

Chapter 1: An Introduction to Chemical-Scale Neuroscience

1.1 Toward a Chemical-Scale Understanding of the Brain

For centuries, the human brain has inspired generations of scientists and non-scientists alike. With a hundred billion nerve cells wired together to form an estimated million billion synapses that endow our species with thought, emotion, and consciousness, it is the most complex structure, natural or artificial, on earth.¹ As such, unraveling its mysteries remains a formidable challenge for the 21st century and beyond. Until relatively recently, most systematic studies of brain function took a "black box" approach. Theories were formulated and tested using behavioral endpoints with the goal of predicting or treating behavior, ignoring how the brain creates that behavior. To accomplish a more complete understanding of the human brain and move beyond behavior-based therapies, scientists had to break open the veritable black box within our skulls and interrogate brain function on the cellular and molecular levels.

This odyssey began just over a century ago, with the development of a rudimentary appreciation of how cells in the brain are connected.² It is now well established that nerve cells in the brain, or neurons, have branchlike extensions called axons and dendrites that they use to communicate with one another. Information is passed from one neuron to another through a combination of electrical and chemical signals. A nerve impulse travels down the axon of one neuron until it reaches the synapse, or gap between neurons. The electrical signal then triggers the release of small-molecule neurotransmitters that diffuse across the synapse and bind to receptors on the neighboring neuron. In binding to and activating receptors on the neighboring neuron, these neurotransmitters initiate a new electrical signal that then further propagates the message. This process is called synaptic transmission, and its regulation is believed to underlie

much of higher brain function. As a result, much work in modern neuroscience is devoted to understanding the synapse.

1.2 The Nicotinic Acetylcholine Receptor: The Prototypical Cys-Loop Receptor

Arguably some of the most interesting synaptic proteins are those involved in mediating neuronal communication by binding neurotransmitters. The neurotransmitters that mediate synaptic transmission activate two classes of receptors: G protein-coupled receptors (GPCRs) and ligand-gated ion channels. While GPCRs are critical in modulating synaptic transmission, the majority of fast synaptic transmission in the brain is mediated by ligand-gated ion channels. These receptors respond directly to the binding of small-molecule neuroreceptors by opening an integral pore within 1-100 ms.

The Cys-loop superfamily of ligand-gated ion channels constitutes an important class of these fast-acting neuroreceptors.³⁻⁵ Among this superfamily of ion channels are the receptors that bind serotonin, acetylcholine, and nicotine, and their functions are implicated in a variety of neurological disorders. The Cys-loop receptors are comprised of a pentamer of subunits arranged around a central ion-conducting pore. Subunits further share a homologous superstructure, including a large extracellular domain, which is involved in agonist binding; four transmembrane domains (M1-M4), which form the central ion-conducting pore; and a short extracellular carboxy terminus. Early studies on the Cys-loop family were made possible in large part because the abundance of one member of this family, the muscle subtype of the nicotinic acetylcholine receptor (nAChR) found in the cells of electric organs of the *Torpedo* electric ray.⁶ For this reason, the muscle nAChR has historically been the best-characterized member of the Cys-loop family and is generally considered the prototypical Cys-loop receptor.

The muscle nAChR consists of four distinct subunits with the stoichiometry $\alpha_2\beta\gamma\delta$.⁷ This affords the receptor with two nonequivalent binding sites formed at the interface between the α -subunit (principal component) and the γ - or δ -subunit (complementary component). Several noncontiguous regions of both the principal and complementary subunits contribute amino acids to the compact pocket that is the agonist binding site. The structure of the nAChR is illustrated in figure 1.1A-E. These regions are referred to as loops A, B, and C (principal) and D, E, and F (complementary) (Figure 1.1B,C). Five highly conserved residues form what is known as the aromatic binding box (Figure 1.1E) and include α Y93 (loop A), α W149 (loop B), α Y190 and α Y198 (loop C), and γ W55 or δ W57 (loop D). The face of α W149 makes a cation- π interaction with the endogenous agonist, acetylcholine, constituting a major non-covalent binding interaction.⁸ Other residues in the aromatic binding box have been proposed to play a role in shaping the agonist binding site and transmitting binding events to channel opening events;⁹⁻¹² a process referred to as gating. Furthermore, the motions of the C-loop are strongly implicated in the gating process.^{10, 13-16}

