CHAPTER 1: Introduction

1.1 Chemical Signaling in the Brain

The adult human brain uses an intricate process to communicate between its more than one-hundred billion (10^{11}) neurons. This process begins when an electrical signal, called an action potential, is generated in one neuron termed the presynaptic nerve cell. The action potential travels down the axon of the presynaptic neuron until it reaches the synaptic cleft (the space between two nerve cells). This triggers the mobilization of vesicles that contain small-molecule neurotransmitters. These vesicles ultimately fuse to the terminal of the presynaptic nerve cell, releasing their neurotransmitter contents into the synaptic cleft. Neurotransmitters diffuse across the synaptic cleft and bind a special class of integral membrane proteins, called ligand-gated ion channels, on a postsynaptic neuron. Upon binding the neurotransmitter, the ligand-gated ion channel undergoes a conformational change that opens an ion-conducting pore. This event enables ions to diffuse across the otherwise impermeable membrane of the postsynaptic nerve cell, thereby generating a new electrical signal to propagate the message that originated in the presynaptic cell. This process is called synaptic transmission, and its regulation is central to many important processes including memory, thought, sensory perception, and awareness.

Our lab is interested in using physical organic chemistry to understand the chemical component of synaptic transmission– the activation of a ligand-gated ion channel by a small molecule.

1.2 Nicotinic Acetylcholine Receptors (nAChRs): The Prototype of the Cys-Loop Superfamily of Ligand-Gated Ion Channels

The Cys-loop (or pentameric receptor) superfamily is a large and important class of neurotransmitter-gated ion channels. Among this superfamily are receptors for the neurotransmitters acetylcholine (nicotinic acetylcholine receptors), γ-aminobutyric acid ($GABA_A$ and $GABA_C$), glycine (GlyR), and serotonin ($5\text{-}HT_3$).[1] The family is essential for proper brain function and is also implicated in an assortment of neurological disorders including Alzheimer's disease, Parkinson's disease, schizophrenia, and depression.[2, 3]

The Cys-loop receptors are pentamers composed of five subunits arranged around a central ion-conducting pore. Subunits share a common structure consisting of a large, N-terminal extracellular domain that contains the agonist binding site and also the signature disulfide loop, four transmembrane α-helices (M1-M4) that line the ion pore, and a short extracellular C-terminus (**Figure 1.1**). Nicotinic acetylcholine receptors (nAChRs) are arguably the best-characterized members of the family and are therefore generally considered the prototypical Cys-loop receptor.[1-3]

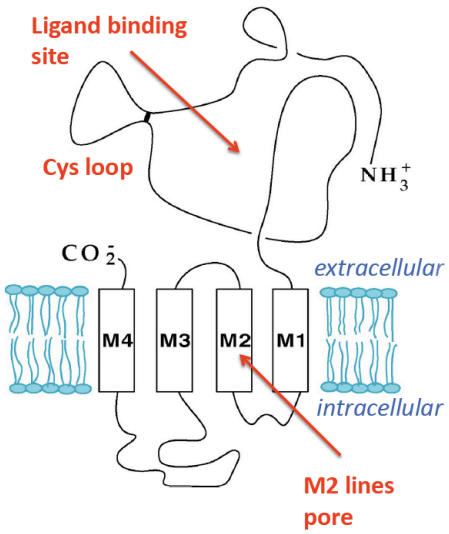

Figure 1.1. Topology of a Cys-loop receptor subunit.

The nAChRs mediate rapid synaptic transmission in the central and peripheral nervous systems.[1, 4, 5] They are activated endogenously by the neurotransmitter acetylcholine and also, coincidentally, by nicotine. There are 16 mammalian genes that code for 16 nAChR subunits, termed α1-α7, α9, α10, β1-β4, γ, δ, ε. These subunits arrange as pentamers to form more than 20 active and pharmacologically distinct nAChR subtypes in humans. Of these subtypes, $\alpha1_2\beta1\gamma\delta$ is the best studied owing to its precise subunit stoichiometry (**Figure 1.2**) and its abundance in the electric organs of eels and rays.[1, 4, 5] In humans, the $\alpha1_2\beta1\gamma\delta$ subtype, often called the "muscle-type" receptor, is expressed postsynaptically at neuromuscular junctions in the peripheral nervous system. Many other nAChR subtypes are expressed in the central nervous system at nerve synapses. The most abundant of these are likely the α4β2 and α7 subtypes.[2, 6] The α4β2 subtype is the neuroreceptor most associated with nicotine addiction[2, 6] and also the intended target of Pfizer's smoking cessation drug Chantix® (varenicline).[7-9] This subtype assembles into two viable stoichiometries, $\alpha4_2\beta2_3$ and $\alpha4_3\beta2_2$ (**Figure 1.2**), but the $\alpha4_2\beta2_3$ stoichiometry is more sensitive to nicotine and is upregulated during chronic nicotine exposure.[10] The α7 subtype is a homopentamer that is often linked to schizophrenia and Alzheimer's disease.[3]

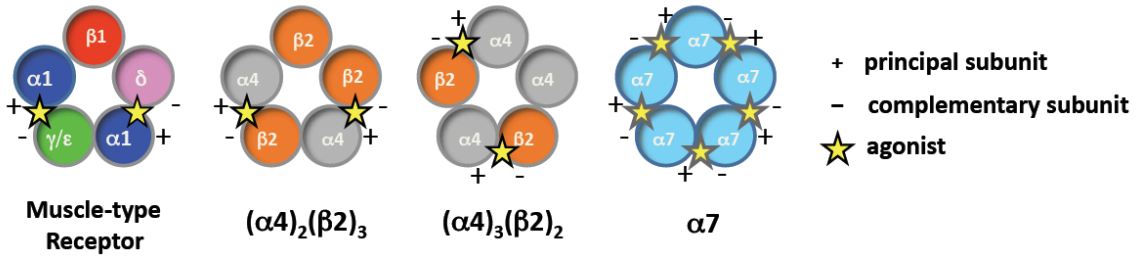

Figure 1.2. Stoichiometries of several nAChRs.

