Chapter 1

Introduction

1.1 CHEMICAL SCALE NEUROBIOLOGY

One of the most challenging topics facing modern biology is understanding the complexities of neurobiology. The brain uses an intricate network of neurons to communicate information. The process of propagating information from one neuron to the next, termed synaptic transmission, is depicted in **Figure 1.1**. In this process, the axon of a pre-synaptic neuron sends an electrical signal, or action potential, to release a small molecule neurotransmitter (ligand) across a small synaptic cleft. A membrane receptor, or ligand gated ion channel, located on the dendrite of the post-synaptic neuron binds the ligand and undergoes a conformational change to allow the passage of ions through the otherwise impermeable cell membrane. Thus, the electrical signal of the action potential is converted to a chemical signal through the release of a neurotransmitter that is then converted to an electrical signal through the gating of a ligand gated ion channel (LGIC).

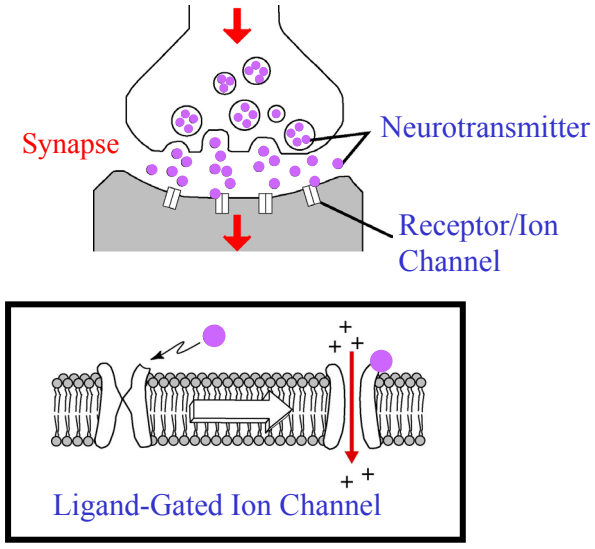


Figure 1.1. Synaptic Transmission. Neurotransmitters in an axon are released from vesicles across the synaptic cleft and bind to receptors on post-synaptic dendrites. Ligand gated ion channels are one type of receptor that binds the neurotransmitter, undergoing a conformational change to allow the passage of ions through the otherwise impermeable cell membrane.

Ligand gated ion channels (LGIC) are transmembrane proteins implicated in Alzheimer's disease, Schizophrenia, drug addiction, and learning and memory.[1,2] The ability of neurotransmitters to bind and induce a conformational change in these dynamic proteins is not fully understood. A number of studies have identified key interactions that lead to binding of small molecules at the agonist-binding site of LGICs. High-resolution structural data on neuroreceptors are only just becoming available,[3-8] yet functional data are still needed to further understand the binding and subsequent conformational changes that occur during channel gating.

The primary focus of the present work is to gain a chemical scale understanding of the ligand-receptor binding determinants of LGICs. In particular, these studies explore drug-receptor interactions at the nicotinic acetylcholine receptor (nAChR), the most extensively studied members of the Cys-loop family of LGICs, which include γ-aminobutyric, glycine, and serotonin receptors. Remarkably, several ligands are known to bind to the same region of the protein while eliciting different responses in protein activity, raising interesting chemical recognition questions. In addition, nAChRs are interesting therapeutic targets, and natural products, nicotine, epibatidine, and cytisine, serve as lead compounds for drug discovery.[2] For example, cytisine served as the lead compound for Pfizer's smoking cessation drug candidate, Varenicline, that targets nAChRs.[9] Therefore, chemical scale insights into drug-receptor interactions at the nAChR are interesting both from a chemical recognition perspective and from a drug discovery perspective.[2]

1.2 UNNATURAL AMINO ACIDS

In Vivo Nonsense Suppression

The present study performs chemical scale investigations of nAChR agonist activity though incorporation of unnatural amino acids. The ability to incorporate an unnatural amino acid site specifically into proteins is achieved through a method termed *in vivo* nonsense suppression.[10,11] This powerful tool has enabled successful structure-function studies of several ion channels including nicotinic acetylcholine receptors,[10] Shaker potassium channels, $K_V2.1$ potassium channels,[12] $5HT_3$ serotonin receptors,[13] and more recently $GABA_C$ receptors.[14] The present study applies this well-established method of incorporating unnatural amino acids to probe drug-receptor interactions at the nAChR.