Gating culminates in the opening of the central ion-conducting pore within the transmembrane domain. The second transmembrane α -helix lines the channel pore, with each subunit contributing several highly conserved hydrophobic residues to the gate of the channel. Of these, the L9' residue (simplified transmembrane numbering system for the M2 domain¹⁷) resides at approximately the halfway point and comprises the narrowest constriction point in the Cryo-EM structure of the *Torpedo* nAChR (Figure 1.1D).¹⁶ It has been demonstrated to play a critical role in gating, and mutating this residue to a more polar amino acid stabilizes the pore in an open, ion-conducting conformation.^{18, 19}

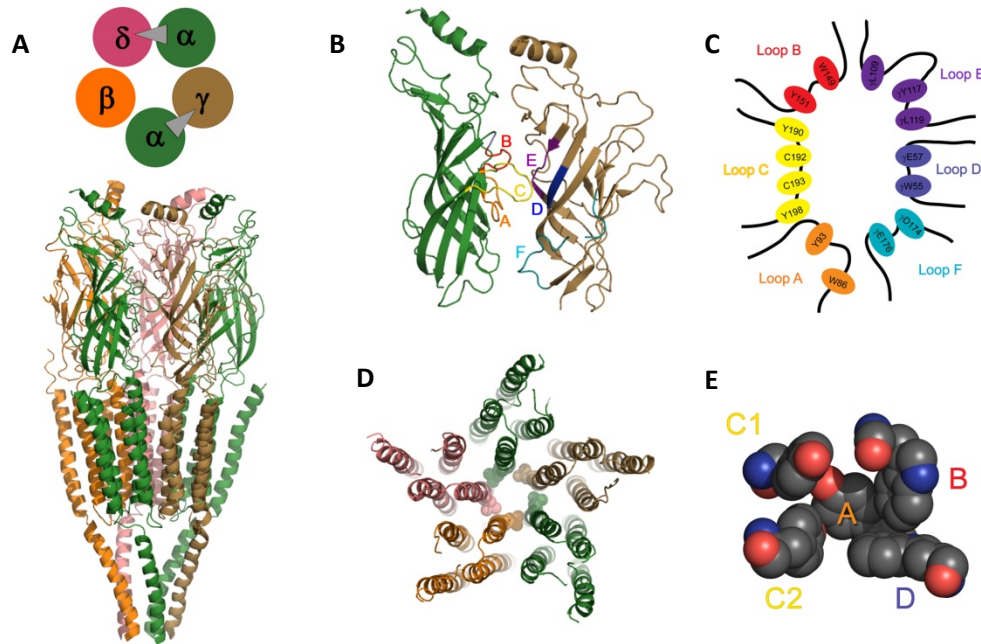


Figure 1.1. The general topology of the muscle nAChR. (A) The muscle nAChR has two α_1 (green), one β_1 (orange), one γ (brown), and one δ (pink) subunit arranged around a central ion-conducting pore. The binding sites (grey triangles) are at the $\alpha\gamma$ and $\alpha\delta$ interfaces. (B) The binding sites are located within the largely β -sheet extracellular domain with contributions from six loops: A (orange, primary subunit), B (red, primary subunit), C (yellow, primary subunit), D (blue, complementary subunit), E (purple, complementary subunit), and F (cyan, complementary subunit). (C) Amino acid contributions to the agonist binding site from the loops. (D) The transmembrane region is comprised of four transmembrane-spanning helices from each subunit. The second transmembrane helix (M2) lines the ion-conducting pore. The L9' residue is highlighted as space-filling. (E) The structure of the aromatic binding box, with contributions from loops A, B, C (primary subunit), and D (complementary subunit).