Much of our understanding of the structure of the nAChR comes from early mutagenesis studies,[11, 12] and affinity labeling studies,[1,13-16] which provided information about the location of the agonist-binding site and the residues involved.[1, 4, 5] No high-resolution structure of a nAChR exists, but a great deal of relevant structural information has become available within the last 20 years. A major advance came in the early 2000s with the identification and structural characterization of a family of snail acetylcholine binding proteins (AChBPs).[17-22] The AChBPs are soluble, pentameric proteins that share 20−25% sequence identity with the extracellular ligand-binding domain of the nAChRs. As such, their X-ray crystallography structures have served as structural templates for many of our studies of the residues involved in agonist binding in the nAChRs. Note, however, that the AChBPs are not neurotransmitter-gated ion channels; they are simply soluble proteins that evolved to contain a binding site. As such, they offer little information about the activation/gating pathway of the nAChRs. There is also a cryo-electron microscopy (EM) structure of the *Torpedo californica* ray nAChR.[23, 24] This structure is of lower resolution than the AChBP structures (many amino acid side chains cannot be resolved), but it does provide a general picture of the overall secondary structural layout of a full-length nAChR (**Figure 1.3A**). Another major breakthrough in Cys-loop receptor research has come very recently with the publication of X-ray crystallography images of GluCl, an anion-selective invertebrate Cys-loop receptor that binds inhibitory neurotransmitters.[25] This protein does not bind ACh or other nicotinic agonists, and so it is almost certain that there will be interesting differences in its agonist binding site and also in its channel pore owing to its preference for anions (where nAChRs prefer cations). A key goal of research in the Dougherty lab is to test the

relevance of these model structures to the nAChRs and to try to use these static images to infer information about the conformational changes responsible for receptor activation and channel gating. The studies presented in this thesis primarily focus on residues in the agonist binding site, and so the AChBP structures have heavily guided this research.

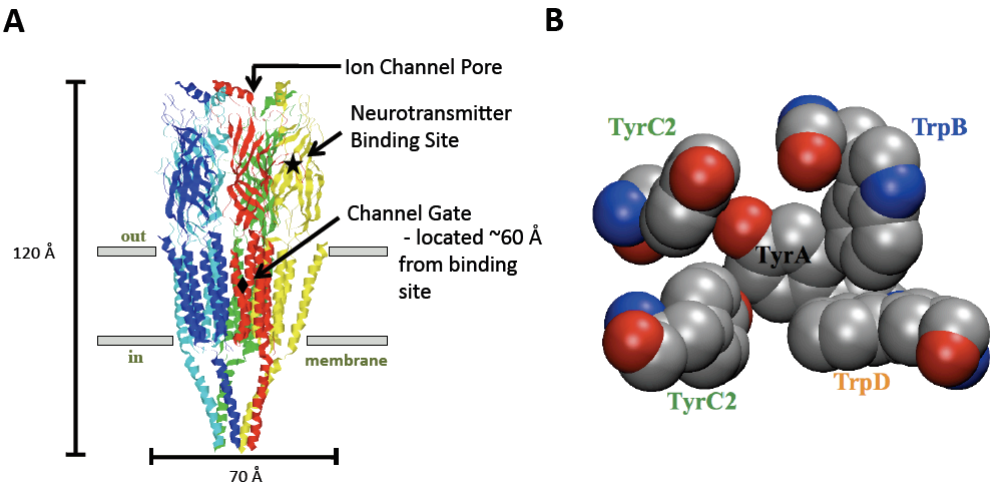

Figure 1.3. nAChR structure. (A) Structure of the nAChR based on cryo-EM structure of the *Torpedo* receptor (pdb: 2BG9). (B) Enlargement of aromatic box from AChBP (pdb: 119B).

Agonists bind at the interface of adjacent subunits in the nAChR pentamer.[1, 4, 5] The agonist binding site is a compact pocket comprised of amino acids from several noncontiguous regions from the "principal" (always an α subunit) and "complementary" (*e.g.*, γ or δ in $\alpha 1_2\beta 1\gamma\delta$ or β2 in α4β2) subunits. These regions are referred to as loops A, B, and C from the principal subunit and loops D, E, and F from the complementary subunit. The α subunits are defined by a universally conserved vicinal disulfide between C192 and C193 (using $\alpha 1_2\beta 1\gamma\delta$ numbering). This disulfide has played a pivotal role in a number of pioneering studies in nAChR research,[13, 14, 26-31] but its functional role in native receptors remains uncertain. Five conserved aromatic residues – α1Y93 (loop A), α1W149 (loop B), α1Y190 (loop C), α1Y198 (loop C), and γW55/δW57 (loop D) – form

what is known as the aromatic binding box. For simplicity, these residues are often referred to as TryA, TrpB, TyrC1, TyrC2 and TrpD representing the loop in which they reside (**Figure 1.3B**). Several aromatic box residues (particularly TrpB) have been shown to play roles in agonist binding to the nAChRs[32-34] while others are predicted to play a role in shaping the agonist binding site or relaying conformational changes that are initiated upon agonist binding.

The ion channel pore is lined by the M2 helix from each subunit of the pentamer (**Figures 1.1 and 1.3A**). Each M2 helix contributes several highly conserved hydrophobic residues that constitute the channel gate. Of these, the L9' residue (where 9' represents the ninth residue from the cytoplasmic end of the transmembrane helix) comprises the narrowest constriction point in the *Torpedo* cryo-EM structure and sits at the approximate midpoint of the M2 helix.[24, 34] This residue has been shown to play a critical role in channel gating, and when mutated to a more polar amino acid, the pore is stabilized in an open, ion-conducting conformation.[35, 36] Given the 60 Å distance separating the L9' residue and the agonist binding site, it has been a bit of a mystery in nAChR research as to how structural changes that occur upon agonist binding are communicated to the channel gate. It has been suggested that a 'conformational wave' is responsible for transmitting conformational changes that initiate at the agonist binding site to the L9' residue.[37] Movements of the C loop, which contains the vicinal disulfide and two of the five aromatic box residues, have been strongly implicated[38-40] in the gating process and are likely to be involved in the proposed conformational wave. Chapter 5 discusses a potential role for the vicinal disulfide and other residues of loop C in communicating conformational changes of the receptor.