This method is outlined in **Figure 1.2**. The mRNA encoding the ion channel is mutated to contain a UAG Amber stop codon. An orthogonal suppressor $tRNA_{CUA}$ containing a chemically ligated unnatural amino acid recognizes the UAG codon. The endogenous translational machinery incorporates the unnatural amino acid into the protein at the site of interest. The incorporation of unnatural amino acids into ion channels utilizes *Xenopus* oocytes[10] or mammalian CHO cells[15] as the translational machinery host. As shown in **Figure 1.2B**, the synthesized mRNA (***1***) and orthogonal *Tetrahymena thermophila* $tRNA_{CUA}$ (***2***), containing the stop codon and unnatural amino acid respectively, are introduced into the cell (***3***). The translational machinery of the cell then produces a full-length protein with the unnatural amino acid at the site of interest (***4***). This powerful method enables the translation of functional ion channels embedded in the membrane of the cell. The functional characteristics of the mutated membrane proteins are monitored through sensitive two-electrode voltage clamp assays (***5***).

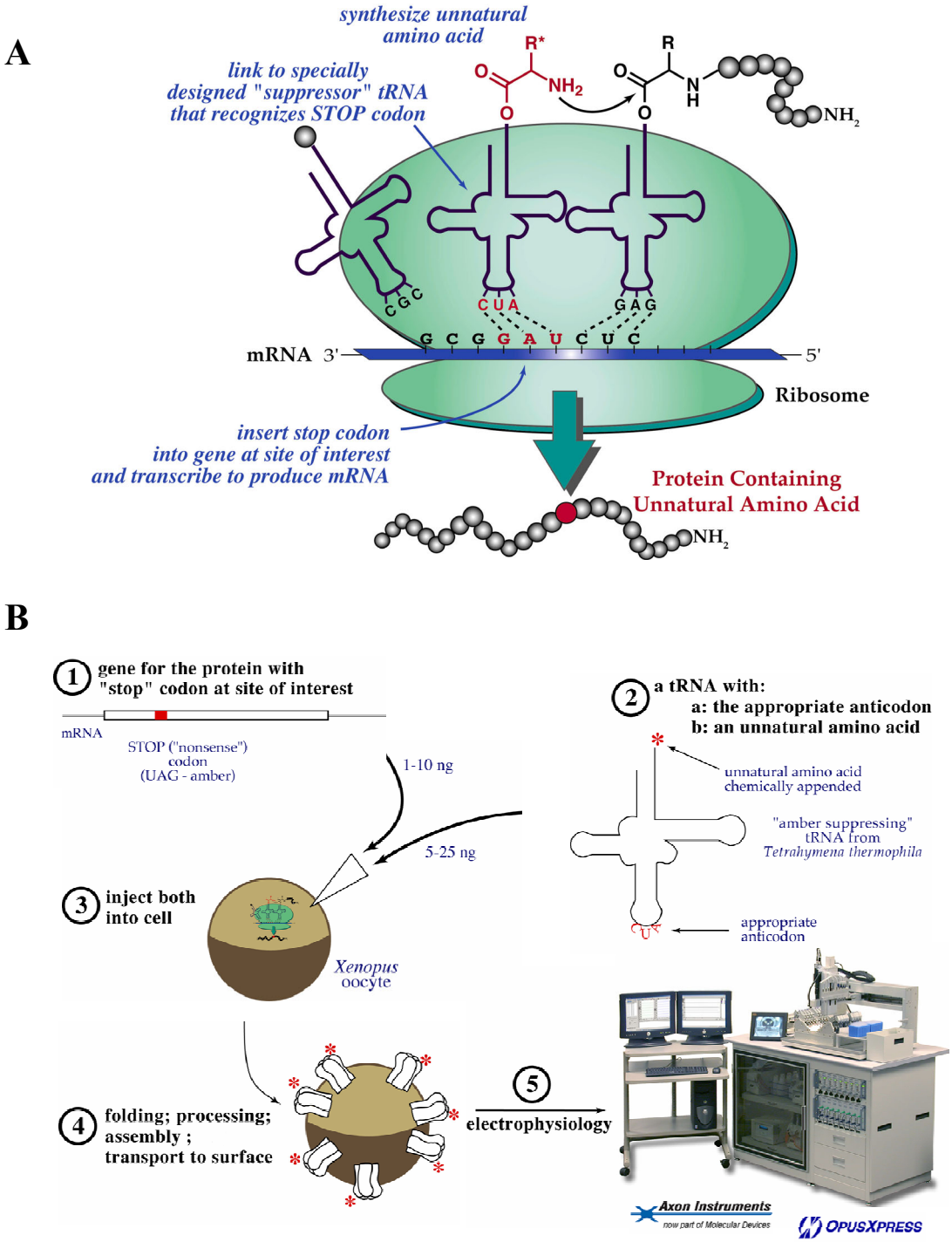


