic and 5-pyr-nic analogs were similar as measured for rat and human α4β2 (Table 3-2) as may be expected based on their similar structures. Thus, only the enantiomers of the 5-phe-nic were tested for their ability to

bind to rat α4β2. The enantiomers of both the 5-phe-nic and 1'-N-ethyl-nor have yet to be identified as to which peak corresponds to the (R) or (S)-form, therefore the enantiomers are labeled as peak 1 or 2. It may be expected that peak 1 is the (S)-enantiomer based upon data from the chiral column indicating that (S)-nicotine elutes before its (R)-enantiomer (Armstrong *et al*. 1998). Peak 1 of 5-phe-nic had a K_i value of 0.17 μM as compared to the K_i value of 3.6 μM for peak 2. The difference in affinities of the enantiomers was significantly different for rat α4β2 ($P = 0.0104$). The enantiomeric selectivity ratio (ESR), calculated by dividing the K_i of peak 2 by the K_i of peak 1, gives a value of 21. The K_i value for peak 1 of 1'-N-ethyl-nor was 0.24 μM and 2.3 μM for peak 2 was also significantly different ($P = 0.0015$). The higher affinity of peak 1 for rat α4β2 of both analogs was anticipated based upon previous findings (Copeland *et al*. 1991; Zhang and Nordberg 1993).

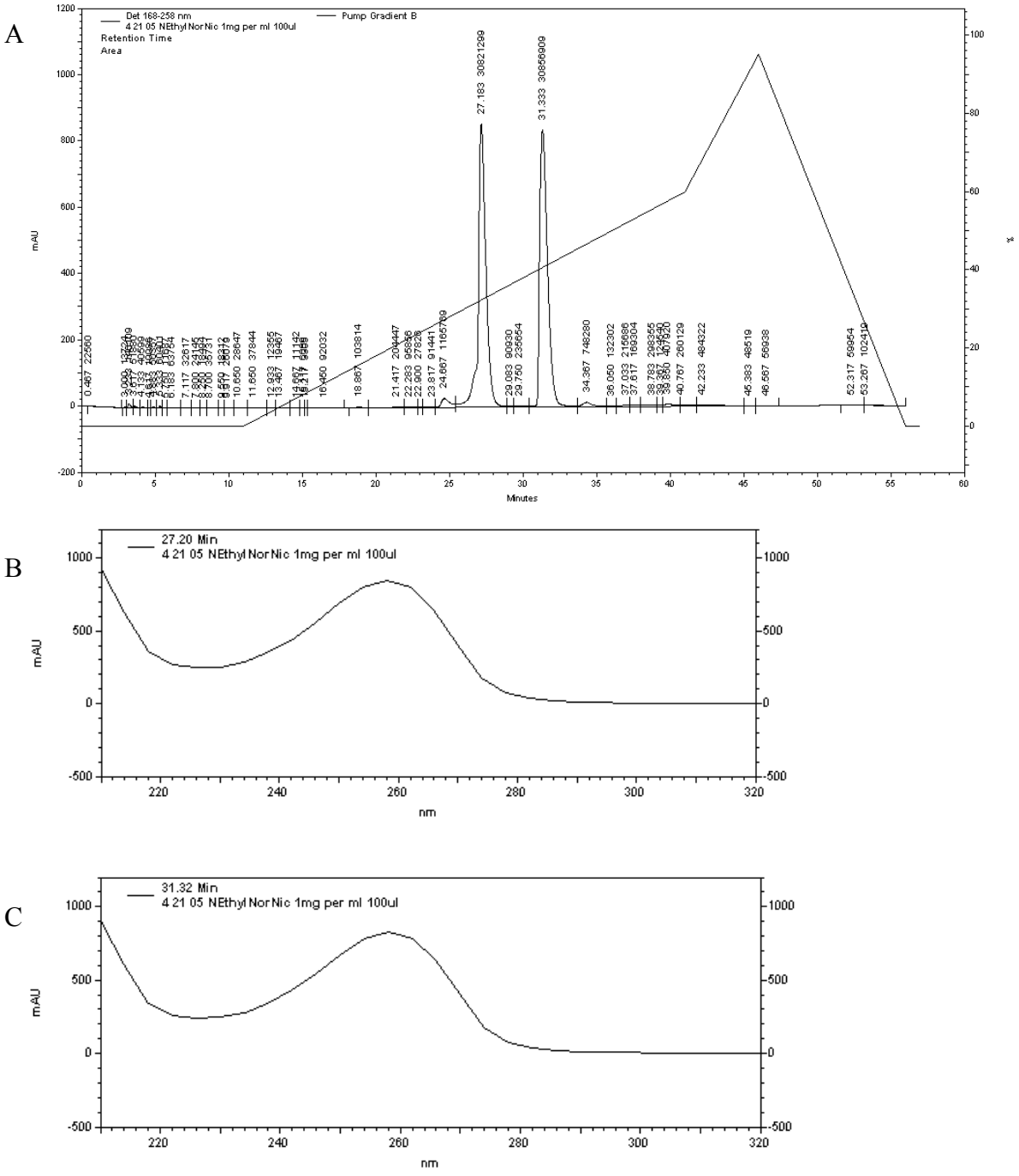


Figure 4-1. HPLC chiral separation of 1'-N-ethyl-(S,R)-nornicotine. A) The chromatogram with retention times for the separation of presumed 1'-N-ethyl-(S)-nor (peak 1) and presumed 1'-N-ethyl(R)-nor (peak 2). B) The spectra of peaks one and two indicate an absorbance at 260 nm.

Table 4-1. Inhibition of ^{3}H-cytisine binding to rat brain or to human α4β2 expressing tsA201 cell membranes by nicotine enantiomers.

Compound Name or Abbreviation	**K_i for α4β2 (μM)**	
(S)-Nicotine	0.0089 ± 0.0039	(human)
(R)-Nicotine	0.011 ± 0.0013	(human)
(S)-Nornicotine	0.48 ± 0.26	(human)
(R)-Nornicotine	0.042 ± 0.025	(human)
1'-N-Ethyl-Nor (peak 1)	0.24 ± 0.060	(rat)
1'-N-Ethyl-Nor (peak 2)	2.3 ± 0.26	(rat)
5-Phe-Nic (peak 1)	0.17 ± 0.023	(rat)
5-Phe-Nic (peak 2)	3.6 ± 0.76	(rat)

K_i values were calculated according to the Cheng Prusoff equation. The concentration of radioligand was 1 nM. Each value represents the mean ± SEM of three separate experiments. Concentrations for each experiment were done in triplicate. The K_d values for ^{3}H-cytisine binding to rat brain membranes was 0.92 ± 0.1 nM and 0.48 nM ± 0.2 for tsA201 membranes.

The binding of the enantiomers of either nicotine or nornicotine to human α4β2 nAChRs interestingly did not differ significantly as measured by ^{3}H-cytisine displacement. The ESR for the two nicotine enantiomers on human α4β2 was 1.2. Since previously published data indicates that a stereospecificity exists for nicotine and the high-affinity nAChR, this result was unexpected. The K_i value for (R)-nornicotine (0.042 μM) was also not significantly different (P value = 0.1619) from the (S)-enantiomer (0.48 μM) for human α4β2. The large difference in standard error may account for the lack of significance; further replicates will decrease the standard error and may make apparent a significant difference. The nornicotine data agrees with previous binding data obtained with rat brain in which there was no significant difference between (S)- and (R)-nornicotine (Copeland *et al*. 1991; Zhang and Nordberg, 1993).

