PHARMACOLOGICAL PROPERTIES OF α4β2 and α3β4 NICOTINIC ACETYLCHOLINE RECEPTORS: LIGAND BINDING, ACTIVATION, DESENSITIZATION, AND INTERSPECIES DIFFERENCES

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PHARMACOLOGICAL PROPERTIES OF α4β2 AND α3β4 NICOTINIC ACETYLCHOLINE RECEPTORS: LIGAND BINDING, ACTIVATION, DESENSITIZATION, AND INTERSPECIES DIFFERENCES

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ABSTRACT

Smoking cessation medications using nicotinic ligands, including nicotine, cytisine, and varenicline, have traditionally revolved around selective activation of α4β2 nicotinic acetylcholine receptors (nAChRs). However, nAChR desensitization may be the primary action of nicotine and may be a viable approach for smoking cessation medication. The α3β4 nAChR subtype has been implicated in side effects of nicotinic drugs as well as nicotine addiction. The purpose of the work here is to investigate pharmacological properties of these two nAChR subtypes, with a focus on mechanisms of ligand binding, activation, desensitization, and interspecies differences between human and rat receptors.

The first aim of this dissertation was to dissect the properties of six ligands (nicotine, cytisine, sazetidine-A, varenicline, epibatidine, and 5-I-A85380) at α4β2 nAChRs. We developed a method for determining the dissociation rates of unlabeled ligands. These ligands are more potent at desensitizing than at activating α4β2 nAChRs. A correlation was found between duration of desensitization and binding affinity.

The second aim was to develop methods for using microfluidic laminar stream solution exchange (MLSSE) to study nAChRs expressed in HEK293 cell lines. The
variables considered in this technique were drug exposure time and washout time. We demonstrated that MLSSE can be applied to a broad range of nAChRs.

The final aim studied interspecies differences in α3β4 drug affinity. All tested drugs besides acetylcholine have higher potency for human α3β4 nAChRs than rat nAChRs. AT-1001, a new high affinity α3β4 ligand, does not select for desensitization over activation in α3β4 or α4β2 nAChRs. Furthermore, the differences in affinity of AT-1001 between α3β4 and α4β2 nAChRs result in longer desensitization in α3β4 nAChRs compared to α4β2 nAChRs. This is consistent with the affinity of a ligand for a receptor subtype being the major determinant for desensitization length.

The investigations in my dissertation contribute to a better understanding of the pharmacological properties of α4β2 and α3β4 nAChRs. The methods developed will help in future studies. Moreover, the results provide important insight in developing new nicotinic therapeutics based on nAChR desensitization and indicate that investigators should be aware of species differences in studying nicotinic ligands.
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I’ve had the incredible fortune of having the opportunity to work with two amazing mentors, Kenneth J. Kellar and Yingxian Xiao. Both have contributed hugely to my growth as a scientist while simultaneously encouraging me to have a rich personal life outside of work. Ken and Yingxian have exemplified, in my mind, a perfect and healthy work-life balance, and I hope to one day be as successful as they are at both a personal and professional level. I’m very grateful to both of them for having me in the lab and for taking me under their wings.

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CHAPTER I: INTRODUCTION
Brief history of tobacco use and control

When people in the future observe cultural conventions of present society, the worldwide use of tobacco will be a difficult phenomenon to comprehend. Tobacco originates in South America and has been used by Native American societies for more than 2,000 years in ceremonies and rituals (Godlaski, 2013). After Columbus introduced tobacco to Europe, snuff and pipe tobacco were soon used by many Europeans (Samet, 2013). But it was cigarettes marketed by the modern tobacco industry that pushed tobacco use to an epidemic level: more than 1 billion people smoke cigarettes everyday currently even though it has been widely known since the 1960s that the health effects of smoking are very serious.

It has been widely accepted that tobacco use is the most important preventable cause of early morbidity and mortality in industrialized countries (CDC, 1997; Fiore, 2000). Cigarette smoking is associated with increased risks for lung cancers, other cancers, coronary heart disease, and stroke, accounting for up to 1 in 5 deaths in the United States and 1 in 10 deaths worldwide, according to the World Health Organization (Danaei et al, 2009; Office of Smoking and Health, 2014; World Health Organization, 2014).

Since the beginning of tobacco use in European countries, many attempts have been made by authorities to control tobacco use. One way to control tobacco usage is by placing restrictions on where tobacco may be used. Interventions intended to curtail the use of tobacco date back as far as 1575, when the Roman Catholic Church banned tobacco use in all Mexican churches. Since then, many other smoking bans have been
instated across the world at various points in history. These bans, however, were not based on health concerns and were instead related to either aversion to the smell of tobacco or fear of the fires caused by burning tobacco. In the late 1930s and early 1940s, the German government banned smoking in all universities, hospitals, government, and other public places based on the health concerns of smoking (Proctor, 2001). In 1975, Minnesota became the first US state to restrict smoking in close public spaces. Since then, many states have followed suit – to date 28 of the 50 states have endorsed legislation banning smoking in enclosed public spaces. Currently, 43 out of 60 of the most populated cities in the United States have now banned smoking in both bars and restaurants. These legislative efforts have been successful in ensuring that the population is not exposed to second hand smoke against its will and in making smoking a significantly more inconvenient habit. Altogether, the laws cover 81.3% of the US population (American Nonsmokers’ Rights Foundation, 2014).

Another method that governments use to discourage tobacco use is to heavily tax tobacco products. Early attempts to tax tobacco products were not motivated by public health. Alexander Hamilton’s idea to enforce an excise tax on tobacco in 1794 was not motivated by any knowledge of the consequences of habitual tobacco usage, but rather by the potential tax revenue such a tax would generate. Although this legislation did not pass at the time, the financial burden of the Civil War soon resulted in excise taxes on many goods, including tobacco, being enforced in 1862. This federal tax on tobacco was not repealed after the Civil War ended. Instead, it has steadily increased on a near annual basis since then and has become a stable source of revenue for the government. In 1880, before the advent of the income tax, the tobacco excise tax accounted for 31% of total
federal tax revenue. In 1910, Iowa followed the federal government’s example and started taxing tobacco at the state level and by 1969 all 50 states had state taxes on tobacco products (McGrew, 1972). It wasn’t until 1983 that growing public concerns over tobacco’s negative health consequences resulted in enough pressure to warrant the federal government levying taxes on tobacco with the express purpose of reducing tobacco use. States followed the federal government’s example, resulting in an increase of up to 200% on tobacco taxes from 1980 to 1990. These initiatives were, by tobacco companies’ own admission, successful at reducing the overall usage of tobacco in the United States (Chaloupka et al, 2002).

Perhaps the most important event in the history of United States tobacco control was the release in 1964 of the first report by the Surgeon General’s Advisory Committee on Smoking and Health (Alberg, 2014). After reviewing more than 7,000 articles relating to smoking and disease, the Advisory Committee concluded that cigarette smoking is a cause of lung cancer and chronic bronchitis. Looking back, the release of this report started many of the measures taken by the US, state, and local governments, and society as a whole to diminish the negative impact of tobacco use on the health of the American people (Alberg, 2014; Giovino, 2007).

More recently, in the 1990s, 46 states sued the four largest tobacco companies to recover tobacco-related health-care costs for state governments (Jones, 2010). In 1998, these states entered the Tobacco Master Settlement Agreement (MSA) with the tobacco companies, which agreed to pay a minimum of $206 billion over the first 25 years of the agreement. In addition to the MSA money, tobacco companies agreed to certain restrictions in marketing tobacco products.
In the United States, the efforts to control tobacco use by governments and society have driven down the smoking rates among adults from 42.4% in 1965 to 19.0% in 2011 (CDC, National Health Interview Survey, 1965–2011), which is highly significant indeed. However, in 2011, more than 43.8 million of US adults were still smokers, despite the well-known deleterious health effects of smoking. Therefore, tobacco use is still the leading public health problem of the United States and a major cause of early mortality.
Nicotine: the addictive agent in tobacco

Tobacco use, the majority of which is cigarette smoking, represents a significant health risk to both those that partake and the people around them. In addition, smoking is increasingly more expensive and inconvenient, both logistically and socially. So, why then, do smokers continue to smoke? Stripped of social motivations, mounting evidence supports a widely accepted explanation: tobacco use is an addictive behavior sustained by nicotine.

More than 4,800 compounds have been identified in tobacco smoke (Hoffman, 2001). Among these compounds, many are very harmful to humans and cause lung cancer and other cancers (PAH, NNK, NNN, NNAL, etc.), cardiovascular diseases and chronic obstructive lung diseases (carbon monoxide, nitrogen oxides, hydrogen cyanide, etc.). These carcinogens and other toxic substances in tobacco smoke are explanations for smoke related diseases but have little effect on forming the habit of smoking. Many lines of evidence indicate that nicotine is the major addictive substance in tobacco.

Named after the tobacco plant, *Nicotiana tabacum*, nicotine was initially isolated by two German scientists, Wilhelm Heinrich Posselt and Karl Ludwig Reimann (Henningfield and Zeller, 2006). The chemical structure of nicotine was later elucidated by another two German scientists, Adolf Pinner and Richard Wolffenstein (Pinner and Wolffenstein, 1891; Pinner, 1893). Nicotine was later synthesized by Amé Pictet and A. Rotschy (Pictet and Rotschy, 1904). These advancements ultimately allowed for the use of nicotine by John Langley in his seminal studies that laid the foundations on the nature of neurotransmission. As part of this work, he identified what would later become known
as the peripheral nervous system’s nicotinic acetylcholine receptor network (Langley, 1905). Based on these discoveries and others, Louis Lewin remarked in his classic pharmacology novel, *Phantastica*, that the presence of nicotine in tobacco accounted for its psychoactive effects (Lewin, 1924). Johnston and Glasg took these conclusions a step further by investigating the effects of intravenously injecting nicotine into people, concluding that nicotine was indeed the culprit behind the behavioral effects of tobacco (Johnston and Glasg, 1941; Henningfield and Zeller, 2006).

Through the 1960s and 1970s, a large amount of new data emerged reinforcing the idea that nicotine is the addictive agent in tobacco. Critical research during this period of time helped to define the psychopharmacological and pharmacokinetic properties of nicotine. By the late 1970s, the National Institute of Drug Abuse was actively focusing on nicotine. Through the concerted efforts of many prominent addiction researchers during this period of time, enough data was collected to conclusively infer that nicotine is addictive and behind the habit-forming quality of tobacco (Henningfield and Zellar, 2006). These studies culminated in tobacco dependence being added into the DSM-III in 1980 and in then-NIDA director William Pollin’s address to congress in 1982 and 1983 regarding nicotine as an addictive substance (American Psychiatric Association, 1980; Henningfield and Zellar, 2006). The presence of the addictive and psychoactive substance, nicotine explains why many begin and continue to use tobacco despite the obvious health and social implications behind its continued use. Based on many studies over several decades, the 1988 US Surgeon General Report, *The Health Consequences of Smoking: Nicotine Addiction*, concluded that cigarettes and other forms of tobacco are
addicting and that nicotine is the drug in tobacco that causes addiction. Moreover, because of its pharmacokinetics, nicotine is especially addictive when smoked.
Nicotinic acetylcholine receptors: distribution, physiology, and pathology

Tobacco addiction is mediated by neuronal nicotinic acetylcholine receptors (nAChRs) in brain. These receptors did not develop in human neuronal systems for carrying out the addictive effects of nicotine. Nicotinic receptors are widely distributed in the central nervous system (CNS) as well as in the peripheral nervous system (PNS). Neuronal nAChRs are stimulated by the neurotransmitter, acetylcholine (ACh), the endogenous ligand of nAChRs, which initiates many physiological functions carried out by the receptors (Kellar and Xiao, 2007).

A key fundamental step in neurotransmission is the initial depolarization of neurons, which reaches a threshold level of depolarization in the cell and opens voltage dependent channels, resulting in an action potential. Action potentials are mediated by voltage dependent sodium channels that open in response to membrane potentials more positive than resting membrane potentials. Manipulation of the voltage across the cell membrane is required to depolarize the cell membrane enough to open these voltage dependent channels and initiate the action potential. Nature has evolved several ways to manipulate the probability of action potentials, whether it is to depolarize (thus increasing the probability of an action potential) or hyperpolarize (thus decreasing the probability of an action potential) the cell. One way that nature manipulates membrane potentials is through ligand-gated ion channels, which open as a pore in cell membranes in response to either neurotransmitters or other chemicals to allow the flow of ions. Allowing cation flow (particularly Na$^+$ and Ca$^{2+}$) depolarizes the cell, while anion flow (particularly Cl$^-$) hyperpolarizes the cell.
Nicotinic acetylcholine receptors (nAChRs) are in plasma membranes and are permeable to Na\(^+\), K\(^+\), and Ca\(^{2+}\). Endogenously, the receptors respond to the neurotransmitter ACh and open to allow cations to pass, thus depolarizing the cells they are expressed in. At the neuromuscular junction, muscle type nAChRs mediate signaling between motor neurons and muscle fibers (Del Castillo and Katz, 1954a; Del Castillo and Katz, 1954b; Del Castillo and Katz, 1955). In the peripheral nervous system, nAChRs are expressed in both sympathetic and parasympathetic ganglia (Skok, 2002).

In the brain, nAChRs are located post-synaptically, pre-synaptically, and preterminally on axons. Post-synaptic nAChRs serve a straight-forward purpose: mediating fast ACh-evoked excitation and generating action potentials in the cells they are expressed in (Frazier et al, 1998; Jones et al, 1999). Acetylcholine released into the synaptic cleft can be detected by these receptors at the soma or dendrites of the post-synaptic cell, depolarizing the cell and exciting the cell directly (Mulle et al, 1991; Yang et al, 1996; E.X. Albuquerque, 1997). However, one of the main roles of nAChRs in the brain may be at the pre-synapse (McGehee et al, 1995, Kellar and Xiao, 2007). Here, nAChRs modulate neurotransmitter release by affecting the local environment of vesicular release machinery in the bouton. Nicotinic acetylcholine receptors presumably do this by allowing enough Na\(^+\)-influx to activate voltage-gated calcium channels, although some suggest that the calcium permeability of nAChRs alone may be enough to alter vesicular release (C.J. Hillard, 1992; Soliakov et al, 1995; Gray et al, 1996). Pre-terminal nAChRs are located on axons of neurons, where they control the depolarization of the nerve fiber, either by directly depolarizing the cell and generating an action
potential or by affecting existing action potentials as they propagate down the axon (Léna et al, 1993).

With their widespread distribution within the CNS and PNS, it is unsurprising that nAChRs are players in many physiological functions and that dysfunctions of nAChRs are involved in many disorders. Neuronal nicotinic receptors are believed to be involved in attention, learning, memory, consciousness, and development (Aramakis and Metherate, 1998; Perry et al, 1999; Aramakis et al, 2000; Ji et al, 2001; Ge et al, 2005). Diseases with possible nAChR related pathology include Parkinson’s, Alzheimer’s, autosomal dominant nocturnal frontal lobe epilepsy, and schizophrenia, as well as various drug addictions, including nicotine (Lena et al, 1998; Court et al, 2001; Leonard et al, 2001; Picciotto and Zoli, 2002; Raggenbass and Bertrand, 2002; Gotti and Clementi, 2004; Dani and Harris, 2005).
Nicotinic receptors: structure and pharmacology

Nicotinic acetylcholine receptors are part of the cys-loop superfamily of ligand-gated ion channels. Each nAChR is composed of 5 subunits assembled into a pentamer, forming a pore in the center. These pentamers are assembled from a variety of different subunits – α(1-10), β(1-4), γ, δ, and ε – that are differentially expressed in various parts of the body; in the CNS and PNS, only α(2-10) and β(2-4) subtypes are expressed. Each subunit is a protein formed from a single polypeptide with four transmembrane domains (TM1 – TM4). Notably, the TM2 domain is the primary contributor to the lining of the transmembrane pore, forming the channel that allows passage of cations. Both terminals of this single polypeptide reside in the extracellular domain; the N-terminal is long whereas the C-terminal short. Two adjacent cysteine residues in the N-terminal domains of all α subunits differentiate them from the other subunits, most notably β subunits, which do not possess these adjacent cysteine residues (Lukas et al, 1999). Ligand binding domains in assembled receptors form a pocket at the interface of an α subunit and another adjacent subunit (Karlin, 2002; Dani and Bertrand, 2007). With the only requirement for functional receptors being the inclusion of at least two α subunits, a large amount of possible pentamer compositions are possible, although only certain compositions exist in native tissue.

The α1, β1, γ, δ, and ε subunits assemble as muscle-type nAChRs, while α (2-10) and β (2-4) assemble as neuronal nAChRs. Note that α8 subunit has only been found in chick and is not found in mammalian tissue. Muscle-type nAChRs, which are at the neuromuscular juncture in skeletal muscles, are initially composed of (α1)2β1γδ nAChRs.
during embryonic development, but in mammals are replaced by \((\alpha 1)_2\beta 1\delta\varepsilon\) at the mature neuromuscular junction. These receptors are an integral component of the neuromuscular junction, where the muscle-type nAChRs initiate muscle contraction by depolarizing the sarcolemma enough to activate voltage-gated sodium channels, thus causing an action potential that results in muscle contraction.

Neuronal-type nicotinic receptors, the focus of my dissertation, are significantly more variable in composition. Neuronal nAChRs assemble both heteromerically (as with \(\alpha 4\beta 2^*\) nAChRs) and homomerically (as with \(\alpha 7\)). Here, and subsequently, the use of an asterisk (*) in nAChR subunit composition denotes that other subunits may be involved in pentamers of this type (e.g., \(\alpha 4\beta 2^*\) can refer to a pentamer of composition \((\alpha 4)_2(\beta 2)_3\), \((\alpha 4)_3(\beta 2)_2\), or \((\alpha 4)_2(\beta 2)_2\alpha 5\)). The diverse variety of neuronal nAChR subtypes is reflected in an equally diverse pharmacology and distribution in differing compositions of subunits.

The focus of neuronal nAChR research over the past three decades has been \(\alpha 4\beta 2\), \(\alpha 3\beta 4\), and \(\alpha 7\) nAChRs (Kellar and Xiao, 2007). These three subtypes effectively demonstrate the vast possibility of variation in properties that can result from differing subunit composition in nAChRs. Amongst the three, the \(\alpha 4\beta 2\) subtype has highest affinity for ACh, has relatively fast activation kinetics, is the least permeable to Ca\(^{2+}\), desensitizes at a moderate rate, and desensitizes for the longest periods of time following the cessation of desensitizing ligand application (more explanation on this concept to follow). The \(\alpha 3\beta 4\) subtype has moderate affinity for ACh, the slowest activation kinetics, moderate permeability to Ca\(^{2+}\), slowest desensitization kinetics, and desensitizes for moderate periods of time following the cessation of desensitizing ligand application.
Compared to $\alpha 4\beta 2$ and $\alpha 3\beta 4$ subtypes, the $\alpha 7$ subtype has the lowest affinity for ACh, the fastest activation kinetics, the highest permeability to $\text{Ca}^{2+}$, the fastest desensitization kinetics, and does not remain desensitized following cessation of desensitizing ligand application (Lax et al, 2002). In spite of the broad array of pharmacological and functional variation that are possible with just these three subtypes, many more subtypes have already been identified in the brain, and some may have unique pharmacological and functional characteristics. For instance, the inclusion of $\alpha 5$ in heterologously expressed $\alpha 3\beta 4^*$ nAChRs results in receptors with higher $\text{Ca}^{2+}$ permeability and faster desensitization kinetics than $\alpha 3\beta 4$ nAChRs (Gerzanich et al, 1998). Inclusion of $\alpha 5$ in $\alpha 4\beta 2^*$ also increases $\text{Ca}^{2+}$ and accelerates desensitization kinetics (Tapia et al, 2007; Kuryatov et al, 2008).

