Chapter 1

Using Chemical Biology to Study the Brain

1.1 Chemical Signaling in the Brain

As scientists, we continually strive to understand complex biological and chemical systems with the ultimate goal of comprehending the human condition and improving human health. From Hippocrates and Aristotle to the modern scientist, we have been fixated on studying the most complex organ in the human body – the brain. The adult human brain contains approximately 10^{11} neurons, and each neuron forms thousands of connections to other neurons through junctions called synapses. As such, the resulting 10^{14} to 10^{15} synapses form the complex neural network responsible for the intricacies of cognition and behavioral function. Efficient communication between neurons is facilitated by neuroreceptors located at these synapses. Modern neurobiology aims to understand the relationship between the properties of these fundamental brain components and cognitive function/dysfunction.

Neurons communicate via synaptic transmission; a process in which a presynaptic neuron produces a signal and a postsynaptic neuron receives this signal (**Figure 1.1A**). This process begins when the presynaptic nerve cell receives information from other neurons via its dendrites. This information is processed and the presynaptic neuron fires an electrical signal, called an action potential, which travels down the axon of the presynaptic neuron. Upon reaching the axon terminal, the action potential triggers the release of vesicles containing small-molecule neurotransmitters into the synaptic cleft, the space between neurons. Neurotransmitters diffuse across the synaptic cleft and bind to receptors embedded within the postsynaptic membrane, the so-called neurotransmitter-

gated ion channels. Upon neurotransmitter binding, the receptor undergoes a conformational change from a closed (non-conducting) state to an open (ion-conducting) state allowing the flow of ions across the postsynaptic membrane (**Figure 1.1B**). Thus, an electrical signal (the action potential) is converted into a chemical signal (the neurotransmitter) and subsequently back into an electrical signal (ion flow across the membrane), thereby completing the transmission of information from one cell to another.