The binding results for the enantiomers of nicotine and nornicotine are made more complex when compared to the activity of these compounds on human α4β2 receptors as measured by changes in membrane potential (Figure 4-2). Both (S)- and (R)-nicotine are full agonists as compared to the maximum ACh response of human α4β2 nAChRs. Although there was no significant difference in the measured K_i values for the nicotine enantiomers as measured by binding experiments, there was however a difference in potency, although it was not statistically significant (P = 0.16). The EC_{50} value of (S)-nicotine (2.3 ± 1.2 μM) was 7.0 fold lower than that of (R)-nicotine (16 ± 8.8 μM). The calculated EC_{50} for (S)-nornicotine was 8.5 ± 1.8 μM as compared to an EC_{50} of 39 ± 5.4 μM for (R)-nornicotine. The difference between the EC_{50} values of the nornicotine enantiomers was significant (P = 0.0016). Both forms of nornicotine appear to be functioning as partial agonists as compared to nicotine at the concentrations tested. (R)-Nornicotine appears to be more efficacious than (S)-nornicotine. Further replicates at higher concentrations would be needed to determine if the maximum effects and EC_{50} values obtained by fitting the concentration response curve are consistent.

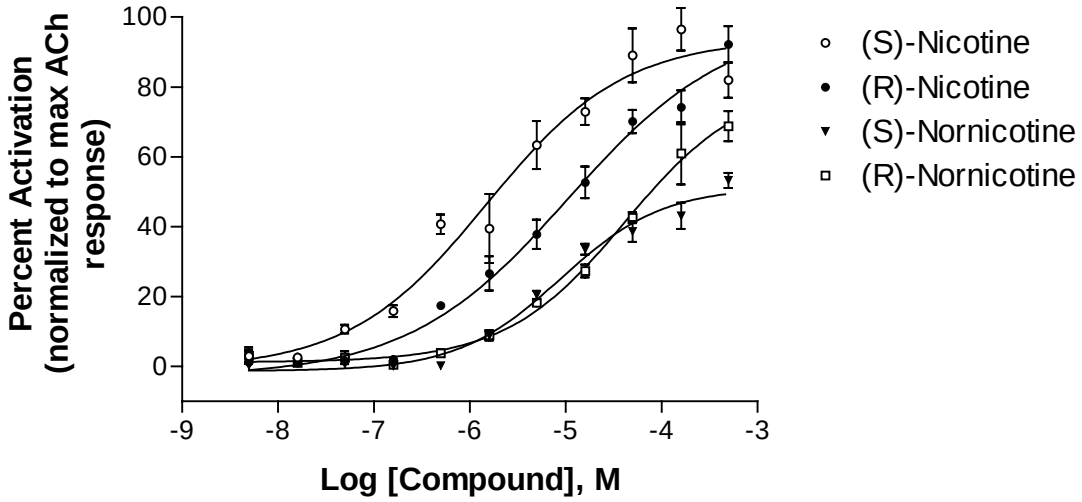


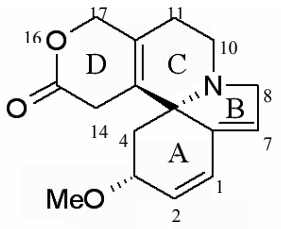
Figure 4-2. Concentration response curves for the enantiomers of nicotine and nornicotine for human α4β2 receptors expressed in tsA201 cells as measured by changes in membrane potential. Responses were normalized to the maximum ACh response (500 μM). Each point represents the average ± SEM of four separate wells. The (S)-nornicotine data is also in Figure 3-3.

CHAPTER 5
RESULTS

Erythrina Alkaloids

Plant alkaloids extracted from the genus *Erythrina* share a common heterocyclic ring system (Figure 5-1). Sheridan *et al*. (1986) determined that the essential aromatic groups of dihydro-β-erythroidine (DHβE) superimpose with those of nicotine indicating that they may share a similar conformation. The affinities and inhibition of the *Erythrina* alkaloids for nAChRs have been less well characterized *in vitro* than the nicotine analogs. The only published literature on these alkaloids and their interaction with nicotinic receptors pertains primarily to DHβE (Williams and Robinson, 1984; Anderson and Arneric, 1994; Harvey and Luetje, 1996; Harvey *et al*. 1996). Erysodine is the only other alkaloid that has been studied for its interaction with the α4β2 nAChR (Decker *et al*. 1995). Aromatic erysodine was determined to have a higher apparent affinity for the receptor (5 nM) than DHβE (35 nM) in rat brain membranes as measured by ^{3}H-cytisine displacement (Decker *et al*. 1995). Erysodine also had a selectivity 1800-fold greater for rat α4β2 than rat α7 whereas DHβE had a selectivity only 114-fold greater for rat α4β2 than rat α7 (Decker *et al*. 1995).

Since the early 1940s it has been known that curare-like effects result upon administration of *Erythrina* alkaloids *in vivo* (Lehman, 1936; Folkers and Major, 1937). The alkaloids were apparently producing their primary effects through blockade of neuromuscular transmission, but ganglionic blocking effects were also observed.



β-Erythroidine

Figure 5-1. The common heterocyclic structure of *Erythrina* alkaloids. The rings are designated A through D and atoms numbered accordingly.

In 1984 Williams and Robinson first studied the effects of DHβE on nAChRs. Their results indicated that it was binding with high affinity (2 nM) to a neuronal nicotinic receptor in rat brain and that its distribution of binding was similar to the binding of ^{3}H-nicotine. Since then DHβE has been referred to and used as a competitive antagonist for selective inhibition of β2-containing nAChRs. Decker *et al*. (1995) determined that DHβE was inhibiting (S)-nicotine-evoked ^{3}H-dopamine release from slices of rat striatum with an IC_{50} of 58 nM. DHβE also interacts with other β2-containing receptors. Electrophysiological measurements on oocytes expressing human α4β2 indicate that DHβE is 14.7-fold less potent of an inhibitor for the α3β2-subtype (Chavez-Noriega *et al*. 1997).

In order to further understand the interaction of antagonists with α4β2 nAChRs our goal was to create a structure activity relationship for *Erythrina* alkaloids and the α4β2 receptor with the ultimate goal of designing a selective probe for use *in vivo* and *in vitro* to study the resting state of the receptor as well as a possible smoking cessation drug. To accomplish this goal several natural and semi-synthetic alkaloids were studied for their ability to displace ^{125}I-α-btx and ^{3}H-cytisine from rat brain membranes and ^{3}H-cytisine

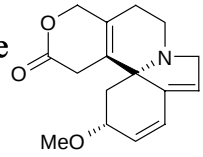
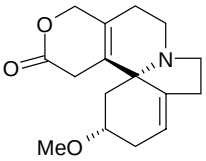
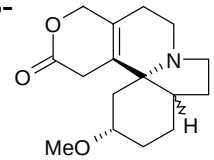
from tsA201 cells expressing human α4β2 nAChRs. The inhibitory properties of these alkaloids were then characterized using tsA201 cells expressing human α4β2 nAChR and a membrane potential dye. Our hypothesis was that alterations to the D-ring of the *Erythrina* alkaloids would have a large affect on affinity and potency for α4β2 and that the nitrogen is critical for binding.

β-Erythroidines

The β-erythroidine compounds contain an unconjugated lactone group in their D-ring and thus lack D-ring aromaticity. β-Erythroidine contains two conjugated double bonds, one in the A ring and another in the B ring. Its K_i value as measured for rat α4β2 was 1.1 μM and its selectivity ratio (SR = K_i α7/K_i α4β2) was 45 (Table 5-1). The two double bonds in β-E have been reduced to one in DHβE so the resulting double bond is now between the 1- and 6-positions, which still maintains approximate co-planarity of the A and B rings. Interestingly, this reduction resulted in an increased affinity for rat α4β2. The affinity of DHβE for rat α4β2 (K_i = 0.14 μM) was eight times greater than that of β-E. Reducing the remaining double bond of DHβE produces THβE. The absence of double bonds in the A and B rings greatly reduced the affinity of this compound for rat α4β2. THβE had an affinity (K_i = 7.4 μM) that was about 7-fold less than that of β-E. Neither DHβE nor THβE displaced α-btx at concentrations up to 50 μM (SR = 377 and 6.76 respectively), indicating that they have a selectivity for rat α4β2 over rat α7. The affinity of β-erythroidine for human α4β2 was not determined. The decrease in affinity of THβE as compared to DHβE was also observed in binding to human α4β2. There was

a 4.4-fold difference in the affinity of DHβE for rat versus human α4β2. THβE had a 2.7-fold difference in affinity between rat and human α4β2.

