Chapter 1: An Introduction to Chemical-Scale Neuroscience

1.1 Chemistry and the Brain

No object in nature is more complex than the human brain. The average adult brain contains more than one-hundred billion (> 10^{11}) neurons. Each neuron connects with one- to ten-thousand (10^3–10^4) other neurons through specialized junctions, called synapses. There are, therefore, an unfathomable quadrillion (10^{15}) synapses in the human brain—a quantity that eclipses the number of stars in our galaxy (~ 400 billion). This intricate web of cells draws massive amounts of energy: the brain uses 25% of the body's glucose and 20% of its oxygen[1]. This energy consumption fuels the processing of information that regulates mental and physical actions, such as locomotion, social behavior, learning, and memory. How can an organ as complex as the human brain be understood, in part, through the properties and interactions of its chemical components?

A chemical-scale understanding is possible because, at a basic level, the brain processes information through chemical signals transmitted between neurons. This exchange is called synaptic transmission (Figure 1.1). Synaptic transmission begins with an electrical signal, called an action potential, traveling down a neuron's axon. To communicate with another neuron, this signal must move towards an axon terminal that has formed a synapse with the dendrite of the other neuron (***i*** in Figure 1.1b) This first cell is referred to as the presynaptic cell and the second cell as the postsynaptic cell. At

the axon terminal, the electrical signal stimulates the mobilization of vesicles containing neurotransmitters. These vesicles fuse with the cell membrane and release their contents into the space between the two cells, the synaptic cleft (***ii*** in Figure 1.1b). These chemicals diffuse across the synaptic cleft and bind to neuroreceptors on the postsynaptic cell. Activated neuroreceptors either directly or indirectly produce electrical signals in the postsynaptic cell, which can then promote or inhibit the generation of an action potential in this cell (***iii*** in Figure 1.1b). Thus an electrical signal in the presynaptic cell is translated into a chemical signal that the postsynaptic cell decodes back into an electrical signal.

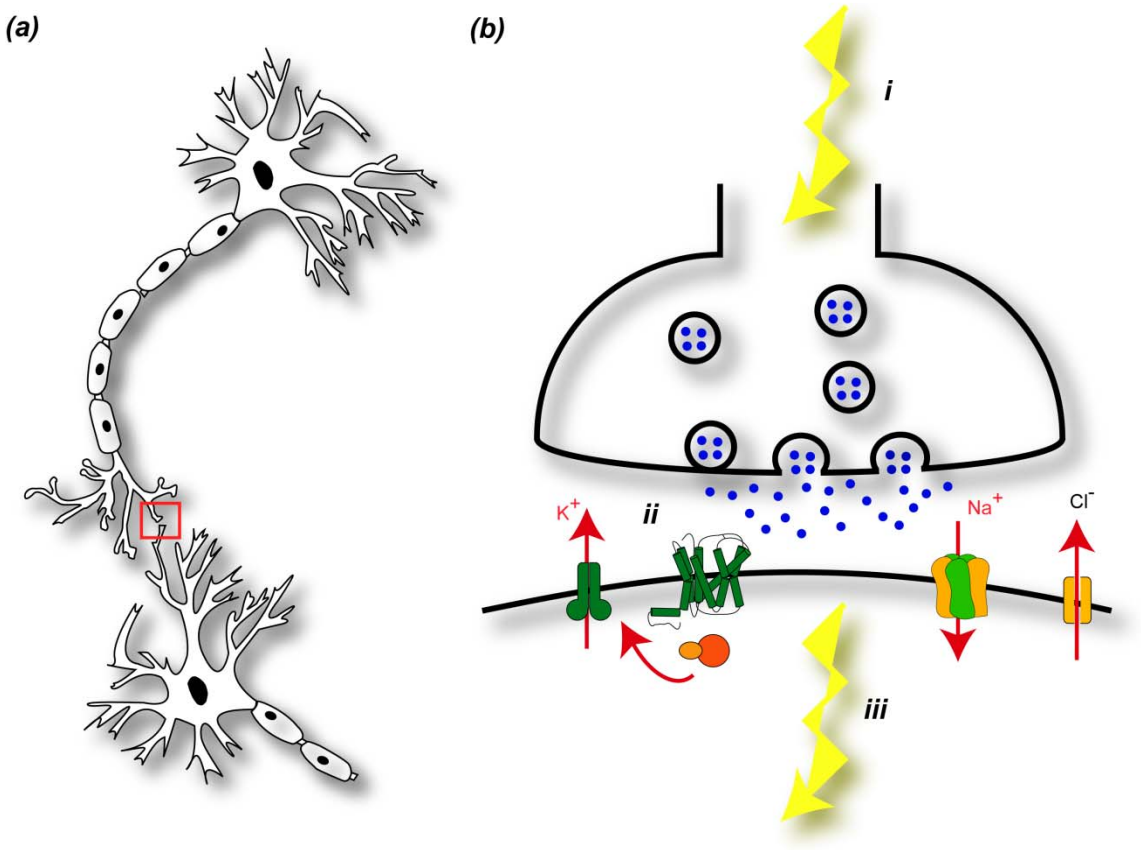

Figure 1.1. Synaptic transmission. ***(a)*** Two neurons making a connection: an axon terminal of the presynaptic cell (*top*) forms a synapse (red box) with a dendrite from the postsynaptic cell (*bottom*). ***(b)*** Scheme of synaptic transmission