One important aspect of nAChR pharmacology is the tendency of $\alpha 4\beta 2$ to be upregulated in vivo during chronic nicotine exposure. Near concurrently, our lab and another discovered that chronic nicotine application resulted in upregulated nicotinic binding sites in rat and mice (Schwartz and Kellar, 1983; Marks et al, 1983). These initial observations were made by using $[^3\text{H}]-\text{acetylcholine}$ and $[^3\text{H}]-\text{nicotine}$; therefore, they did not identify the population subtype of receptors that was being upregulated. However, these observations did serve as one of the earliest physiological markers for nicotine addiction and tolerance. These receptors were later identified as the $\alpha 4\beta 2^*$ subtype by immunoprecipitation of receptors after labeling them with $[^3\text{H}]-\text{cytisine}$ and $[^3\text{H}]-\text{epibatidine}$ binding (Flores et al, 1997). This upregulation is also observed in humans, where upregulated receptors were also identified in the post-mortem brains of smokers (Benwell et al, 1988; Breese et al, 1997; Perry et al, 1999).
Nicotinic receptors: receptor binding, activation, and desensitization

Nicotinic acetylcholine receptors are allosteric proteins that exert their function by shifting between various conformations to allow or disallow the passage of ions through the central pore of the protein (Changeux et al, 1984). According to observations in whole cell current measurements, there seem to be at least four conformations (or states) of nAChRs: open, resting, intermediate desensitized, and deep desensitized (Figure 1). In the resting state of the receptor, the central pore of the receptor is not open, but the receptor itself is amenable to shifting to the open state in the presence of ligand. In the open state, the central pore is open and ions are permitted to flow through. The intermediate desensitized state is similar to the closed state in that it does not allow for the passage of ions, but also is does not shift into the open state even in the presence of ligand. This is the type of desensitization that is responsible for the decay of current present during sustained drug application to nAChRs, representing a stabilization of the state by the drug being applied. Deep desensitized state occurs during more prolonged applications of drug. Similar to the intermediate desensitized state, receptors in deep desensitization do not allow opening of the central pore even in the presence of drug. Deep desensitization has a higher affinity for drugs; therefore, receptors that are stabilized in the deep desensitized state recover from this state at a much slower rate than receptors stabilized in the intermediate state. Since the deep desensitized state more accurately reflects receptors that have been exposed to drugs for long periods of time in vivo, subsequently, “desensitization” will refer to this deep desensitized state, unless stated otherwise.
in the resting state, no ion flow is observed. When agonist is applied, the receptor shifts to the open state, allowing ions to flow. As the agonist is applied, the population of receptors undergoes a rapid shift towards the intermediate desensitized state as receptors are stabilized in the state, which they recover from rapidly. However, during this agonist application, some population is also stabilized in the deep desensitized state, which has a much higher affinity for agonist. This is denoted by the decrease in maximal current between the first and second applications of 1 mM ACh. Recovery from the deep desensitized state is much slower and subsequent exposure to the same concentration agonist results in a diminished response due to receptors still in the deep desensitized state.

Although it is accepted that ligand binding and nAChR channel opening coincide, it is not currently fully understood how ligand binding relates to channel conformation. Two models, the induced-fit model and selected fit model, have have been proposed to explain how ligand binding relates to receptor conformation and how these events relate to activation of the receptors. These two models therefore deal with receptor-ligand interactions and how they relate to the state of the receptor.
In the induced-fit model (Koshland, 1958), ligand binding to the receptor precedes the opening of the channel pore (Figure 2A). In this model, ligand binding causes allosteric modifications within the structure of the receptor, which opens the channel pore. Thus, in this model, ligand binding is a required step for channel opening, as well conformational shifts to the desensitized state. Therefore, in this model, both agonists and competitive antagonists bind to the resting state of the receptor. Agonist binding results in conformational shifts in the receptor that first shift the receptor into the open state, followed by shifts to the desensitized state. Competitive antagonists bind to the receptor in the same orthosteric site, but do not induce conformational changes to the protein that open the channel.

In the selected-fit model, also known as the Monod-Wyman-Changeux model, receptors idly isomerize between the different states of the receptor (Figure 2B). The addition of ligand therefore serves to preferentially stabilize individual states of the receptor when the ligand binds to it (Changeux, 2012). Therefore, rather than altering the conformations of the receptors themselves, ligands in the selected-fit model serve to shift the equilibrium of the receptor population towards the conformation that they stabilize. In this model, a classical agonist has low affinity for the resting state, a higher affinity for the open state, and the highest affinity for the desensitized state. Therefore, the application of an agonist will cause the population of receptors to shift towards both the open and desensitized state of the receptor, but because of higher affinity for the desensitized states, will eventually cause an equilibrium that greatly favors desensitized receptors. Antagonists, on the other hand, stabilize the closed state of the receptor, thus shifting equilibrium of receptors to the closed conformation.
Figure 2. Two models of ligand binding. (A) The induced-fit model of ligand binding. Ligand entering into the binding pocket causes the receptor to undergo conformational changes that result in allosteric modification of the protein structure. (B) The selected-fit model of ligand binding. Protein structure shifts spontaneously. The addition of ligand binds with high affinity to specific conformations of the receptor and stabilizes them in that conformation.

It is important to note that neither of these models have been unequivocally proven to be correct and that both continue to be only models that serve to guide our understanding of receptor binding and channel function. Both models fail to explain readily observable experimental phenomena and therefore likely do not represent the truth individually. For instance, the induced-fit model does not account for the ability for ligands to desensitize receptors seemingly without activating them or for the existence of spontaneous single channel openings in the absence of agonist (Paradiso and Steinbach, 2003). In this model, channel opening and desensitization are sequential events that must
begin with agonist binding to induce structural changes in the protein. Neither of the
aforementioned phenomena should be possible in this context. In contrast, the selected-fit
model does not adequately explain the rapid transitions between the closed and open
states that occur during agonist exposure at high concentrations. In such an otherwise
fine-tuned system of ion channels, full activation of receptors would require near
simultaneous “random” isomerization to the open state of the receptor followed by
coincidence binding of agonist on a mass scale in order to generate a single concerted
current that results from agonist application. In fact, some have speculated that receptor
binding is a mixture of these two models, with the selected-fit mechanism being the
predominant mechanism at lower concentrations of ligand and the induced-fit mechanism
being the predominant mechanism at higher concentrations (Weikl and von Deuster,
2008). This type of combined theory would in fact account for aforementioned
phenomena. *Because a large portion of the work here focuses on the desensitization of
nAChRs, I will be discussing results in the context of the selected-fit model, since this
model more accurately accounts for the desensitization properties of nAChRs.*
Nicotinic receptors: addiction, smoking cessation, and targeting desensitization

The role of nicotinic receptors in the formation and maintenance of nicotine addiction has been well-defined. As with several other substance dependencies, the mesolimbic pathway has been heavily implicated in nicotine addiction. Early studies demonstrated that nicotine self-administration is the result of nicotine activity in the ventral tegmental area (VTA), which contains dopaminergic neurons that project into the nucleus accumbens (Corrigall et al, 1994). Within the VTA, the critical nature of both α4 and β2 subunits in nicotine self-administration have been demonstrated in subunit specific knock out studies followed by selective re-expression in the VTA of these subunits (Picciotto et al, 1998; Maskos et al, 2005; Pons et al, 2008).

However, where do desensitization and upregulation of receptors fit into this picture? It is clear that desensitization of nAChRs by nicotine plays an important role in addiction (Hulihan-Giblin et al., 1990; Xiao et al., 2006; Kellar and Xiao, 2007; Picciotto et al, 2008; Buccafusco et al., 2009). As mentioned previously, one of the hallmarks of α4β2 nAChRs is that they are upregulated when exposed to chronic nicotine. How might this upregulation mediate the addictive effects of nicotine? The chronic and habitual consumption of nicotine results in withdrawal symptoms when nicotine administration is ceased. Withdrawal symptoms, such as anxiety (Turner et al, 2011), anhedonia (De Biasi and Salas, 2008), anger, difficulty concentrating, sleep disturbance, and increased appetite (De Biasi and Dani, 2011) may be mediated by the increased nicotinic drive produced by the higher numbers of re-sensitized α4β2 nAChR subtype. Nicotine-intake,
therefore, is not meant to activate these α4β2 nAChRs, but rather desensitize them, thus subduing this overactive cholinergic drive (Xiao et al., 2006; Hussmann et al., 2012).

One possible example of this relationship between upregulation and desensitization may manifest itself in the expression pattern of α4β2* and α7 nAChR subtypes within the VTA and the upregulation of α4β2* there. In the VTA, both α7 and α4β2* nAChRs are expressed. However, α4β2 nAChRs are expressed post-synaptically on dopaminergic (DA) neurons and pre-synaptically on GABAergic interneurons (Figure 3A), whereas α7 receptors are the dominant type expressed pre-synaptically on glutamatergic afferents onto DA neurons (Arroyo-Jimenez et al., 1999; Klink et al., 2001; Marubio et al., 2003; Wooltorton et al., 2003). Under baseline conditions, GABAergic interneurons and glutamatergic afferents maintain a basal dopamine tone in the nucleus accumbens via VTA DA neurons. The activity of these GABAergic interneurons and glutamatergic afferents partially controlled by α4β2* and α7 nAChRs, respectively (Pidoplichko et al., 2004). However, chronic nicotine preferentially upregulates the α4β2* receptors that are expressed at the GABAergic interneurons, but does not affect the α7 expression at pre-terminal glutamatergic afferents, thus increasing the overall activity of the GABAergic output to DA neurons and lowering the basal dopamine tone in the nucleus accumbens (Figure 3B) (Zhang et al., 2012). In order to return DA tone in the nucleus accumbens to its natural basal state, nicotine is administered by smoking cigarettes. The brain concentration of nicotine following a cigarette (50-300 nM) is sufficient to desensitize ~80% of α4β2 nAChRs, but does not desensitize or activate α7 nAChRs (Benowitz et al., 1989; Henningfield et al., 1993; Gourlay and Benowitz, 1997; Wooltorton et al., 2003). Selectively desensitizing α4β2* nAChRs, but not α7 nAChRs,
thus allows the smoker to return his/her natural basal dopamine tone in the nucleus accumbens (Figure 3C). The smoker then goes about his/her day until desensitization at α4β2* wears off and the smoker is left craving another cigarette.
Figure 3. Schematic representation of desensitization in the VTA. Filled squares represent active α7 nAChRs. Open circles represent desensitized α4β2 nAChRs. Filled circles represent active nAChRs. Lightning bolts represent (legend continues next page)
Figure 3 legend (continued): relevant neurotransmitter release at synapses. (A) A regular VTA DA neuron receives input from glutamatergic afferents and GABAergic interneurons. (B) Smoking induced upregulation results in increased expression of α4β2 nAChRs. As a results, increased GABAergic transmission inhibits DA neuron activity and reduces DA transmission to the nucleus accumbens. (C) Smoking administers nicotine that desensitizes α4β2 nAChRs, but not α7 nAChRs. This restores natural DA transmission to the nucleus accumbens. Resensitization of α4β2 nAChRs sends the system back to (B).

There are several lines of evidence suggesting that targeting desensitization may be an effective avenue for smoking cessation therapy. One is that nicotine is more potent at producing desensitization at α4β2* nAChRs than it is at activating them (Hulihan-Giblin et al, 1990; Paradiso and Steinbach, 2003). Therefore, although smoking tobacco rapidly raises the plasma concentration of nicotine, nAChRs will still be exposed to desensitizing concentrations of nicotine before they are exposed to excitatory concentrations of nicotine, suggesting that the primary mode of action of nicotine is desensitization. This is reinforced by PET data indicating that β2* occupancy by nicotine is ~88% following 1 cigarette, with plasma levels of nicotine that correspond to 50% desensitization of α4β2 nAChRs (Brody et al, 2006). An example of nicotine mediated desensitization in vivo is the observations that both acute and chronic nicotine administrations abolish acute nicotine mediated prolactin release in rats (Hulihan-Giblin et al, 1990).

There is also evidence that many nAChR antagonists elicit similar behavioral and physiological responses in animals as nicotine. Behaviorally, direct infusion of the α4β2 nAChR competitive antagonist dihydro-β-erythroidine (DHβE) into the VTA will reduce
nicotine seeking behavior in rats that have been trained to self-administer nicotine (Corrigall et al, 1994). In electrophysiological slice recordings, the nAChR antagonists d-tubocurarine and α-bungarotoxin produce excitatory responses in rat hippocampal slices that resemble those of nicotine (Ropert and Krnjevic, 1982).

The underlying brain activity that drives smoking may therefore be targeted desensitization of α4β2* nAChRs. Under this paradigm, an ideal smoking cessation compound will be selective for α4β2* nAChRs, potently and efficaciously desensitize α4β2* nAChRs, and desensitize α4β2* nAChRs for extended periods of time (Xiao et al, 2006).

The purpose of the present work was to more thoroughly define the property of desensitization. During the course of this, I developed several tools to explore nAChR pharmacology. More specifically, I explore the ability of ligands to selectively desensitize α4β2 and α3β4 nAChRs, as well as the ligand properties that cause long lasting desensitization. Also part of this project was defining the interspecies differences in α3β4 nAChR pharmacology, which has important implications in selectivity of ligands for α4β2 nAChRs and also provides interesting insight into the mechanisms of long lasting desensitization.
CHAPTER II: EXPERIMENTAL METHODS AND MATERIALS
Drugs

Unless stated otherwise here, all drugs were purchased from Sigma-Aldrich (St. Louis, MO) \[^{3}\text{H}\]-epibatidine (\[^{3}\text{H}\]-EB) was purchased from Perkin-Elmer Life Sciences (Waltham, MA). Sazetidine-A and DHβE were provided by Research Triangle Institute (Research Triangle Park, NC). AT-1001 was synthesized and supplied to us by Dr. Andrew Horti of Johns Hopkins University (Baltimore, MD). Varenicline tartrate was generously given to us by Hans Rollema of Pfizer (Groton, CT).
Cell culture and stable cell lines

Unless stated otherwise, cell culture materials were purchased from Invitrogen Corporation (Carlsbad, CA). Fetal bovine serum and horse serum were purchased from Gemini Bio-Products (Woodland, CA). SGM-7 medium consisted of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 µg/ml streptomycin, and 0.7 mg/ml Geneticin (G418). GH medium consisted of minimum essential medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 µg/ml streptomycin, 0.7 mg/ml G418 (Geneticin), and 0.1 mg/ml hygromycin B.

Four cell lines were used over the course of the experiments described presently. KXα3β4R2 and KXα4β2R2 are HEK293 cell lines that stably express rat α3β4 and rat α4β2, respectively (Xiao et al., 1998; Xiao et al., 2004). Both KXα3β4R2 and KXα4β2R2 were cultured in SGM-7 medium. YXα3β4H1 and YXα4β2H1 are HEK293 cell lines that stably express human α3β4 and human α4β2 nAChRs, respectively. These two cell lines were established recently (Xiao et al., unpublished data). Both YXα3β4H1 and YXα4β2H1 were cultured in in GH medium.
Binding assays

**Cell homogenate preparation.** Cultured cells at ~90-100% confluence were removed from their flasks with a cell scraper and placed in 50 mM Tris-HCl buffer (pH 7.4, 4 °C). The cell suspension was centrifuged at 1,000 x g for 10 min and the pellet was collected. The cell pellet was then homogenized with a Polytron homogenizer in Tris-HCl buffer (pH 7.4, 4 °C) and centrifuged at 30,000 x g for 10 min at 4 °C. The membrane pellet was resuspended in fresh buffer. This was repeated for a total of 3 times before the final pellet was resuspended in 50 mM Tris-HCl and used in subsequent ligand binding experiments.

**Saturation binding experiments.** Saturation radioligand binding assays were performed with [³H]-EB and [³H]-cytisine as described previously in 50 mM Tris-HCl, pH 7.4 (Xiao et al, 1998). The radioligand was incubated (4 hr, room temperature) with membrane preparations in concentrations up to ~3 nM ([³H]-EB) or ~10 nM ([³H]-cytisine). Non-specific binding was determined by binding radioligand in the presence of 300 µM (-)-nicotine. Free and bound radioligand were then filtered through Whatman GF/C filters treated with 0.5% polyethylenimine using a Brandel 48-sample, Semi-Auto Harvester and bound radioligand was determined by liquid scintillation counting of filters. Specific binding was normalized to protein concentration as determined by a standard BSA protein assay. Receptor density (B<sub>max</sub>) and dissociation constant K<sub>d</sub> were then determined using nonlinear least square regression analysis in GraphPad Prism 5.
In order to determine the nature of AT-1001’s binding, saturation radioligand binding assays were performed with \[^3\text{H}\]-EB in the presence of AT-1001. \[^3\text{H}\]-EB, at increased concentrations of up to \(~3\) nM (rat \(\alpha_3\beta_4\), human \(\alpha_4\beta_2\), and rat \(\alpha_4\beta_2\)) or \(~20\) nM (human \(\alpha_3\beta_4\)), was incubated with membrane preparations at a single concentration of AT-1001.

**Competition binding experiments.** Competition binding assays were performed to determine the affinity of non-radiolabeled ligands at all four receptor subtypes. Ligands, at various concentrations were incubated with \(~0.4\) nM \[^3\text{H}\]-EB for two hours at room temperature. As described above, binding samples were harvested and data were processed to determine \(K_i\) values of each ligand.

**Determining association rate constant (\(k_{\text{on}}\) of \[^3\text{H}\]-EB.** The association rates of \[^3\text{H}\]-EB at \(~2.5\) nM and \(~6.5\) nM were tested. This was performed by adding \[^3\text{H}\]-EB to a final concentration of \(~2.5\) nM or \(~6.5\) nM into test tubes containing membrane homogenates of YX\(\alpha_4\beta_2\)H1 and incubating for set periods of time before harvesting to detect the amount of associated \[^3\text{H}\]-EB for that amount of time. In experiments with \(~2.5\) nM \[^3\text{H}\]-EB, time points were spread out across 30 minutes. In experiments with \(~6.5\) nM, time points were spread out across 10 minutes. As described above, binding samples were harvested and data were processed to determine \(k_{\text{on}}\) value of \[^3\text{H}\]-EB.