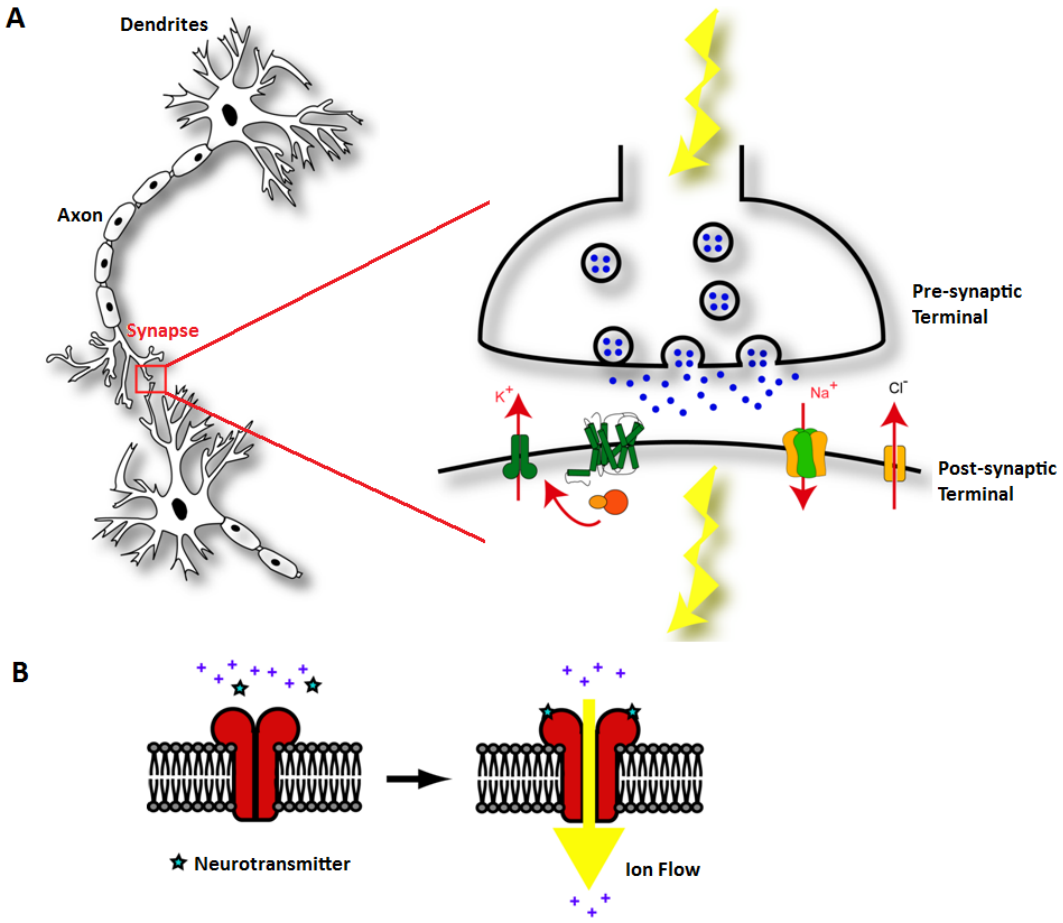

Figure 1.1. Synaptic transmission. A. The communication between two neurons occurs at the synapse, boxed in red. The presynaptic neuron receives a message from other neurons and transmits this information to the postsynaptic neuron. Enlargement of the synapse shows that various receptors are embedded within the postsynaptic membrane. These neuroreceptors recognize and bind neurotransmitters (blue circles). B. Upon agonist activation, the ion channel undergoes a conformational change from a closed (non-conducting) state to an open (ion-conducting) state, thereby propagating the signal.

These neuroreceptors are among the molecules of sensory perception, learning, and memory, and can function at either the presynaptic or the postsynaptic neuron. If located at the presynaptic terminal, the ligand-gated ion channel usually has a regulatory function, such as facilitating neurotransmitter release, whereas postsynaptic receptors propagate rapid electrical signal transmission between neurons.[1-3] Regardless of synaptic location of the ligand-gated ion channel, neurotransmitter binding and receptor activation are chemical-scale events essential to proper receptor function. As chemists, we are interested in developing chemical strategies to understand specific chemical interactions that mediate the structure/function relationship of these complex proteins. We employ chemical neurobiology to understand the process by which small-molecule neurotransmitters activate these much larger neuroreceptors proteins.

1.2 Nicotinic Acetylcholine Receptors: The Longest Known and Best-Studied Neuroreceptor

The nicotinic acetylcholine receptor (nAChR) represents a class of neurotransmitter-gated ion channels belonging to the Cys-loop superfamily of neuroreceptors, which also includes the γ-aminobutyric acid type A and type C ($GABA_A$ and $GABA_C$), glycine (Gly), and serotonin type 3 ($5\text{-}HT_3$) receptors.[4] As a family of complex transmembrane proteins, nAChRs are activated by the endogenous neurotransmitter acetylcholine. Coincidentally, nAChRs are also activated by the addictive lipophilic alkaloid, nicotine and other structurally related molecules (**Figure 1.2**). This class of neuroreceptors is essential to rapid synaptic transmission in the mammalian central nervous system (CNS) and peripheral nervous system (PNS).[4-6]

Given the abundant source of nAChRs available from the *Torpedo* electroplax, the nAChR has become the best-studied and prototypical Cys-loop receptor.[4-8]

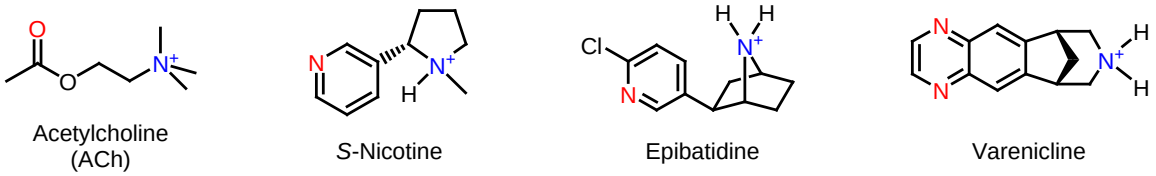

Figure 1.2. nAChR agonists studied in this dissertation. Structures are shown for acetylcholine (ACh), S-nicotine, epibatidine, and varenicline (Chantix®).

Over the past 20 years, several studies have greatly increased our understanding of nAChR structure. To begin with, a cryo-electron microscopic structure of the *Torpedo californica* nAChR (4 Å) obtained by Unwin *et al.* revealed the gross topology of a full-length nAChR.[9, 10] As shown in **Figure 1.3**, nAChRs are composed of five homologous subunits arranged pseudosymmetrically around a central ion-conducting pore. Each subunit contains a large, principally β-sheet extracellular N-terminal domain, four transmembrane α-helices (M1-M4), and a small extracellular C-terminal domain. The agonist binding site resides within the N-terminal extracellular domain, whereas the channel gate is located 60 Å away in the transmembrane domain. The M2 helix from each subunit lines the ion-conducting pore and the L9' residue has been identified as the channel gate.[9] (In Cys-loop receptors, these highly homologous M2 sequences are numbered from the cytoplasmic end, termed position 1'.)