1.3 Heterologous Expression of Synaptic Proteins and Electrophysiological Characterization

The synapse itself is a dizzyingly complex structure. It not only contains the receptors and ion channels that bind various neurotransmitters, but also numerous scaffolding proteins that control spatial arrangements and signaling proteins involved in downstream cascades beyond the simple further propagation of the electrical signal. Many of these proteins interact with each other and these interactions are constantly in flux, both spatially and temporally. Untangling this web of interactions is the focus of cellular neuroscience and is an immensely important goal for eventually understanding brain function. To fully do this, however, we must understand something about the synaptic proteins on a molecular level. The most efficient way

of interrogating the structure and function of these synaptic proteins is to isolate them from the seeming chaos of the synapse. Thus, studying the molecules of the synapse requires us to leave the synapse.

Heterologous expression of proteins provides an elegant way of expressing these synaptic proteins in a controlled native like environment without the overwhelming complexity of the synapse. In particular, the large size of a *Xenopus oocyte* (1 mm) makes it particularly advantageous as a vessel for the expression of synaptic proteins such as ligand-gated ion channels. The size affords relative ease both in terms of (1) the delivery of the genetic material that encodes the synaptic protein and (2) characterizing the behavior of the expressed protein. When the expressed protein is an ion channel, electrophysiology provides a sensitive assay that is a direct measure of function. In the synapse, the creation of an electrical current by the flow of ions through the ion channel (Figure 1.2A) allows for the further propagation of the signal in synaptic transmission. In the case of a heterologously expressed ion channel, however, generated current provides a convenient and extremely sensitive readout of function.

Receptor function can be assayed at the whole-cell level, using the response of all the ion channels being expressed in the cell membrane, or on the single-channel level, looking at a single channel opening and closing. For whole-cell characterization, the aggregate passage of ions through the expressed ion channels produces a cell current measurable by two-electrode voltage clamp.^{20, 21} When the concentration of agonist increases, the net current also increases as more receptors are in an active ion-conducting state (Figure 1.2B). Thus, a range of agonist concentrations is used to construct a dose-response relationship for the ion channel (Figure 1.2C). This is, in turn, fitted to the Hill equation to give the EC_{50} , or effective concentration at half maximum response, which is a convenient metric for assessing ion channel function. A loss

of ion channel function is marked by an increase in the whole-cell EC_{50} , where higher concentrations of agonist are necessary to evoke the same response (Figure 1.2C, green curve). Conversely, if the ion channel opens in response to lower concentrations of agonist (gain-of-function), this would be reflected by a lower EC_{50} value (Figure 1.2C, blue curve).

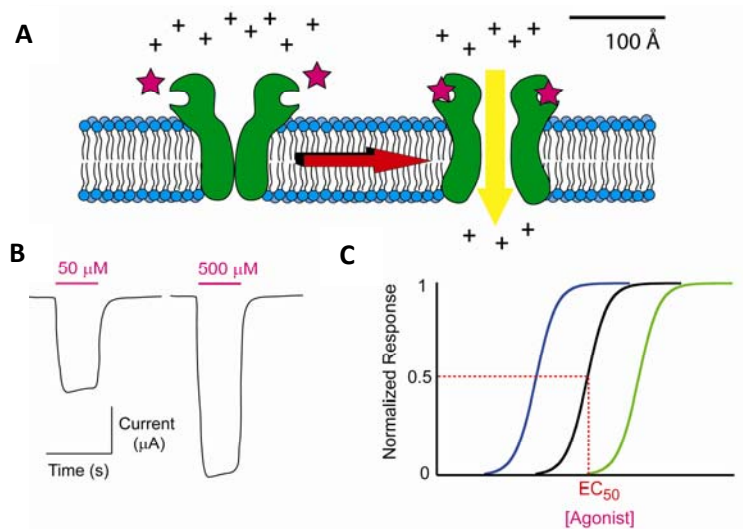


Figure 1.2: Basics of an electrophysiology assay. A) Agonist (pink stars) binding to the ion channel promotes channel opening. Once open, the channel allows the passage of ions across an otherwise impermeable membrane, producing a measurable current. **B)** Examples of current response to varying agonist concentrations. **C)** Example dose-response curves. The black curve represents the dose-response relation of an unmodified (wild-type) receptor. The blue and green curves show shifts in this relationship in response to gain-of-function and loss-of-function mutations, respectively.