**Determining dissociation rate constants (\(k_{\text{off}}\) of radioligands.** The dissociation rates of \[^3\text{H}\]-EB and \[^3\text{H}\]-cytisine were determined by direct observation of radioligand
dissociation from receptors. In [³H]-EB experiments, receptors were initially incubated with [³H]-EB at ~0.4 nM for 2 hours to allow binding to reach equilibrium. Nicotine was then added to a final concentration of 300 µM at progressive time points over 4 hrs. [³H]-cytisine experiments were performed similarly, but with [³H]-cytisine at a concentration of ~15 nM and nicotine additions over the course of 1 hr. As described above, binding samples were harvested and data were processed to determine $k_{off}$ value of radioligands.

*Determining dissociation rate constants ($k_{off}$) of unlabeled ligands.* The dissociation rates of unlabeled ligands, including epibatidine, saz-A, varenicline and 5-I-A85380, were determined by using a newly developed procedure (Xiao et al., unpublished data). A cold ligand was incubated with membrane homogenates for two hours. Then, [³H]-EB was added to a final concentration of ~2.5 nM or higher at progressive time points. At each of these time points, [³H]-EB binding was inhibited by cold ligand still bound, resulting in a sub-$B_{max}$ binding ($B_i$). For epibatidine, saz-A, and 5-I-A85380, dissociation was determined over 4 hours. For nicotine, cytisine, and varenicline, dissociation was determined over 1 hour. For every time point where [³H]-EB was added to membrane homogenates with cold ligand, [³H]-EB was also added to a tube containing only membrane homogenates and buffer to determine total binding possible ($B_{max}$) at that time point. This served two purposes: one was to ensure that [³H]-EB binding was not still in its association phase at its given time point, the other was to determine the averaged total binding throughout the experiment, which was then used for calculations of dissociation. At the end of the experiment, binding samples were harvested as described above. The percentage of cold ligand that was still bound at each
time point was determined by finding the difference in total binding and dissociating binding divided by the total binding:

\[
f_{\text{cold bound}} = \frac{\text{Average } B_{\text{max}} - B_i}{\text{Average } B_{\text{max}}} \times 100\%
\]

All data was fitted to a one-phase exponential decay in GraphPad Prism 5 with a constrained plateau. One-phase exponential decay model was used because affinity binding results independently indicated that all ligands bound to one-site. When a fitting is done, the plateau is constrained because of the small difference between concentration of dissociating cold ligand and displacing radiolabeled ligand. In this method, the amount of displacing radioligand (\(\text{[}^3\text{H}]\)EB at \(\sim 2.5 \text{ nM} - \sim 6.5 \text{ nM}\)) compared to the amount of cold ligand (0.25 – 100 nM) is not as exaggerated as in the direct observation method (where \(\sim 0.4 \text{ nM} [^3\text{H}]\)EB is being displaced by 300 \(\mu\text{M}\) nicotine). Therefore, even at infinite time, where the dissociating and displacing ligands have reached equilibrium, there will still be a significant amount of dissociating ligand bound. Since the concentrations of both unlabeled dissociating ligand and hot displacing ligand were know, a plateau at which the binding reaction will reach equilibrium can be calculated.

The value at which to constrain the plateau is based on the following classical pharmacology equation, which determines the receptor binding of a cold ligand in the presence of a hot competing ligand:

\[
B_i = \frac{B_{\text{max}}[I]}{[I] + K_i (1 + [L]/K_D)}
\]
By defining $B_{\text{max}}$ as 1, $B_i$ is the fraction of receptors that would be bound by cold ligand $I$ at concentration $[I]$ at equilibrium, or at time infinite, in the presence of radioligand $L$ with an affinity of $K_D$ at concentration $[L]$. Therefore the fraction of receptors that will remain bound by dissociating cold ligand ($f$) at equilibrium, and the point at which we set our plateau, is:

$$f = \frac{[C]}{[C]+K_C(1+[H]/K_H)} \times 100\%$$

Where $[C]$ is the known concentration of cold ligand used, $K_C$ is the $K_i$ of the cold ligand, $[H]$ is the concentration of the hot displacing ligand, and $K_H$ is the $K_D$ of the hot displacing ligand.
\[ {^{86}\text{Rb}}^{+}-\text{efflux assays} \]

\[ {^{86}\text{Rb}}^{+} \text{ efflux assays} \] were carried out as reported previously (Xiao et al., 1998). Briefly, cells were cultured in Nunc Nunclon surface 200 mL flasks to \( \sim 80\% \) confluence. The culture media was aspirated and replaced with fresh media. The following day, cell confluence was observed to be 90-100\% and cells were plated in 1 mL culture media onto Nunc Nunclon Surface 24 well plates coated with poly-D-lysine. Efflux experiments were performed the following day. Experiments throughout were performed using 15 mM HEPES buffer solution (pH 7.4). In all experiments, “stimulation buffer” was added for 2 min as a final step. This stimulation buffer was then pipetted out and into liquid scintillation tubes and \( {^{86}\text{Rb}}^{+} \) efflux was assessed using Cerenkov counting on a Beckman-Coulter LS6500 Multi-Purpose Scintillation Counter.

\[ \text{Agonist dose-response for activation.} \] To test dose-dependent activation by agonists, cells were first loaded with \( {^{86}\text{Rb}}^{+} \) by incubating them with 0.5 mL media containing roughly 100,000 dpm \( {^{86}\text{Rb}}^{+} \) for 2 – 4 hrs. Cells were then rinsed 4 times over 10 min with 1 mL buffer each time and then stimulated with buffer containing drug for 2 min. Background efflux was determined by stimulating cells with buffer containing no drug, while maximal response was determined using buffer containing 100 \( \mu \)M nicotine. Agonist stimulation was normalized such that background efflux was 0\% activation and 100 \( \mu \)M nicotine stimulated efflux was 100\% activation.
Agonist dose-response for desensitization. To test dose-dependent desensitization, cells were first loaded with 0.5 mL media containing roughly 100,000 dpm $^{86}$Rb$^+$ for 2–4 hrs. Following the loading period, the $^{86}$Rb$^+$ containing media was removed and cells were rinsed gently 3 times over ~2 min with 1 mL buffer each time and were treated for 10 min with desensitizing drug in 0.5 mL buffer. After 10 min treatment, the drug-containing buffer was aspirated and cells were immediately stimulated with 0.5 mL buffer containing 100 µM nicotine for 2 minutes. In analysis, $^{86}$Rb$^+$ efflux after desensitizing pretreatments was normalized with background $^{86}$Rb$^+$ efflux as 0% efflux and un-desensitized $^{86}$Rb$^+$ efflux as 100% efflux.

The recovery of function after desensitization was assessed using a modified version of the aforementioned protocol. After 10 minute pretreatment with desensitizing drug, cells were submitted to three wash steps: once immediately following the 10 minute desensitization, once 30 min later, and once two hours after the initial 10 minute desensitization. Each wash consisted of 3 rinses with 1 mL media each time and a final addition of 1 mL media that was left in until the next wash. After the wash at two hours post-desensitization, 0.5 mL $^{86}$Rb$^+$ loading buffer was added. After another 1 hr and 50 min, cells were washed over 10 min and stimulated over 2 min in the same manner as cells as previously described. Background and un-desensitized $^{86}$Rb$^+$-efflux were determined the same way as described above and were also used to normalize response the same way.

DHβE effect on desensitization. After loading cells with $^{86}$Rb$^+$, loading medium was removed and cells were pretreated with either buffer, 10 nM Saz-A, 10 µM DHβE,
10 nM Saz-A + 10 µM DHβE, 50 nM Saz-A, or 50 nM Saz-A + 10 µM DHβE for 10 min. Cells were then rinsed 4 times with 1 mL buffer over 10 min. Then, cells were stimulated with 0.5 – 300 µM nicotine, thus forming a nicotine dose response curve. Background $^{86}\text{Rb}^+$-efflux was determined by pre-treating with buffer and stimulating with buffer sans nicotine. Maximal $^{86}\text{Rb}^+$- efflux was determined by pre-treating with buffer and stimulating with 100 µM nicotine. Data was normalized such that background efflux was 0% and maximal efflux was 100%.

Potency of mecamylamine at inhibiting $^{86}\text{Rb}^+$-efflux. Cells were loaded with $^{86}\text{Rb}^+$ as previously described. After washing the cells 4 times with buffer over 10 minutes, either 100 µM nicotine or 3 µM sazetidine-A were co-applied with 0 – 100 µM mecamylamine in 0.5 mL buffer to stimulate for 2 min. This buffer was then removed and $^{86}\text{Rb}$-efflux was assessed. Results were normalized such that background efflux (stimulation buffer was plain buffer) was 0% and maximal efflux (stimulation buffer contained 100 µM nicotine) was 100%.

Wash-out of mecamylamine. After loading with $^{86}\text{Rb}^+$ as previously described, loading buffer was removed and cells were pre-treated with 0.3 – 100 µM mecamylamine for 10 min and separated into three groups: cells that were washed for (A) 10 min, (B) 30 min, and (C) 60 min. In all treatment groups, background and maximal efflux controls were not exposed to any mecamylamine. Background condition cells were subsequently stimulated with plain buffer and maximal efflux cells were stimulated with 100 µM nicotine after wash period.
(A) Cells were rinsed 4 times over 10 min with buffer and stimulated for 2 min with 100 µM nicotine.

(B) Cells were rinsed 4 times over 10 min with media, with the final rinse containing ~100,000 dpm $^{86}\text{Rb}^+$. After this 10 min wash, another 10 min wash of the same type was repeated. After this second 10 min wash, a final wash consisting of four rinses with buffer over 10 min was performed and cells were stimulated with buffer containing 100 µM nicotine.

(C) Cells were rinsed 4 times over 10 min with media, with the final rinse containing ~100,000 dpm $^{86}\text{Rb}^+$. After this 10 min wash, a second wash of 3 rinses with media was performed, after which media containing ~100,000 dpm was added. After 40 min had passed after the first wash, a third wash consisting of 4 rinses with buffer over 10 min was performed and cells were stimulated with buffer containing 100 µM nicotine.

The effect of mecamylamine on desensitization. After loading with $^{86}\text{Rb}^+$, cells were pretreated with 0.3 – 3 µM sazetidine-A, either co-applied with 30 µM mecamylamine or not, for 10 min. After 10 min, cells were washed according to wash procedure (C) as described above over 60 min and cells were stimulated with 100 µM nicotine. Again, background $^{86}\text{Rb}^+$-efflux was determined by pre-treating with buffer and stimulating with buffer sans nicotine. Maximal $^{86}\text{Rb}^+$-efflux was determined by pre-treating with buffer and stimulating with 100 µM nicotine.
Patch-clamp electrophysiology with microfluidic laminar stream solution exchange (MLSSE)

Cell culture and plating. Prior to plating, cells were maintained as described above. Cells were considered ready for plating when they reached 90-100% confluency. Cells were plated onto glass coverslips (not treated with poly-D-lysine) in a 6-well dish containing the appropriate culture medium. Although both (A) α3β4 cell lines are highly functional and did not require any pretreatment, both (B) α4β2 cell lines required a carbachol pretreatment.

(A) KXα3β4R2 and YXα3β4H1: When cells were observed to be ~90-100% confluent in culture flasks, medium was aspirated out and cells were dissociated with 5 mL of Gibco Versene (an EDTA based dissociation reagent). After 2-3 min, cells were observed to be dissociated from the surface of the flask and to quench the dissociation reaction, 5 mL of culture medium was added. This slurry was then gently triturated 5 times with a 10 mL pipette and 5 drops were distributed into each well of the 6-well dish containing glass coverslips with a flame polished Pasteur pipette. These cells were used the following day in patch-clamp experiments.

(B) KXα4β2R2 and YXα4β2H1: When cells were ~50% confluent, culture medium was aspirated out and replaced with culture medium containing 1 mM carbachol. The following day, cells would be observed to be ~90-100% confluent and would be plated in a manner similar to (A). All culture medium used during the plating process (e.g., in the 6-well dish, 5 mL used to quench
the dissociation reaction) were supplemented with 1 mM carbachol. On the day of experiment, cells were washed with fresh media (without carbachol) for at least 1 h prior to patch clamp electrophysiology.

Electrophysiology. Glass coverslips plated with HEK293 cells were placed into the recording chamber of a microfluidic chip (Dynaflow Resolve, Cellectricon Inc., Sweden) containing extracellular solution composed of (in mM): 130 NaCl, 5 KCl, 2 CaCl$_2$, MgCl$_2$, 10 dextrose, and 10 HEPES. The chip was attached to a motorized X-Y stage of an inverted microscope (Olympus IX 70). Cells of interest were identified visually and by a CCD camera (Dage S-75). A 40X objective was used for identifying and approaching the cell.

Whole-cell voltage clamp ($V_{\text{hold}}$ = -70mV) recordings were made with patch electrodes (~5-8MΩ) containing a solution (pH 7.2) composed of (in mM): K gluconate 145; EGTA 5; MgCl$_2$ 2.5; HEPES 10; ATP.Na 5; and GTP.Na 0.2. The patch pipette was coupled to an amplifier (Axopatch 200B; Axon Instruments Inc.) and its signal filtered (5kHz), digitized (Digidata 1440A; Axon Instruments Inc.) and stored on a computer running the pClamp 10 software (Axon Instruments Inc.) for later analysis. Series resistance was typically <10 MΩ and was continuously monitored. All experiments were performed at room temperature (23–25°C).
Figure 4. Photomicrographs of a typical HEK293 cell that serves as a good candidate for electrophysiology using the MLSSE system. Cells are chosen that are round and lightly adhere to the glass coverslip (A). To form a gigaohm seal, the tip of the glass pipette is lowered to the level of the cell, which is then approached and patched from the side. Prior to drug exposure, each ‘patched’ cell is positioned in the stream of the first channel at a distance that approximates the width of the channel opening (B).

Cell selection, approach, whole-cell configuration and relocation: Cells were selected for patching that were spherical, had a well-defined appearance and lightly adhered to the surface of the non-coated coverslip (Figure 4). [Note: Except for some, the majority of the spherical cells adhere lightly to the surface of the non-coated glass coverslips.] A cell thus selected is approached with the patch pipette from the side (Figure 5A). After establishing a gigaohm seal and whole-cell configuration, the cell is then lifted up while applying gentle suction. Once free of the glass surface, it is sent to the opening of the first microfluidic channel via a command signal to the X-Y motorized stage (Figure 5B). Here, it is then lowered to rest at the center of the laminar flow column (visually estimated from the surface and base of the channel opening, see Figure 5B). Once positioned, the infusion pump (26 µL/min) is turned on for 3 min to establish laminar flow in each channel. This is then followed by initiating the drug application
protocol that allows for a single 'patched' cell to be exposed to various drugs or their concentrations.

Figure 5. A schematic illustrating the steps involved in using the MLSSE in conjunction with patch clamp electrophysiology. (A) A well-defined ‘round’ cell is chosen, patched, and lifted off the coverslip with gentle suction. (B) The cell is positioned in front of the channel using a computerized protocol. (C) The cell is lowered to the focal plane of the channel and its distance adjusted to approximate the width of the channel. (D) The pump is turned on for 3 minutes to establish laminar flow in each channel. Once laminar flow is established, the chip is then moved underneath the cell using a preset protocol to record whole-cell responses.
Drug Application: Subsequent to whole-cell configuration, relocation and prior to commencement of a drug (s) exposure protocol (see above), a steady-state microfluidic laminar flow in each channel needs to be established. This is accomplished by an infusion pump (26 µl/min) after 3 min using an attached 2 ml syringe that is connected to the microfluidic 'chip' (Dynaflow Resolve, Celletricon, Sweden). Once the steady-state flow is reached, the drug application protocol is initiated, which allows for a single 'patched' cell to be exposed to various drugs or their concentrations. The parameters for the stage speed (assigned in the Celletricon Dynaflow Commander Pro 1.3 software under settings) were 5 mm/s$^2$ for maximum scan speed and 0.8 mm/s$^2$ for maximum scan acceleration.

Agonist dose-response. After laminar flow had been established, dose-response protocol was initiated using Celletricon Dynaflow Commander Pro 1.3. In general, agonist protocols moved cell first into a stream with drug (we call this the exposure) and then into a stream with buffer (we call this the recovery). The first exposure was always 1 mM ACh, followed by exposures to the relevant drug. After each exposure to the relevant drug and the required recovery, cell was exposed to the next highest concentration of drug, followed by the required recovery, and so on until an entire dose-response was recorded. The final exposure of drug (the highest concentration) was always performed twice in order to confirm that no desensitization was occurring between exposures. Note that for each cell line and drug, different exposure times (time a cell spends in a drug stream) and interstimulus interval (time a cell spends in a buffer stream to allow receptors
to recover from agonist-mediated desensitization) were necessary due to the varying channel kinetics in nAChRs and differing desensitizing properties of drugs.

*Recovery from desensitization mediated by AT-1001.* An AT-1001 concentration equal to 100 times the DC$_{50}$ of the corresponding cell line was chosen to ensure full desensitization of receptor function: [AT-1001] = 11 µM for YXα3β4H1 (human α3β4), [AT-1001] = 120 µM for KXα3β4R2 (rat α3β4), and [AT-1001] = 330 µM for YXα4β2H1 (human α4β2). Cells were first exposed to two applications of 1 mM ACh separated by 1 min, followed by 10 min of AT-1001, and then 1 mM ACh every minute immediately following the 10 min AT-1001 exposure until full recovery of function was observed. Exposure times of 500 ms (YXα3β4H1 and KXα3β4R2) and 200 ms (YXα4β2H1) were used.

*Data analysis:* Elicited peak currents in each individual cell were normalized to the peak current of a 1 mM ACh response in the same cell. Data was fitted in Graphpad Prism 5.
CHAPTER III: RESULTS

PART 1

CHARACTERIZING THE PHARMACOLOGY OF HUMAN α4β2: LIGAND BINDING, ACTIVATION, AND DESENSITIZATION
**Saturation binding in YXα4β2H1 cell homogenates**

YXα4β2H1, the cell line expressing human α4β2 nAChRs was newly established (Xiao *et al.*, 2011) and, as such, needed to be thoroughly characterized before use. The binding affinity of epibatidine (EB) and cytisine were determined by performing saturation binding experiments with [³H]-epibatidine ([³H]-EB) and [³H]-cytisine ([³H]-cyt). The results of these studies are presented in Figure 6 and Table 1. The $K_d$-values for [³H]-EB (23 ± 4 pM) and [³H]-cyt (292 ± 58 pM) are similar to values that have been reported both internally within the lab (for rat α4β2, $K_d = 46$ pM for EB and $K_d = 1.5$ nM for cytisine) and externally by other research groups (for human α4β2, $K_d = 10$ pM for EB and $K_d = 1$ nM for cytisine) (Eaton *et al.*, 2003; Slater *et al.*, 2003; Xiao *et al.*, 2004). The $B_{max}$-values demonstrated that YXα4β2H1 expresses human α4β2 nAChRs at very high density. Note that although the $B_{max}$, as determined by [³H]-EB saturation binding, and the $B_{max}$, as determined by [³H]-cytisine saturation binding, are different, that this difference is not statistically significant. This inconsistency, however, is still quite large, and could be explained by either variability in receptor densities among different passages cells or by incorrectly noted specific activity of the radioligands.
Figure 6. Representative graphs for saturation binding of $[\text{H}]$-EB and $[\text{H}]$-cyt in YXα4β2H1 cell homogenates. Close circles represent radioligand binding at that concentration of drug; open circles represent non-specific binding as determined by drug co-incubated with 300 µM nicotine.