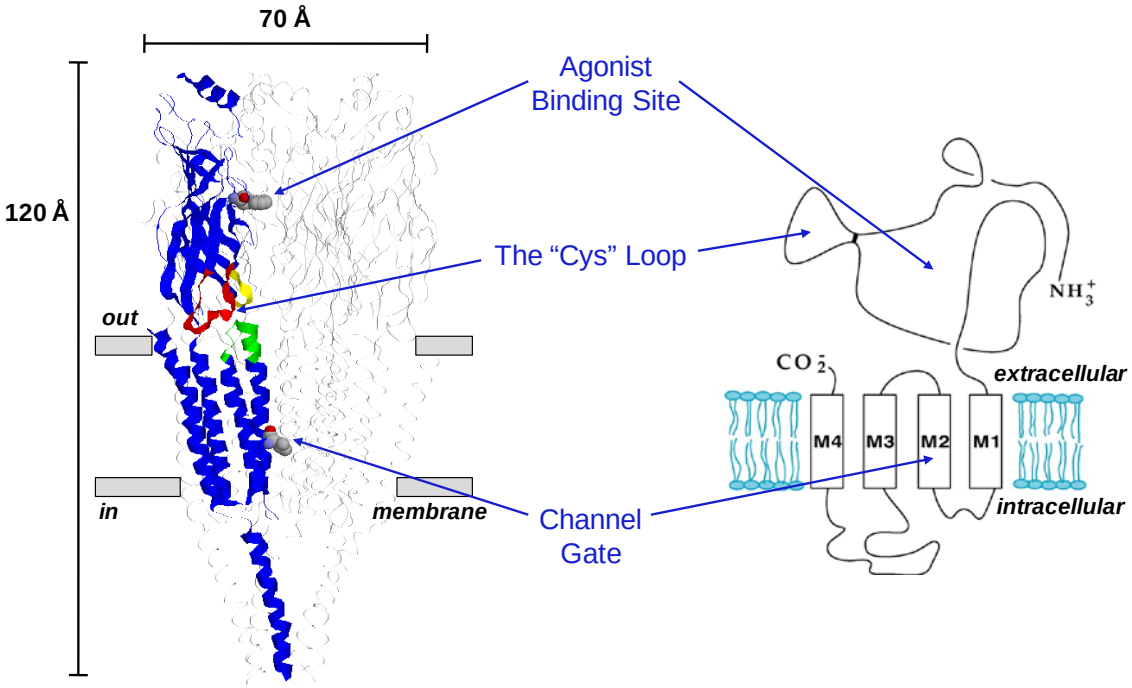

Figure 1.3. nAChR structure. Detailed view of nAChR with one subunit highlighted in blue. Structure is based in Unwin's model of the *Torpedo* receptor (left, pdb file 2BG9).[9] The highlighted subunit is compared to a cartoon (on right) describing the topology of a Cys-loop receptor subunit.

A significant advance in the field of nAChR research was the discovery of the water-soluble, snail acetylcholine binding proteins (AChBPs).[11-16] The pentameric AChBPs are structural surrogates for the extracellular ligand-binding domain of the nAChR, sharing 20%–25% sequence identity with the extracellular domain of the significantly larger ion channel proteins.[11] As such, crystal structures of AChBPs bound to various ligands have guided our structure-function studies of the nAChR binding site presented herein. The major caveat, however, is that AChBPs are not a neurotransmitter-gated ion channel, like the nAChR. Instead, AChBPs are merely proteins that contain an acetylcholine binding site, and therefore offer little guidance regarding the nAChR gating mechanism.

There are 16 mammalian genes that encode nAChR subunits, termed α1–α7, α9, α10, β1–β4, δ, γ, and ε. nAChRs are modular in nature, so these subunits arrange into pentamers to form as many as 25 different nAChR subtypes in humans.[7, 8] The muscle-type receptor is the prototypical nAChR with its precise stoichiometry of $(\alpha1)_2\beta1\gamma\delta$, fetal form (the adult form is $(\alpha1)_2\beta1\varepsilon\delta$)) (**Figure 1.4**).[4-6] This nAChR subtype is localized at the neuromuscular junction and mediates electrical transmission responsible for skeletal muscle tone. The neuronal nAChRs comprise the remaining nAChR subtypes, which are formed by combinations of α2–α7, α9, α10 and β2–β4 subunits (**Figure 1.4**).[7] The diverse array of neuronal subtypes is involved in maintaining multiple cognitive processes such as learning, memory, reward, and motor control.[7, 8]