1.3 Using Physical Organic Chemistry to Study Ion Channels: The Power of Unnatural Amino Acids

The overarching goal of the Dougherty lab is to obtain "chemical-scale" information – on a distance scale that is interesting to a physical organic chemist – on the structure and function of these receptors. By this we mean information about the functional groups, noncovalent interactions or conformational changes that are important for the activation and gating of these complex integral membrane proteins.

To obtain such an understanding, we could take a classical pharmacological approach and mutate the small molecule agonists of the receptor. This is certainly a viable strategy that we have used in several chapters of this thesis, but to obtain an understanding of the residues affected by mutation of the agonist, we must also mutate the receptor. A total chemical synthesis or even semisynthesis of a multisubunit, many kDA protein like the nAChR would certainly be quite a feat, made even more difficult if multiple variations in receptor structure are needed. But even if they were synthetically accessible, the proteins would need to be reconstituted into their native conformation and embedded into an appropriate membrane. An appropriate solution is to use conventional mutagenesis combined with heterologous expression of the protein. However, the extent of structural perturbations that can be performed by this methodology is limited by the structures of the side chains of the 20 naturally occurring amino acids.

As an illustration of these limitations, consider a study of a cation-π interaction in a protein. A cation-π interaction is a stabilizing, noncovalent (primarily electrostatic) interaction between a π system and a cation.[41-44] Nearly all neurotransmitters and ligands that bind to receptors and ion channels contain a positive charge,[45] and nature has equipped proteins with suitable π systems to bind these charges in the form of the

aromatic amino acids Phe, Tyr and Trp. In the Protein Data Bank, there is one cation-π interaction for every 77 amino acids, and approximately 26% of all Trp residues are involved in cation-π interactions.[42]

To probe a cation-π interaction by conventional mutagenesis, the aromatic side chain of interest could be completely ablated by incorporation of an Ala residue, but this is a fairly dramatic mutation that doesn't directly probe the electrostatic component of the interaction and could affect the structure/function of the protein in a number of ways. Alternatively, the residue could be modified to one of the other two aromatic residues. However, although there are differences in cation-π binding energies between the aromatic amino acids, there are also many substantial structural differences that could also impact the protein's structure/function.

The use of an amino acid that is not found in nature – an unnatural amino acid – could provide a more productive probe of the cation-π interaction. For example, a Trp residue can be replaced with Trp derivatives bearing progressively fluorinated side chains. Due to the electronegativity of the atom, each fluorine added to an aromatic side chain diminishes the cation-π binding ability of the ring in an additive fashion. Moreover, the innate cation binding ability of the mutant side chain (**Figure 1.4**) can be plotted versus a functional measure of the protein (discussed below) to produce a "fluorination plot" (**Figure 1.5**). A linear trend in the fluorination plot is the signature of a cation-π interaction. The structural change incurred from replacing a hydrogen with a fluorine should also be quite subtle, certainly more subtle than the structural perturbations available in conventional mutagenesis. This thesis uses the fluorination strategy to probe cation-π interactions in the nAChR.

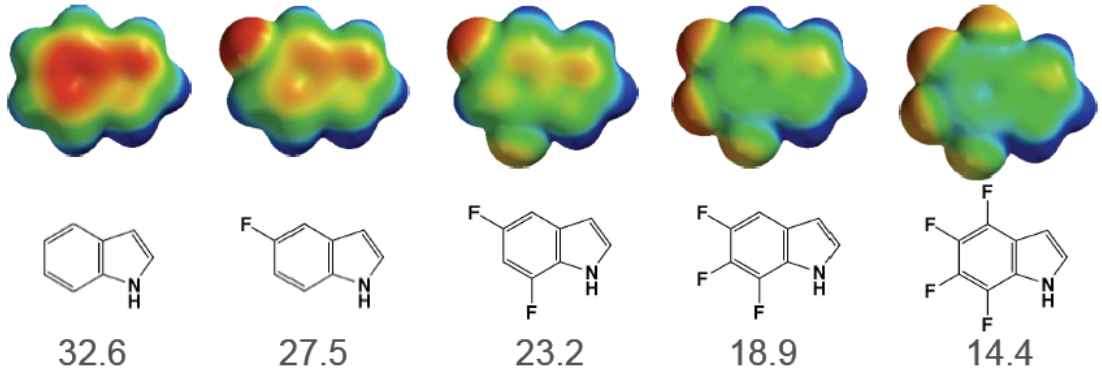

Figure 1.4. Fluorinated Trp side chains (indole rings) and calculated cation-π binding energies. Binding energies (kcal/mol) are from gas-phase *ab initio* calculations of the interaction between an indole side chain and a Na^+ ion.[33] Electrostatic potential maps show negative potentials (regions that a positive charge is likely to bind) in red and positive potentials in blue.