The goal of taking the ion channels out of the synapse is to characterize their structure and function, which can be accomplished using conventional site-directed mutagenesis. Replacing a single amino acid residue with one of the other 20 canonical amino acids can A) provide a sense of the importance of a particular residue and B) provide clues as to the role of that amino acid in overall protein function. However, the structural variation available in the 20 natural amino acids is neither broad nor systematic enough to enable an in depth, chemical-scale understanding of protein structure and function. For instance, if we wish to understand the role of a glutamate residue in a protein- its participation in hydrogen bonding, for example- we could conventionally mutate to an alanine residue to completely remove the side chain

functionality. A less dramatic mutation would involve replacing the carboxylate of glutamate with an amide (e.g., glutamine).

However, this alters both the hydrogen bonding ability and the steric properties of the side chain (Figure 1.3). Interpreting changes in function is thus complicated by effects that are secondary to the question at hand. If we want to understand the role of charge, a nitro functionality (nitrohomoalanine, in this

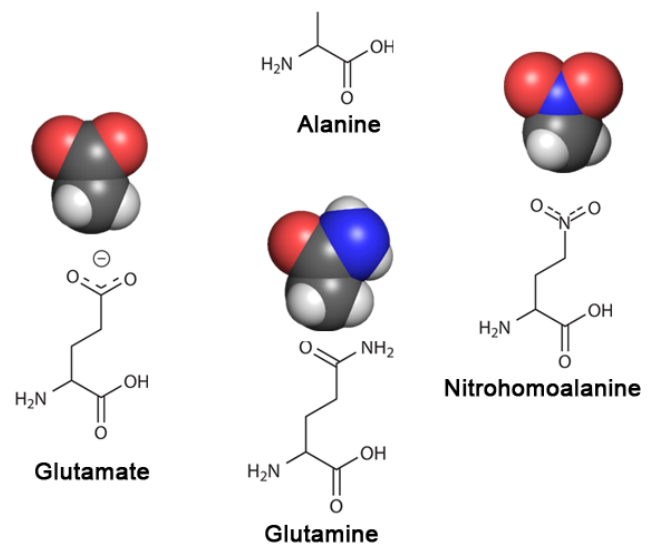


Figure 1.3: Natural and unnatural amino acids for testing the function of the glutamate side chain.

case) would provide a sterically similar, but charge-neutral alternative to the carboxylate of glutamate. Of course, the nitro functionality is absent among the natural amino acids.

In addition to the limited chemical functionality of the twenty natural amino acids, some structural modifications, such as altering the peptide backbone, are impossible using conventional mutagenesis. Unnatural amino acid mutagenesis allows us to move past these limitations and achieve the precise control over structural variation that we desire.

1.4 Unnatural Amino Acid Incorporation

The nonsense suppression methodology was developed in the late 1980s to allow researchers to site specifically incorporate unnatural amino acids.²²⁻²⁴ In normal protein synthesis, the ribosome decodes messenger RNA by matching set three-nucleotide codes, or codons, with the anticodon region of a tRNA molecule bearing the specified amino acid. The nonsense suppression method borrows one of the cell's stop codons (typically UAG or UGA) to

serve as the codon for the unnatural amino acid. An orthogonal suppressor tRNA with a complementary anticodon that recognizes the stop codon bears the desired unnatural amino acid. The result is that instead of terminating at the appropriated stop codon, the ribosome incorporates the unnatural amino acid into the growing polypeptide chain (Figure 1.4).