Figure 1.2. ***In Vivo*** **Nonsense Suppression.** **A**) Nonsense Suppression. mRNA containing a stop codon (UAG) is recognized by an orthogonal suppressor $tRNA_{CUA}$ that contains a chemically acylated unnatural amino acid (shown in red). The translation machinery incorporates the unnatural amino acid into the protein to produce full-length protein containing a single unnatural amino acid at the site of interest. **B**) *In Vivo* Nonsense Suppression in *Xenopus* Oocytes. mRNA and tRNA are injected into *Xenopus* oocytes, protein translation occurs, and protein function is monitored using high throughput electrophysiology.

The chemical acylation of an unnatural amino acid to *in vitro* transcribed orthogonal tRNA is shown in **Figure 1.3**. First, amino acids must be prepared by protecting the α-amine group with a photo-labile or I_2 cleavable protecting group to prevent destabilization of the free amine. It is often unnecessary to protect the α-hydroxy group of hydroxy acid analogues.[16] The carboxylic acid is then activated to react with dCA as a cyanomethyl ester.[10,16] The α-N-protected cyanomethyl ester or the α-hydroxy cyanomethyl ester is coupled to dCA dinucleotide and then ligated to a truncated 74 base $tRNA_{CUA}$ with T4 RNA ligase to yield the amino-acylated 76 base $tRNA_{CUA}$ (aa-tRNA). Immediately prior to injection into *Xenopus* oocytes, the α-N-protecting NVOC group or 4PO group is deprotected with 350 nm light or I_2, respectively.

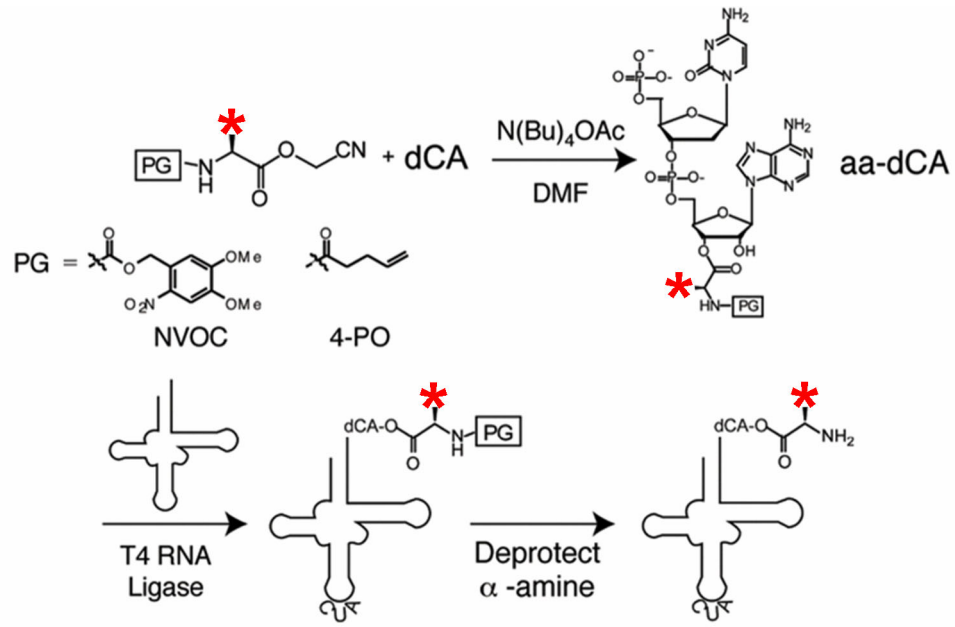


Figure 1.3. Preparation of Aminoacyl tRNA.[17]