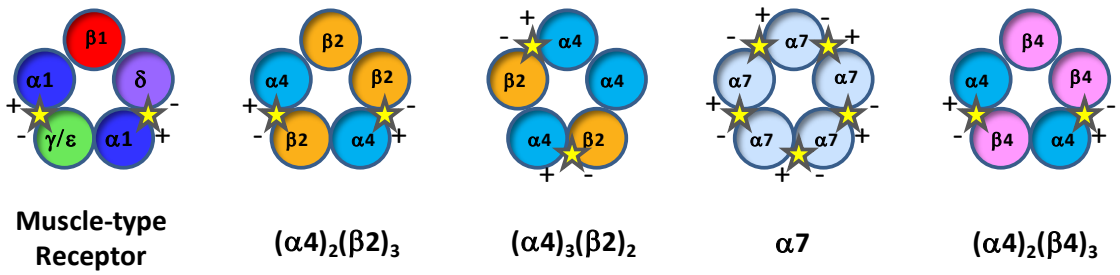

Figure 1.4. nAChR subtypes studied in this dissertation.

In the CNS, the two major neuronal subtypes are the α4β2 and α7 receptors.[8, 17] The α4β2 receptor accounts for over 90% of the high affinity nicotine binding sites in the brain,[7, 18-20] and as such, Pfizer's smoking cessation drug varenicline was designed to target this receptor.[21-23] The α4β2 receptor exists in two stoichiometries, $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$, and chronic exposure to nicotine leads to upregulation of $(\alpha4)_2(\beta2)_3$, the form most relevant to nicotine addiction.[24, 25] The homopentameric α7 receptor has been implicated in schizophrenia and is considered a treatment target for Alzheimer's disease

and other cognitive disorders.[7] The last nAChR subtype discussed in this thesis is the α4β4 subtype, which like α4β2, can arrange in variable stoichometries and is associated with nicotine addiction.[7, 26, 27]

Agonists bind at select subunit interfaces[4-6] originally identified by photoaffinity labeling and mutagenesis experiments.[11, 28-32] The agonist binding site is formed from several loops contributed by the principal and complementary binding interfaces. The principal binding site (an α subunit) contributes loops A, B, and C, while the complementary binding site (a non-α subunit, *e.g.*, γ, δ, β2, β4) contributes loops D, E, and F. The cationic moiety of agonist molecules interacts with a cluster of five aromatic amino acids, termed TyrA, TrpB, TyrC1, TyrC2, and TrpD (**Figure 1.5**). These residues are conserved for all nAChR subtypes and named according to the loop on which they reside. A major focus of this thesis focus is TrpB, a residue shown to bind agonists via a cation-π interaction in several nAChR subtypes.[33-35]

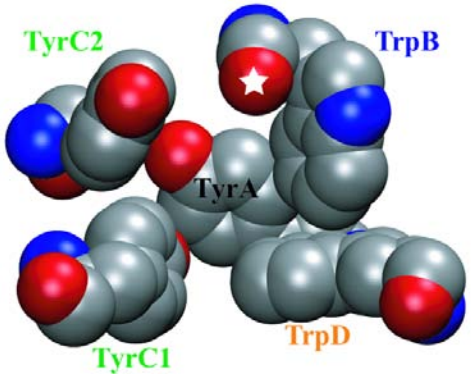

Figure 1.5. A model of the nAChR agonist binding site from AChBP (pdb file1I9B).[11] Residues are labeled according to the loop on which they reside. Residues TyrA, TrpB, TyrC1, and TyrC2 are from the α subunit, but residue TrpD is from a non-α subunit.

1.3 The Nonsense Suppression Methodology: An Invaluable Tool

Nonsense suppression is a broadly applicable technique that can dissect the structure-function relationship of various complex proteins,[33-36] and is especially useful for understanding proteins in the absence of a crystal structure. The major advantage of this approach is the ability to introduce minimal structural modifications to amino acid side chains, thereby allowing for more accurate interpretations of the effects of a specific perturbation. This strategy is complementary to conventional site-directed mutagenesis which can more globally alter or completely abolish side chain functionality within the confines of the naturally occurring amino acids.

Conventional mutagenesis can severely limit the ability to probe the functionality of a multifunctional residue. Consider a tyrosine residue, which may serve as a hydrogen bond donor, a hydrogen bond acceptor, a site for a cation-π interaction, or a source of steric bulk (**Figure 1.6**). When Tyr is mutated to an Ala, all possible side-chain functionality is abolished, and this mutation can only determine if Tyr is essential for protein function. Alternatively, mutating the Tyr to either Phe or Ser can probe for a hydrogen bonding interaction. Both mutations can only establish if the hydroxyl group is necessary for proper function, which can act as either a hydrogen bond donor or acceptor. The Tyr to Phe mutation is more conservative as it leaves the π system of the phenyl ring intact. The Tyr to Ser mutation, however, is more complicated due to the significant size discrepancy between Ser and Tyr. This mutant may result in decreased protein function, which could be attributed to the smaller size of the Ser residue unable to provide an OH group at the same point in space as that provided by Tyr.