Table 5-1. Structures, nomenclature and binding results for β-Erythroidine, dihydro-β-erythroidine and tetrahydro-β-erythroidine.

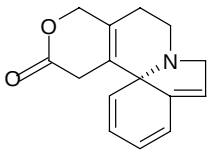
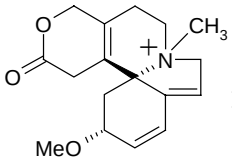
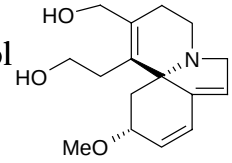
	K_i (μM)		
	Rat		Human
Compound Name	**^{125}I-α-Btx (α7)**	**^{3}H-Cytisine (α4β2)**	**^{3}H-Cytisine (α4β2)**
β-Erythroidine NP β-E*	 >50	1.1 ± 0.84	Not Determined
Dihydro-β-Erythroidine SS DHβE*	 >50	0.14 ± 0.018	0.62 ± 0.17
Tetrahydro-β-Erythroidine SS THβE*	 >50	7.4 ± 4.2	>20

K_i values were calculated according to the Cheng Prusoff equation. The concentration of each radioligand was 1 nM. Each value represents the mean ± SEM of ≥ three separate experiments unless otherwise indicated. Concentrations for each experiment were done in triplicate or quadruplicate. The K_d values for ^{125}I-α-btx and ^{3}H-cytisine binding to rat brain membranes were 0.32 nM ± 0.04 and 0.92 ± 0.1 nM respectively. The K_d value for ^{3}H-cytisine binding to tsA201 cell membranes was 0.48 nM ± 0.2. NP indicates a natural product. SS indicates a semi-synthetic compound. Abbreviations for the analogs are indicated by an asterisk.

3-Des-Met-βE differs from β-E in that it contains two double bonds in the A ring and one in the B ring resulting in an even more extensive conjugation between these two rings (but this is accompanied by movement of the C and D rings with respect to the A and B rings). As may be predicted from the results of β-E and DHβE, the addition of the

third double bond was accompanied by a decrease in affinity for rat α4β2 (Table 5-2). This reduction in affinity also may be due to the loss of the methoxy group at the 3-position. 3-Des-Met-βE did not displace either ^{3}H-cytisine or ^{125}I-α-btx at concentrations up to 20 μM.

Table 5-2. Structures, nomenclature and binding results for 3-Desmethoxy-β-Erythroidine, N-Methyl-β-erythroidine and β-erythroidinediol.

		K_i (μM)		
		Rat		Human
Compound Name		**^{125}I-α-Btx (α7)**	**^{3}H-Cytisine (α4β2)**	**^{3}H-Cytisine (α4β2)**
3-Desmethoxy-β-Erythroidine SS 3-Des-Met-βE*		>20	>20	Not Determined
N-Methyl-β-Erythroidine SS Me-βE*		>20	>20	Not Determined
β-Erythroidinediol SS βE-Diol*		>50	0.31 ± 0.12	0.24 ± 0.015

Parameters were the same as in Table 5-1. Abbreviations for the analogs are indicated by an asterisk.

Me-βE has a quaternary ammonium in place of the tertiary amine in β-E. The presence of this positively charged nitrogen decreased the affinity of Me-βE (K_i > 20 μM) for rat α4β2 at least 20-fold as compared to β-E. Me-βE had no interaction with α7 at concentrations up to 20 μM. The final β-erythroidine studied, βE-Diol, contains an

open D-ring. It may be expected that opening the D-ring would decrease the affinity of βE-Diol as compared to β-E. The results however are just the opposite. The K_i value of βE-Diol for rat α4β2 was 0.31 μM, 3.5-fold greater than that of β-E. βE-Diol had a minimum selectivity ratio (SR) of 161.

To ensure that DHβE was not producing any activation of the human α4β2 receptors expressed by tsA201 cells as measured using the membrane potential fluorescent dye, it was administered in the absence of agonist and as expected there was no measurable activation (Figure 5-2). The ability of the other *Erythrina* alkaloids to produce activation was also measured and the results indicated that all the alkaloids were functioning only as antagonists.

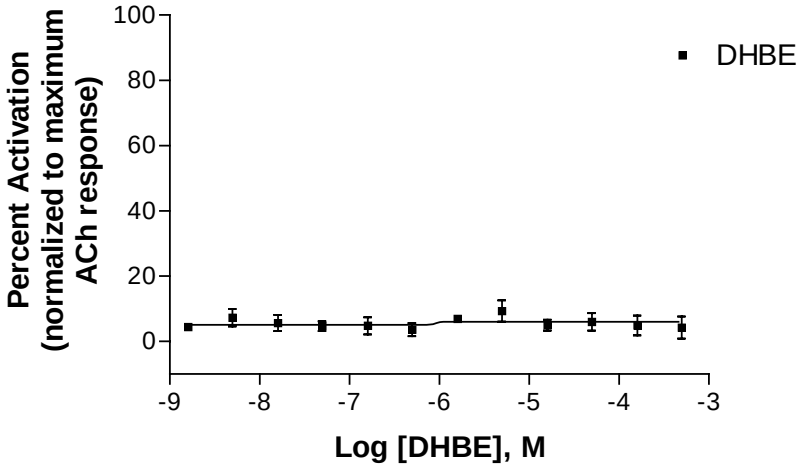


Figure 5-2. Concentration response curve of DHβE for human α4β2 receptors expressed in tsA201 cells as measured by changes in membrane potential. The response was normalized to the maximum ACh response. The result represents an average of four wells.

To verify that DHβE was acting as a competitive antagonist in our system, measurements were made to determine EC_{50} values for ACh in the presence and absence of 1 μM DHβE. A competitive antagonist would shift the concentration response curve

to the right and as the results indicate DHβE is acting as a competitive antagonist (Figure 5-3). The EC_{50} of ACh in the presence of 1 μM DHβE was 298 ± 29 μM versus the EC_{50} of ACh alone, 2.33 ± 0.94 μM.

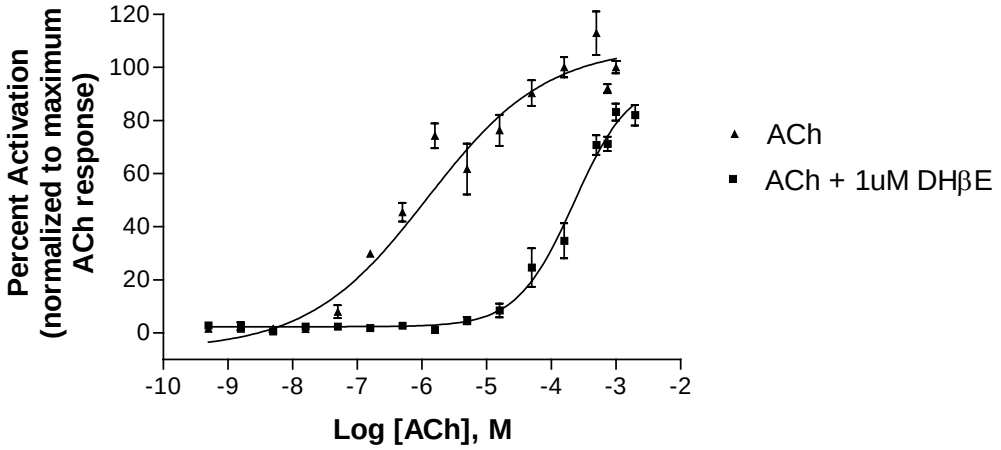


Figure 5-3. Concentration response curve of ACh in the presence and absence of 1 μM DHβE for human α4β2 receptors expressed in tsA201 cells as measured by changes in membrane potential. The responses were normalized to the maximum ACh response. The result represents an average of 3 experiments for ACh and 8 experiments for ACh + 1 μM DHβE.