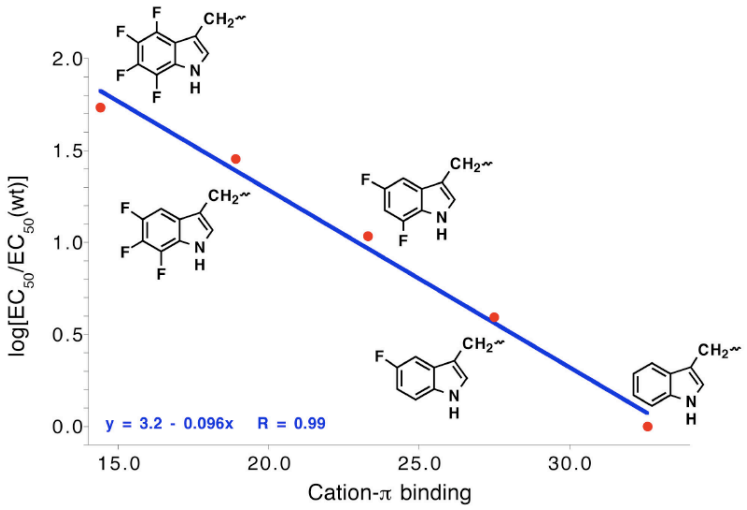

Figure 1.5. An example fluorination plot giving a linear trend indicative of a cation-π interaction. This plot was used to establish the cation-π interaction between an agonist and the $\alpha 1_2\beta 1\gamma\delta$ receptor.[33]

Another testament of the usefulness of unnatural amino acids is seen in the study of hydrogen bonding interactions to the backbone amide bond of a protein. Since conventional mutagenesis is limited to side chain modification, it is difficult (if not impossible) to directly probe the importance of an amide bond by this methodology.

Backbone amide bonding can be probed using an unnatural amino acid strategy by replacing the residue that contributes the backbone NH with its α-hydroxy acid analog (**Figure 1.6**).[46-49] This mutation converts the amide bond to an ester with two major effects on backbone hydrogen bonding. The backbone NH is replaced with an O and can therefore no longer serve as a hydrogen bond donor. This mutation also attenuates the hydrogen bonding ability of the *i-1* CO by converting it to an ester CO, which is well-established to be a poorer hydrogen bond accepter than an amide CO. Thus, incorporation of an α-hydroxy acid can probe the hydrogen bonding ability of the associated amide NH and amide CO. This mutation can also potentially introduce an unfavorable electrostatic interaction by the introduction of an electronegative O, but this effect has been shown to be quite small in model studies, with an estimated 0.3–0.4 kcal/mol energetic consequence.[50, 51] Overall, the amide-to-ester mutation results in a relatively subtle change in protein structure that maintains the original side chain properties and backbone conformational preferences (*e.g.*, bond lengths, bond angles and *cis*-trans conformational preferences) of the parent amide backbone.

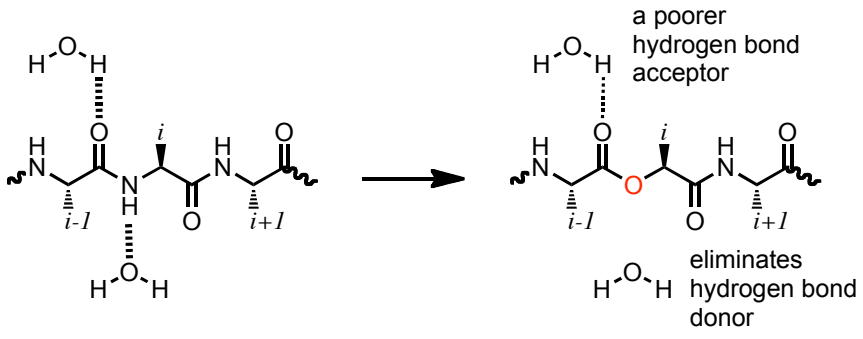

Figure 1.6. Amide-to-ester mutation.

Thus, unnatural amino acids enable subtle probes of protein structure and function that are not limited by the chemical diversity of conventional amino acid side chains.

Instead these studies are limited only by the boundaries of chemical synthesis and also by the diversity of structures that can be incorporated into proteins by a cellular ribosome via the nonsense or frameshift suppression methodology described below.

1.4 Incorporation of Unnatural Amino Acids Through Nonsense or Frameshift Suppression Methodology

Nonsense suppression methodology was developed in the late 1980s for the site-specific incorporation of unnatural amino acids into proteins.[52-54] In normal protein synthesis, mRNA is decoded by a ribosome and matched by its three nucleotide codon to its cognate tRNA. The nonsense suppression method employs a stop codon (UAG, UGA or UAA), which does not code for any natural tRNAs and is instead used to signify a stop (termination) in protein synthesis. The unnatural amino acid is appended to an "orthogonal" suppressor tRNA with the corresponding anticodon. An orthogonal tRNA is one that is not recognized by any of the endogenous aminoacyl-tRNA synthetases, the enzymes that append natural amino acids onto their cognate tRNAs. During protein synthesis, the ribosome incorporates the unnatural amino acid into the polypeptide sequence at the site of interest (the location of the stop codon) (**Figure 1.7**).