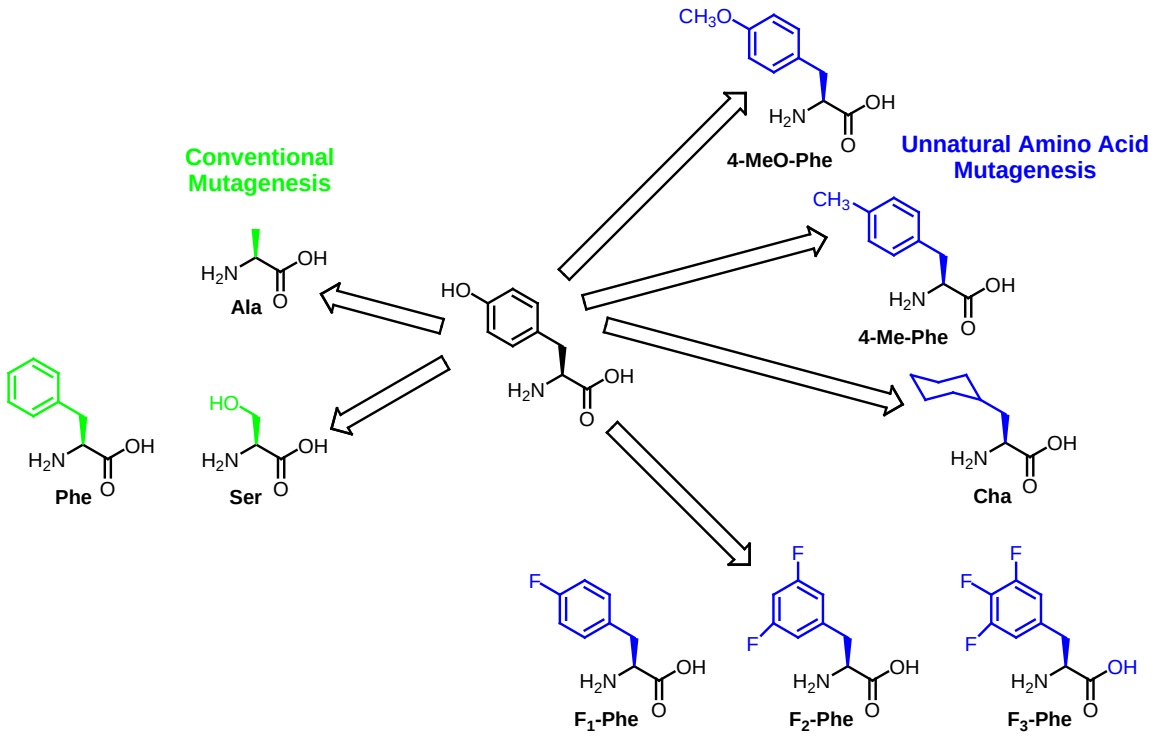

Figure 1.6. Mutation of tyrosine: Comparison of conventional mutagenesis and unnatural amino acid mutagenesis.

Unnatural amino acid mutagenesis, however, can address all of these issues by expanding the repertoire of amino acids offered by nature (**Figure 1.6**). For instance, incorporation of 4-methoxy phenylalanine (4-MeOPhe) allows one to determine whether the residue of interest acts as a hydrogen bond acceptor. The issue of steric bulk can be examined by incorporation of 4-methyl phenylalanine (4-MePhe), a residue that would occupy the same relative space as Tyr. Incorporation of cyclohexylalanine (Cha) can establish if the aromatic nature of Tyr is important to proper function. Additionally, incorporation of fluorinated Phe derivatives can investigate the presence of a cation-π interaction, a non-covalent interaction between the face of an electron-rich π system and a cation.[37-40]

We use the nonsense suppression methodology, developed by Schultz in 1989,[41] to site-specifically incorporate unnatural amino acids into proteins heterologously

expressed in *Xenopus laevis* oocytes.[42, 43] Using this method, our lab has successfully determined the ligand binding mechanism and channel gating properties of numerous ion channels and neuroreceptors.[33-35, 44-52]

In normal protein synthesis, the ribosome is a multisubunit complex of RNAs and proteins that functions to decode a template mRNA strand and generate a specific protein target. The mRNA sequence contains a series of codons that directs the succession of "charged" tRNA molecules (*i.e.*, tRNA with an amino acid appended to the 3' end) containing the appropriate anticodons. Amino acids are linked together via peptide bonds to form the growing polypeptide chain. Termination of protein synthesis occurs when the ribosome encounters a STOP or nonsense codon (*e.g.*, UAA, UAG, or UGA), after which, the polypeptide chain is released.