Table 1. Saturation binding of $[\text{H}]$-Epibatidine and $[\text{H}]$-Cytisine in YXα4β2H1 cell homogenates. Values shown are mean ± S.E.M of 3 – 5 independent experiments.

<table>
<thead>
<tr>
<th></th>
<th>$K_d$ (pM)</th>
<th>$B_{max}$ (fmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[\text{H}]$-Epibatidine</td>
<td>23 ± 4</td>
<td>3537 ± 538</td>
</tr>
<tr>
<td>$[\text{H}]$-Cytisine</td>
<td>292 ± 58</td>
<td>2117 ± 272</td>
</tr>
</tbody>
</table>
Competition binding in YXα4β2H1 cell homogenates

Competition binding experiments were performed to determine the binding affinity ($K_i$) of nicotine, sazetidine-A (Saz-A), varenicline, and 5-I-85380. Data was fitted to a one-site competition model using GraphPad Prism 5 to yield $K_i$-values (Figure 7, Table 2). Again, the values determined here are comparable to those determined using KXα4β2R2 internally by the lab, with the exception of the values determined for Saz-A (Xiao et al., 2004; Xiao et al., 2006). For Saz-A, the $K_i$-value reported previously (0.64 nM) is more than 5 times higher than the value reported currently (0.12 nM). This difference has been attributed to incorrect quantification of the amount of Saz-A yielded in synthesis during the previous study (Xiao, personal communication). This resulted in an incorrect $K_i$ being reported; the current value reported here is therefore taken as the actual $K_i$ of Saz-A.

Figure 7. Competition by nicotine, Saz-A, 5-I-A85380, and varenicline for nAChRs binding sites in YXα4β2H1 cell homogenates labeled by $[^3H]$-EB.
Table 2. Comparison of binding affinities of nicotinic ligands at human α4β2 subtype. Values shown are the mean ± S.E.M. of n = 3 – 6 independent experiments.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_i$ (nM)</th>
<th>$n_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>6.6 ± 0.4</td>
<td>0.96 ± 0.03</td>
</tr>
<tr>
<td>Sazetidine-A</td>
<td>0.12 ± 0.013</td>
<td>1.02 ± 0.07</td>
</tr>
<tr>
<td>Varenicline</td>
<td>0.33 ± 0.006</td>
<td>0.97 ± 0.04</td>
</tr>
<tr>
<td>5-I-A85380</td>
<td>0.082 ± 0.007</td>
<td>1.08 ± 0.06</td>
</tr>
</tbody>
</table>
Dissociation rate of ligands from α4β2 nAChRs

Our plan for using the indirect method of determining the dissociation rate constant, $k_{\text{off}}$, was dependent on the association rate of [3H]-EB being very rapid compared to the dissociation rate of the unlabeled ligand being tested. Thus, association rates were first determined for the concentrations at which [3H]-EB would later be used in “indirect method” dissociation rate measurements. [3H]-EB at ~2.5 nM fully saturates receptors within 2 minutes of addition and 6.5 nM fully saturates receptors within 1 minute of addition (Figure 8).

![Graphs showing time dependent saturation of receptors by [3H]-EB.](image)

Figure 8. Time dependent saturation of human α4β2 nAChRs by [3H]-EB. When [3H]-EB is added at (A) ~2.5 nM, receptors are saturated by 2 minutes. When [3H]-EB is added at (B) ~6.5 nM receptors are fully saturated by 1 minute.

Two methods, the “direct method” and “indirect method,” were used to determine the dissociation rate (“off-rate”) of ligands from receptors and results were fit using a one-phase exponential decay model in GraphPad Prism. The rate constant, $k_{\text{off}}$, and half-time (the time it took for 50% of initially bound ligand to dissociate from receptors), $t_{1/2}$, were derived from the equation for one-phase exponential decay ($Y = Y_0 e^{-kt}$).
The “direct method” could only be used to determine the off-rates of radiolabeled ligands. In this case, only the off-rates of \([^3\text{H}]\)-EB and \([^3\text{H}]\)-cyt were determined (Figure 9, Table 3).

![Graph of [^3\text{H}]\)-EB and [^3\text{H}]\)-cyt dissociation](image)

**Figure 9.** Determination of dissociation rates of \([^3\text{H}]\)-EB and \([^3\text{H}]\)-cyt by direction measurement of radioligand dissociation.

**Table 3.** Comparison of dissociation rate constants and half-times determined by direct method and indirect method. Values shown are the mean ± S.E.M. of 4 – 8 independent experiments.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Direct Determination</th>
<th>Indirect Determination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(k_{\text{off}}) (min(^{-1}))</td>
<td>(t_{1/2}) (min)</td>
</tr>
<tr>
<td>Epibatidine</td>
<td>0.0224 ± 0.0004</td>
<td>31.0 ± 0.7</td>
</tr>
<tr>
<td>Cytisine</td>
<td>0.229 ± 0.018</td>
<td>3.17 ± 0.27</td>
</tr>
</tbody>
</table>
In contrast to the direct method, the “indirect method” for determining dissociation rate can be used to determine the off-rate of any unlabeled ligand at any receptor, so long as a high affinity, high selectivity radioligand exists for the receptor in question (Tuan et al, 2010). This method was used to determine the dissociation rate for EB, cytisine, nicotine, Saz-A, varenicline, and 5-I-A85380 (Figure 10). The results for EB and cytisine as determined using this method were compared to those of [³H]-EB and [³H]-cytisine from direct determinations using a two-tailed unpaired t-test with Welch’s correction (Table 3). We found that there was no significant difference between results obtained using the direct and indirect methods.

The results for all indirectly determined off-rates are summarized in Table 4.

Surprisingly, saz-A had the slowest dissociation rate out of all drugs tested, despite having a lower predicted affinity compared to EB and 5-I-A85380. All other drugs tested had off-rates that correlated to their affinity (i.e., lower $K_D/K_i$ = slower dissociation).

### Table 4. Dissociation rate constants and half-times determined by indirect method.

Values shown are the mean ± S.E.M. of 4 – 7 independent experiments. $K_d/k_{off}$ indicates the $k_{on}$ of the ligand.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Off-rate $t_{1/2}$ (min)</th>
<th>$k_{off}$ (min⁻¹)</th>
<th>$K_d/k_{off}$ (nM·min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>0.94 ± 0.30</td>
<td>1.12 ± 0.44</td>
<td>5.9</td>
</tr>
<tr>
<td>Cytisine</td>
<td>2.6 ± 0.6</td>
<td>0.337 ± 0.059</td>
<td>0.9</td>
</tr>
<tr>
<td>Saz-A</td>
<td>49.7 ± 4.7</td>
<td>0.015 ± 0.002</td>
<td>8.0</td>
</tr>
<tr>
<td>Varenicline</td>
<td>14.4 ± 0.8</td>
<td>0.049 ± 0.003</td>
<td>6.7</td>
</tr>
<tr>
<td>Epibatidine</td>
<td>34.3 ± 3.8</td>
<td>0.022 ± 0.002</td>
<td>1.0</td>
</tr>
<tr>
<td>5-I-A85380</td>
<td>33.8 ± 3.0</td>
<td>0.021 ± 0.002</td>
<td>3.9</td>
</tr>
</tbody>
</table>
Figure 10. Determination of the dissociation rates of unlabeled ligands. (A) Off-rate of epibatidine, cytisine, nicotine, Saz-A, varenicline, and 5-I-A85380 determined by indirect method – representative graphs. (B) Summary graph of averaged data from all experiments for all drugs.
Activation of human α4β2 nAChRs

YXα4β2H1 cells express high density of functional human α4β2 nAChRs. Therefore, the cell line is an excellent cell model for studying the activation of these nAChRs using $^{86}\text{Rb}^+$ efflux experiments, generally yielding a signal to background (100 µM nicotine elicited-to-buffer elicited efflux) of ~3. We found that cytisine, Saz-A, varenicline, and 5-I-A85380 acted as partial agonists compared to nicotine. Epibatidine acted as a super agonist compared to nicotine. All results were normalized to 100 µM nicotine efflux (Figure 11, Table 5).

Table 5. Activation of human α4β2 nAChRs by various ligands. All $E_{\text{max}}$-values represent the efficacy of the respective drugs when compared to 100 µM nicotine stimulation. Please note that nicotine’s $E_{\text{max}}$ is higher than 100% because 300 µM nicotine elicits slightly higher $^{86}\text{Rb}^+$-efflux when compared to 100 µM nicotine. Values shown are the mean ± S.E.M. of 3 – 4 independent experiments.

<table>
<thead>
<tr>
<th>Drug</th>
<th>$EC_{50}$ (nM)</th>
<th>$E_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>1829 ± 26</td>
<td>110%</td>
</tr>
<tr>
<td>Cytisine</td>
<td>1676 ± 861</td>
<td>38%</td>
</tr>
<tr>
<td>Sazetidine-A</td>
<td>20 ± 9</td>
<td>40%</td>
</tr>
<tr>
<td>Varenicline</td>
<td>1236 ± 352</td>
<td>50%</td>
</tr>
<tr>
<td>Epibatidine</td>
<td>34 ± 5</td>
<td>142%</td>
</tr>
<tr>
<td>5-I-A85380</td>
<td>14 ± 5</td>
<td>60%</td>
</tr>
</tbody>
</table>
Figure 11. Summarized activation dose response curves of agonists. Activation of human α4β2 nAChRs by six agonists was determined using ⁸⁶Rb⁺-efflux assays. Each curve represents an average of 3 – 4 experiments.
Desensitization of human α4β2 nAChRs and recovery from desensitization

All six drugs tested here were efficacious at desensitizing nicotinic receptors following a 10-minute incubation using $^{86}\text{Rb}^+$-efflux. Briefly, cells were exposed to varying concentrations of ligands for 10-minutes and $^{86}\text{Rb}^+$-efflux elicited by 100 µM nicotine was compared to that of control cells that were exposed to buffer for 10-minutes. Here and henceforth, the concentration at which a compound desensitizes 50% of the 100 µM nicotine-stimulated $^{86}\text{Rb}^+$-efflux will be referred to as the “$\text{DC}_{50}$”. The potency of ligands for desensitizing α4β2 nAChRs followed the same order as for activation (from highest potency to lowest): EB, 5-I-A85380, Saz-A, varenicline, cytisine, and finally nicotine. The recovery of receptors from desensitization by these ligands was assessed at the lowest concentrations tested at which full desensitization was induced: nicotine at 10 µM, cytisine at 30 µM, Saz-A at 0.1 µM, varenicline at 1 µM, EB at 0.1 µM, and 5-I-A85380 at 0.1 µM. Recovery from this desensitization was variable. Some drugs (nicotine, cytisine, varenicline) fully recovered from full desensitization after the 4-hour period, even when even higher concentrations were used, while other drugs (Saz-A, epibatidine, 5-I-A85380) did not fully recover function following this period (Figure 12, Table 6).
**Figure 12. Summarized desensitization dose response curves of agonists.** Open circles represent normalized $^{86}\text{Rb}^+$-efflux elicited by 100 µM nicotine immediately following 10 minute pretreatment with drug. Closed circles represent normalized $^{86}\text{Rb}^+$-efflux elicited by 100 µM nicotine 4 hours after 10 minute pretreatment. All results are normalized to the efflux elicited by 100 µM nicotine in cells that received no pretreatment. Each curve represents an average of 4 independent experiments. Each curve represents an average of 6 – 14 experiments.
Table 6. Desensitization of human α4β2 nAChRs by various ligands. Recovery from desensitization is expressed as a percentage of the $^{86}\text{Rb}^+$-efflux elicited from cells that were not pretreated with a testing ligand, but otherwise received the same handling as desensitized cells. Bracketed ([ ]) numbers represent the concentration at which the recovery is reported and are the lowest concentration at which full desensitization of nAChR function were observed. Values shown are the mean ± S.E.M. of 6 – 14 independent experiments.

<table>
<thead>
<tr>
<th>Drug</th>
<th>DC$_{50}$ (nM)</th>
<th>Recovery from desensitization After 4 hrs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>39.2 ± 5.8</td>
<td>97 [10 µM]</td>
</tr>
<tr>
<td>Cytisine</td>
<td>28.3 ± 9.1</td>
<td>95 [30 µM]</td>
</tr>
<tr>
<td>Sazetidine-A</td>
<td>1.4 ± 0.3</td>
<td>76 [0.1 µM]</td>
</tr>
<tr>
<td>Varenicline</td>
<td>5.8 ± 1.6</td>
<td>86 [1 µM]</td>
</tr>
<tr>
<td>Epibatidine</td>
<td>1.5 ± 0.05</td>
<td>49 [0.1 µM]</td>
</tr>
<tr>
<td>5-I-A85380</td>
<td>1.3 ± 0.2</td>
<td>55 [0.1 µM]</td>
</tr>
</tbody>
</table>
Effects of DHβE on desensitization

Nicotine-induced dose response of $^{86}$Rb$^+$-efflux was measured in YXα4β2H1 cells following 10 minute pretreatment with 10 nM Saz-A, 10 nM Saz-A + 10 µM DHβE, 50 nM Saz-A, or 50 nM Saz-A + 10 µM DHβE and was compared to a control, the efflux elicited from untreated YXα4β2H1 (Figure 13). The response of cells treated with these conditions were compared to the control at each concentration of nicotine applied using two-way repeated measures ANOVA with Bonferroni post-test. Cells pretreated with 10 nM Saz-A had significantly (p<0.05 – p<0.001) less nicotine induced $^{86}$Rb$^+$-efflux from control condition between nicotine concentrations 19.2 – 300 µM. Similarly, cells pretreated with 50 nM Saz-A had significantly (p<0.05 – p<0.001) less nicotine induced $^{86}$Rb$^+$-efflux from control pretreatment from concentrations 3.1 – 300 µM. Co-application of DHβE with Saz-A attenuated this effect: no significant difference was observed between the control condition and cells pretreated with 10 µM DHβE, 10 nM Saz-A + 10 µM DHβE, or 50 nM Saz-A + 10 µM DHβE.
Figure 13. Pre-treatment with Saz-A reduces nicotine evoked $^{86}$Rb$^+$-efflux, an effect that is attenuated by DHβE. Cells were loaded with $^{86}$Rb$^+$, washed, and subsequently pretreated with either wash buffer (control), 10 nM Saz-A, 10 μM DHβE, 10 nM Saz-A + 10 μM DHβE, 50 nM Saz-A, or 50 nM Saz-A + 10 μM DHβE. Following this 10-minute pretreatment, pretreatment was removed and cells were immediately stimulated with 0.5 – 300 μM nicotine. $^{86}$Rb$^+$-efflux was normalized to the efflux evoked by 100 μM nicotine in cells that had not been pretreated with any drug. In cells pretreated with 10 nM Saz-A, significant differences from control were observed for nicotine concentrations of 19.2 – 300 μM. In cells pretreated with 50 nM Saz-A, significant differences from control were observed for nicotine concentrations of 3.1 – 300 μM. No significant differences from control were observed in any conditions where DHβE was co-applied with Saz-A or where DHβE was applied by itself.
Effects of mecamylamine on desensitization

First, a concentration of mecamylamine that completely inhibited $^{86}$Rb$^+$-efflux was chosen. Mecamylamine at various concentrations (3 – 100 µM) was co-applied with 100 µM nicotine or 3 µM Saz-A and $^{86}$Rb$^+$ efflux was measured (Figure 14A). The amount of time it took for cells to fully recover function, or the amount of time it took for mecamylamine to wash out, from a 10 min application of these concentrations of mecamylamine was also measured over an hour (Figure 14B). The goal of these studies was to determine a concentration that would fully inhibit Saz-A function when co-applied and then to determine the amount of time required for complete washout of this concentration of mecamylamine. Based on results from these two studies, 30 µM mecamylamine along with a 1 hr wash out was used in the subsequent experiment (Figure 14C).

YX$\alpha 4\beta 2$H1 cells were desensitized with 0.3 µM, 1 µM, or 3 µM Saz-A with or without 30 µM mecamylamine. These cells were then washed for 1 hr, and the amount of desensitization elicited by Saz-A with or without 30 µM mecamylamine co-application was assessed (Figure 14C). Co-application with 30 µM mecamylamine did not result in any statistically significant differences in the desensitization induced by Saz-A at any concentration tested.
(A) $^{86}$Rb$^{+}$ efflux (% 100 μM nicotine stimulation)

(B) $^{86}$Rb$^{+}$ efflux (% 100 μM nicotine stimulation, no mecamylamine treatment)

(C) Control

30 μM mecamylamine co-applied
Figure 14. Mecamylamine does not block Saz-A desensitization (previous page).

These experiments were performed to determine whether the passage of ions through nAChRs is necessary to mediate nAChR desensitization. Mecamylamine was used to allosterically block the passage of ions through the pore of human α4β2 nAChRs while Saz-A was co-applied to desensitize the nAChRs via the orthosteric site. (A) Concentration-dependent inhibition of $^8$Rb$^+$-efflux when mecamylamine is co-applied with 100 µM nicotine and 3 µM saz-A. Based on this data, 30 µM mecamylamine was chosen to block function in later experiments. (B) Washout of various concentrations of mecamylamine over 1 hr. Recovery of function was observed as mecamylamine was washed out from cells and based on this data, a 1 hr wash was chosen for the subsequent experiment. (C) Mecamylamine has no effect on Saz-A mediated desensitization. Cells that were desensitized with Saz-A co-applied with 30 µM mecamylamine exhibited the same level of desensitization as cells desensitized with saz-A alone an hour later.
CHAPTER III: RESULTS
PART 2
CHARACTERIZATION OF HUMAN α4β2 NICOTINIC ACETYLCHOLINE RECEPTORS USING MICROFLUIDIC LAMINAR STREAM SOLUTION EXCHANGE (MLSSE)
After studying functional properties of human α4β2 nAChRs expressed by YXα4β2H1 cells using ⁸⁶Rb⁺-efflux assays, the pharmacological properties of the nAChR subtype were further studied using microfluidic laminar stream solution exchange (MLSSE). These studies also served as a platform for MLSSE method development.

The amount of time a cell stays in the flow column with drug is termed the “exposure time” while the amount of time the cell stays in a flow column containing just buffer is termed the “interstimulus interval” (ISI).