History of *In Vivo* Nonsense Suppression

The methodology of incorporating unnatural amino acids in biological systems was developed by Peter Schultz in 1989.[18] The Schultz group combined several experimental observations in the field of nonsense suppression to develop this method. They utilized prior knowledge of the ability of amber suppressor tRNAs to recognize amber nonsense TAG stop codons and block translation suppression. In addition, studies demonstrating the ability of tRNA recognition to be independent of amino acid identity were utilized in combination with knowledge of the ability of the ribosome to incorporate a broad range of amino acid side chains. Precedent for the chemical strategy of aminoacylating suppressor tRNAs with amino acids was set by the work of Hecht and Brenner. The combination of these observations led to the first example of site-specific incorporation of unnatural amino acids into proteins.[18]

More recently, this method was adapted at Caltech and optimized for *in vivo* nonsense suppression of unnatural amino acids to probe ion channel structure-function relationships in *Xenopus* oocytes[10,11] and mammalian cells.[15] A limitation of this method is the small quantity of protein produced in cells. The translational host can only produce as much protein as the amount of aminoacylated tRNA, a stoichiometric reagent, present in the cell. Fortunately, studies of LGICs utilize a highly sensitive electrophysiology assay where protein amounts produced from nonsense suppression experiments are sufficient to monitor channel function. To date, Dougherty and co-workers have incorporated 100 unnatural amino acids into 20 different proteins at 140 different sites using this method. Structures of many of these successfully incorporated unnatural amino acids are shown in **Figure 1.4**.