Nonsense suppression, however, "hijacks" the endogenous translational machinery of the *Xenopus* oocyte (**Figure 1.7**). In this process, either a nonsense codon[35, 41] or four-base codon[50-52] (*e.g.*, TAG or GGGT) is placed at the amino acid position of interest in DNA containing the subunit gene. Naturally occurring tRNAs do not recognize these codons, and as such these codons would normally elicit termination of protein synthesis or a frameshift mutation, respectively. Instead, we employ a special suppressor tRNA that contains the correct anticodon and is charged with the unnatural amino acid of choice linked through a highly reactive ester bond.[51-53] The fidelity of this method relies on the orthogonality of the suppressor tRNA, meaning that the tRNA is not recognized by the endogenous aminoacyl-tRNA synthesizes of the cell and thereby avoids recharging with natural amino acids.

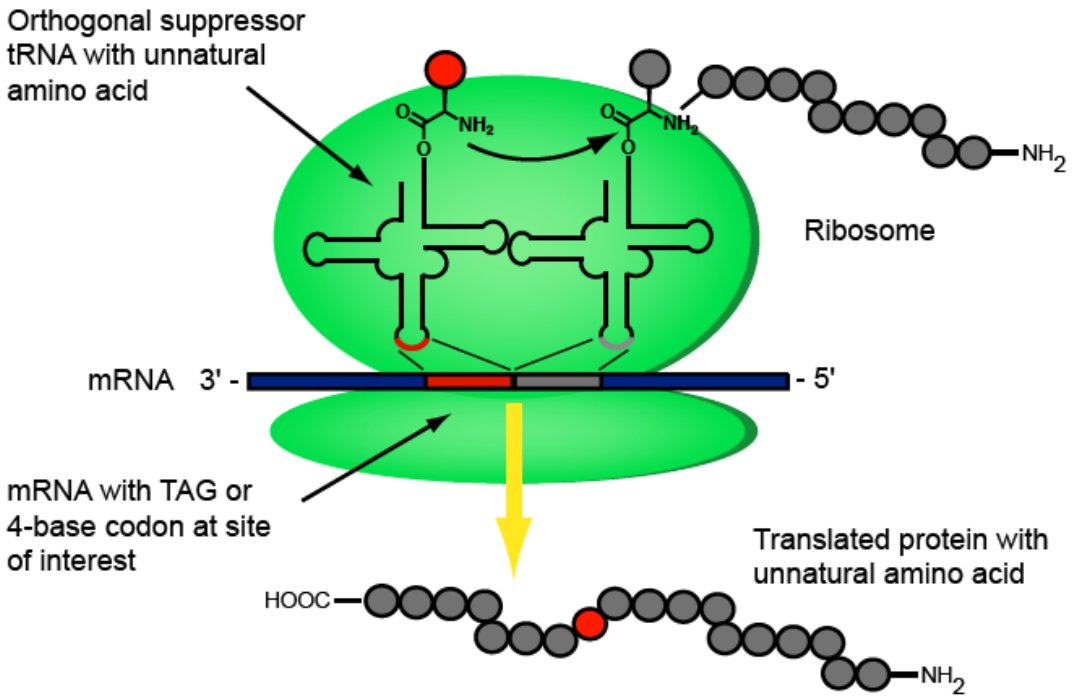

Figure 1.7. Hijacking protein translation: Using nonsense suppression to incorporate unnatural amino acids.

A full-length, 76 nucleotide suppressor tRNA is made through a non-trivial process using a combination of synthetic and molecular biology techniques.[42, 43, 53, 54] First, the suppressor tRNA is *in vitro* transcribed as a 74-nucleotide fragment, which is missing the last two nucleotides of the acceptor stem (cytosine; C and adenine; A). The deoxy-C and A (dCA) dinucleotide is synthesized and chemically acylated with the unnatural amino acid (UAA) of choice. Chemical ligation of the dCA- UAA to the 74mer tRNA produces a complete tRNA-UAA molecule.

In the last step of nonsense suppression, *in vitro* transcribed mRNA containing the nonsense codon and the tRNA-UAA are coinjected into a *Xenopus laevis* oocyte, an unfertilized frog egg (**Figure 1.8**). The endogenous translational machinery of the oocyte completes this process by synthesizing, processing, and exporting the desired protein to the surface of the cell membrane. We then examine the functional properties of the novel

protein, which can be attributed to the subtle structural perturbation induced by the unnatural amino acid.