**The relationship between activation kinetics and drug concentrations**

Dose response curves for ACh, nicotine, and Saz-A were generated for human α4β2 nAChRs in YXα4β2H1 using the MLSSE platform. Predictably, we found that lower concentrations of drug resulted in a slower time to peak response for current. Saz-A induced current noticeably slower than other ligands, an effect that is most obvious at 0.1 µM. At this concentration, saz-A induces peak current after 453 ms and nicotine induces peak current after 147 ms (Figure 15). Concentration of drug versus time to peak current was fit to an one-phase exponential model. Higher concentrations of drug result in shorter times to peak. Furthermore, currents elicited by Saz-A reach peak response much sooner than currents elicited by ACh and nicotine, especially at lower concentrations.
Figure 15. Relationships of concentration of drug being used and time to peak in whole-cell current response from YXα4β2H1 cells. The resulting $k$ values are: ACh, $8.12 \pm 1.59 \times 10^5$ M$^{-1}$; nicotine, $2.02 \pm 0.28 \times 10^6$ M$^{-1}$; and Saz-A, $4.95 \pm 0.60 \times 10^6$ M$^{-1}$. The resulted $Y_0$ values were $157 \pm 11$ ms, $160.6 \pm 12$ ms, and $699 \pm 24$ ms for ACh, nicotine, and Saz-A, respectively. The plateau values were $46 \pm 2$ ms, $42 \pm 8$ and $68 \pm 12$ ms, for ACh, nicotine, and Saz-A, respectively. Higher concentrations result in faster time to peak current. Notably, saz-A induces peak currents that reach peaks slower than nicotine and ACh, even at similar concentrations.
Peak response amplitude is dependent on exposure time

One of the main variables to decide upon when designing an experiment using MLSSE is the amount of time a cell stays in each laminar flow column, termed here the exposure time. It was evident over the course of our experiments here that different exposure times can result in differing peak currents. This is exemplified in experiments where a single cell was used to generate ACh dose response curves with varying exposure times (Figure 16). Whereas at lower concentrations of ACh (0.3 – 3 µM), whole cell currents did not reach the “true peak” with shorter exposure times, (26 and 100 ms, Figure 16), at higher concentrations of ACh (30 µM – 1 mM) these same shorter exposure times resulted in higher peak currents. To put it another way, at lower concentrations of ACh, peak currents were reached while cell was still in the laminar stream containing ACh, while at higher concentrations of ACh, peak currents were reached when the cell had already been removed from the laminar stream containing ACh and into a wash stream.
Figure 16. Exposure time versus peak response for ACh induced current from YXα4β2H1 cells. Superimposed recordings represent 26 ms (red), 100 ms (green), and 200 ms (blue) exposures of ACh in the same cell.
Long desensitizing compounds necessitate increased ISI between stimulations.

In each series of dose-response curves, the final (highest) concentration is applied twice with the assigned ISI between them in order to determine whether desensitization from the first application affects the recorded peak current of the second (Figure 16, 1 mM ACh). With ACh and nicotine, only a 200 ms exposure time and 1 min ISI were required. However, we anticipated that working with Saz-A would require longer ISI due to its previously defined desensitizing properties (Xiao et al, 2006; presently, Results: Part 1). This effect was likely exacerbated by the longer exposure times (1000 ms) necessitated by results observed in earlier in the present study (Figure 15). Initially, a 2.5 min ISI was used, but substantial desensitization was observed (Figure 17, left column). However, increasing the ISI to 4 min allowed receptors to achieve full functional recovery of function. This is evidenced by the clear dose-dependent increase of current, as well as the equal peak currents in both applications of 30 µM Saz-A (Figure 17, right column).
Figure 17. Duration of inter-stimulus interval (ISI) influences the amplitude of Saz-A induced whole-cell currents in human α4β2 nAChRs. **A**: Whole-cell currents induced by sequential exposure to increasing concentrations of Saz-A (100 µM) at 2.5 min intervals. **B**: Saz-A induced currents in response to the drug series in **A** at 4.0 min inter-stimulus interval. **Note**: At the 2.5 min ISI, an additive desensitizing effect is observed compared to an ISI of 4 min duration. This is particularly noteworthy at the 100 µM Saz-A dose, which is repeatable at the 4 min ISI.
Agonist activity of ACh, nicotine, and Saz-A at human α4β2 nAChRs

The methods developed over the course of this study were used to determine the activation dose-response of ACh, nicotine, and Saz-A in human α4β2 nAChRs. Currents for nicotine, Saz-A, and lower concentrations of ACh were normalized to current elicited by 1 mM ACh and the results were fitted using GraphPad Prism 5 (Figure 18, Table 7).

Figure 18. Dose response of ACh, nicotine, and Saz-A in YXα4β2H1 cells. Responses from each cell were normalized to the response of that same cell to 1 mM ACh. Curves represent the average responses of n = 5 – 9 cells.

Table 7. Agonist activities of ACh, nicotine, and Saz-A at α4β2 nAChRs as determined using MLSSE. Values shown are the mean ± S.E.M. of x – y independent experiments.

<table>
<thead>
<tr>
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<th>EC₅₀ (µM)</th>
<th>Eₘₐₓ (% 1 mM ACh Response)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh</td>
<td>50 ± 13</td>
<td>100</td>
</tr>
<tr>
<td>(-)-Nicotine</td>
<td>9.5 ± 3.7</td>
<td>90 ± 8</td>
</tr>
<tr>
<td>Saz-A</td>
<td>0.037 ± 0.006</td>
<td>23 ± 4</td>
</tr>
</tbody>
</table>
CHAPTER III: RESULTS
PART 3
COMPARING PHARMACOLOGICAL PROPERTIES OF HUMAN $\alpha_3\beta_4$, RAT $\alpha_3\beta_4$, AND HUMAN $\alpha_4\beta_2$ NICOTINIC ACETYLCHOLINE RECEPTORS: INTERSPECIES DIFFERENCES IN $\alpha_3\beta_4$ RECEPTORS
A thorough and complete comparative study of human and rat α3β4 nAChRs including many different ligands is currently in progress. Since the cloning of most nAChR subunit genes 20 years ago, pharmacological properties of the two predominant heteromeric subtypes, α4β2 and α3β4, have been studied extensively. In these studies, the rat and human receptors showed similar pharmacological properties for many nicotinic ligands. However, some nicotinic ligands were found to have significant species selectivity (Young et al., 2007; Zwart et al., 2008). Since establishing two stable cell lines expressing human α3β4 and α4β2 nAChR subtypes (Xiao et al., 2011), ligand binding and functional properties of typical nicotinic ligands as well as newly developed nicotinic ligands, including Saz-A and its analogs, have been investigated at these receptors. These studies have revealed interspecies differences between the human nAChR subtypes and their rat counterparts in α3β4 nAChRs (Xiao et al., 2012; Xiao et al., 2013). The studies indicate that there are no obvious species differences in human and rat α4β2 nAChRs for nicotine, ACh, other classical nicotinic ligands, or other more newly developed nicotinic ligands. However, there are some nicotinic ligands that showed significant interspecies differences between human and rat α3β4 nAChRs. For example, there is a 10 fold difference in the $K_i$ values of MLA between human α3β4 and rat α3β4 receptors. Varenicline and Saz-A showed even bigger interspecies difference in their binding affinities between the two species, 15 fold and 200 fold, respectively. There are also clear differences in functional properties of some ligands. For example, Saz-A has very low efficacy in stimulating $^{86}$Rb$^+$ efflux from KXα3β4R2 cells, which expresses rat receptors. In contrast, Saz-A has very high efficacy, almost similar to that of nicotine,
in stimulating $^{86}\text{Rb}^+$ efflux from YX$\alpha$$3\beta$4H1 cells, which express human receptors. Therefore, for those new nicotinic ligands in development, it is important to investigate their interspecies differences between human and rat $\alpha$3$\beta$4 receptors.

Here, the results of such studies for several nicotinic ligands are presented, including a complete dissection of the pharmacology of the $\alpha$3$\beta$4-selective compound AT-1001 are presented.

**AT-1001: receptor binding properties**

The binding properties of AT-1001 were tested in two ways at four receptor subtypes: human $\alpha$3$\beta$4, rat $\alpha$3$\beta$4, human $\alpha$4$\beta$2, and rat $\alpha$4$\beta$2. First, the affinity of AT-1001 for receptors was determined using competition binding. Then, the nature of AT-1001’s binding was explored using $[^3\text{H}]$-EB saturation curves in the absence and presence of AT-1001.

In competition binding studies, AT-1001 competed for $[^3\text{H}]$-EB binding sites in all four nAChR subtypes tested (Figure 19). AT-1001 has higher affinity for both human and rat $\alpha$3$\beta$4 nAChRs over human and rat $\alpha$4$\beta$2 nAChRs (Figure 19, Table 8). The difference in AT-1001’s affinity for $\alpha$3$\beta$4 and $\alpha$4$\beta$2 nAChRs is significant both in human (p<0.0001) and rat (p<0.0001). Surprisingly, AT-1001 also exhibits significantly (p<0.0001) higher affinity for human $\alpha$3$\beta$4 over rat $\alpha$3$\beta$4 nAChRs (Rat $K_i$/Human $K_i$ = 20). Interestingly, all Hill co-efficients for these binding experiments are less than 1; this difference is statistically significant for all four receptor subtypes. One-site and two-site
modeling were performed and compared in GraphPad Prism 5 on individual binding experiments. However, competition curves from individual experiments did not consistently fit to the two-site model.

Figure 19. Competitive binding curves of AT-1001 to human α3β4, human α4β2, rat α3β4, and rat α4β2 nAChRs. Data shown are representative of 5 competition binding experiments. See Table 8 for a summary and analysis of data from all experiments.
Table 8. The binding affinities of AT-1001 for human α4β2, rat α4β2, human α3β4, and rat α3β4 nAChRs as determined by competition binding with [3H]-epibatidine.

The $K_d$ values of $[^3H]$-EB (pM) used for calculating $K_i$ values of AT-1001 were: 64 for human α3β4, 102 for rat α3β4, 16 for human α4β2, and 21 for rat α4β2. Selectivity ratio refers the ratio of $K_i$ α4β2/$K_i$ α3β4 for each species nAChRs. Values are mean ± standard error of 5 independent experiments.

<table>
<thead>
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<th>$K_i$ (nM)</th>
<th>Hill Coefficient</th>
<th>Selectivity ratio</th>
</tr>
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<tbody>
<tr>
<td>Human α3β4</td>
<td>0.092 ± 0.008</td>
<td>0.74 ± 0.05</td>
<td>989</td>
</tr>
<tr>
<td>Human α4β2</td>
<td>91 ± 8</td>
<td>0.72 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Rat α3β4</td>
<td>1.9 ± 0.1</td>
<td>0.79 ± 0.02</td>
<td>41</td>
</tr>
<tr>
<td>Rat α4β2</td>
<td>78 ± 10</td>
<td>0.82 ± 0.05</td>
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</table>

The competition binding results are consistent with the hypothesis that AT-1001 binds to the orthosteric binding site of α4β2 and α3β4 nAChRs, but the possibility of allosteric binding still existed, especially since Toll et al had previously reported non-competitive binding of AT-1001 (Toll et al., 2012). To evaluate this possibility, we performed $[^3H]$-EB saturation curves in the presence of various concentrations at all four receptor types. Concentrations of AT-1001 used were chosen according to the affinity of AT-1001 at that subtype. As shown in Figure 20 and Table 9, an AT-1001 concentration dependent shift in observed $K_d$ was seen in all four receptors. However, a significant reduction in observed $B_{max}$ was seen in human and rat α3β4 nAChRs; this reduction in $B_{max}$ was not seen in human or rat α4β2. Therefore, it is conceivable that in addition to the orthosteric site, AT-1001 may also bind to a non-competitive allosteric binding site in α3β4 nAChRs. It is important to note that AT-1001 concentrations used in α4β2 experiments were not as high as those used in experiments with α3β4 relative to their
respective $K_i$. Specifically, the highest concentration tested with human $\alpha 3\beta 4$, rat $\alpha 3\beta 4$, human $\alpha 4\beta 2$, and rat $\alpha 4\beta 2$ were 30 nM, 300 nM, 1 $\mu$M, and 1 $\mu$M, respectively. These concentrations are 326 times, 158 times, 11 times, and 13 times the respective $K_i$ of AT-1001 at human $\alpha 3\beta 4$, rat $\alpha 3\beta 4$, human $\alpha 4\beta 2$, and rat $\alpha 4\beta 2$ nAChRs. A similar reduction in $B_{\text{max}}$ may therefore be observed if higher concentrations of AT-1001 were used in experiments with $\alpha 4\beta 2$. However, given the limitation in [$^3$H]-EB concentration ranges used in these experiments, the $B_{\text{max}}$ values derived from specific binding curves with higher AT-1001 concentrations are not conclusive. In fact, subsequent Schild analysis of this data indicates that results with $\alpha 3\beta 4$ are in accordance with competitive binding and that the reduction in $B_{\text{max}}$ is the result of insufficiently high concentrations of [$^3$H]-EB being used. However, cost makes use of higher [$^3$H]-EB concentrations prohibitively expensive.

Table 9. The $K_d$ and $B_{\text{max}}$ of [$^3$H]-epibatidine in the presence of varying concentrations of AT-1001 at human $\alpha 3\beta 4$, rat $\alpha 3\beta 4$, human $\alpha 4\beta 2$, and rat $\alpha 4\beta 2$ nAChRs. Values shown are the mean ± S.E.M. of 3 – 5 independent experiments.

<table>
<thead>
<tr>
<th>[AT-1001] (nM)</th>
<th>Human $\alpha 3\beta 4$</th>
<th>Rat $\alpha 3\beta 4$</th>
<th>Human $\alpha 4\beta 2$</th>
<th>Rat $\alpha 4\beta 2$</th>
<th>Human $\alpha 3\beta 4$</th>
<th>Rat $\alpha 3\beta 4$</th>
<th>Human $\alpha 4\beta 2$</th>
<th>Rat $\alpha 4\beta 2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>64 ± 5</td>
<td>102 ± 16</td>
<td>16 ± 1</td>
<td>21 ± 3</td>
<td>8666 ± 662</td>
<td>6258 ± 1094</td>
<td>2688 ± 755</td>
<td>520 ± 78</td>
</tr>
<tr>
<td>0.5</td>
<td>271 ± 18</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>7867 ± 851</td>
<td>NT</td>
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<td>NT</td>
</tr>
<tr>
<td>3</td>
<td>670 ± 15</td>
<td>292 ± 26</td>
<td>NT</td>
<td>NT</td>
<td>5463 ± 1155</td>
<td>4703 ± 618</td>
<td>NT</td>
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<tr>
<td>30</td>
<td>2088 ± 290</td>
<td>1215 ± 309</td>
<td>NT</td>
<td>NT</td>
<td>2604 ± 290</td>
<td>3619 ± 606</td>
<td>NT</td>
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<tr>
<td>300</td>
<td>NT</td>
<td>4920 ± 194</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>2125 ± 116</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>1000</td>
<td>NT</td>
<td>NT</td>
<td>141 ± 10</td>
<td>233 ± 11</td>
<td>NT</td>
<td>NT</td>
<td>2319 ± 10</td>
<td>543 ± 30</td>
</tr>
</tbody>
</table>
Figure 20. $[^3\text{H}]-\text{epibatidine}$ saturation binding performed in the presence of AT-1001 in (A) human $\alpha_3\beta_4$, (B) rat $\alpha_3\beta_4$, (C) human $\alpha_4\beta_2$, and (D) rat $\alpha_4\beta_2$. Concentrations in legends refer to concentration of AT-1001 that was co-incubated with $[^3\text{H}]-\text{EB}$. Data shown are representatives of 3 – 5 independent experiments. See Table 9 for a summary and analysis of data from all experiments.
AT-1001: activation of nAChRs

The agonist activity of AT-1001 was assessed using both patch-clamp electrophysiology/MLSSE (Figure 21) and $^{86}\text{Rb}^+$-efflux (Figure 22). In experiments where activity of AT-1001 was detected (namely, with human and rat $\alpha_3\beta_4$ nAChRs), the differences in EC$_{50}$ detected are insignificant (Table 10).

In patch-clamp experiments, agonist activity is detected for all four nAChR receptor subtypes. Unfortunately, the low inherent expression of functional rat $\alpha_4\beta_2$ nAChRs in KX$\alpha_4\beta_2$R2 resulted in low maximal responses ($48 \pm 7$ pA) and even lower maximal AT-1001 induced responses ($7.2 \pm 4.6$, n = 3). Such small activity does not allow for accurate fitting of dose-response and, as such, no dose-response relationship between AT-1001 and rat $\alpha_4\beta_2$ nAChRs is presented here. Of the other three subtypes, AT-1001 has the highest potency at human $\alpha_3\beta_4$ nAChRs, lower potency at rat $\alpha_3\beta_4$ nAChRs, and lowest potency at human $\alpha_4\beta_2$ nAChRs (Figure 21, Table 10). The efficacies of AT-1001 at human and rat $\alpha_3\beta_4$ are similar, but is much lower at human $\alpha_4\beta_2$. 


Figure 21. Activation dose response curves of AT-1001. The agonists activity of AT-1001 was determined by measuring whole-cell currents of YXα3β4H1, KXα3β4R2, and YXα4β2H1 cells with the MLSSE method. Each curves represents the averaged results from 6 – 9 cells. See Table 10 for a summary of analyses of data from all experiments.

Table 10. Summary of AT-1001 activation pharmacology as determined by patch-clamp electrophysiology and by $^{86}$Rb$^+$-efflux. E$_{\text{max}}$-values for patch-clamp experiments are expressed in % of a 1 mM ACh response in the same cell. E$_{\text{max}}$-values for $^{86}$Rb$^+$-efflux are expressed in % of 100 µM nicotine induced efflux. $^k$Statistically significant (p<0.05) differences. $^\eta$ $^\psi$Statistically significant (p<0.0001) differences. $^\nu$Statistically significant (p<0.01) differences. $^\rho$Statistically significant (p<0.05).

<table>
<thead>
<tr>
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<th>Patch-clamp electrophysiology</th>
<th>$^{86}$Rb$^+$-efflux</th>
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<tr>
<td></td>
<td>EC$_{50}$ (µM)</td>
<td>E$_{\text{max}}$ (%)</td>
</tr>
<tr>
<td>Human α3β4</td>
<td>0.4 ± 0.1$^k$</td>
<td>70 ± 6$^\nu$</td>
</tr>
<tr>
<td>Rat α3β4</td>
<td>1.4 ± 0.2$^k$</td>
<td>65 ± 4$^\nu$</td>
</tr>
<tr>
<td>Human α4β2</td>
<td>4.3 ± 1.2$^k$</td>
<td>18 ± 3$^\nu$</td>
</tr>
</tbody>
</table>
Similar results were observed using $^{86}$Rb$^+$-efflux for human and rat $\alpha_3\beta_4$ cells but not for human $\alpha_4\beta_2$ nAChRs (Table 10, Figure 22). No $^{86}$Rb$^+$-efflux is detectable in KX$\alpha_4\beta_2$R2, so $^{86}$Rb$^+$-efflux experiments were not performed in this cell line. Again, statistically significant greater potency was observed at human $\alpha_3\beta_4$ ($EC_{50} = 0.1 \pm 0.003 \mu M$) compared to rat $\alpha_3\beta_4$ ($EC_{50} 1.5 \pm 0.4 \mu M$) (Table 10). Unexpectedly, the efficacies of AT-1001 at human (59 ± 0.3%) and rat (36 ± 4%) $\alpha_3\beta_4$ nAChRs were significantly ($p < 0.001$) different in $^{86}$Rb$^+$-efflux assays, whereas they were not in patch-clamp.