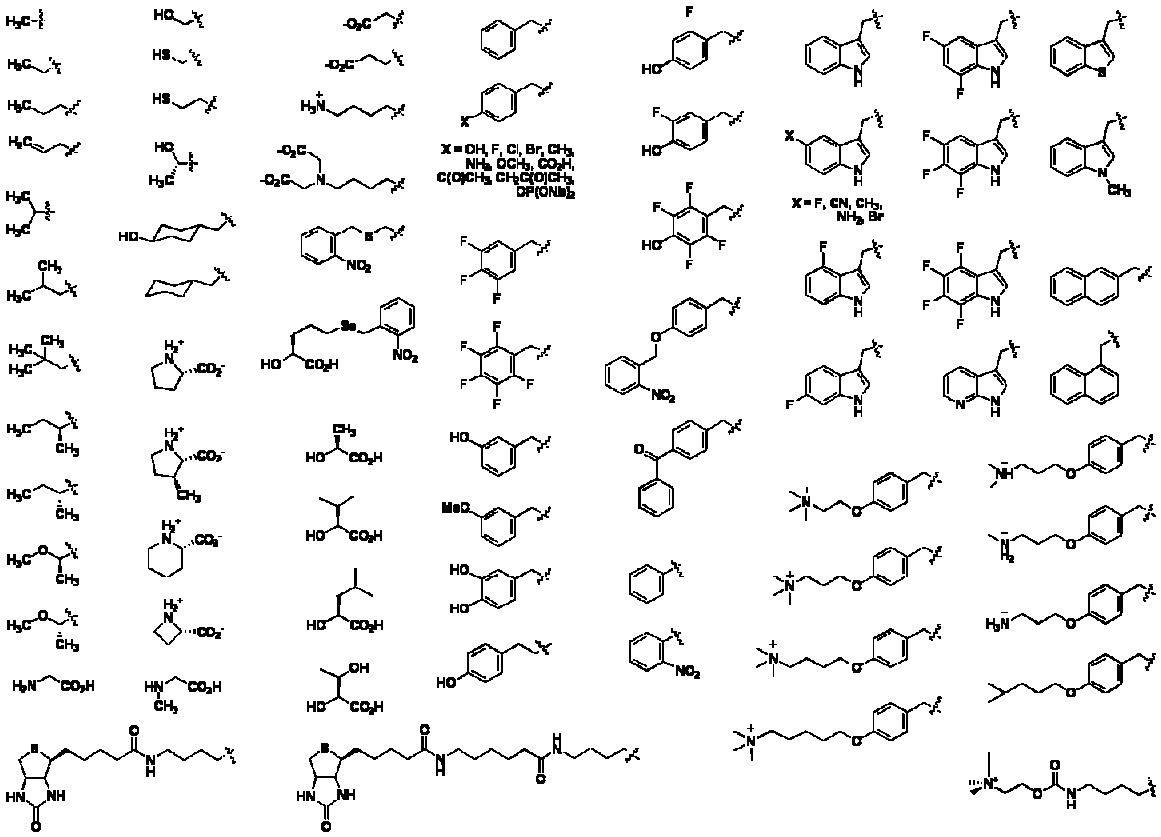


Figure 1.4. Unnatural Amino Acids Incorporated into Ion Channels Using *In vivo* Nonsense Suppression.

Alternate Methods of Incorporating Unnatural Amino Acids

While the present work utilizes *in vivo* nonsense suppression methodology in *Xenopus* oocytes, it is worth mentioning other methods for incorporating unnatural amino acids into proteins. The auxotrophic strain method of mutagenesis of Tirrell and co-workers and the evolution of orthogonal tRNA/synthetase pairs of Schultz and co-workers are described in detail in recent review articles[19-21] and are briefly discussed below. The auxotrophic strain method allows for residue-specific replacement of amino acids with unnatural residues in *E. coli*. By depleting an auxotrophic bacterial host of the natural amino acid and supplementing the system with the unnatural analogue, the Tirrell

method enables chemical modification of proteins at multiple sites.[20] This method is able to produce significantly more protein than the above-mentioned *in vivo* nonsense suppression methodology, but is limited to studies where residue-specific mutations are desired.

Recent advances for the site-specific introduction of unnatural amino acids by Schultz and co-workers significantly improve protein expression efficiency in comparison to *in vivo* nonsense suppression. In this method, the evolution of an orthogonal aminoacyl-tRNA synthetase to misaminoacylate a suppressor tRNA with an unnatural amino acid in *E. coli*, results in high protein yields.[19,21] This method has not been optimized for residues such as α-hydroxy acids that are metabolized in the cell and therefore are unable to acylate the suppressor tRNA *in vivo*.[21] Recently, Dougherty,[22] Schultz,[21] and Sisido[23] achieved site-specific incorporation of two different unnatural amino acids into the same protein by utilizing two different quadruplet stop codons. These methods provide powerful tools for conducting structure-function studies of proteins and potentially for creating proteins with enhanced therapeutic properties.

1.3 NICOTINIC ACETYLCHOLINE RECEPTORS

Nicotinic acetylcholine receptors (nAChR) are the most extensively studied members of the Cys-loop family of LGICs. The embryonic mouse muscle nAChR is a transmembrane protein composed of five subunits, $(\alpha_1)_2\beta_1\gamma\delta$ (**Figure 1.5**). Each subunit contains an extracellular ligand-binding domain at the N-terminus and four transmembrane domains (TM1-4). The second transmembrane domain, TM2, lines the interior of the channel pore. Early biochemical studies identified two agonist-binding

sites at the α/γ and α/δ interfaces on the muscle type nAChR that are defined by an aromatic box of conserved amino acid residues.[24,25] The principal face of the agonist-binding site contains four of the five conserved aromatic box residues, while the complementary face contains the remaining aromatic residue.