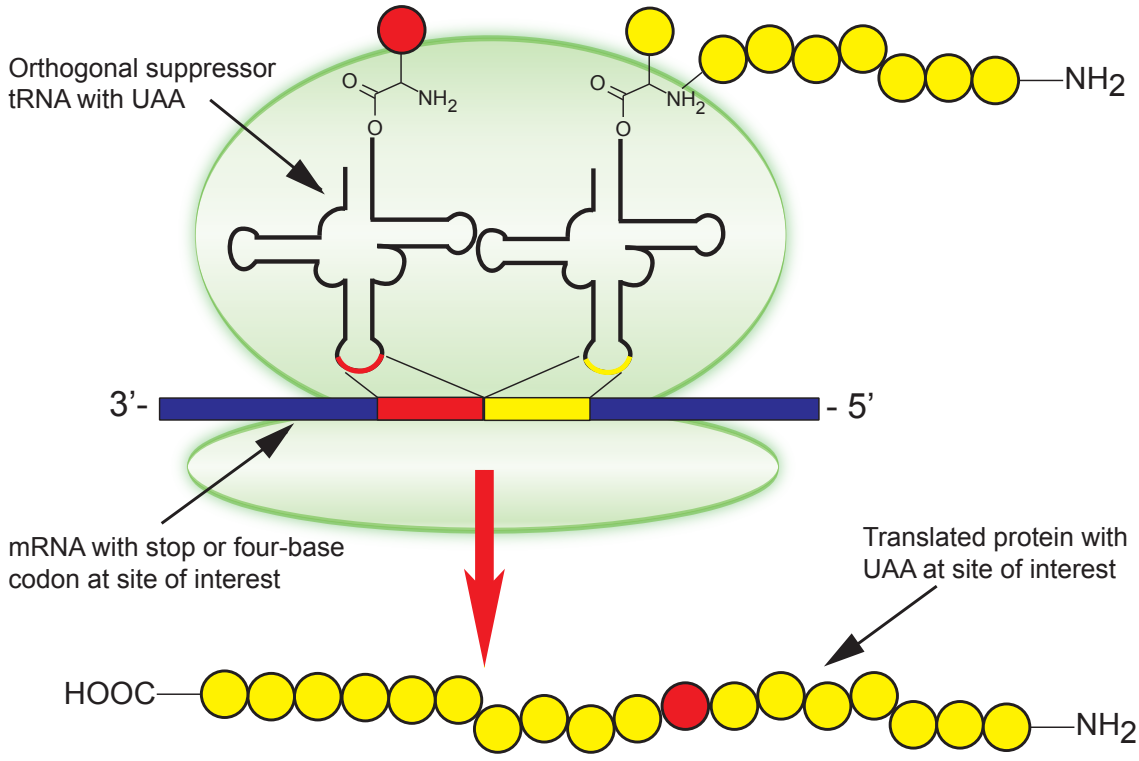

Figure 1.7. An overview of the nonsense and frameshift suppression methodologies used to incorporate unnatural amino acids (UAAs).

An alternative method for incorporating unnatural amino acids is frameshift suppression, which uses a four-base codon (GGGT) instead of a stop codon.[55, 56] This method employs a suppressor tRNA with the corresponding four-base anticodon to incorporate the unnatural amino acid and, as a result, suppresses the reading frame shift that would normally occur when the ribosome encounters a four-base codon. By using combinations of stop (TAG or TGA) and four-base (GGGT) codons, multiple unnatural amino acids can be incorporated into a single protein.[57]

Both methodologies require a combination of techniques in chemical synthesis and molecular biology.[45, 58, 59] Standard mutagenesis protocols are used to mutate a stop codon (or four base codon) into the gene of the protein being studied at the site of

interest. The suppressor tRNA is transcribed without the last two nucleotides of the acceptor stem (C and A). The deoxy-C and A (dCA) dinucleotide is then chemically synthesized and used as an adapter to append an unnatural amino acid to truncated suppressor tRNA (**Figure 1.8**). Unnatural amino acids are chemically synthesized and their α-amino groups are protected by a photo- or I_2- labile protecting group (α-hydroxy acids are not protected). The free carboxylate of the amino acid is then activated as a cyanomethyl ester to facilitate acylation onto the dCA dinucleotide. This complex is then enzymatically ligated onto the acceptor stem of the truncated suppressor tRNA to yield amino-acylated tRNA.

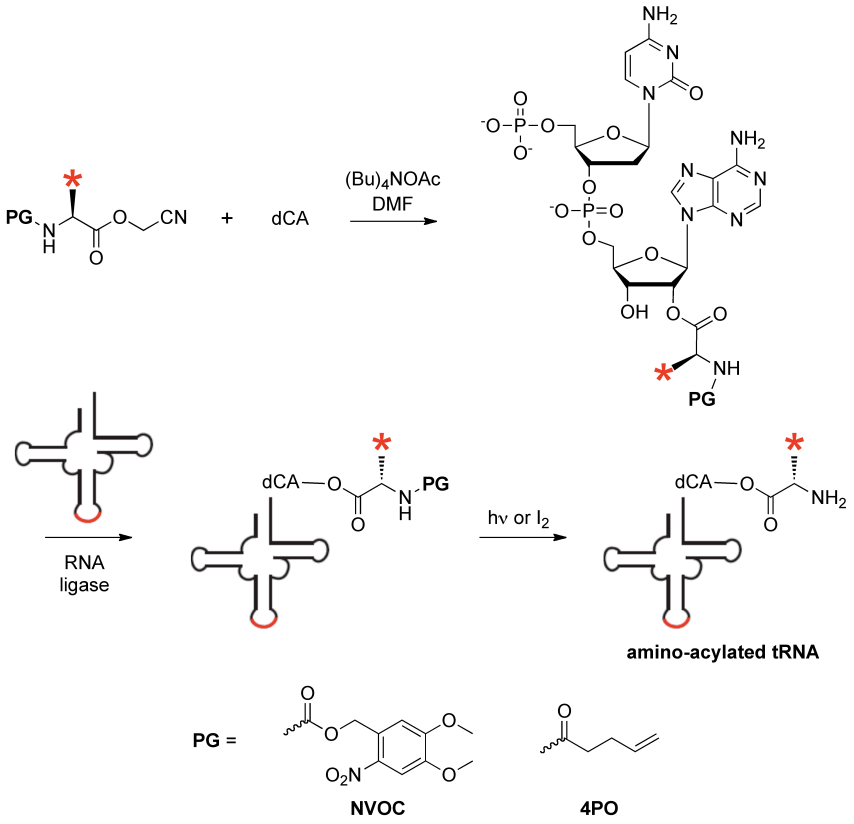

Figure 1.8. Schematic of the production of amino-acylated tRNA. The nitroveratryloxycarbonyl (NVOC) protecting group is photolabile and the 4-PO protecting group is removable by treatment with I_2.