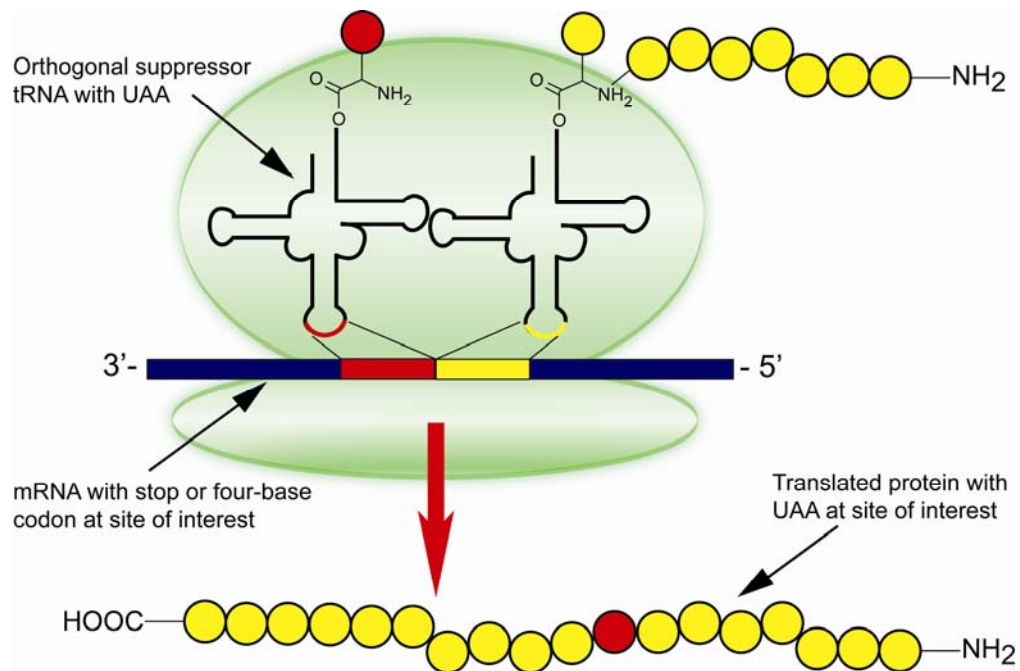


Figure 1.4: Overview of unnatural amino acid incorporation using the nonsense and frameshift suppression methodologies.

A related, alternative method for unnatural amino acid incorporation uses a four-base codon instead of a stop codon.^{25, 26} This method uses a suppressor tRNA with a matching four-base anticodon to incorporate the unnatural amino acid and suppress the shift in reading frame that would normally occur when the ribosome encounters a four-base codon. Appropriately, this method is known as frameshift suppression.

Both methods require the same basic combinations of simple molecular biology and chemical synthesis (Figure 1.5).²⁷⁻²⁹ Using standard mutagenesis protocols, the gene for the target protein is mutated to contain a stop or four-base codon at the site of interest and

subsequently used to transcribe the mRNA transcript. The suppressor tRNA lacking the final two nucleotides of the acceptor stem (C and A) is likewise transcribed *in vitro*. These final nucleotides of the acceptor stem are used as a handle for the unnatural amino acid, both of which are chemically synthesized separately before being chemically joined. Once prepared, the dCA-amino acid is ligated onto the truncated tRNA body using the T4 RNA ligase. Injecting the prepared mRNA and tRNA into the *Xenopus oocyte* provides the necessary genetic material for the cellular machinery to translate, process, and transport the target protein to the cell membrane. An incubation period of 18- 48 hours is required for this process, after which the impact of the unnatural amino acid at the site of interest can be assayed.