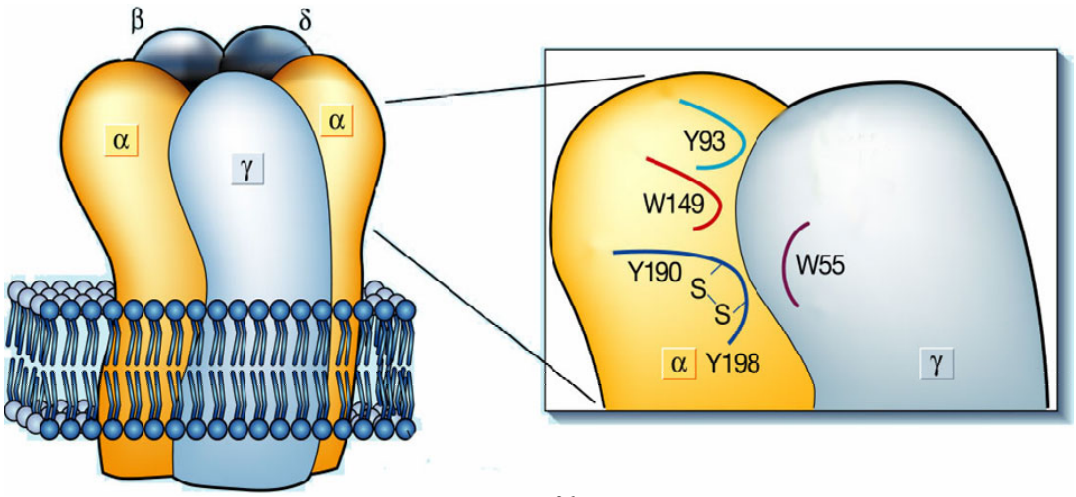


Figure 1.5. nAChR Subunit Arrangement.[26] Overall layout of the mouse muscle nAChR showing $(\alpha_1)_2\beta_1\gamma\delta$ subunits. The bindings sites reside at the interface of α/γ and α/δ subunits, where the majority of the binding site residues reside on the primary α subunit. Figure adapted from reference 26 by permission from Macmillan Publishers Ltd: Nature, copyright (2001).

In the past 5 years, knowledge of the ligand-binding domain has been dramatically advanced by the discovery[27] and crystallization of the acetylcholine-binding protein (AChBP).[5] AChBP is a homopentamer isolated from the snail *Lymnaea stagnalis* and it shares approximately 20 % sequence homology with the nAChR extracellular ligand-binding domain. In 2001, a 2.7 Å crystal structure of the acetylcholine-binding protein (AChBP)[5] confirmed early biochemical studies and provided additional structural information on the ligand-binding domain (**Figure 1.6 A, B**). Sixma and co-workers also

published a 2.2 Å nicotine-bound AChBP structure and a 2.5 Å carbamylcholine-bound AChBP structure in 2004.[28] In addition, the crystal structures of AChBP were solved in 2005 from the *Bulinus truncatus*[29] and *Aplysia californica* snail species.[30] These structures greatly impacted the field by providing insight for studies examining ligand-receptor interactions and by aiding in drug discovery.

While high resolution crystallographic data is difficult to obtain for transmembrane nAChRs, studies by Unwin and co-workers shed light onto the structure of the full-length receptor.[6,31-33] The structure of full-length nAChR was determined at 9 Å [31] and later at 4.6 Å resolution using cryo-electron microscopy.[32] More recently, Unwin generated a refined model at 4.0 Å resolution of *Torpedo* nAChR using insights from the AChBP structures (**Figure 1.6 C, D**).

These structures represent only static pictures and do not provide information on how these dynamic proteins transition from one conformation to another. Thus, more knowledge of protein transitions on the atomic level is still needed to fully understand structural rearrangements that occur when ligand binding at the agonist-binding site leads to the gating of channel residues nearly 60 Å away. The structural models of the full-length nAChR provide insights for recent studies that make significant advances towards understanding the gating mechanisms of these membrane proteins.[13,34,35] The complete mechanism, however, that couples ligand binding to channel gating of Cys-loop receptors is not fully understood and remains an important topic in molecular neuroscience.