Figure 5-4 represents the signals of simultaneous applications of 5 μM ACh with increasing concentrations of DHβE as recorded using membrane potential fluorescent dye and human α4β2 expressing tsA201 cells. The signals of the 5 μM ACh response decrease with increasing concentrations of DHβE. The 50 μM concentration of DHβE produces a response that is identical to that of the blank (which did not receive 5 μM ACh), indicating that at 50 μM DHβE the ACh response was completely inhibited.

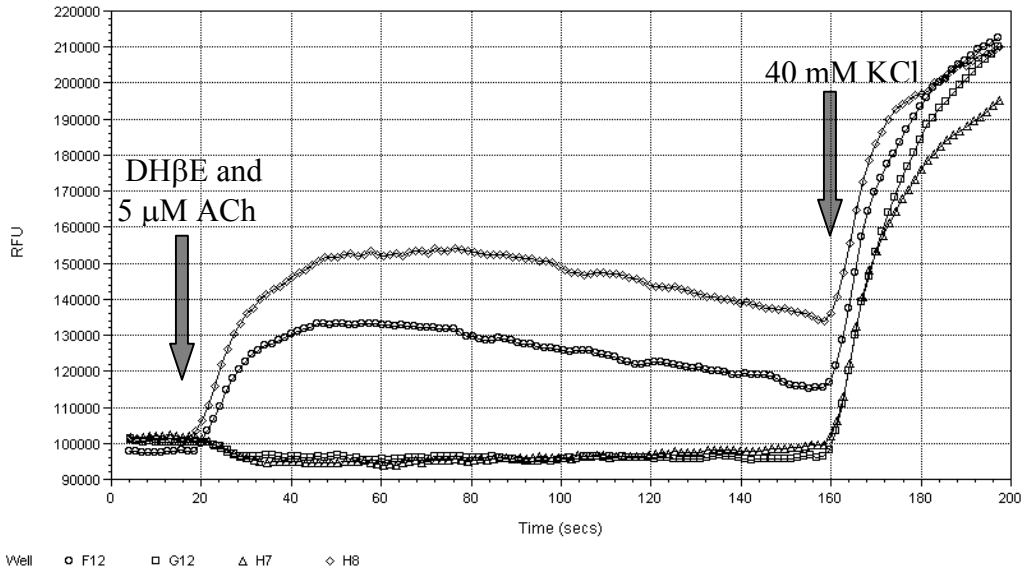


Figure 5-4. Signal acquired for α4β2 nAChRs upon simultaneous application of DHβE and ACh. During the first 17 seconds baseline fluorescence was measured for each well. At 18 seconds well H8 (◊) received 0.0005 μM DHβE + 5 μM ACh, well F12 (o) received 0.05 μM DHβE + 5 μM ACh, well G12 (□) received 50 μM DHβE + 5 μM ACh and well H7 (Δ) received only 20 mM HEPES/HBSS. All wells received a final concentration of 40 mM KCl at 160 seconds

All of the β-erythroidine alkaloids except for 3-Des-Met-βE and Me-βE were able to fully inhibit cell response to 5 μM ACh (Table 5-3). Me-βE had no interaction with rat α4β2 receptors at concentrations up to 20 μM as measured by ^{3}H-cytisine displacement and it also did not inhibit the human α4β2 ACh response at concentrations up to 50 μM. 3-Des-Met-βE also had no measured binding to rat α4β2 at concentrations up to 20 μM but it did inhibit the human α4β2 ACh response by 50% at the highest concentration tested (50 μM). The potency of DHβE to inhibit the human α4β2 ACh response is 5.5-fold greater than that for β-E as indicated by their IC_{50} values of 0.067 μM and 0.37 μM respectively. THβE, with all double bonds in the A and B rings reduced, is the least potent of the β-erythroidines on human α4β2 with a measured IC_{50}

of 2.5 μM. After DHβE, βE-Diol had the second highest affinity (for both rat and human α4β2) of the β-erythroidine compounds. βE-Diol was also the second most potent of the erythroidines on human α4β2 with an IC_{50} value of 0.065 μM.

Table 5-3. Inhibition of human α4β2 receptor ACh responses by natural product and semi-synthetic β-erythroidines.

Compound Name or Abbreviation	**IC_{50} (μM)/Percent Inhibition for Human α4β2**
Erythroidine compounds	
β-E	0.37 ± 0.055
DHβE	0.067 ± 0.0035
THβE	2.5 ± 0.20
3-Des-Met-βE	50% Inhibition at 50 μM
Me-βE	No inhibition up to 50 μM
βE-Diol	0.065 ± 0.012

The compounds and ACh (5 μM) were administered simultaneously. Each value represents the mean ± SEM of ≥ four separate wells.

To determine the importance of the C-ring for binding to rat α4β2 a homoerythrinan compound from another plant genus, *Phelline*, was studied for its ability to displace ^{3}H-cytisine. This compound, named O-methylisophellibiline, is similar in structure to DHβE except that it has a seven membered C-ring, which increases the size of the molecule and alters the 3-dimensional position of the lactone carbonyl oxygen (Figure 5-5). Enlarging the C-ring resulted in a decreased affinity for rat α4β2. There was over 50% displacement of ^{3}H-cytisine from rat α4β2.

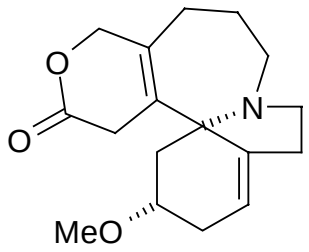


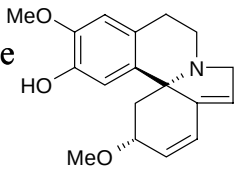
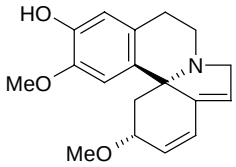
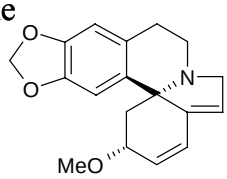
Figure 5-5. Structure of a *Phelline* alkaloid, O-methylisophellibiline. This alkaloid is similar in structure to DHβE except for its seven-membered C-ring.

Aromatic Alkaloids

Erythraline (ELA), an aromatic alkaloid, lacks the D-ring lactone moiety of the erythroidines and instead has a benzenoid ring (Table 5-4). ELA has an additional (1,3-dioxolane) ring fused to the D-ring. The measured affinity (K_i value = 0.056 μM) of ELA is greater than that of DHβE (K_i value = 0.14 μM) for rat α4β2. ELA does have an affinity, although low, for the rat α7 receptor (SR = 46).

The aromatic alkaloids, erysovine and erysodine (ERV and ERD), are isomers (Table 5-4). ERV contains a hydroxy group at position 15 and a methoxy group at position 16 on its D-ring. In ERD the hydroxy and methoxy attached to the D-ring are reversed. The reversal of those two substituents has a large influence upon the affinity for rat α4β2. ERV has a high affinity for rat α4β2 (12 nM), which is 18 times greater than the affinity of DHβE for rat α4β2. ERD, with the hydroxy and methoxy groups reversed, had a much lower affinity than ERV, 23-fold less, for rat α4β2 and 8.9-fold less for human α4β2. ERV also exhibited an increased affinity for rat α7, with a selectivity ratio of 50.8. ERD had no measurable interaction with rat α7 at up to 20 μM.