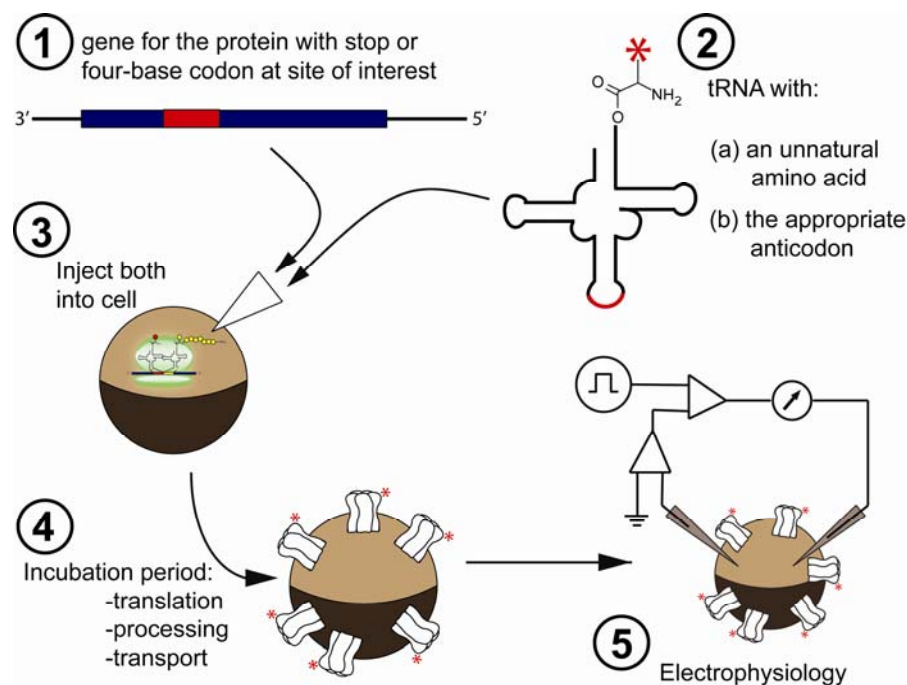


Figure 1.5: Implementation of the nonsense or frameshift methodologies for incorporating unnatural amino acids into ion channels in *Xenopus laevis* oocytes.

Control experiments are simultaneously performed to ensure the fidelity of unnatural amino acid incorporation. Injecting mRNA alone tests for readthrough of the stop or four-base codon by the ribosome. The impact of misacylation, a process in which the cell's synthetases

append a natural amino acid onto the exogenous tRNA body, can be controlled for by injecting the mRNA along with suppressor tRNA with no amino acid appended to the acceptor stem. Both readthrough and misacylation can lead to a heterogeneous population of proteins, obscuring the impact of the unnatural amino acid at the site of interest. Finally, wild-type recovery experiments, in which the natural amino acid is appended to the suppressor tRNA, ensure that normal protein function can be recovered using the nonsense or frameshift suppression methodology.

1.5 α -Hydroxy Acid Incorporation for Probing the Peptide Backbone

The power of unnatural amino acid mutagenesis is that it allows us to ask, and answer, otherwise inaccessible questions about protein structure and function. One prime example of this is the ability to modify the peptide backbone by incorporating unnatural α -hydroxy acids. Upon incorporation, α -hydroxy acids confer backbone flexibility by removing the amide bond rigidity and disrupting the hydrogen bonding ability of a residue. Hydrogen bonding ability is critical for secondary protein structure, as it is a key component of both α -helices and β -sheets (Figure 1.6). In addition, individual backbone-to-side chain and backbone-to-backbone hydrogen bonds can play a significant role in modulating protein function.

By replacing an amino acid with an α -hydroxy acid, the critical NH is replaced by an oxygen, thus eliminating the ability of the residue to act as a hydrogen bond donor. This mutation also diminishes the ability of the neighboring carbonyl to act as a hydrogen bond acceptor, as the carbonyl of an ester is a weaker hydrogen bond acceptor than that of an amide. This mutation also potentially introduces unfavorable electrostatic interactions between the ester oxygen and the amide carbonyl (Figure 1.6). Overall, however, the amino-to-hydroxy acid

mutation is subtle, maintaining the original side chain properties and the amide backbone conformational properties (i.e., bond lengths, angles, cis/trans preferences).