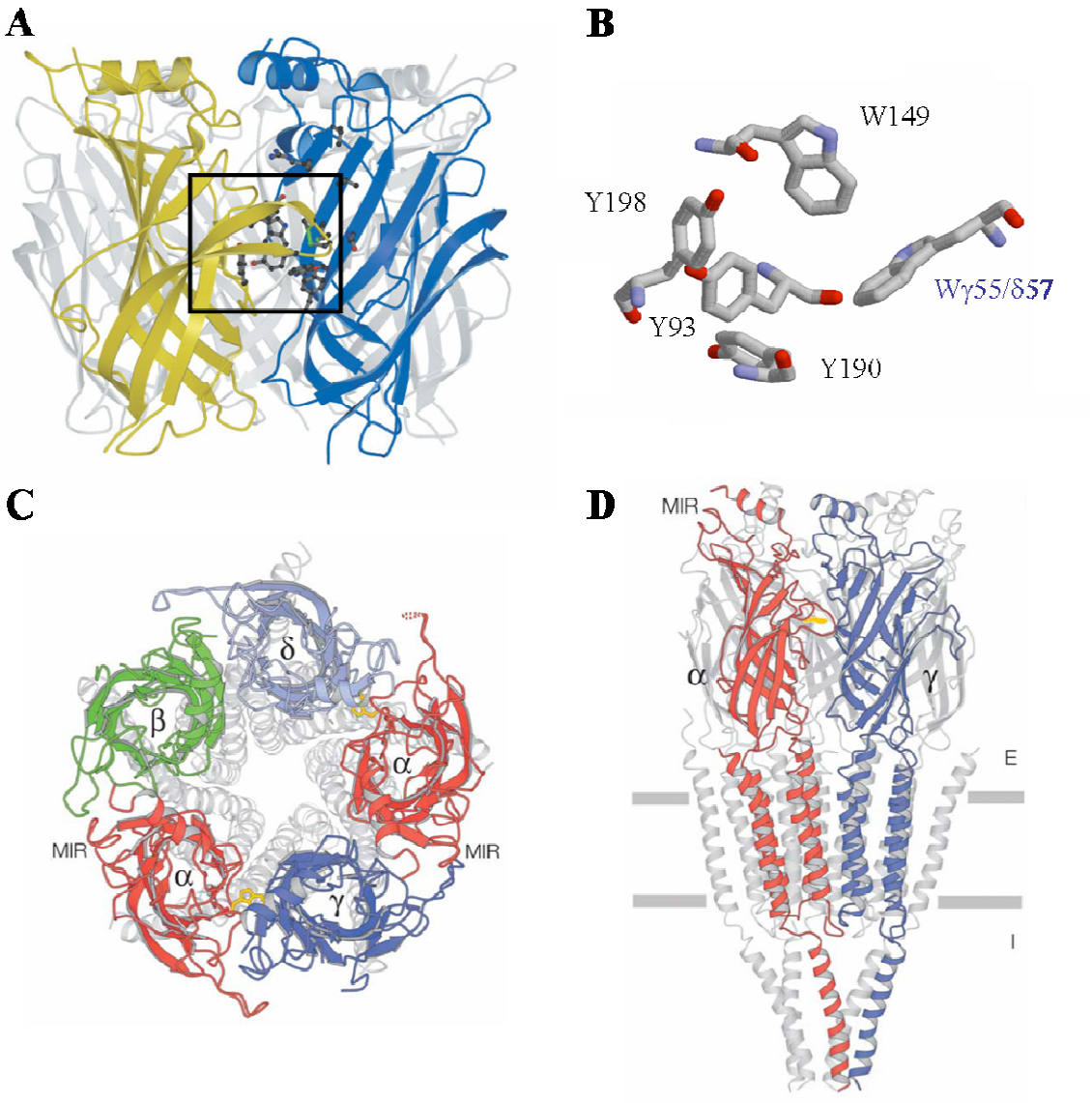
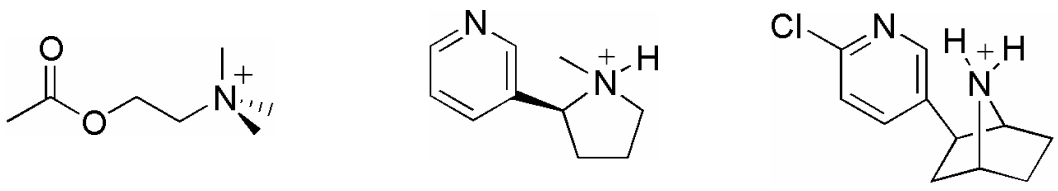