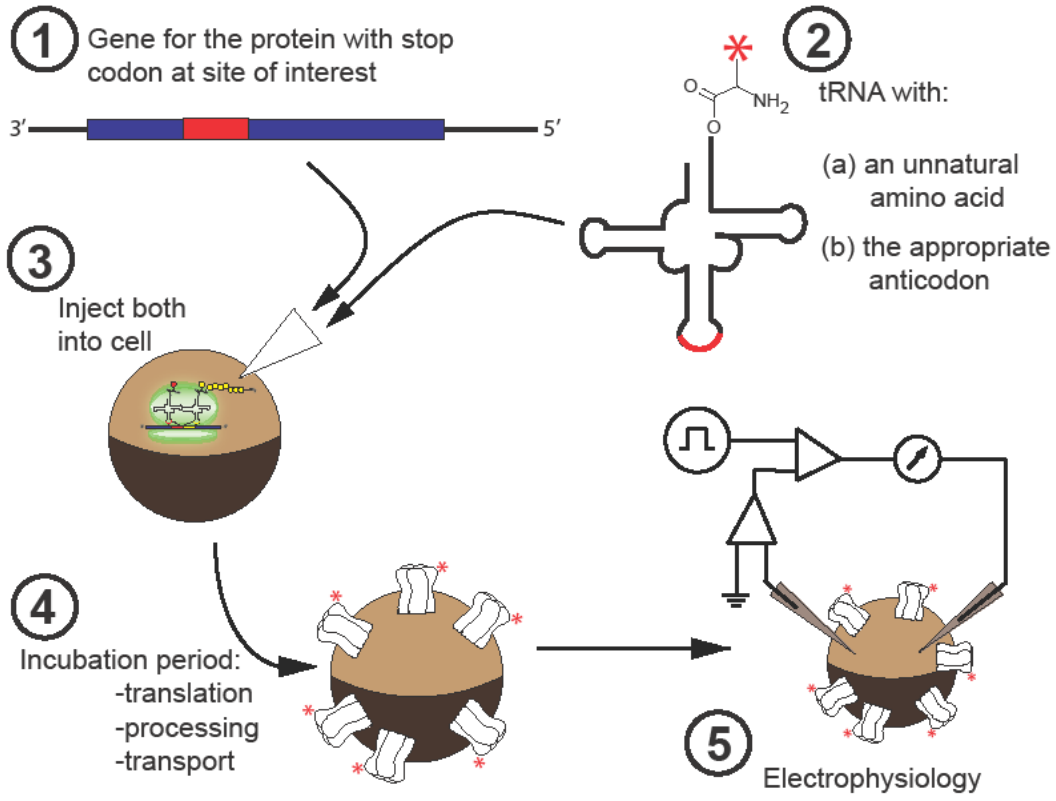

Figure 1.8. Illustration of the *in vivo* nonsense suppression technique, a method for incorporating unnatural amino acids into ion channels expressed in *Xenopus* oocytes.

1.4 Electrophysiology: A Sensitive Assay of Receptor Function

The nAChR proteins are ion channels, and as such they mediate the flow of ions (*e.g.*, an electrical current) across a cell membrane. Conveniently, this electrical signal can be readily measured and reports the functionality of the protein under study. Recall, however, that the orthogonal suppressor tRNAs are a stoichiometric reagent – once they deliver their unnatural amino acid to the protein, they are not recharged with additional unnatural amino acid. Additionally, the efficiency of nonsense suppression is inherently variable due to mRNA surveillance mechanisms of the cell (*e.g.*, nonsense-mediated

decay).[55, 56] These processes complicate suppression of a nonsense codon and therefore limit the production of the desired protein. We overcome these potential challenges by using a very sensitive assay of ion channel function, termed two-electrode voltage clamp electrophysiology.