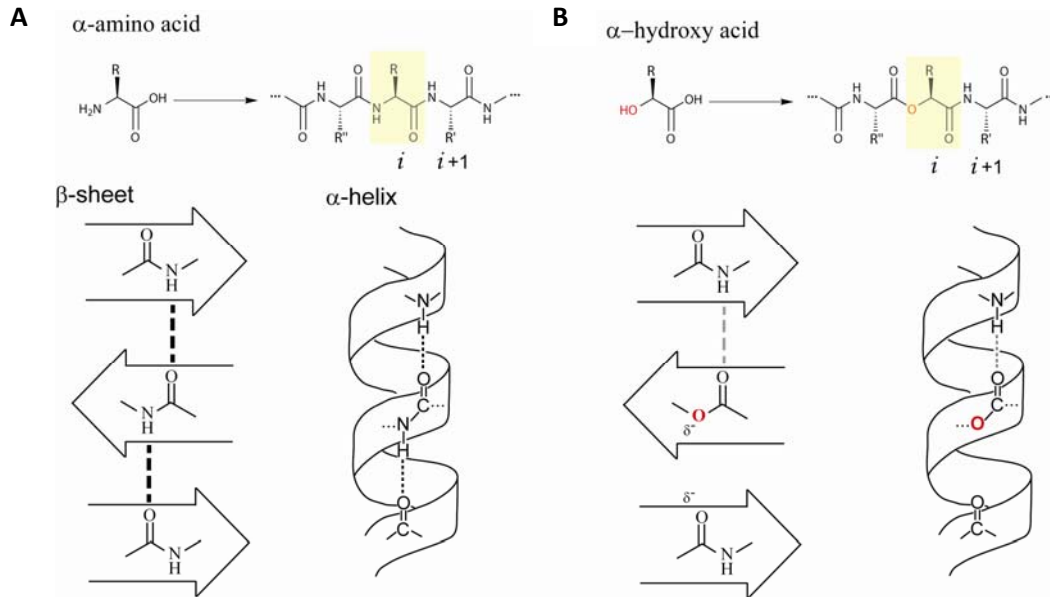


Figure 1.6: Amide-to-ester mutations. A) Normal α -amino acid incorporation in a polypeptide and its hydrogen bonding patterns in β -sheets and α -helices. B) The impact of α -hydroxy incorporation on hydrogen bonding in α -helices and β -sheets.

1.6 Dissertation Research

This dissertation describes one *in silico* and three experimental studies that consider the impact of ester backbone mutations in the extracellular domain of the muscle nAChR. One additional chapter describes a method for using whole-cell data to more fully characterize the functional impact of a mutation.

Chapter 2 describes the discovery of a specific intersubunit hydrogen bond between the backbone of a residue in the C-loop and the side chain in the complementary binding subunit. This resolved the role of a conserved asparagine residue originally thought to directly participate in agonist binding. In chapter 3, the role of the peptide backbone is considered more broadly in

the context of the outer β -sheet that connects the agonist binding site to the transmembrane domain. We determined that single α -hydroxy mutations were well tolerated in this region, causing only minor functional perturbations. However, the presence of two nearby backbone esters was extremely detrimental to receptor function. Chapter 4 discusses the ELFCAR methodology for using readily obtainable whole-cell data to determine whether a particular mutation impacts channel gating. This method is particularly useful for elucidating gating function in residues that are physically removed from the channel gate, such as those in and around the binding site. Chapter 5 discusses the impact of amide-to-ester mutations in the agonist binding box. Finally, chapter 6 discusses the complementary use of molecular dynamics to understand the effect of α -hydroxy acid incorporation.