**Figure 22. AT-1001 dose response determined by $^{86}$Rb$^+$-efflux.** (A) Signal-to-background of $^{86}$Rb$^+$-efflux experiments. Background was determined by basal levels of $^{86}$Rb$^+$ released by plain buffer. Maximal efflux (“signal”) was determined by measuring $^{86}$Rb$^+$-efflux elicited by 100 µM nicotine. Human $\alpha_3\beta_4$ had a signal-to-background ratio of 15; rat $\alpha_3\beta_4$ had a signal-to-background ratio of 18; human $\alpha_4\beta_2$ had a signal-to-background ratio of 4. (B) Dose response activation of nAChRs. No AT-1001 activity is detected for human $\alpha_4\beta_2$ in this assay, despite the cell line YX$\alpha_4\beta_2$H1 exhibiting robust signal-to-background in response to 100 µM nicotine. Each curve represents the averaged result from 4 experiments. See Table 10 for a summary of all experiments.
**AT-1001: desensitization of nAChRs**

The potency and efficacy of AT-1001 for desensitization was assessed using 100 µM nicotine elicited $^{86}\text{Rb}^+$-efflux following a 10 min AT-1001 desensitizing pre-treatment. The concentration that desensitizes receptors to half of the baseline, undesensitized 100 µM nicotine elicited $^{86}\text{Rb}^+$-efflux will be referred to as the “DC$_{50}$”. Again, rat α4β2 nAChRs were not tested in the assay due to the low $^{86}\text{Rb}^+$-efflux of KXα4β2R2 cells.

AT-1001 completely desensitizes human α3β4, rat α3β4, and human α4β2 nAChRs, but with varying potency (Figure 23). Predictably, the potency of AT-1001 for desensitizing nAChRs mimicks the results observed in both binding and activation studies (Table 11). AT-1001 has the highest potency for desensitizing human α3β4 (DC$_{50}$ = 0.11 ± 0.01 µM), followed by rat α3β4 (DC$_{50}$ = 1.2 ± 0.1 µM), and finally human α4β2 (DC$_{50}$ = 3.3 ± 0.4 µM). The potencies at all three receptors were compared using a two-tailed unpaired t-test. We found that the differences in potencies between all three receptor subtypes are significantly different (p<0.0001 for human α3β4 vs rat α3β4; p = 0.0003 for human α3β4 vs rat α3β4; p = 0.0024 for rat α3β4 vs human α4β2).

Interestingly, there are no significant difference between the potencies of AT-1001 for activating nAChRs and the potencies of AT-1001 for desensitizing them.
Figure 23. AT-1001 potently desensitizes human α3β4 (n=4), human α4β2 (n=4), and rat α3β4 (n=3) nAChRs. See Table 11 for a summary and analyses of data from all experiments.

Table 11. Desensitization of nAChRs by AT-1001 as determined by $^{86}$Rb$^+$-efflux assays.

<table>
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<tr>
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<th>DC50 (µM)</th>
<th>Hillslope</th>
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<tbody>
<tr>
<td>Human α3β4</td>
<td>0.11 ± 0.01</td>
<td>1.4 ± 0.05</td>
</tr>
<tr>
<td>Rat α3β4</td>
<td>1.2 ± 0.1</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Human α4β2</td>
<td>3.3 ± 0.4</td>
<td>1.6 ± 0.1</td>
</tr>
</tbody>
</table>
Given the potency of AT-1001 for desensitizing nAChRs, we decided to also determine the rate at which receptors recovered from desensitization. This was determined using MLSSE. Initially, a cell was chosen and patched into whole-cell configuration. The maximal function of this cell was tested by two applications of 1 mM ACh, separated by 1 min. The second application of 1 mM ACh was performed in order to confirm that 1 min is enough time for receptors to recover from a 1 mM ACh application by observing no difference in peak current between the first and second stimulations. Cells were then exposed to 10 min of AT-1001 at 100 times the DC50 of the corresponding receptor subtype in order to induce full desensitization of receptors. Each cell was then washed by fresh recording solution and exposed to 1 mM ACh every minute until full recovery was observed (Figure 24). The rates at which nAChRs recover from AT-1001 desensitization correspond to the results predicted by binding, activation, and desensitization studies: human α3β4 recovers the slowest, followed by rat α3β4, and human α4β2, which recovers the fastest (Table 12). All curves were fit to both one-phase and two-phase models, but all three fit to the one-phase model ($Y = 1 - e^{-kt}$).

<table>
<thead>
<tr>
<th></th>
<th>$t_{1/2}$ (min)</th>
<th>$k$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human α3β4</strong></td>
<td>9.7</td>
<td>0.10</td>
</tr>
<tr>
<td><strong>Rat α3β4</strong></td>
<td>3.3</td>
<td>0.31</td>
</tr>
<tr>
<td><strong>Human α4β2</strong></td>
<td>0.68</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Table 12. Kinetics of recovery from desensitization induced by 10 min application of AT-1001. Values represent the variables present in the equation $Y = 1 - e^{-kt}$.
Figure 24. Nicotinic receptor recovery of function following a 10 min application of AT-1001 at a concentration of 100 times the DC₅₀ of the respective cell line. Results were normalized to the maximal response of 1 mM ACh. All curves fit to a one-phase exponential model. Each curve represents the averaged results from 3 – 5 cells. See Table 12 for a summary and analyses of data from all experiments.
Interspecies differences of α3β4 nAChRs: classical and newly developed nicotinic ligands

Pharmacological properties of several classical nicotinic ligands and new developed ligands, including ACh, nicotine, cytisine, varenicline, Saz-A, VMY-2-95 and YL-2-203, have been investigated using ligand binding assays and of $^{86}\text{Rb}^+$ efflux assays in the four stable cell lines expressing human and rat α3β4 and α4β2 subtypes (Xiao et al., 2012; Xiao et al., 2013). To further study interspecies differences of nicotinic ligands, the agonist activities of nicotinic ligands were evaluated using patch-clamp electrophysiology in conjunction with MLSSE for human α3β4, rat α3β4, and human α4β2 nAChRs (Figures 24 – 30, Table 13). Nicotine and acetylcholine are not species selective: they both exhibit similar potency and efficacy at human and rat α3β4 (Figure 25, 26). Saz-A, varenicline, and YL-2-203 all have highest potencies at human α4β2 (Figure 27, 28, and 29). These three ligands also exhibit marked differences in both potency and efficacy at human α3β4 and rat α3β4. Cytisine exhibits relatively similar potency at both species of α3β4, but acts as a full agonist at the human α3β4 and a super agonist at rat α3β4 (Figure 30). VMY-2-95 exhibits much higher potency and efficacy at human α3β4 than at rat α3β4 nAChRs (Figure 31).
Figure 25. Activation dose response curves of ACh. Whole-cell recordings were generated by sequential application of ACh to single cells using MLSSE. Peak amplitudes of currents generated by each concentration were measured and normalized to the peak current elicited by 1 mM ACh. An exposure time of 500 ms with ISI of 1 min was used in experiments with human and rat α3β4; an exposure time of 200 ms with ISI of 1 min was used in experiments with human α4β2. Each curve represents the averaged results from 4 – 5 cells. See Table 13 for a summary and analyses of data from all experiments.
Figure 26. Activation dose response curves of nicotine. Whole-cell recordings were generated by sequential application of nicotine to single cells using MLSSE. Peak amplitudes of currents generated by each concentration were measured and normalized to the peak current elicited by 1 mM ACh. An exposure time of 1000 ms with ISI of 1.5 min was used in experiments with human and rat α3β4; an exposure time of 200 ms with ISI of 4 min was used in experiments with human α4β2. Curves here represent an average of n = 3 – 6 cells. Each curve represents the averaged results from 3 – 6 cells. See Table 13 for a summary and analyses of data from all experiments.
Figure 27. **Activation dose response curves of Saz-A.** Whole-cell recordings were generated by sequential application of Saz-A to single cells using MLSSE. Peak amplitudes of currents generated by each concentration were measured and normalized to the peak current elicited by 1 mM ACh. An exposure time of 1000 ms with ISI of 1.5 min was used in experiments with human and rat α3β4; an exposure time of 800 ms with ISI of 4 min was used in experiments with human α4β2. Each curve represents the averaged results from 3 – 6 cells. See Table 13 for a summary and analyses of data from all experiments.
Figure 28. Activation dose response curves of varenicline. Whole-cell recordings were generated by sequential application of varenicline to single cells using MLSSE. Peak amplitudes of currents generated by each concentration were measured and normalized to the peak current elicited by 1 mM ACh. An exposure time of 1000 ms with ISI of 1.5 min was used in experiments with human and rat α3β4; an exposure time of 800 ms with ISI of 4 min was used in experiments with human α4β2. Each curve represents the averaged results from 4 – 6 cells. See Table 13 for a summary and analyses of data from all experiments.
Figure 29. Activation dose response curves of YL-2-203. Whole-cell recordings were generated by sequential application of YL-2-203 to single cells using MLSSE. Peak amplitudes of currents generated by each concentration were measured and normalized to the peak current elicited by 1 mM ACh. An exposure time of 1000 ms with ISI of 1.5 min was used in experiments with human and rat α3β4; an exposure time of 800 ms with ISI of 4 min was used in experiments with human α4β2. Each curve represents the averaged results from 4 – 6 cells. See Table 13 for a summary and analyses of data from all experiments. Note that although experiments were performed in rat α3β4 cells, YL-2-203 did not elicit any responses at concentrations up to 1 mM. These same cells exhibited robust signals in response to 1 mM ACh.
Figure 30. Activation dose response curves of cytisine. Whole-cell recordings were generated by sequential application of cytisine to single cells using MLSSE. Peak amplitudes of currents generated by each concentration were measured and normalized to the peak current elicited by 1 mM ACh. An exposure time of 1000 ms with ISI of 1.5 min was used in experiments with human and rat α3β4. Each curve represents the averaged results from 4 cells. See Table 13 for a summary and analyses of data from all experiments.
Figure 31. Activation of α3β4 nAChRs by VMY-2-95. Whole-cell recordings were generated by sequential application of VMY-2-95 to single cells using MLSSE. Peak amplitudes of currents generated by each concentration were measured and normalized to the peak current elicited by 1 mM ACh. An exposure time of 1000 ms with ISI of 1.5 min was used in experiments with human and rat α3β4. Each curve represents the averaged results from 3 – 10 cells. See Table 13 for a summary and analyses of data from all experiments.
<table>
<thead>
<tr>
<th>Ligand</th>
<th>Human α3β4</th>
<th>Rat α3β4</th>
<th>Human α4β2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh</td>
<td>216 ± 88</td>
<td>2.16 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Nicotine</td>
<td>12.4 ± 1.6*</td>
<td>39.0 ± 4.1</td>
<td>9.54 ± 3.65</td>
</tr>
<tr>
<td>Saz-A2</td>
<td>0.99 ± 0.12*</td>
<td>69.1 ± 5.6</td>
<td>5.06 ± 2.55*</td>
</tr>
<tr>
<td>VMY-203</td>
<td>3.55 ± 1.09*</td>
<td>38.6 ± 3.6</td>
<td>2.35 ± 0.69*</td>
</tr>
<tr>
<td>Cytisine</td>
<td>52.9 ± 14.5</td>
<td>92.3 ± 5.3</td>
<td>52.9 ± 14.5</td>
</tr>
<tr>
<td>YL-2-201</td>
<td>5.06 ± 2.55*</td>
<td>26.3 ± 5.6</td>
<td>5.06 ± 2.55*</td>
</tr>
</tbody>
</table>

**Table 13. Summary of activation pharmacology of ligands at human α3β4, rat α3β4, and human α4β2 nAChRs.**

*Values where a statistically significant difference exists between human α3β4 and rat α3β4. Results are expressed as a mean ± SE from n = 3 – 10.

Note: Where a *value is indicated, the difference is statistically significant at p < 0.05.
CHAPTER IV: DISCUSSION
PART 1
CHARACTERIZING THE PHARMACOLOGY OF LIGANDS AT HUMAN α4β2 NICOTINIC ACETYLCHOLINE RECEPTORS: BINDING, ACTIVATION, AND DESENSITIZATION
The overarching purpose of this portion of work was to determine and correlate various pharmacological properties of both classical and newer nicotinic ligands and, by doing this, to determine the properties of ligands that make a ligand a desensitizer that desensitizes for a long time. As such, various properties of ligands were determined at human α4β2 nAChRs using a newly developed HEK293 cell line, YXα4β2H1, which was also characterized throughout the course of these studies. The ligand properties that were studied were binding affinity, dissociation rate, potency for activation, potency for desensitization, and recovery from desensitization. When one reads further into this dissertation, the question may come up, “Why did you not use MLSSE to determine recovery from desensitization?” The answer to this is rather simple, the MLSSE platform was not acquired until far later into my time here. However, there may be merit to pursuing the determination of desensitization time using the MLSSE platform in conjunction with methods outlined in the “Recovery from desensitization mediated by AT-1001” in Chapter II.

Since YXα4β2H1 is a novel cell line, the first step was to define the receptors expressed by the cell line and confirm that they are human α4β2 nAChRs. In this cell line, the α4 and β2 subunits were transfected with separate selection markers, G418 and hygromycin B. Therefore, survival of separate clones resulting from these transfections denoted cell lines that could be expressing both α4 and β2 subunits, but did not guarantee that these subunits were being expressed. Since α4 and β2 alone have traditionally been thought to be unable to form functional binding sites on their own, the presence of specific [3H]-EB binding in YXα4β2H1 confirms that both α4 and β2 subunits have been transfected in and that they are forming receptors. It is worth noting here that recent work
by two separate labs have concluded that binding sites can exist at the α4-α4 interface of receptors (Mazzaferro et al., 2011; Harpsøe et al., 2011). However, the binding site formed by the α4-α4 interface is believed to be a low affinity site, whereas the receptors expressed in YXα4β2H1 have extremely high affinity for [3H]-EB (Harpsøe et al., 2011; Xiao et al., 2012). Thus, it can be concluded that YXα4β2H1 is expressing α4β2* nAChRs in high densities.

Since the purpose of this study was to broadly characterize human α4β2* nAChRs various pharmacological properties in a heterologous system, several other compounds were tested in addition to EB in hopes of finding correlates between different properties. These additional compounds were nicotine, cytisine, Saz-A, varenicline, and 5-I-A85380. The $K_d$ or $K_i$-values determined for these compounds in YXα4β2H1 closely resemble the values determined previously for human and rat α4β2 nAChRs (Eaton et al., 2003; Xiao et al., 2004; Xiao et al., 2006; Rollema et al., 2007). These results lends further support to the notion that YXα4β2H1 is expressing α4β2* nAChRs. The rank order of binding affinity among these ligands from lowest to highest was: nicotine, cytisine, varenicline, Saz-A, 5-I-A-85380, and epibatidine. This covers a broad range of binding affinities, from 6600 pM to 23 pM, presumably providing a range of “low-affinity” to “high-affinity” ligands whose properties could be teased apart to provide more insight into the mechanism behind desensitization.

One of the major hypotheses driving this project was that the dissociation rate of a ligand from the desensitized conformation of the receptor is the key property behind how long that ligand desensitizes the receptor. In the selected-fit model of ligand binding, a ligand binds to the desensitized state of the receptor, thus stabilizing the receptor in this
desensitized state. Therefore, as long as the ligand stays bound with high affinity to the desensitized receptor, the receptor will remain in the desensitized state; it follows therefore that the dissociation of the ligand from the desensitized receptor will allow the receptor to isomerize to the resting or opened state. Coincidentally and conveniently, radioligand binding studies at equilibrium measure the binding of ligands to the desensitized state (Heidmann et al, 1983; Picciotto et al, 2008). Thus, radioligand binding is the ideal tool for studying the dissociation rate of ligands from α4β2 nAChRs.

Although studying the dissociation rates of the radiolabeled ligands ([3H]-EB and [3H]-cyt) using radioligand binding was simple, determining the dissociation rates of the non-radiolabeled ligands, nicotine, Saz-A, varenicline, and 5-I-A85380, was trickier. Determining the dissociation of a radioligand from receptors is straight-forward: after incubating radioligand until equilibrium binding has been reached, addition of a high amount of displacing ligand (in this case 300 µM nicotine) will cause radioligand binding to decrease over time (Figure 32). This decrease in binding represents radioligand dissociating from receptors and being blocked from reassociating by the high concentration of displacing ligand. Measuring the dissociation rate of an unlabeled ligand has been described previously (Motulsky and Mahan, 1984; Contreras et al, 1986). In contrast to determining the dissociation of radioligands, this method for determining dissociation of cold ligands is time-consuming, tedious, and requires extensive computation. (Note from author: I met Harvey Motulsky in 2011. After telling him about my research and referencing his paper, he described this 1984 paper as “[his] boringest paper.”) Due to the time-consuming nature of this method, it was impractical for
applications such as ours where the dissociation rates of multiple drugs were to be determined and compared.

Figure 32. Cartoon schematic of using the direct method to determine the dissociation rate of $[^3\text{H}]$-EB from a receptor.
We therefore developed a more convenient method for determining the dissociation rate of non-radiolabeled ligands. The method is described in depth in Results: Part I. Briefly, a saturating concentration of non-labeled ligand is incubated with receptors until binding equilibrium has been reached. A large concentration of \([{}^3\text{H}]\)-EB (or any high affinity radioligand) is added and as cold ligand dissociates from receptors, radioligand occupies receptors that cold ligands had previously occupied. With time, the increasing radioligand bound represents the dissociation of cold ligand and the dissociation rate of the cold ligand can be inferred (Figure 33).

**Figure 33. Cartoon schematic of using \([{}^3\text{H}]\)-EB to determine the dissociation rate of saz-A from a receptor.**
This indirect method was used to determine the dissociation rates of cold epibatidine and cold cytisine. The results were compared to the dissociation rates determined by the direct method using $[^3\text{H}]$-EB and $[^3\text{H}]$-cyt (Table 3). The dissociation rates that were determined from radioligand displacement by the indirect method are not significantly different from those determined using the traditional direct method. With confidence that this new method was accurately determining dissociation rates of cold ligands, the method was then used to determine the dissociation rates of nicotine, Saz-A, varenicline, and 5-I-A85380, of which there are no available labeled ligands.