Figure 1.6. Structural Information for AChBP and nAChR. A) AChBP structure with two subunits highlighted and the binding site residues indicated.[5] Figure reprinted from reference 5 by permission from Macmillan Publishers Ltd: Nature, copyright (2001)**. B**) The aromatic binding site of AChBP with muscle nAChR numbering. Black numbers indicate α subunit residues while blue numbers indicate non-α subunit residues. **C**) Top view of Unwin's refined 4 Å cryo-electron model.[6] **D**) Side view of Unwin's 4 Å model indicating the extracellular, transmembrane, and intracellular regions.[6] Figures C & D reprinted from reference 6 with permission from Elsevier.

1.4 nAChR DRUG-RECEPTOR INTERACTIONS

The primary focus of the present work is to gain an understanding of ligand-receptor interactions at the mouse muscle nAChR. We utilize chemical scale investigations to identify mechanistically significant drug-receptor interactions at the muscle-type nAChR as predicted by AChBP structures. Interestingly, structurally similar nAChR agonists acetylcholine (ACh), nicotine, and epibatidine (**Figure 1.7**) are known to bind to the same region of the protein while eliciting different responses in protein activity. These three agonists also display different relative activity among different nAChR subtypes. A better understanding of residues that play a role in determining agonist activity and specificity would provide insight into mechanisms that underlie agonist binding and channel gating. This information could also aid in designing nAChR therapeutics.



Acetylcholine **Nicotine** **Epibatidine**

Figure 1.7. Structures of nAChR Agonists.

The goals of this thesis are threefold. First, the study incorporates unnatural amino acids at the ligand binding site to probe agonist binding determinants that differentiate acetylcholine, nicotine, and epibatidine agonist nAChR activity. Second, the study identifies residues in the shell of amino acids immediately surrounding the agonist binding box that are important in shaping the ligand binding site for all three agonists.

Third, the study examines residues surrounding the agonist-binding site that contribute to ACh, epibatidine, and nicotine specificity.

1.5 DISSERTATION SUMMARY

The work presented in this thesis centers on drug-receptor interactions at the mouse muscle nAChR. Chapter 2 describe studies that probe the binding of three distinct agonists–acetylcholine (ACh), nicotine, and epibatidine–to the nAChR using unnatural amino acid mutagenesis. Results from these studies reveal how three structurally similar agonists bind to the same binding site through quite different non-covalent binding interactions to activate the receptor. This chapter is based on a *Journal of the American Chemical Society* paper written in collaboration with E. James Petersson. James Petersson conducted computational modeling studies to supplement the experimental data.

The work in Chapter 3 describes the ability of conserved residues immediately outside of the aromatic binding box to interact with binding site residues and to play a role in determining nAChR activity. Part A of this work examines a network of hydrogen bonds between an outer shell residue and residues in the aromatic box. These studies demonstrate an important role for this residue in stabilizing the agonist-binding site. These studies were performed in collaboration with Michael Torrice who designed an unnatural amino acid to probe the importance of charge in this region. Part B of this work examines a highly conserved residue immediately surrounding the agonist binding box that is proposed to reposition its side chain upon ligand binding. With additional

evidence from other recent advances, this site is proposed to be important in initiating the nAChR channel gating pathway.

The work in Chapter 4 utilizes computational protein design to probe residue positions that affect nAChR agonist specificity for acetylcholine, nicotine, and epibatidine. Results from these studies identify mutations that enhance nAChR specificity for nicotine, over ACh and epibatidine compared to wild-type receptors. This project was conceptualized through collaboration with Jessica Mao in Steve Mayo's lab who generated the computational predictions.

Finally, Chapter 5 reflects studies conducted prior to candidacy in Peter Dervan's lab. Part A of this work evaluates the ability of a series of cationic polyamides to enhance polyamide affinity while maintaining specificity by varying the number, relative spacing, and linker length of aminoalkyl side chains. These studies were performed in collaboration with Ben Edelson who synthesized *N*-aminohexyl and *N*-aminodecyl pyrrole containing polyamides. Part B of this work examines the nuclear uptake properties of these polyamides in mammalian cells, also performed in collaboration with Ben Edelson.

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