1.7 References:

1. Green, T., Heinemann, S.F. & Gusella, J.F. Molecular neurobiology and genetics: investigation of neural function and dysfunction. *Neuron* **20**, 427-44 (1998).
2. Ramón y Cajal, S., Pasik, P. & Pasik, T. v. <1, 3 > (Springer, Wien ; New York, 1999).
3. Miller, P.S. & Smart, T.G. Binding, activation and modulation of Cys-loop receptors. *Trends Pharmacol Sci.*
4. Dougherty, D.A. Cys-loop neuroreceptors: structure to the rescue? *Chem Rev* **108**, 1642-53 (2008).
5. Sine, S.M. & Engel, A.G. Recent advances in Cys-loop receptor structure and function. *Nature* **440**, 448-55 (2006).
6. Eldefrawi, M.E. & Eldefrawi, A.T. Characterization and partial purification of the acetylcholine receptor from Torpedo electroplax. *Proc Natl Acad Sci U S A* **69**, 1776-80 (1972).
7. Taly, A., Corringer, P.J., Guedin, D., Lestage, P. & Changeux, J.P. Nicotinic receptors: allosteric transitions and therapeutic targets in the nervous system. *Nat Rev Drug Discov* **8**, 733-50 (2009).
8. Zhong, W. et al. From ab initio quantum mechanics to molecular neurobiology: a cation- π binding site in the nicotinic receptor. *Proc Natl Acad Sci U S A* **95**, 12088-93 (1998).
9. Celie, P.H. et al. Crystal structure of acetylcholine-binding protein from *Bulinus truncatus* reveals the conserved structural scaffold and sites of variation in nicotinic acetylcholine receptors. *J Biol Chem* **280**, 26457-66 (2005).
10. Hansen, S.B. et al. Structures of *Aplysia* AChBP complexes with nicotinic agonists and antagonists reveal distinctive binding interfaces and conformations. *EMBO J* **24**, 3635-46 (2005).
11. Rucktooa, P., Smit, A.B. & Sixma, T.K. Insight in nAChR subtype selectivity from AChBP crystal structures. *Biochem Pharmacol* **78**, 777-87 (2009).
12. Williams, D.K., Stokes, C., Horenstein, N.A. & Papke, R.L. Differential regulation of receptor activation and agonist selectivity by highly conserved tryptophans in the nicotinic acetylcholine receptor binding site. *J Pharmacol Exp Ther* **330**, 40-53 (2009).
13. Gao, F. et al. Solution NMR of acetylcholine binding protein reveals agonist-mediated conformational change of the C-loop. *Mol Pharmacol* **70**, 1230-5 (2006).
14. Mukhtasimova, N., Lee, W.Y., Wang, H.L. & Sine, S.M. Detection and trapping of intermediate states priming nicotinic receptor channel opening. *Nature* **459**, 451-4 (2009).
15. Shi, J., Koeppe, J.R., Komives, E.A. & Taylor, P. Ligand-induced conformational changes in the acetylcholine-binding protein analyzed by hydrogen-deuterium exchange mass spectrometry. *J Biol Chem* **281**, 12170-7 (2006).
16. Unwin, N. Refined structure of the nicotinic acetylcholine receptor at 4Å resolution. *J Mol Biol* **346**, 967-89 (2005).
17. Miller, C. Genetic manipulation of ion channels: a new approach to structure and mechanism. *Neuron* **2**, 1195-205 (1989).
18. Filatov, G.N. & White, M.M. The role of conserved leucines in the M2 domain of the acetylcholine receptor in channel gating. *Mol Pharmacol* **48**, 379-84 (1995).
19. Labarca, C. et al. Channel gating governed symmetrically by conserved leucine residues in the M2 domain of nicotinic receptors. *Nature* **376**, 514-6 (1995).

20. Hodgkin, A.L. & Huxley, A.F. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J Physiol* **117**, 500-44 (1952).
21. Hodgkin, A.L. & Huxley, A.F. Movement of sodium and potassium ions during nervous activity. *Cold Spring Harb Symp Quant Biol* **17**, 43-52 (1952).
22. Bain, J.D. et al. Site-specific incorporation of nonnatural residues during in vitro protein biosynthesis with semisynthetic aminoacyl-tRNAs. *Biochemistry* **30**, 5411-21 (1991).
23. Ellman, J., Mendel, D., Anthony-Cahill, S., Noren, C.J. & Schultz, P.G. Biosynthetic method for introducing unnatural amino acids site-specifically into proteins. *Methods Enzymol* **202**, 301-36 (1991).
24. Noren, C.J., Anthony-Cahill, S.J., Griffith, M.C. & Schultz, P.G. A general method for site-specific incorporation of unnatural amino acids into proteins. *Science* **244**, 182-8 (1989).
25. Hoshika, T., Ashizuka, Y., Taira, H., Murakami, H. & Sisido, M. Incorporation of nonnatural amino acids into proteins by using various four-base codons in an Escherichia coli in vitro translation system. *Biochemistry* **40**, 11060-4 (2001).
26. Rodriguez, E.A., Lester, H.A. & Dougherty, D.A. In vivo incorporation of multiple unnatural amino acids through nonsense and frameshift suppression. *Proc Natl Acad Sci U S A* **103**, 8650-5 (2006).
27. Dougherty, D.A. Physical organic chemistry on the brain. *J Org Chem* **73**, 3667-73 (2008).
28. Nowak, M.W. et al. In vivo incorporation of unnatural amino acids into ion channels in Xenopus oocyte expression system. *Methods Enzymol* **293**, 504-29 (1998).
29. Nowak, M.W. et al. Nicotinic receptor binding site probed with unnatural amino acid incorporation in intact cells. *Science* **268**, 439-42 (1995).