The dissociation rates determined for these drugs are largely correlated with their $K_i$, with the exception of Saz-A. Specifically, despite having a higher $K_i$ than the $K_d$ of EB and the $K_i$ of 5-A-85380, Saz-A exhibits a slower dissociation rate than both EB and 5-A-85380 ($t_{1/2} = 49.7 \text{ min for Saz-A vs 34.3 min for EB and 33.8 min for EB}$). Since the affinity of a ligand ($K_d/K_i$) is defined as the ratio of the dissociation rate constant and association rate constant ($k_{off}/k_{on}$), the slower dissociation of Saz-A compared to EB and 5-I-A85380, despite having lower affinity, implies that Saz-A also has a slower association rate than EB and 5-I-A85380. This is strange, since generally the $k_{on}$ of ligands is considered a property that remains roughly the same between different ligands. More evidence of the relatively slow association rate of Saz-A was later functionally observed in patch clamp experiments (Figure 15). Functional assays were performed to probe for correlates between binding and function.

It has been proposed that agonists have a higher affinity for the desensitized conformation of the receptor than for the opened conformation of the receptor (Quick and Lester, 2002; Kellar and Xiao, 2007), a theory that is reinforced here by the observation
that all agonists tested have higher potencies for desensitization than for activation (Table 5, Table 6). However, the ligands have differing degrees of “functional selectivity” between activation and desensitization. Specifically, varenicline has a DC$_{50}$ (defined as the concentration at which a compound desensitizes 50% of the 100 µM nicotine-induced $^{86}$Rb$^+$-efflux) that is much lower than its activation EC$_{50}$ – varenicline is essentially very functionally selective for desensitization. Meanwhile, epibatidine, 5-I-A85380, and Saz-A have a much lower EC$_{50}$/DC$_{50}$ ratio and are not very selective for desensitization (Table 14). It is notable that Saz-A had a similar EC$_{50}$/DC$_{50}$ to those of epibatidine and 5-I-A85380, despite having a higher $K_i$. That different ligands exhibit differing degrees of selectivity between activating nAChRs and desensitizing them implies that the desensitized state of α4β2 nAChRs differs significantly from the resting and opened states. Therefore, it is possible for a ligand to be so functionally selective for desensitizing that it effectively CAN be the so-called “silent desensitizer” (Xiao et al., 2006). No such ligand has been discovered so far, however. This functional selectivity between the two states does not seem to be correlated in any way to other properties of the ligands. Please note the discussion here is only for potencies. Functional selectivity has another important aspect: efficacy of activation, which will be discussed later.
Table 14. Ratios of the EC\textsubscript{50} to DC\textsubscript{50}. EC\textsubscript{50} and DC\textsubscript{50} values used in these calculations were determined using \textsuperscript{86}Rb\textsuperscript{+}-efflux assays. EC\textsubscript{50} and DC\textsubscript{50}-values are from Table 5 and Table 6.

<table>
<thead>
<tr>
<th>Drug</th>
<th>EC\textsubscript{50}/DC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>47</td>
</tr>
<tr>
<td>Cytisine</td>
<td>59</td>
</tr>
<tr>
<td>Sazetidine-A</td>
<td>14</td>
</tr>
<tr>
<td>Varenicline</td>
<td>213</td>
</tr>
<tr>
<td>Epibatidine</td>
<td>23</td>
</tr>
<tr>
<td>5-I-A85380</td>
<td>17</td>
</tr>
</tbody>
</table>

Despite being highly selective for desensitizing receptors, varenicline does not desensitize receptors for a particularly long time compared to the other ligands tested here. Recovery of function 4 hours following a desensitizing treatment was determined at the lowest concentration at which full desensitization was observed (Table 6). Using this rubric, we found that the recovery from desensitization is closely correlated to the binding affinity of ligands, but not necessarily the dissociation rate. The rank order (from slowest to fastest) for recovery from desensitization is: EB, 5-I-A85380, Saz-A, varenicline, cytisine, and nicotine. While these results indicate a correlation between binding affinity and desensitization, they do not reinforce our initial hypothesis that desensitization time is a result of dissociation rate from receptors, since according to this hypothesis Saz-A induced desensitization should have recovered the slowest of all the ligands.
The ability of DHβE, a competitive antagonist, to protect receptors from Saz-A mediated desensitization further supports the role of binding in the induction of desensitization. As a competitive antagonist, DHβE presumably stabilizes receptors in the resting conformation, rather than desensitized or opened conformation, thus preventing Saz-A from ever stabilizing the desensitized conformation. To put it another way, agonist binding is a required step to induce desensitization and DHβE prevents this.

It is important to remember that agonists affect the equilibrium of receptor conformations and that this equilibrium, in turn, affects ion passage through the channel of the receptors as well as ion concentrations within the cell. It can therefore be argued that the actual flow of ions through the pore is what generates desensitization. For instance, mutations in the M2 pore-lining domain of both α4 subunits alter desensitization kinetics without affecting binding affinity, while intracellular messengers such as Ca\(^{2+}\) can alter the rate of recovery from desensitization (Matsushima et al., 2002; Khiroug et al., 1998; Khiroug et al., 2003). Therefore, we performed experiments designed to address this concern.

By blocking channels with the non-competitive antagonist mecamylamine while applying Saz-A, we prevented ion flux through nAChRs while simultaneously allowing Saz-A to bind to receptors. The purpose of this experiment was to induce desensitization using Saz-A while simultaneously preventing receptor function to see if the desensitization caused by Saz-A was affected. Blocking function of nAChRs did not affect the recovery from desensitization by Saz-A, meaning that neither ion-flux nor altered intracellular ion concentration are necessarily for desensitization. We can thus
conclude that the properties of the ligand, receptor, and receptor-ligand complex are what define desensitization and desensitization length in α4β2 nAChRs.
Conclusions

Several important results came from this part of my research. We developed a method for determining the dissociation rates of unlabeled ligands. This method was applied to determine the dissociation rates of several nicotinic ligands and has subsequently been adapted in methods to measure receptor occupancy of ligands *ex vivo* (Hussmann *et al.*, 2012). Although we did not successfully demonstrate the relationship between dissociation rate and recovery from desensitization, Saz-A has a similar potency to EB and 5-A85380 for desensitizing α4β2 nAChRs despite having a substantially lower potency than EB and 5-I-A85380 in binding assays, which suggests that dissociation rates may still play a role in desensitization.

The data also confirms that recovery from desensitization is not simply a nAChR subtype specific property. By testing the recovery from desensitization by several compounds at α4β2 nAChRs, we demonstrate here that recovery from desensitization is ligand dependent, an observation that has already been alluded to in the past with nicotine and ACh (Paradiso and Steinbach, 2003).

Finally, the results here confirm that binding is the critical step that results in the desensitization of α4β2 nAChRs. Some have proposed separate sites for activation and desensitization (Lopez-Hernandez *et al.*, 2004; Giniatullin *et al.*, 2005). However, DHβE blockade of ligand binding precludes the possibility of desensitization of receptors, but mecamylamine does not, which confirms that ligand occupation of the receptor’s orthosteric site is the only requirement for receptor desensitization.

The results here do not answer the initial question that we set out to answer: what properties of a ligand determine how long it will desensitize receptors? Recovery from
and potency for desensitization follow the same trends as binding affinity, but the reasons behind these correlations are currently unknown. One possibility is that ligands interact with the N-terminal binding pocket in different ways such that the binding and gating mechanisms are uncoupled to different degrees (Zhang et al., 2011). However, this is purely conjecture. The disconnect between observations made about binding, desensitization, and activation underscore the poor understanding we have of the exact mechanistic changes that receptors undergo in order to function properly. The prospect of targeting desensitization in drug development is an exciting one that may yield useful therapeutic applications (Xiao et al., 2006), but creating functionally-selective, long-lasting desensitization of nAChRs is currently a process that seems to rely more on luck than knowledge.
CHAPTER IV: DISCUSSION

PART 2

CHARACTERIZATION OF HUMAN α4β2 NICOTINIC ACETYLCHOLINE RECEPTORS USING MICROFLUIDIC LAMINAR STREAM SOLUTION EXCHANGE (MLSSE)
The platform used for microfluidic laminar stream solution exchange described here is the Dynaflow system developed by the Swedish company Cellectricon. The system was a generous gift from Dr. Ruin Moaddel of the National Institute on Aging (Baltimore, MD). As mentioned previously, the system is a microfluidic chip fixed to a motorized platform. Using a syringe driven pump, the microfluidic chip creates laminar columns of different solutions and the motorized platform can move the chip below a lifted cell in whole-cell configuration, thus exposing the cell to different solutions and recording the response. This system differs from the vast majority of current “automated” patch platforms such as the Fluxion IonFlux in that the operator is still manually “patching” the cell into whole-cell configuration. While this means that the throughput of method is considerably lower than automated systems, the Dynaflow system allows an extra degree of customizability since the user can visually select cells to patch, adjust experiments on the fly, and design more complex experiments than may be possible with automated platforms (see: Figure 24. Nicotinic receptor recovery of function). Although these qualities do not necessarily make the system an ideal tool for high throughput drug screening, they do make the system an effective method for studying compounds and receptors more in-depth in an academic setting.

Because the usage of microfluidics in the context of patch-clamp electrophysiology is such new technology, there is a lack of literature on how to use this particular set up. One publication has outlined the usage of the system with α7 nAChRs, but focused on rapid drug delivery/washout applications (to avoid desensitization of α7 at peak amplitude) as well as technical details that did not aid in actual usage of the system (Fedorov et al, 2012). The protocols presented in this publication do not translate well to
work with HEK293 cells, which are the type that our lab predominantly works with, or with nAChRs with slower overall kinetics, such as α4β2 and α3β4. The goal of this part was therefore to define parameters to use with HEK293 cell lines that the lab possesses.

Designing experiments with MLSSE can be a tricky prospect. Every variable used in the method involves some degree of compromise. Cells need to be attached to cover slips, but not so much that they cannot be lifted. Exposure times need to be long enough in order for peak concentrations to be reached, but longer exposure times also result in a higher amount of desensitization of receptors, necessitating longer wash-out times (here, termed interstimulus interval, ISI.)

Traditional HEK293 cell preparations for patch are designed to encourage cell adherence to a surface. Generally, cells are plated onto glass coverslips that are coated with poly-D-lysine, thus strongly adhering to the coverslips and providing a smooth surface for the formation of a gigohm seal. However, due to the strong adherence of this type of preparation, cells cannot be lifted following the formation of a gigaohm seal and therefore cannot be used for MLSSE. Cellectricon recommends dissociation of cells from their culture medium and direct addition into the recording chamber. However, we found that cells prepared in this manner were too loosely adhered to the recording chamber floor and thus were very difficult to achieve gigaohm seals on. With this preparation, even cells where gigaohm seals were formed were difficult to break into whole-cell configuration.

By plating cells onto uncoated glass coverslips ~18 hrs prior to experiments, we found that cells adhere tightly enough to coverslips that we can consistently form gigaohm seals and subsequently break into whole-cell configuration. Cells prepared in
this manner are also easily lifted off of the coverslip for repositioning. This preparation provides the added benefit of facilitating the use of multiple cell lines in one day's experiment, whereas direct addition of dissociated cells to the recording chamber necessitates thorough cleaning of the recording chamber before addition of another cell line to remove any of the previously used cells, the preparation described presently allows one to simply exchange the coverslip from the recording chamber with a coverslip containing a different cell line.

Cells prepared in this manner exhibited variable morphologies. Some of the cells looked like cells prepared in the more traditional, poly-D-lysine coated coverslip method – flattened morphologies with small processes extending outwards. Others more resembled cells prepared in the Cellectricon mandated method – round morphologies with the appearance of being loosely attached. Although both morphologies were easily patched and lifted, we chose to patch round cells. This is because the fluid exchange surrounding a cell in a laminar stream is subject to Stokes law and, thus, rounder cells will result in less turbulent fluid flow around the cell and more thorough fluid exchange. Furthermore, the turbulent flow generated by a less-than-round cell will disrupt the stability of the gigaohm seal, whereas the laminar flow present during Stokes flow helps to stabilize the gigaohm seal.

Once a patched cell is positioned in front of the microfluidic channels, it can be exposed to streams containing varying concentrations of drug contingent on two variables: the exposure time and the ISI. Both these variables need to be fine-tuned to both the receptor type being studied and the ligand being applied.
Exposure time needs to be fine-tuned in order to generate the optimal response at lower concentrations while still maintaining a relatively low ISI. At lower concentrations of agonist, agonist association to receptor is slower, resulting in slower attainment of peak current at these concentrations (Hulme and Trevethick, 2010). When the cell is removed from a stimulating stream too early, maximal binding to receptors is not achieved and the “true” peak response of the cell at that concentration is not measured correctly. This is readily observable in the case of ACh, where exposure times of 26 ms and 100 ms do not reach the same peak current as when ACh was applied for 200 ms (Figure 16).

An interesting phenomenon emerged over the course of these studies with ACh. While shorter exposure times of drug at lower concentrations resulted in sub-maximal measured peak currents, shorter exposure times (26, 100 ms) at higher concentrations (30 µM – 1 mM) resulted in higher peak currents than longer exposure times (200 ms). An analogous phenomenon has been observed in α7 nAChRs, where faster applications result in higher peak currents (Papke et al, 1998; Fedorov et al, 2012). A lower peak current results from longer application times because, at higher concentrations, ACh rapidly binds to the desensitized conformation of the receptor, resulting in a sizable population of receptors that are stabilized in the desensitized conformation before peak activation has occurred. Consequently, a lower amount of receptors can be stabilized in the open conformation and a lower elicited response is observed. Although this is an interesting observation, it is not physiologically or pharmacologically relevant as drugs do not distribute or disseminate at the speed that would be necessary for this phenomenon to occur *in vivo*. 
Length of exposure time must be balanced by an appropriate ISI. By increasing the amount of time receptors are exposed to a drug, more receptors will be stabilized in a desensitized state, thus disrupting subsequent measurements of current (Reitstetter et al, 1999). The ISI to be used with a ligand is therefore an important, but complicated, variable that needs to be considered, since both higher concentrations and longer exposure times necessitate an increase in ISI. This is especially true with ligands at α4β2 nAChRs, which are considered to be the high affinity subtype of nAChR. The example ligand that is used for demonstrating this concept here is Saz-A (Figure 17). Since Saz-A has a high potency at α4β2 nAChRs, the concentrations that are used to generate a dose response curve are low. However, as mentioned previously, lower concentrations of drug result in slower rates of association with receptors, demanding longer exposure times in order to correctly measure peak current at lower concentrations. These longer exposure times, along with the propensity for Saz-A to desensitize receptors for long periods of time (Table 6), results in significant desensitization with each application. Inappropriately short ISI will therefore result in progressive desensitization from exposure to exposure (Figure 17, left column), ultimately obscuring currents that should be generated during experiments (Figure 17, right column). However, cells do not remain healthy indefinitely in whole-cell recording conditions nor do solution chambers in the MLSSE chip contain infinite solution; therefore ISI’s must be kept as short as possible while still maintaining full receptor function recovery between exposures. Generally speaking, each dose-response protocol includes two applications of the highest concentration of drug in order to confirm that an appropriately long ISI has been chosen that allows for full recovery from desensitization between exposures. In the case of experiments presented here, 100
µM Saz-A is applied twice with an ISI of 4 mins. This generated equivalent peaks, confirming that no desensitization was occurring between exposures to lower concentrations, either (Figure 17, right column).

During the development of protocols for using the MLSSE system, we focused on the use of three compounds: ACh, nicotine, and Saz-A. We used ACh and nicotine because they are canonical nAChR ligands that have been well defined throughout the literature. We used Saz-A because prior experiments from Part 1 predicted that this would be among the most difficult compounds we could work with – if protocols could be constructed that worked properly with Saz-A, then any other compound would be deemed feasible for use with the system. Ultimately, all the method development for the MLSSE platform is useless if the method does not accurately determine pharmacological properties of the ligands in question. The EC50-values determined using MLSSE here are similar to values that were reported previously (Table 7, Figure 18). The Emax-values in literature indicate that both ACh and nicotine are full agonists at human α4β2 nAChRs, which matches with our experimental results from MLSSE. Although the Emax-value for Saz-A determined with MLSSE is close to the value determined in Part II with efflux assays, it differs significantly from those determined in both Zwart et al and Carbone et al (Table 7). These differences, however, can be readily explained by differences in the stoichiometry of α4β2 receptors in the current cell line and the stoichiometry of α4β2 expressed in the oocyte system (Zwart et al, 2008; Carbone et al, 2009). Saz-A acts as a full agonist or near-full agonist at the high sensitivity (α4)2(β2)3 stoichiometry and has almost very low efficacy, if any, at the low affinity (α4)3(β2)2 stoichiometry (Carbone et al, 2009). YXα4β2H1, as a stable cell line, likely expresses a variable ratio of high
sensitivity versus low sensitivity stoichiometries. According to data here, the cell line can express 23 - 40% composition of the high sensitivity stoichiometry. By contrast, both Zwart et al and Carbone et al tested efficacy of Saz-A at nAChRs expressed in an oocyte system. Both studies purposefully injected subunit cDNA or cRNA in ratios that highly preferred expression of exclusively high sensitivity or exclusively low sensitivity α4β2 nAChRs, between which Saz-A exhibits a huge difference in efficacy. Therefore, the experiments performed here with Saz-A likely reflect exclusive characterization of the high affinity (α4)2(β2)3 stoichiometry. The functional characteristics of ACh and nicotine in the present study also reflect the properties of these ligands in a population of mixed stoichiometry.
Conclusion

The α4β2 subtype is probably the most difficult nAChR to work with. Due to the high potencies of ligands for the receptor, lower concentrations of drugs are used, thus demanding longer exposure times, which in turn dictate longer ISIs. This contrasts starkly with earlier studies with α7 nAChRs, where rapid desensitization kinetics necessitate shorter exposure times, but fast recovery from desensitization following agonist exposure allows for very short ISIs. These two receptor subtypes represent a range of properties that ligand gated ion channels can possess. Our ability here to use MLSSE with α4β2 nAChRs demonstrates the possibility for MLSSE techniques to be applied to a broad range of ligand gated ion channels with varying properties. Subsequently, in experiments for Part 3, MLSSE was used to study the pharmacology of several compounds at α4β2 nAChRs, as well as α3β4 nAChRs, which have qualities intermediary to α4β2 and α7 nAChRs.
CHAPTER IV: DISCUSSION

PART 3

COMPARING PHARMACOLOGICAL PROPERTIES OF HUMAN α3β4, RAT α3β4, AND HUMAN α4β2 NICOTINIC ACETYLCHELONE RECEPTORS: INTERSPECIES DIFFERENCES IN α3β4 RECEPTORS
Whereas the $\alpha 4\beta 2$ subtype is primarily expressed in the brain, the $\alpha 3\beta 4$ nAChR subtype is expressed both in the brain and in the peripheral nervous system. In the brain, although $\alpha 4\beta 2$ and $\alpha 7$ nAChRs are considered to dominate the forebrain and $\alpha 3\beta 4$ nAChRs to dominate the mid- and hindbrain, $\alpha 3\beta 4$ nAChRs are co-expressed with $\alpha 4\beta 2$ in several regions of the brain that are believed to be involved in nicotine addiction, such as the ventral tegmental area, the interpeduncular nucleus, and the medial habenula (Perry et al., 2002; Gotti et al., 2006). Because of this distribution, traditionally hitting off-target at $\alpha 3\beta 4$ is deemed undesirable when designing drugs that target $\alpha 4\beta 2$, especially given that the function of $\alpha 3\beta 4$ in these brain regions is still poorly understood.