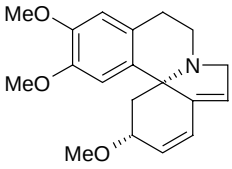
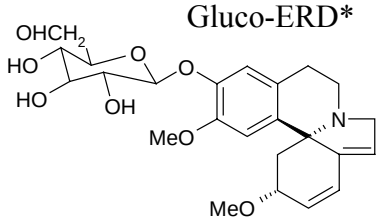
Table 5-4. Structures, nomenclature and binding results for the aromatic alkaloids erysovine, erysodine and erythraline.

Compound Name	K_i (μM) Rat ^{125}I-α-Btx (α7)	Rat ^{3}H-Cytisine (α4β2)	Human ^{3}H-Cytisine (α4β2)
Erysovine NP ERV* 	0.61 ± 0.13	0.012 ± 0.0032	0.027 ± 0.0032
Erysodine NP ERD* 	>20	0.28 ± 0.087	0.24 ± 0.074
Erythraline NP ELA* 	2.6 ± 1.4	0.056 ± 0.0035	0.081 ± 0.018

Parameters were the same as in Table 5-1. Abbreviations for the analogs are indicated by an asterisk.

ERT has a methoxy group at both position 15 and 16 on the D-ring (Table 5-5). The presence of the two methoxy groups resulted in an affinity of 0.33 μM for rat α4β2, which was similar to that of ERD for rat α4β2. It did however have a minimum of 26.6-fold greater affinity for rat α7 as compared to ERD. Gluco-ERD is ERD with a glucose molecule attached. Gluco-ERD is a natural product and like other phenolic D-ring *Erythrina* compounds is attached to large sugar molecules until they are liberated by application of an acid. Even with this large substituent Gluco-ERD had an affinity (K_i value = 0.032 μM) 4.4-fold greater than that of DHβE for rat α4β2. It had no measurable affinity for rat α7 at concentrations up to 20 μM (SR = a minimum of 625).

Table 5-5. Structures, nomenclature and binding results for the aromatic alkaloids erysotrine, and glucoerysodine.

Compound Name	K_i (μM) Rat ^{125}I-α-Btx (α7)	Rat ^{3}H-Cytisine (α4β2)	Human ^{3}H-Cytisine (α4β2)
Erysotrine NP ERT* 	0.75 ± 0.20	0.33 ± 0.070	0.86 ± 0.22
Glucoerysodine NP Gluco-ERD* 	>20	0.032 ± 0.0085	♦ 2.2 ± 0.74

Parameters were the same as in Table 5-1. Abbreviations for the analogs are indicated by an asterisk. The diamond indicates a significant difference between the K_i values for rat and human with ^{3}H-cytisine.

Interestingly, the only alkaloid that had a significantly different K_i value ($P < 0.043$) between rat (K_i value = 0.032 μM) and human (K_i value = 2.2 μM) α4β2 nAChR was Gluco-ERD, which is a natural product. The other aromatic alkaloids had similar rat and human α4β2 affinities.

Table 5-6. Inhibition of human α4β2 receptor ACh responses by natural product aromatic *Erythrina* alkaloids.

Compound Name or Abbreviation	**IC_{50} (μM) for Human α4β2**
Aromatic compounds	
ERV	0.054 ± 0.00049
ERD	0.092 ± 0.0035
ELA	0.13 ± 0.0049
ERT	1.6 ± 0.52
Gluco-ERD	0.43 ± 0.079

The compounds and ACh (5 μM) were administered simultaneously. Each value represents the mean ± SEM of ≥ four separate wells.

Several of the aromatic compounds were as potent as DHβE in inhibiting human α4β2 response to ACh. ERV was the most potent of all the alkaloids, it had an IC_{50} value of 0.054 μM for human α4β2 (Table 5-6). As indicated earlier, ERV also had the highest affinity for both the rat and human α4β2-receptor. Although ERD had a 8.9-fold decrease in affinity (for human α4β2) as compared to ERV, it was the second most potent aromatic compound with an IC_{50} of 0.092 μM on human α4β2. ELA was the third most potent of the aromatic compounds (on human α4β2) with and IC_{50} value of 0.13 μM for human α4β2. ERT was the least potent (IC_{50} = 1.6 μM) of the aromatic alkaloids on human α4β2. The final aromatic compound, Gluco-ERD was the second to last potent with an IC_{50} value of 0.43 μM.

CHAPTER 6
DISCUSSION

Nicotine and dihydro-β-erythroidine (DHβE) have both been used extensively as tools for studying nAChRs. The reason being, that nicotine binds to and activates various subtypes of nAChRs with its highest affinity and potency for α4β2 nAChRs, while DHβE is one of the only antagonists with a relative selectivity for β2-containing receptors. Although their functional effects for the receptor differ, both are competing for the acetylcholine (ACh) binding site. The role of the α4β2 receptor in nicotine addiction as well as its involvement in several neurological dysfunctions warrants further investigation of the interactions of these compounds with β2-subunit containing receptors.

There are various methods that have been employed to study the interaction of the α4β2 nAChR with specific compounds in order to elucidate properties of the binding site. The α4β2 receptor has undergone mutagenesis and chimeric studies in order to determine residues important in allowing for activation or inhibition. Another approach to understanding ligand and receptor interaction is to study the ligand directly. Through alteration of the ligand's structure the consequential effects upon binding and function tell a story about requirements of a molecule for interacting with α4β2 (structure activity relationship approach). The goal of this dissertation was to determine structure activity relationships to understand what aspects of their structures allow for nicotine and DHβE to bind and either activate or inhibit the α4β2 receptor. Determination of ligand

structural properties important for receptor interaction will provide a basis for further manipulation of these molecules to develop higher affinity selective probes for *in vitro* and *in vivo* studies of the α4β2 nAChR as well as possible drugs to treat nicotine addiction.

Substituents at Four Different Positions on Nicotine Decrease Affinity for the α4β2 nAChR and Confer Partial Agonist and Antagonist Properties

This structure activity relationship study of nicotine analogs built on previously published data of several substituted nicotine analogs as well as explored several novel nicotine compounds. The previous studies (Damaj *et al*. 1996; Dukat *et al*. 1996; Glassco *et al*. 1994; Kim *et al*. 1996; Lin *et al*. 1994) however only examined the ability of the nicotine analogs to bind to the α4β2 nAChR as measured with either ^{3}H-cytisine or ^{3}H-nicotine. The studies conducted in this dissertation further explore the ability of nicotine analogs to bind to the other high affinity neuronal nAChR, α7, for the first time. We also investigate the effects of these compounds on the human α4β2 receptor expressed in a mammalian cell line.

Substitutions were made at the 5'-, 1'-, and 3'-pyrrolidine positions as well as at the 5-pyridyl position on nicotine. The substituents at the 5-pyridyl position, either a phenyl or pyridyl ring, were large. They decreased the affinity of these compounds for rat and human α4β2 receptors as compared to that of (S)-nicotine. The presence of the tertiary nitrogen in the 5-pyridyl ring did not increase the affinity of this compound for either the rat or human α4β2 receptor as compared to the nitrogen lacking phenyl ring. It may have been expected that the tertiary nitrogen would provide an additional group for possible hydrogen bonding or other additional electrostatic interactions with the binding site and thus increase the affinity, but this was not the case. The K_i values for the

interactions of these two compounds with the rat α7 receptor were greater than 20 μM. This was an important finding because the selectivity ratios (K_i α7/ K_i α4β2) for both compounds were greater than that of (S)-nicotine. Thus, the large substituents added at the 5-position resulted in an enhanced selectivity for the high affinity receptor (as seen for the rat α7 and α4β2 receptors). The 5-pyridyl substituted analogs were the only nicotine analogs with significantly different K_i values for rat and human α4β2 receptors ($P = 0.0326$ for 5-phe-nic and $P = 0.0334$ for 5-pyr-nic).