With the mRNA bearing the stop codon and the amino-acylated tRNA in hand, we use heterologous expression to express the proteins in a native-like membrane environment (**Figure 1.9**). In our studies, we use *Xenopus* oocytes, egg cell precursors from an African frog, as our heterologous expression system. Upon injection of mRNA and amino-acylated tRNA, the oocyte synthesizes, folds, assembles, and transports the protein to the surface of the cell membrane. When expressed in a *Xenopus* oocyte, the pharmacology and physiology of the proteins are indistinguishable from those found in native environments.[45]

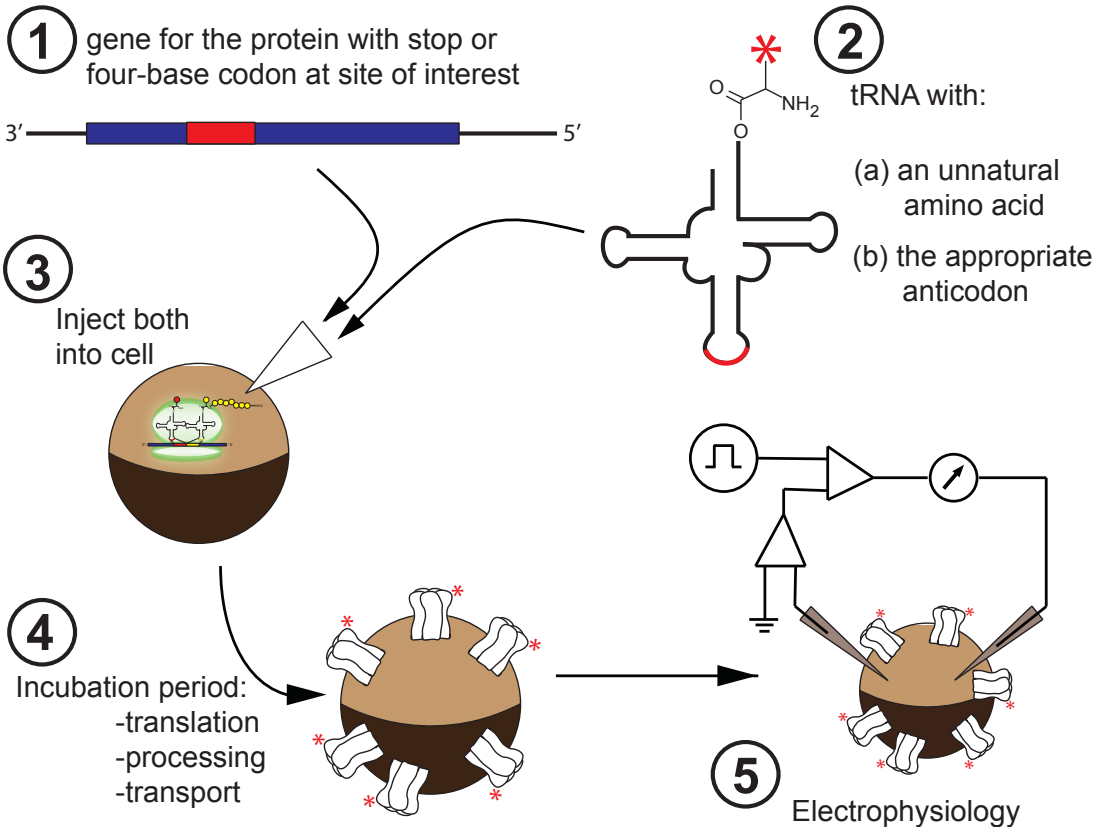

Figure 1.9. Implementation of the nonsense or frameshift suppression methodology for incorporating unnatural amino acids into ion channels expressed in *Xenopus* oocytes.

1.5 Electrophysiology as an Assay of Receptor Function

The suppressor tRNA used in these applications is a stoichiometric reagent. After incorporation of the unnatural amino acid into the protein, the tRNA cannot be amino-acylated with more unnatural amino acid within the cell. As such, protein yields cannot exceed the amount of injected tRNA. A sensitive assay is therefore needed to assess the function of mutant proteins. Because we are studying ligand-gated ion channel proteins that enable the flow of ions across the cell membrane, we can use the highly sensitive assay of two-electrode voltage clamp electrophysiology to monitor the impact of each mutation on protein function by measuring changes in agonist-induced current (**Figure 1.10**). In these assays, increasing concentrations of agonist are applied to the cell, which in turn, induce increasing currents (**Figure 1.10B**). From this, a dose-response relationship is generated, which is fit to the Hill equation (**equation 1**) to yield the EC_{50}– the concentration of the agonist that induces a half-maximal current or the midpoint of a dose-response curve (**Figure 1.10C**). We use EC_{50} as a convenient metric to compare ion channel function. Mutations that disrupt the function of a protein (loss-of-function mutations) will result in an increase in EC_{50}, because higher concentrations of agonist are required to evoke the same response. In contrast, a mutation that improves the function of the protein (a gain-of-function mutation) will require lower concentrations of agonist to achieve the same response and will therefore result in a lower EC_{50} value.

$$I_{Response} = \frac{I_{Max\ Response}}{1 + \left(\frac{EC_{50}}{[Agonist]}\right)^{n}} \quad \textbf{(1)}$$