However, an increasing body of evidence suggests that the $\alpha 3\beta 4^*$ subtype may be involved in nicotine addiction. At the epidemiological level, allelic variations within the $\alpha 5$-$\alpha 3$-$\beta 4$ gene cluster have been associated with smoking addiction within European populations (Berrettini et al., 2008; Fowler et al., 2011). In rodents, $\alpha 3\beta 4$ nAChRs have been implicated in both nicotine reinforcement and withdrawal (Salas et al., 2004; Glick et al., 2011; McCallum et al., 2012). This emerging body of evidence highlights the need for ligands that are selective for $\alpha 3\beta 4$ nAChRs over other subtypes, but also underscores the importance of selectivity in $\alpha 4\beta 2$ selective compounds.

The development of both $\alpha 3\beta 4$-selective and $\alpha 4\beta 2$-selective ligands is complicated by reports that significant pharmacological differences exist between rat and human $\alpha 3\beta 4$ nAChRs. An in-depth study observed the interspecies difference between human and rat $\alpha 3\beta 4$ nAChRs for the compound TMAQ (Young et al., 2007). The study, using mutagenic techniques, found that of the 17 individual differences between humans and rats in the amino acid sequences of the $\beta 4$ subunit, two differences in residues at
positions 55 and 56 accounted for the interspecies difference in activity of TMAQ. Although these residues may not be the cause of all interspecies differences, the study does confirm that structural differences exist between human and rat α3β4 nAChRs that result in differences in pharmacology between the species for the nAChR subtype. Similar interspecies differences have been reported in cytisine, varenicline and Saz-A. (Zwart et al., 2008; Stokes and Papke, 2012).

In the present study, we aimed to characterize the pharmacologies of several compounds at human α3β4, rat α3β4, and human α4β2. These compounds were ACh, nicotine, Saz-A, varenicline, YL-2-203, cytisine, VMY-2-95, and AT-1001. YL-2-203 and VMY-2-95 are experimental compounds selective for α4β2 nAChRs developed as possible smoking cessation aids in conjunction with Dr. Milton Brown’s lab and the Drug Discovery Program at Georgetown University. AT-1001 is an α3β4-selective compound that was previously described as a competitive/non-competitive antagonist (Toll et al., 2012). Because of inconsistencies between the previously reported properties of AT-1001 with what we observed, a more thorough investigation on the properties of AT-1001 was performed that included binding as well as desensitization. For other compounds, comparison was restricted to activation pharmacology, although more thorough investigations of the compounds are in progress.

ACh has similar pharmacology at human and rat α3β4 nAChRs, but is significantly more potent at human α4β2 nAChRs (Table 12, Figure 25). ACh also exhibits a Hill coefficient that is significantly less than 1 in experiments with human α4β2 nAChRs, reflecting the presence of both high sensitivity, (α4)2(β2)3, and low sensitivity, (α4)3(β2)2, stoichiometries of the receptor subtype in the receptor population
expressed by YXα4β2H cells. Curve fitting, however, was unable to consistently estimate the \(EC_{50}\) and percent composition of the two forms. The relatively close \(EC_{50}\) (1.5 \(\mu\) M and 61 \(\mu\) M, Table 7) of the two stoichiometries makes discerning the two overlapping dose response curves of the stoichiometries difficult without a greater number of data points on the dose response curve. This type of experiment may be performed later, but is beyond the current scope of this study.

Nicotine demonstrates full agonist activity at both human α3β4, rat α3β4, and human α4β2. It has highest potency at human α4β2. It differs slightly in potency between human α3β4 and rat α3β4 nAChRs. This difference is negligible (~2 fold) and likely has very little physiological relevance (Table 12, Figure 26).

Saz-A, predictably, has highest potency at human α4β2 nAChRs. However, Saz-A is also significantly more potent (70-fold) and efficacious (23-fold) at activating human α3β4 nAChRs compared to rat α3β4 nAChRs. As a result, although Saz-A is 1,800-fold selective for human α4β2 over rat α3β4 nAChRs, it is only 26-fold selective for human α4β2 over human α3β4 nAChRs (Table 12, Figure 27).

Varenicline, as expected, has a higher potency at human α4β2. It exhibits significantly higher potency (11-fold) at human α3β4 nAChRs than at rat α3β4 nAChRs. As a result, although varenicline is 33-fold selective for human α4β2 nAChRs over rat α3β4, it is only 3-fold selective for human α4β2 over human α3β4. In contrast to Saz-A, however, varenicline has higher efficacy (1.7-fold) at rat α3β4 nAChRs than at human α3β4 nAChRs (Table 12, Figure 28). While a prior study has found similar differences in the \(EC_{50}\)-values of human α3β4 and rat α3β4 nAChRs, no such difference has been reported for the efficacy (Stokes and Papke, 2012).
YL-2-203 is 32-fold selective for human α4β2 over human α3β4 nAChRs. Importantly, however, is that YL-2-203 has extremely low efficacy at both these receptor subtypes and no efficacy at rat α3β4 nAChRs even when applied at up to 1 mM (Table 12, Figure 29). The low efficacy at both α4β2 and α3β4 of YL-2-203 and high binding affinity (Xiao et al, 2013) suggest that YL-2-203 may be a promising lead compound for specifically targeting α4β2 nAChR desensitization.

Cytisine does not exhibit any difference in potency at human and rat α3β4 nAChRs, but has significant differences in efficacy (Table 12, Figure 30). While cytisine acts as roughly a full agonist (92% of 1 mM ACh response) at human α3β4 nAChRs, it acts as a superagonist (158% of 1 mM ACh response) at rat α3β4 nAChRs. These results are congruent with results reported previously in oocytes (Stokes and Papke, 2012).

VMY-2-95, another experimental compound like YL-2-203, again demonstrates differences in both potency and efficacy. The compound is more potent (8-fold) and efficacious (3.7-fold) at human α3β4 than at rat α3β4 nAChRs (Table 12, Figure 31). Despite having comparable potency off-target at α3β4, VMY-2-95 is less promising than YL-2-203 as a lead compound because it has more potential to affect α3β4 nAChRs due to its higher efficacy at this subtype.

Although AT-1001 already was an interesting ligand, as a reported α3β4-selective compound, the initial observations that piqued our interest in AT-1001 and encouraged more thorough investigation of the compound were made by Dr. Stefano Vicini, Dr. John Partridge, and Ruixi Luo. In electrophysiological recordings they noticed what appeared to be agonist activity when applying the compound. Follow up studies within the lab confirmed these observations (Figure 21, Figure 22).
Both patch-clamp electrophysiology and $^{86}\text{Rb}^+$-efflux assays confirmed that AT-1001 is a strong partial agonist with relatively high potency for human and rat $\alpha_3\beta_4$ nAChRs; electrophysiology also found that AT-1001 acts as a weak partial agonist at human $\alpha_4\beta_2$ nAChRs, but is significantly less potent than both rat and human $\alpha_3\beta_4$ nAChRs. Agonist activity was detected at rat $\alpha_4\beta_2$ nAChRs, although no dose-response curve could be fit from rat $\alpha_4\beta_2$ data due to low activity. The difference in potency between human $\alpha_3\beta_4$ and human $\alpha_4\beta_2$, although statistically significant, is only ~3 fold. This difference is even smaller between the potency of AT-1001 at rat $\alpha_3\beta_4$ and human $\alpha_4\beta_2$. Practically speaking, this means that any concentration of AT-1001 that affects $\alpha_3\beta_4$ nAChRs will also affect $\alpha_4\beta_2$ nAChRs in both humans and rats.

An interspecies difference for the EC$_{50}$ at $\alpha_3\beta_4$ was confirmed for AT-1001 using both assays. However, an interspecies difference in efficacy of AT-1001 was only observed in results from $^{86}\text{Rb}^+$-efflux, while no significant difference between human and rat was observed when using patch-clamp (Table 10). This inconsistency could be a result of the ~2-fold difference in the potency of nicotine at human and rat $\alpha_3\beta_4$ nAChRs (Table 13). Thus, whereas 100 $\mu$M nicotine may elicit a maximal response from rat $\alpha_3\beta_4$ nAChRs, the same concentration nicotine may elicit a submaximal response from human $\alpha_3\beta_4$ nAChRs. When normalizing results, this would result in a higher normalized $E_{\text{max}}$-value in human $\alpha_3\beta_4$ nAChRs when compared to rat $\alpha_3\beta_4$. This type of error would not occur in experiments where 1 mM ACh was used to normalize data, since no significant difference exists between the potencies of ACh at human and rat $\alpha_3\beta_4$ nAChRs.

The difference between human $\alpha_3\beta_4$, rat $\alpha_3\beta_4$, and human $\alpha_4\beta_2$ nAChRs in AT-1001 potency for activation is reflected both in binding and desensitization, as well.
Binding data indicates that AT-1001 is selective for both α3β4 nAChRs over α4β2 nAChRs. Again, human α3β4 nAChRs have higher affinity for AT-1001 than rat α3β4 nAChRs. No such difference exists between the affinity of human and rat α4β2 nAChRs for AT-1001. This suggests that a similar interspecies difference does not exist in α4β2 nAChRs, which is consistent with observations that the interspecies differences are the result of differences in the β4 subunit (Young et al, 2007).

Saturation binding experiments with [³H]-EB in the presence of AT-1001 indicate that AT-1001 acts at an allosteric site as a non-competitive inhibitor for binding in α3β4, but not α4β2 nAChRs. When increasing amounts of AT-1001 were added in saturation binding experiments with human and rat α3β4, significant decreases in the measured Bₘₐₓ resulted. These Bₘₐₓ-values were determined by fitting respective saturation curves while allowing for a floating Bₘₐₓ. Similar results were not made for either species of α4β2, where AT-1001 was added up to a concentration of 1 µM, indicating that no such allosteric binding inhibition takes place in the receptors. Alternatively, allosteric binding sites could exist in α4β2 nAChRs, but the concentrations tested here were not high enough to affect them. However, the allosteric binding of AT-1001 at α3β4 receptors is called into question by Schild analysis: when data (Figure 20, Table 9) are fitted assuming a constant Bₘₐₓ (8666 fmol/mg for human α3β4 and 6258 fmol/mg for rat α3β4), both human and rat α3β4 datasets yield a Schild slope of near 1. This analysis is also flawed, though, since it relies on extrapolation of a large portion of the saturation curve in the 30 nM (in human α3β4) and 300 nM (in rat α3β4) conditions to fit the constant Bₘₐₓ. The only way to completely address this question is to drastically increase the concentration of [³H]-EB used in these conditions to fully define the saturation curve.
in experiments with α3β4 receptors. However, this is logistically impractical due to the prohibitive costs of using large amounts of [3H]-EB and the increase in non-specific binding that will result from higher concentrations of [3H]-EB.

Surprisingly, AT-1001 has the same potency for desensitization as for activation at human α3β4, rat α3β4, and human α4β2. Again, AT-1001 is more potent at human α3β4 nAChRs than at rat α3β4 nAChRs. When compared using two-tailed t-tests, there is no significant difference between the DC50-values and the EC50-values for AT-1001 at any of the three nAChR subtypes. This observation is a unique one and directly contrasts with prior observations that agonists have a higher potency for desensitizing than for activation (Presently, Part 1; Quick and Lester, 2002). While in Part 1, we defined the functional selectivity of several nicotinic compounds at α4β2 nAChRs as being selective for desensitization over activation, in AT-1001 we have found a compound that a non-selective activator/desensitizer at both α3β4 and α4β2 nAChRs.

The interspecies differences between human α3β4 and rat α3β4 AT-1001 pharmacology also manifest themselves during recovery from desensitization. As predicted by binding, activation, and desensitization results, recovery from desensitization occurs the quickest in human α4β2 nAChRs and the slowest in human α3β4 nAChRs. The results here reinforce previous conclusion that binding is a critical determinant for recovery from desensitization. Although the α4β2 subtype nAChR has been thought to be the receptor subtype with the longest recovery kinetics following desensitization, we have demonstrated here that this is largely a function of the high affinity α4β2 nAChRs usually have for ligands. Although α3β4 nAChRs are usually desensitized for shorter periods compared to α4β2, this can be attributed to α4β2 nAChRs

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commonly having a higher affinity for ligands than α3β4 nAChRs. Here, we demonstrate a reversal of this relationship simply by desensitizing with a ligand that has higher affinity for α3β4 nAChRs than α4β2 nAChRs.

It is important to note that although binding seems to be a large determinant of desensitization time, it is likely not the only determinant. This is illustrated when we compare the affinity and desensitization of AT-1001 at human α3β4 and 5-I-A85380 at human α4β2. Specifically, the $K_i$ of AT-1001 at human α3β4 nAChRs is 92 pM and the $K_i$ of 5-I-A85380 at human α4β2 is 82 pM; the DC$_{50}$ of AT-1001 at human α3β4 nAChRs is 0.11 µM and the DC$_{50}$ of 5-I-85380 at human α4β2 nAChRs is 1.3 nM. When human α3β4 nAChRs are desensitized with 11 µM AT-1001 (100x the DC$_{50}$), full function is recovered by 30 mins (Figure 12). In contrast, when human α4β2 nAChRs are desensitized with 0.1 µM 5-I-A85380 (~100x the DC$_{50}$), only 55% function has recovered even 4 hours later (Figure 12, Table 6).
Conclusions

The present study found that almost all compounds besides ACh exhibit some degree of preference for human α3β4 nAChRs over rat α3β4 nAChRs. This is especially true for compounds that are selective for α4β2 nAChRs, such as nicotine, Saz-A, varenicline, and VMY-2-95. This selectivity applies even to AT-1001, which is selective for α3β4 nAChRs over α4β2 nAChRs.

Through our studies with AT-1001, two interesting, novel observations were made. First, AT-1001 does not act as an antagonist at α3β4 nAChRs and instead acts as a strong partial agonist for α3β4 nAChRs. Secondly, AT-1001 desensitizes α3β4 nAChRs for longer that α4β2 nAChRs. This provides evidence that binding affinity is a large determinant of desensitization and that recovery from desensitization is also mediated by binding. Therefore, the commonly held belief that α4β2 nAChRs are the long desensitizing receptor may be due to high affinity for ligands rather than due to strictly structural properties and certain ligands desensitize other subtypes for longer than they do α4β2.
CHAPTER V: OVERALL CONCLUSIONS
Tobacco use and nicotine addiction are an immense public health problem. Cigarette smoke is considered to be the leading preventable cause of early morbidity and mortality in most industrialized countries (Fiore, 2000). According to a recently issued World Health Organization report, tobacco use is responsible for the premature deaths of more than 100 million people worldwide in the 20th century and one billion people may die prematurely due to tobacco-related illnesses in the 21st century unless effective action is taken (WHO, 2008).

Neuronal nAChRs mediate all known pharmacological effects of nicotine, including its addictive effects. Among many subtypes of nAChRs, α4β2 receptors have been widely accepted as the main subtype involving nicotine addiction (Flores et al., 1992, Picciotto et al., 1998; Delay, 2005; Jensen et al., 2005; Kellar and Xiao, 2007). More recently, the receptor subtypes containing α3 and β4 subunits have been implicated in mediating addictive effects of nicotine (Saccone et al., 2007; Berrettini et al., 2008; Bierut et al., 2008; Liu et al., 2010). In addition to the scientific importance, understanding interactions between these nAChR subtypes and nicotinic ligands is essential for developing efficacious smoking cessation therapeutics.

Among the three most common smoking cessation medications in the US, varenicline has better efficacy than nicotine replacement therapy and bupropion. However, the efficacy of treatment with varenicline is modest – only 22% of people using varenicline manage to remain smoke-free 12 months after treatment, which is only a 14% advantage over placebo. Moreover, although varenicline appears to be safe for most people, several modest to severe side-effects, including nausea, abnormal dreams, exacerbation of schizophrenia, and suicidal behavior, have been reported (Gonzalez et al.,
2006; Jorenby et al., 2006; Kohen and Kremen, 2007; Freedman, 2007; Moore et al., 2011). These side effects have been attributed to agonist activity of varenicline at α3β4 and α7 nAChRs (Mihalak et al., 2006; Rollema et al., 2007), as well as agonist activity at 5-HT3 receptors (Lummis, 2011). These side effects underscore the importance of developing new smoking cessation drugs with better pharmacological profiles.

Saz-A is a novel nAChR ligand that is highly selective for α4β2 receptors in ligand binding assays and in desensitization assays. Based on the studies of Saz-A, a new approach was proposed to develop novel smoking cessation drugs based on their ability to selectively desensitize α4β2 receptors (Xiao et al., 2006). Since then, many new analogs of Saz-A have been developed, including VMY-2-95 and YL-2-203 (Yong et al., 2013; Yenugonda et al., 2013), with pharmacological profiles that may be more conducive to smoking cessation therapy than Saz-A.

Over the course of the present study, we demonstrated that ligands are selective for nAChR subtypes in binding assays, but also have varying selectivity for effects such as activation and desensitization. Whereas some ligands, such as varenicline, are over 200 times more selective for desensitization over activation, other ligands such as AT-1001 are non-selective between desensitization and activation. Furthermore, it appears that the primary determinant for length of desensitization is the binding affinity of a ligand for its receptor, although intrinsic properties of the nAChR subtypes also contribute to desensitization time.

Given the importance of specificity in nicotinic drug development, prior and current indications of differences between rat and human α3β4 pharmacology are alarming. Some compounds tested here and previously have demonstrated significantly
higher potency at human $\alpha 3\beta 4$ nAChR when compared to rat $\alpha 3\beta 4$ nAChRs. Rat models are often used to study behavioral activity of nicotinic compounds. However, the current data suggests that these models will not accurately predict effects in humans resulting from $\alpha 3\beta 4$ nAChR activity, while still demonstrating favorable anti-dependence qualities of $\alpha 4\beta 2$ nAChR activity. This applies to any type of activity, whether it is activation or desensitization. This is a serious concern because it will lead researchers to pursue development of compounds that may fail during clinical trials. Conversely, the use of rat models may underestimate the efficacy of treatments that target $\alpha 3\beta 4$ nAChRs, since many selective compounds have higher potency at human $\alpha 3\beta 4$ nAChRs than rat $\alpha 3\beta 4$ nAChRs, meaning they will have more activity at lower concentrations in humans and also have higher selectivity over $\alpha 4\beta 2$ subtypes.

Overall, the work presented here suggests that the pharmacology of ligands at nAChRs is extremely complicated and needs to be approached from many different angles. Although there seem to be correlations between binding, activation, desensitization, and recovery from desensitization, these correlations are, for the most part, overarching trends. In fact, each ligand has nuanced degrees in how they interact with receptors and produce effects, for instance by desensitizing over activating receptors or desensitizing receptors for short or long periods of time. Together, these results suggest that each drug has a unique, multifaceted pharmacological profile at each subtype of nAChR. Therefore, finding the ideal smoking cessation medication is a matter of finding a ligand with the best pharmacological profile available.
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