The compound DMXBA displays a variation in potency between human and animal (rat) forms of a nicotinic receptor, specifically α7 (Meyer *et al*. 1998; Papke and Porter-Papke, 2002). Stokes *et al*. (2004) determined by mutational analysis that this variation in potency between human and rat was due to differences in residues within the C loop (ser184 in human to asn184 in rat; arg186 in human to lys186 in rat) and the F loop (gly167 in human to ser167 in rat) with some influence from the E loop. Although there are two residue differences between the rat and human α4β2 receptors, only one of them is likely to impact binding. The α4 subunit is the primary, or principal subunit, thus loops A, B and C from this subunit interact with loops D, E and F from the β2, or complimentary, subunit. The only residue within the binding loops that might be anticipated to differently influence ligand interaction between rat and human α4β2 is a glutamic acid in the human receptor in place of an aspartic acid in the rat. This residue difference seems small; there is an additional methyl group on glutamic acid, but both residues are polar and charged. There is also the possibility that supporting residues outside of the six binding loops may be influencing (restricting) the ability of the 5-pyridyl analogs to enter the binding site of the human α4β2 receptor as compared to

rat. Another possible explanation of the difference between rat and human K_i values is that in the CNS there exists a heterogenous population of α4β2 receptors. It has been shown that α5-subunits are localized with α4β2 receptors in the chick brain (Conroy and Berg, 1998) as well as in the cortex of rat (Mao *et al*. 2005). Therefore, if ^{3}H-cytisine is labeling both populations of receptors it may explain the differential interaction of several of the nicotine analogs (as well as *Erythrina* alkaloids) between rat brain α4β2 and human α4β2 receptors expressed in the tsA201 cell line.

Both 5-position substituents prevented the ligands from producing any activation of the human α4β2 receptor. However, these compounds displayed antagonistic properties with similar potencies to each other as measured using a membrane potential fluorescent dye. Dukat *et al*. (2002) also observed the influence of smaller substituents at the 5-pyridyl position on the functional properties of rat α4β2 receptors and determined using electrophysiological recordings from *Xenopus* oocytes that modifications resulted in partial agonist and antagonist properties. Also, Carroll *et al*. (2001) found that a phenyl group added at the 3'-position on epibatidine produced similar effects (antagonism).

The method we used to study receptor activation or inhibition by these nicotine analogs involves the measurement of changes in membrane potential using a fluorescent dye. The dye fluoresces upon membrane depolarization resultant from receptor activation. Fitch *et al*. (2003) published data on the effects of nicotinic receptors with membrane potential and calcium dye and measurements from various cell lines expressing nAChRs. They measured an EC_{50} for nicotine of 0.86 μM on the human α4β2 nAChR expressing cell line they utilized (K-177 cells which are a HEK-293

derived cell line). The EC_{50} values measured in their experiments for agonists were 2 to 3-fold less than those reported in the literature for radiolabeled rubidium efflux assays whereas the IC_{50} values for antagonists were higher (5 to 20-fold or greater) for $\alpha3\beta4$ expressing cells. They note that these estimates may possibly be affected by spare receptors, only a sub-maximal occupation of receptors may be required in order to depolarize the membrane resulting in an apparent increase in potency. The cells used for this study are tsA201 cells, a human embryonic kidney cell subclone (HEK-293) obtained from Jon Lindstrom (Kuryatov *et al*. 2005). Since HEK-293 cells endogenously express M1 muscarinic acetylcholine receptors (Mundell and Benovic, 2000), 1 mM atropine (a muscarinic antagonist) was used in the Flexstation assays. Nelson *et al*. (2003) determined that a population of the cells had nicotine and ACh potencies similar to oocytes expressing human $\alpha4\beta2$ receptors. They also determined that the partial agonist cytisine had an efficacy too low to correctly measure potency. Overall, Nelson *et al*. (2003) concluded that the receptors expressed in the tsA201 cells exist in two stoichiometries: the majority exist with three $\alpha4$-and two $\beta2$-subunits, while the minority population possesses two $\alpha4$-subunits and three $\beta2$-subunits.

The substituents at the 5'-pyrrolidine position were methyl groups in either the trans or cis configuration. Kim *et al*. (1996) had determined that the addition of the methyl at the cis-position resulted in a 35-fold decrease in affinity for rat $\alpha4\beta2$ as compared to that of the methyl group in the trans configuration. The results in this dissertation indicated at least a 90-fold difference in affinity between the trans and cis conformations for both the rat and human $\alpha4\beta2$ with the 5'-cis-met-nic having no interaction with the $\alpha4\beta2$ (human and rat) receptor at concentrations up to 20 μM.

5'-Trans-met-nic had a decreased selectivity ratio of 14 as compared to the selectivity ratio of nicotine (SR = 83). 5'-Cis-met-nic also had no interaction with the α7 receptor at concentrations up to 20 μM. The functional measurements of these two analogs corroborate the binding results for human α4β2 in indicating preferential interaction of the trans analog with the receptor and the relative lack of interaction between the receptor and the cis analog. Trans-5'-met-nic produced 50% inhibition (at 50 μM) of the human α4β2 ACh response.

The 1'-N-pyrrolidine analogs provided information on the influence of substituent size on binding and function. Increasing the size of substituents on the nitrogen of the pyrrolidine ring resulted in a decreased affinity for the rat α4β2 receptor. 1'-N-Ethyl-nor had a measured affinity (K_i = 0.14 μM) comparable to (S)-nornicotine (K_i = 0.12 μM) for rat α4β2. It also had a selectivity ratio of 18, which is less than that of (S)-nicotine (SR = 83). There was an inverse relationship between the size of the substituent on the pyrrolidine nitrogen and affinity. As the size of the substituent increased from an ethyl to propyl and cyclopropyl, the affinity for α4β2 decreased. Neither 1'-N-propyl-nor or 1'-N-cyclopropyl-met-nor had measurable binding to rat or human α4β2 or rat α7 receptors at concentrations up to 20 μM. Glassco *et al*. (1994) also measured this trend of decreasing affinity for rat α4β2 with increasing 1'-N-substituent size. They found a 20-fold decrease in affinity (as compared to (S)-nicotine) when the size of the substituent at the 1'-N-position was increased from a methyl to an ethyl, a 369-fold decrease (as compared to (S)-nicotine) when substituting a propyl-group and greater than 7,000-fold decrease when substituting a cyclopropyl-group. Ferretti *et al*. (2003) determined that a

methyl group was optimal for binding to rat α4β2 such that nornicotine or the addition of a methyl or benzyl group to the pyrrolidine nitrogen resulted in reduced affinity.

Interestingly, although the 1'-N-pyrrolidine substituted analogs displayed decreased affinity for the rat α4β2 receptor as compared to (S)-nicotine, they were found (in separate experiments) to both activate and inhibit the human α4β2 receptor. Analogs containing the ethyl or propyl substituent produced about a 40% activation of the receptor as compared to the ACh maximum response for human α4β2. However, it is possible that a higher concentration would produce greater activation. The cyclopropyl-methyl substituent produced about a 75% activation of the receptor as compared to ACh. Upon simultaneous application with ACh, each of the three of the analogs produced an inhibition ranging from 35-80% (1'-N-ethyl-nor = 35%; 1'-N-propyl-nor = 45%; 1'-N-ethyl-nor = 80%). Channel block at high concentrations of these analogs might explain the resultant inhibition, but it does not account for the ability of these compounds to activate the receptor. Also, the greater percent inhibition of human α4β2 as compared to percent activation by the 1'-N-cyclopropyl-nor indicates the involvement of spare receptors. The possible involvement of spare receptors would increase the apparent potency of these compounds resulting in a weak partial agonist appearing more potent and efficacious than if its effects were measured under voltage-clamp conditions. Based upon their sub-maximal activation at high concentrations as well as the predicted influence of spare receptors, these nicotine analogs are most likely functioning as partial agonists.