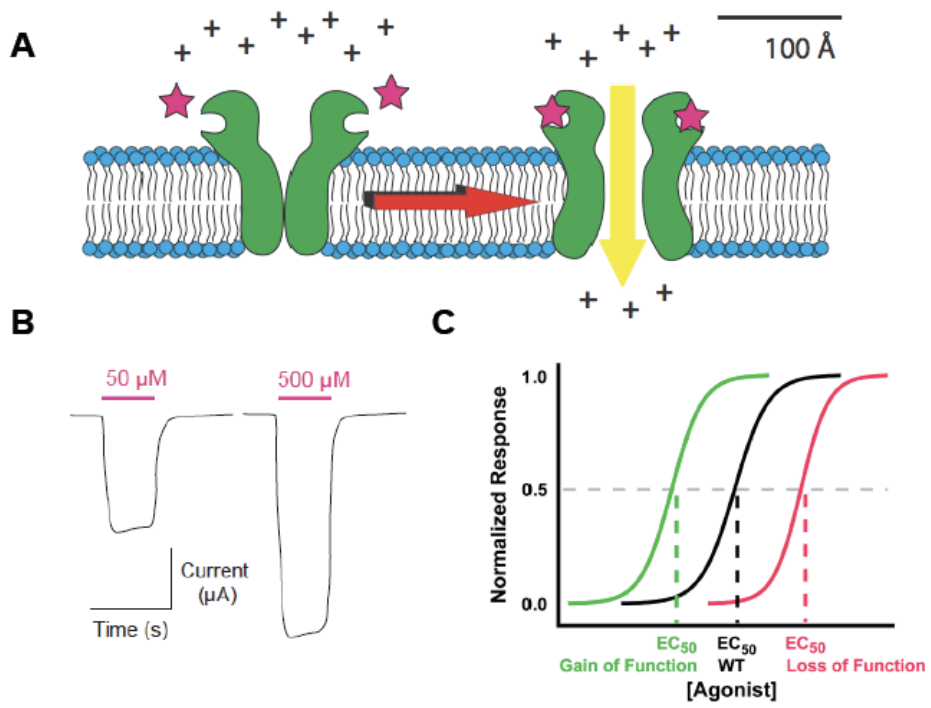

Figure 1.10. The electrophysiology assay. (A) Agonists bind to the ligand-gated ion channel and induce a conformational change in the protein that opens the ion channel pore. This facilitates the passage of ions across the membrane, producing a current that can be measured by electrophysiology. (B) Depiction of an agonist induced current measurement at varying concentrations. (C) Dose-response curves: The black curve represents the dose-response relation for the wild-type protein. The pink and green curves show the dose-response relation for a loss-of-function or gain-of-function mutation, respectively.

EC_{50} is a composite measure of an agonist's potency, which is influenced by the agonist's affinity for the receptor and also its ability to induce opening of the ion channel (efficacy). As such, we use EC_{50} as a metric to evaluate mutations that affect agonist binding or channel gating. Mutations that occur at the agonist binding site (60 Å away from the channel gate) are assumed to mainly affect binding while those farther away are expected to primarily affect gating, although there are exceptions.

1.6 Mutant Cycle Analysis

A common theme in many chapters of this thesis is to use mutant cycle analysis to understand the energetic coupling of a noncovalent interaction. Mutant cycle analysis is

the standard method used to measure the strength of intramolecular or intermolecular interactions in proteins or in protein-ligand complexes.[60] EC_{50}-based mutant cycle analyses have been employed by our lab[61-64] and others[65-67] to investigate many interactions in Cys-loop receptors. In this thesis, mutant cycle analysis is used to study protein-protein interactions between amino acid residues (Chapter 5) and also to study protein-agonist interactions (Chapters 2 and 3). The latter requires mutation of an amino acid of the protein and also "mutation" of the agonist by classical pharmacological strategies.

In the case of two non-interacting residues (or residue and agonist pair), mutation of one site should have no energetic impact on the second site, and so the effect of simultaneous mutation of both sites should be multiplicative.[60] In a double mutant cycle analysis, this is seen in a coupling coefficient (Ω) of 1 (unity), where $\Omega = [EC_{50}(WT) * EC_{50}(\text{double mutant})] / [EC_{50}(\text{mutant 1}) * EC_{50}(\text{mutant 2})]$. If, on the other hand, the two residues (or residue/agonist pair) do interact, then the effect of simultaneous mutation will be greater or less than the product of the individual effects. In our studies, we generally define a significant interaction as having an Ω of <0.2 or >5.

It is also standard practice to convert the coupling coefficient (Ω) into a free energy by the equation $\Delta\Delta G° = -RT\ln(\Omega)$.[60] This is a convenient metric that we consider to be approximately equivalent to the strength of the interaction being studied. A coupling energy of >1 kcal/mol is typically considered indicative of a strong noncovalent interaction.

Mutant cycle analysis can also take on multi-dimensional forms when studying the interaction of three residues in a protein or other higher levels of cooperativity. In

Chapter 5, a three-dimensional mutant cycle analysis is used to characterize the interaction of three amino acids that we believe are important for channel gating.

1.7 The Nicotinic Pharmacophore

The nAChR has the longest known, best-studied pharmacophore of any neuroreceptor. A pharmacophore is an abstract description of the essential chemical features (*e.g.*, functional groups) of a group of structurally related ligands that are required for molecular recognition by a biological receptor. The nicotinic pharmacophore was first discussed in a 1970 publication by Beers and Reich in which two essential components – a cationic nitrogen (N^+) and a hydrogen bond acceptor – were identified in the structures of ACh and nicotine.[68] In later years, this model was revisited by several researchers, notably Barlow and Johnson[69] and Sheridan and co-workers,[70] and from these studies it was suggested that there is an optimal distance that separates the two components called the 'internitrogen' distance (because most nicotinic agonists have a pyridine N and a cationic N). Many of these studies converge around an optimal distance of 4.8 Å by comparing the structures of several agonists, but this number has been a topic of much debate, especially given that many high affinity agonists like epibatidine have larger internitrogen distances.[71] A comparison of the structures and electrostatic potential maps of nicotinic agonists is shown in **Figure 1.11**.