Using this assay, receptors expressed on the cell membrane are exposed to increasing concentrations of agonists. This results in electrical currents proportional to the amount of receptor activation (**Figure 1.9A**). These data are then fit to the Hill equation to generate a dose-response relationship, a curve that plots the agonist concentration against the normalized current responses (**Figure 1.9B**). From this curve, we measure the EC_{50} value, the concentration required to achieve half-maximal activation. EC_{50} is a functional measure of the induced structural perturbation and is used to compare ion channel function. As such, a rightward shift in EC_{50} is a "loss-of-function" mutation, which would require more agonist to activate the channel, whereas a leftward shift in EC_{50} would indicate a "gain-of-function" mutation. We note that EC_{50} is a composite measurement of both agonist binding and receptor gating. As such, the studies presented herein focus mainly on mutation of the agonist binding site. Given that the agonist binding site is separated by a distance of ~60 Å, we assume that such mutations primarily affect the binding parameter of EC_{50}. Additionally, single channel analysis has confirmed that the gating parameter is unaffected by mutations of the agonist binding site.[34]

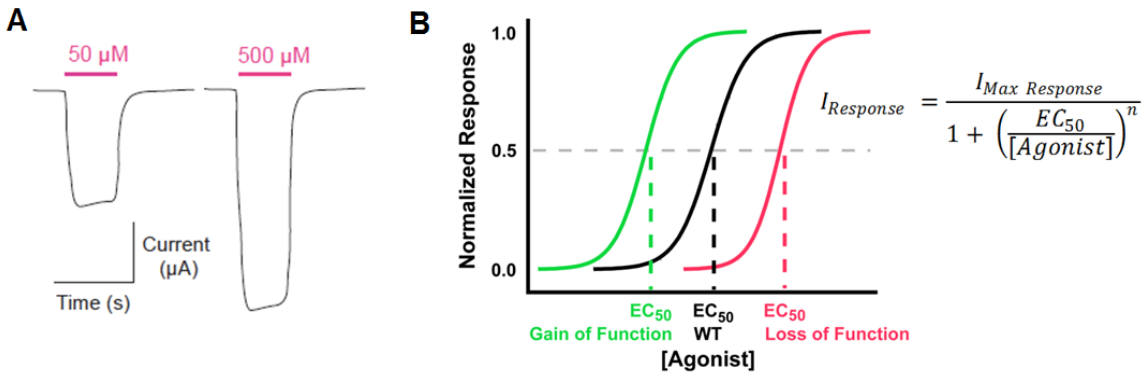

Figure 1.9. Electrophysiology as an assay of ion channel function. A. Illustration of agonist-induced current traces for two agonist concentrations (50 μM and 500 μM). B. Dose-response relationships. The dose-response curve for a wild type protein (in black), a gain-of-function mutation (in green), and a loss-of-function mutation (in pink).

1.5 Summary of Dissertation Work

The following work describes structure-function studies of the nAChR. These studies use unnatural amino acid mutagenesis and electrophysiology to elucidate the molecular determinants for agonist binding in several nAChR subtypes.

In chapter 2, we study the nAChR subtype responsible for nicotine addiction, the α4β2 nAChR. We determine the molecular interactions that differentiate this receptor from other nAChR subtypes and endow it with the ability to mediate nicotine addiction. We report that the high affinity for nicotine at α4β2 is a result of a strong cation-π interaction and a strengthened backbone hydrogen bond to TrpB of this receptor.[34] This result contrasts what was observed in the muscle-type nAChR, where a cation-π interaction was found with ACh, but not with nicotine.[35, 44] We also show that a point mutation near TrpB appears to influence the shape of the agonist binding site, such that it can differentiate the α4β2 and muscle-type receptors' binding mechanisms.[34]

In chapter 3, we further investigate the point mutation near TrpB, termed the "loop B glycine." In three nAChR subtypes (*i.e.*, muscle-type, α4β2, and α7), we show

that the correlation between agonist potency and this loop B site is strong. Low-potency receptor subtypes have a glycine at the loop B site, while high-potency receptors have a lysine at this site. We establish that mutation of this residue can to convert a low-potency receptor to a high-potency receptor and vice versa.

Chapter 4 describes our efforts to understand the agonist binding mechanism of a fourth nAChR subtype, the α4β4 receptor. We confirm that the α4β4 receptor, like α4β2, utilizes a strong cation-π interaction to TrpB for both ACh and nicotine, and nicotine makes a strong hydrogen bond to the backbone carbonyl of TrpB.[33] Additionally, we use chimeric β subunits in an attempt to understand how the complementary binding component can influence agonist binding and receptor pharmacology in the α4β2 and α4β4 receptors. Together, chapters 2-4 identify structural features of the nAChR that contribute to differential receptor pharmacology and hold significant implications for drug discovery efforts seeking to selectively target nAChRs.

Last, chapter 5 takes a shift from the previous chapters and describes a methodology-based project. This chapter focuses on the optimization of unnatural amino acid incorporation into mammalian cells and its application to large-scale imaging techniques, such as the FlexStation 3. We have successfully suppressed an amber stop codon using HSAS, an *in vivo* aminoacylated tRNA, in HEK293T cells. Studies are ongoing to achieve nonsense suppression using *in vitro* aminoacylated tRNAs.

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