The results from the final group of nicotine compounds, the 3'-pyrrolidine substituted analogs, paralleled those of the 1'-N-pyrrolidine substituted analogs. With

increasing substituent size there was a decrease in affinity for both the rat α4β2 and rat α7 receptors. Adding a small substituent (methyl) at the 3'-position resulted in an affinity for the rat α4β2 receptor that was 40-fold less than that of (S)-nicotine for the receptor. This analog also interacted with rat α7 with a K_i value of 4.2 μM (SR for 3'-met-nic = 11 for rat α4β2 and 6.7 for human α4β2). The only other 3'-substitued analog that had measurable binding interaction with rat α4β2 was DMXBN, which had a K_i value of 2.8 μM. The other two 3'-position analogs, like DMXBN, had large benzene ring-containing substituents. The structures of these two compounds (DMXBN and DMXBM) resemble that of DMXBA except that the benzyl or benzylidene moieties are attached to nornicotine and myosmine, respectively, instead of anabaseine. Whereas DMXBA has an affinity of 0.13 μM for rat α7 and 0.25 μM for rat α4β2 (Kem *et al*. 2004a), neither DMXBN nor DMXBM had measurable K_i values for rat α7. The low activity of DMXBM was probably due to its small degree of ionization at physiological pH. Myosmine has a pKa of 5.5, which is well below the pKa (8.05) of nicotine (pKa of nornicotine is 9.12) (Fujita *et al*. 1971); Glennon and Dukat, 2000). DMXBM is predicted to have a pKa no higher than 6.0 (Kem, personal communication), so it would be largely unionized at physiological pH (7.4).

The functional properties of the 3'-substituted analogs upon human α4β2 measured with membrane potential fluorescent dye varied with the compound. Three of the 3'-pyrrolidine substituted analogs produced activation of human α4β2 receptors; DMXBN produced 80% activation, DMXBM produced 20% activation and 3'-met-nic produced 40% activation. The only compound that produced an inhibition of the human α4β2 ACh response was 3'-met-nic (20% inhibition).

Overall, my results for the nicotine analogs indicate that substituents at any of the four positions examined in this dissertation result in a decreased affinity for the human and rat α4β2 nAChR as compared to (S)-nicotine. However, the selectivity ratios for the 5-pyridyl substituted analogs were greater than that of (S)-nicotine. Therefore, substituents at this position confer a greater selectivity for rat α4β2 and merit further investigation for possible use as smoking cessation drug. The substituted nicotine analogs in this study also greatly influence the function of these compounds by converting those that interact with the receptor into partial agonists and antagonists. Rowland *et al*. (2003) also found that 5'-trans-met-nic produced a dose-dependent inhibition of nicotine self-administration in rats.

Previous studies have addressed the influence of pKa for both the pyridyl and pyrrolidine ring nitrogens. Their results indicate that differences in the basicity of these nitrogens could not alone account for variation in affinities of nicotine analogs for the α4β2 receptor (Dukat *et al*. 1998; Copeland *et al*. 1991). To further understand the interaction of nicotine with the ACh binding site, Celie *et al*. (2004) crystallized nicotine bound to the AChBP. Their results indicate that nicotine is primarily in contact with the principal side of the binding site (loops A, B, and C). Also, both nitrogens form hydrogen bonds with residues within these loops. Specifically, the pyrrolidine nitrogen forms a hydrogen bond with tryptophan 143 within the B-loop and the pyridyl nitrogen forms its hydrogen bond with both methionine 114 and leucine 102 within the E-loop. Their results also indicate that the primary differences in the binding site of the AChBP versus the α4β2 receptor are three amino acids. The three residue differences in α4β2 may provide for better contact with nicotine. The nicotine substitutions reported in this

dissertation may be preventing the proper orientation of the analogs within the binding site for establishing the hydrogen bonds necessary for the tight binding. The 5-pyridyl substituted nicotine analogs likely fit the binding site because the large phenyl or pyridyl-ring does not interfere with the primary interactions between the analog and the receptor. This may explain the ability of these analogs to retain an affinity for rat and human α4β2.

Separated Nicotine Analog Enantiomers Display a Difference in Affinity for α4β2 Unlike Nicotine

Interestingly we found that unlike previous studies, there was no significant difference between (S)- and (R)-nicotine in binding to α4β2 (Copeland *et al*. 1991; Zhang and Nordberg, 1993). We measured the affinity for human α4β2 whereas the previous publications were performed using rat brain. It is possible that in a heterogenous population of receptors, as in rat brain, these previous studies were not measuring ^{3}H-nicotine displacement from only α4β2 receptors but that their results were influenced by other subtypes of nAChRs. Our results for the enantiomers of nornicotine agree with those published by Copeland *et al*. (1991) in that the binding affinities for each enantiomer were very similar; their results were done with rat brain tissue whereas our results were measured for the human α4β2-subtype. The higher affinity for the enantiomers of nicotine over those of nornicotine is most likely not due to the differences in pKa of each molecule. Copeland *et al*. (1991) discuss that the difference in the basicity (pKa) of the nornicotine pyrrolidine nitrogen relative to that of nicotine does not produce a difference significant enough to result in the differing affinities of the two compounds. Nicotine is however more lipophilic than nornicotine and the intimate

interaction of nicotine's methyl group may enhance the cation-π interaction with the binding site and account for nicotine's higher affinity.

The results of the nornicotine enantiomers on the activation of human α4β2 receptors expressed in tsA201 cells indicated that they are less efficacious than the nicotine enantiomers. The EC_{50} values for the nornicotine enantiomers indicated that (S)-nornicotine is more potent than its (R)-enantiomer. This contradicts previously published data acquired from dopamine release assays on the nucleus accumbens indicating that (R)-nornicotine is more potent than its enantiomer (Green *et al*. 2001).

The results of the separated nicotine analogs, 1'-N-ethyl-nor and 5-phe-nic are in better agreement with the published literature (Copeland *et al*. 1991; Zhang and Nordberg, 1993) on nicotine for the increased affinity of the (S)-enantiomer (presumably peaks 1 for both analogs). It is important to note however, that both nicotine analogs were studied for their affinities to rat brain membranes like the previously reported findings for nicotine and nornicotine. Our results for the separated enantiomers do indicate that the selectivity of the (S)-enantiomer (presumed peak 1) of 5-phe-nic for rat α4β2 over α7 would be even greater than that of racemic 5-phe-nic (SR = 303).

Substituents on the D-ring of the *Erythrina* Alkaloids Allow for High Affinity Binding to the α4β2 nAChR and Inhibition of the α4β2 Acetylcholine Response

In the literature that has been published on DHβE, it is often referred to as a competitive antagonist selective for the α4β2 receptor (Williams and Robinson, 1984; Anderson and Arneric, 1994; Harvey and Luetje, 1996; Harvey *et al*. 1996). The characterization of DHβE along with several other natural product and semi-synthetic *Erythrina* alkaloids as conducted in these studies have provided a structure activity relationship between the α4β2 receptor and these competitive antagonists. Only one

other *Erythrina* alkaloid has been studied for its effects upon nicotinic receptors. Decker *et al*. (1995) published that the natural product erysodine has a seven-fold higher affinity for rat α4β2 than DHβE. Although our results vary from Decker *et al*. (1995) in that we found DHβE to have a higher affinity (as compared to erysodine) for rat α4β2, it was determined that several structural trends exist among the alkaloids, which result in an increased affinity for the rat and human α4β2 receptors.

The *Erythrina* alkaloids were divided into two groups containing different D-rings: the lactone-bearing erythroidines and the aromatic D-ring compounds. The importance of the double bonds between the A and B rings of the erythroidine compounds was determined from the binding data. By partial reduction, the two double bonds in the A and B rings of β-E are converted to the one double bond of DHβE; there was about an eight-fold increase in affinity for the rat α4β2 receptor. Reduction of the remaining double bond between the A and B ring results in the structure of THβE. This compound had an affinity for the rat α4β2 receptor that was less than that of β-E. These results indicate that the optimal number of bonds between the A and B ring is one. Neither β-E, nor DHβE nor THβE had any binding interaction with rat α7 receptors at concentrations up to 50 μM with DHβE having the highest selectivity ratio (357) of these three β-erythroidine alkaloids.