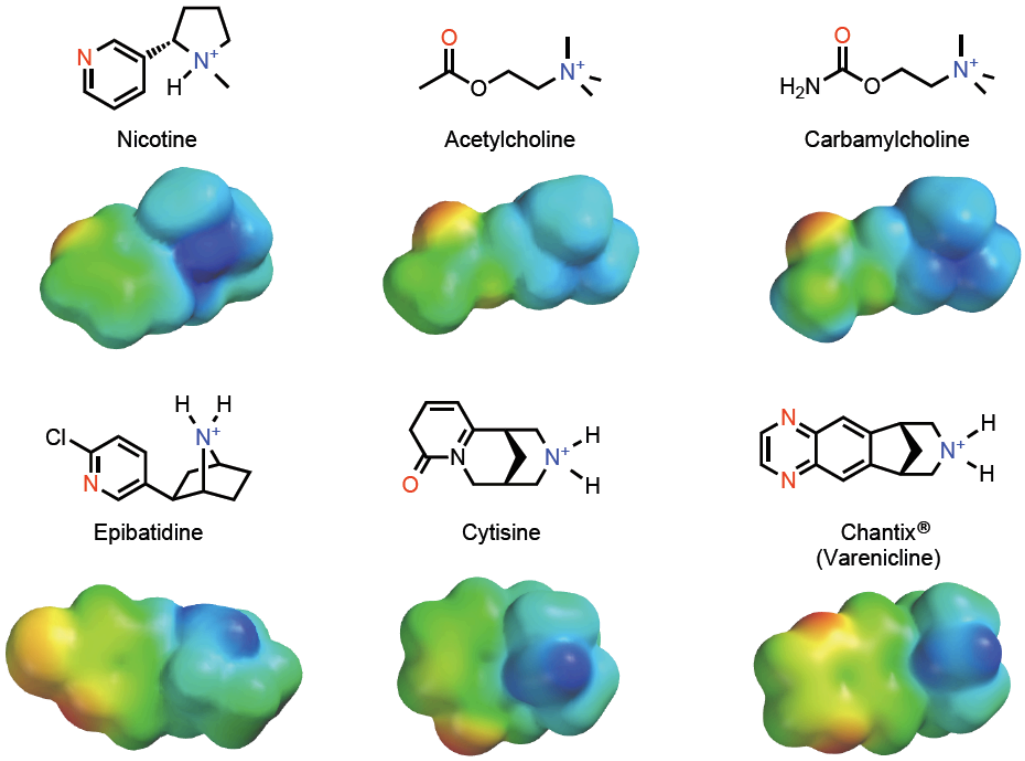

Figure 1.11. Structures and electrostatic potential maps of agonists of the nAChR. All structures contain the essential two-point pharmacophore – a cationic N (blue) and a hydrogen bond acceptor (red). Note that (+)-cytisine is shown in this figure, but (−)-cytisine is the natural isomer and is typically used in studies involving nAChRs.

In 1990, we suggested that the cationic N of acetylcholine could bind to an aromatic residue in the nAChR via a cation-π interaction.[72] Subsequent studies involving incorporation of fluorinated amino acid derivatives (as described above; also see **Figure 1.5**) validated this model in several nAChRs, showing that ACh, nicotine and other agonists make a cation-π interaction to TrpB of the aromatic box (**Figure 1.3B**).[33, 34, 73] AChBP structures with ligands bound confirmed this cation-π interaction and also suggested a second interaction to agonists with protonatable nitrogens (like the N^+ of nicotine) – a hydrogen bond to the backbone CO of TrpB.[18, 21] This interaction was subsequently validated by backbone ester mutagenesis in full-length receptors.[34, 73] The AChBP structures also suggested a hydrogen bonding interaction involving the hydrogen

bond accepting group of the pharmacophore,[18, 74] and this interaction is the focus of Chapters 2 and 3 of this thesis.

1.8 Summary of Dissertation Work

This dissertation primarily describes structure-function studies of the nAChR using a combination of unnatural amino acid mutagenesis, electrophysiology, mutant cycle analysis, and synthetic agonist analogs.

Chapters 2 and 3 describe studies aimed at probing binding interactions of the nicotinic pharmacophore using backbone ester mutagenesis and mutant cycle analyses with a synthetic analog of nicotine. These studies established a hydrogen bond between the pharmacophore's hydrogen bond acceptor, the pyridine N of nicotine or the acetyl CO of ACh, and a backbone NH of a residue in the complementary subunit. This interaction was shown to be important for binding of several agonists in the muscle-type receptor and also in both stoichiometries of α4β2. The only agonist that violates this binding model is Pfizer's smoking cessation drug, varenicline.

Chapter 4 is an application of the pharmacophore binding model that seeks to understand the stereoselectivity of agonist binding by the nAChRs. Given that the nAChR is a chiral molecule, it is surprising that the two epibatidine enantiomers are equipotent. It is known that methylation of the NH of epibatidine negatively impacts the potency of one of the enantiomers at the α4β2 receptor. In an attempt to understand these observations, we characterized the pharmacophore binding interactions of the epibatidine enantiomers and their *N*-methyl derivatives.

Chapter 5 describes studies concerning the vicinal disulfide, a conserved structural unit that defines the nAChR α subunits. We uncovered a hydrogen bond

network that was shown, by mutant cycle analysis, to link the peptide NH of the vicinal disulfide to another amide bond via a β turn and also to a functionally important residue in the complementary subunit. From this we propose that the role of the vicinal disulfide is to distort the β turn and thereby properly position a backbone NH that enables formation of the intersubunit hydrogen bond.

Strategies for the photochemical cleavage of protein and peptide backbones are described in Chapter 6. The first strategy is based on a selenide-mediated cleavage of a backbone ester moiety and utilizes an α-hydroxy acid whose side chain is a *o*-nitrobenzyl caged aryl selenide nucleophile. Studies with a model tripeptide establish the viability of the chemistry, but *in vivo* and *in vitro* studies of this methodology have been challenging. Also described are alternative backbone cleavage strategies based on aniline-mediated intramolecular cyclization and the photochemistry of the (2-nitrophenyl)ethyl (NPE) protecting group.

In Chapter 7, initial studies of a multi-institution collaboration are described. The preliminary studies presented herein seek to develop a small molecule strategy for photo-activation of a voltage-gated potassium channel. The ultimate goal of this collaboration is to develop a small molecule that can restore vision to patients whose retinal photoreceptors are compromised by disease.

Chapter 8 describes work that was completed prior to candidacy in the laboratory of Prof. Robert H. Grubbs in which a strategy was devised to prepare NHC-containing organometallic complexes from the thermolysis of 2-(pentafluorophenyl)imidazolidines. This is a simple, base-free method that could offer access to NHC complexes with functionality that is incompatible with other methods.

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