The possible influence of three double bonds among the A and B rings also was studied using the 3-Des-Met-βE compound. Consistent with the idea that a single double bond among these two rings is optimal for binding, the three double bonds in 3-Des-Met-βE (along with the lack of the 3-methoxy) abolished measurable binding to the rat α4β2 receptor at concentrations up to 20 μM. This also caused a movement of the

C and D rings with respect to the A and B rings. 3-Des-Met-βE also had no measurable binding to the rat α7 receptors at concentrations up to 20 μM.

Me-βE, unlike the other β-erythroidine compounds, has a charged quaternary ammonium group. Me-βE had no interaction with rat α4β2 or α7 at concentrations up to 20 μM. The addition of the methyl group may be preventing the nitrogen from forming the necessary hydrogen bonds required for binding. The remaining β-erythroidine compound, βE-Diol, allowed the importance of the D-ring in binding to be investigated. Interestingly, the opening of the D-ring still allowed for an interaction with rat α4β2 that is comparable to that of DHβE. The opening of the D-ring may allow for additional hydrogen bonding and possibly increase the flexibility of the molecule, thus resulting in a greater affinity than that of β-E. Like the other erythroidine compounds βE-Diol also had no interaction with rat α7 at concentrations up to 50 μM and its selectivity ratio (161) was less than that of DHβE. Alteration in the size of the C-ring (increasing from a 6 to 7-membered ring), as determined with O-methylisophellibiline, resulted in a decreased affinity for rat α4β2. This indicates the importance of the smaller C-ring size for binding to rat α4β2.

All of the aromatic compounds were obtained as natural products. Their D-rings lack the lactone moiety of the erythroidine compounds, and are replaced by a substituted phenyl ring. Several of these alkaloids had measured affinities greater than that of DHβE. ELA and Gluco-ERD were similar in that they both had large substituents on their D-ring. With the increase in affinity for rat α4β2, there was also an increased affinity for the rat α7 receptor. Gluco-ERD, which has a large sugar molecule attached to

the D-ring, had an affinity greater than that of ELA or DHβE for rat α4β2. It did not however have any interaction with the rat α7 receptor at concentrations up to 20 μM. The importance of a hydroxyl group at position 15 was determined from the binding results of ERV, ERD and EST. ERV had the highest affinity for the rat α4β2 receptor of all the *Erythrina* compounds studied. ERD is identical in structure to ERV except that the hydroxy and methoxy groups are reversed. This is important because there is a large decrease in affinity, 23 times less, of ERD for rat α4β2 as compared to ERV. The four fused rings of these alkaloids create rigidity in the 3-dimensional structures. It is possible that when the methoxy group is attached at position 15 there may be repulsion between the methyl of the methoxy group and a side chain of the receptor. Also, it is possible that the 15-hydroxy group is a H-bond donor (methoxy group is not a H-bond donor) that enhances overall binding.

The results from the rat membrane binding studies indicate that one double bond between the A and B ring is optimal for nAChR binding. Various manipulations and substituents to the D-ring still allow for high affinity binding to α4β2. Finally, the aromatic compounds on average have a higher affinity for both the α4β2 and α7 nAChRs.

All of the *Erythrina* alkaloids that had a measurable binding affinity for the α4β2-receptor functioned as antagonists according to measurements performed with the membrane potential fluorescent dye. ERV, which had the highest affinity of the *Erythrina* alkaloids for rat and human α4β2, also had the highest potency for inhibiting the human α4β2 ACh response. Although there was a significant difference (P value = 0.0186) in binding (for human α4β2) between ERV and ERD as measured by ^{3}H-cytisine

displacement, there was less than a two-fold difference in inhibitory potency (for human α4β2) between the two alkaloids.

There exist several published reports on the administration of several *Erythrina* alkaloids *in vivo*. In 1955, Megirian *et al*. found that β-E, DHβE, and 3-Des-Met-βE produced ganglionic and neuromuscular blocking action on mice, rats and cats. Several groups determined the effects of DHβE in the CNS. Corrigall *et al*. (1994) administered DHβE directly to the ventral tegmental area of rats and found that DHβE attenuated self-administration (intravenous) of nicotine. Systemic administration of DHβE can antagonize the locomotor activating effect of nicotine in rats (Stolerman *et al*. 1997). When injected intraventricularly it was determined that DHβE disrupted spatial memory and inhibited nicotine's stimulatory effects (Curzon *et al*. 1996). As an herbal medicine, *Erythrina* leaves and bark (which contain alkaloids) have been found to produce anxiolytic effects (Onusic *et al*. 2003).

The discovery of the acetylcholine binding protein (AChBP) has allowed for the crystallization of ligands bound within the ACh binding sites. Until recently only agonists were crystallized in the binding site. In 2005, two groups crystallized antagonists, an α-conotoxin (PnIA) and an α-neurotoxin (α-cobratoxin), within the binding sites (Celie *et al*. 2005; Bourne *et al*. 2005). Both studies found that the C-loop was in a conformation distinctly different (projects away from the adjacent subunit) from that of the HEPES or nicotine bound AChBP. Both groups suggest that these antagonists are binding to the resting state as opposed to the desensitized or open state of the receptor.

Conclusions

In summary, this dissertation has presented several nicotine analogs and *Erythrina* alkaloids that have varying degrees of affinity for rat and human α4β2 as well as potency for human α4β2 nAChRs. Through alterations of structure we have determined properties important for binding and activation or inhibition, as well as differences in affinity between rat and human α4β2 nAChRs. Whereas the alterations to four different positions on the structure of nicotine result in nicotine analogs with a decreased affinity for α4β2, alterations to the D-ring of the *Erythrina* alkaloids continue to allow tight binding to α4β2. Modifications to the structure of nicotine decrease binding and convert the compounds to partial agonists and antagonists. All *Erythrina* alkaloids that had a measurable affinity for the α4β2 receptor acted as competitive antagonists, like DHβE. This is the first study to provide affinities of several nicotine analogs and *Erythrina* alkaloids for α7 as well as measure the functional effects on human α4β2 nAChRs. These results provide data on several compounds that may be used as leads for the design of partial agonists and antagonists. Partial agonists and antagonists would be useful as smoking cessation drugs. Selective antagonists would be useful as probes to study the resting state of the α4β2 receptor. Future directions, based upon the results of this study, may include; electrophysiological analysis of the effects of nicotine analogs and *Erythrina* alkaloids, comparison of the properties of enantiomers from racemic nicotine analogs, and molecular modeling of several of these compounds to the AChBP and α4β2 human and rat receptors. Some of these compounds may also have quantitatively different binding affinities and functional effects on other naturally-occuring nAChRs.

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BIOGRAPHICAL SKETCH

Kristin Marie Wildeboer was born February 23, 1978, in Grand Haven, Michigan, to Stanley and Judith Wildeboer. She was born 6 minutes after the birth of her twin brother, Todd. Kristin also has a brother, David who is 4 years her junior. When Kristin was 4 years old, her family moved to Ft. Wayne, Indiana where she grew up and attended Snider High School for a year before her family moved to Tampa, Florida. Kristin finished her high school education at Gaither High School, in Tampa. For her college education, Kristin attended Stetson University in Deland, Florida. In Spring 2000, she received her Bachelor of Science degree with a major in biology and a minor in psychology. In the fall, she began her graduate career in the Interdisciplinary Program at the University of Florida. In Spring 2001 Kristin joined the laboratory of Dr. William Kem, to study binding and functional properties of nicotinic acetylcholine receptors.