Through the various neurotransmitters and neuroreceptors at synapses, neurons can send myriad chemical signals. Neurotransmitters can be small organic molecules, peptides, or even fatty acids. Two main types of neuroreceptor proteins exist. Ligand-gated ion channels (LGICs) bind neurotransmitters and directly produce electrical signals. These proteins undergo conformational changes upon ligand binding that produce open protein pores for ions to pass across the cell membrane. G-protein-coupled receptors (GPCRs), the second type of neuroreceptor, activate second messenger systems within the neuron upon neurotransmitter binding. These second messengers can gate ion channels on the neuron surface, but can also intiate other cellular pathways, such as gene transcription.

Chemical-scale studies of the brain analyze the actions of these neurotransmitter and neuroreceptor systems. The goal of chemical-scale neuroscience is to understand the chemistry of the brain through studies of the gating / activation mechanisms of neuroreceptors and the molecular recognition of neurotransmitters by neuroreceptors.

1.2 The Unnatural Amino Acid Methodology

1.2.1 The Power of Unnatural Amino Acids

To probe neurotransmitters and neuroreceptors at the chemical scale, researchers need precise techniques that allow them to investigate these molecules in the brain as chemists would study molecules in a flask. What techniques would allow researchers to perform structure-function studies on these molecules? Neurotransmitter structure and function can easily be probed through chemical synthesis. Medicinal chemists and

pharmacologists have been derivatizing and synthesizing analogs of neurotransmitters for decades to understand how their actions on neurons can be altered through changes to their chemical structures.

Studying neuroreceptors is substantially more difficult. Neuroreceptors are large membrane-bound proteins that often form multi-subunit signaling complexes. These features make them synthetically inaccessible. Even if these proteins could be synthesized chemically, they would need to be investigated in the proper context of the cell to fully assess their structure and function. Conventional mutagenesis combined with heterologous expression provides the proper *in vivo* context and has been used to determine important features of receptor structure. Unfortunately, the changes to protein structure available through conventional mutagenesis are severely limited. The twenty natural amino acids have limited chemical functionalities; there are no ketone, nitro, or ester moieties, to just name a few.

To illustrate this limitation of conventional mutagenesis, consider the cation-π interaction. Inorganic and organic cations have been shown to be stabilized through interactions with the π faces of aromatic rings[2–5]. These interactions are mainly electrostatic in nature. Cations are attracted to the negative charge density of the aromatic π face created by the quadropole moment of the ring. In biological structures, there is one cation-π interaction for every 77 amino acids in the protein data bank and 26% of all Trp residues are involved in cation-π interactions[4].

Despite its significance to protein structure, there is no means to study this interaction through conventional mutagenesis. Although there are differences in cation-π

binding energies between the three aromatic amino acids (Trp, Phe, Tyr), their structual differences are substantial enough that any effect in protein function could not solely be attributed to changes in a cation-π interaction. Ablating aromaticity at the site through Ala mutation would, of course, be even more destructive. An ideal experiment to study a Trp cation-π interaction would involve the progressive replacement of ring hydrogens with fluorines (Figure 1.2). Fluorination would decrease electron density on the π face of the ring through the atom's strong electronegativity. A change from hydrogen to fluorine would also be structurally subtle. Unfortunately, nature has not provided a codon that codes for fluorinated Trp.

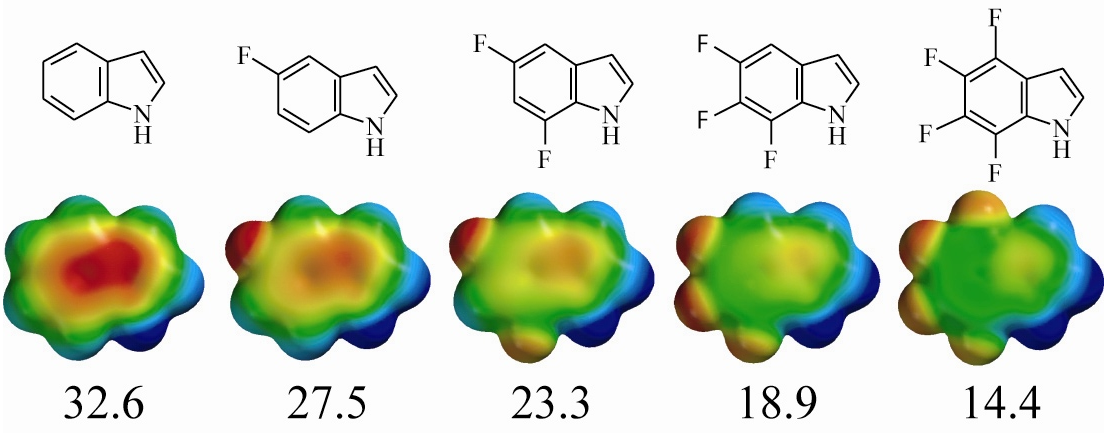

Figure 1.2. Fluorinated Trp analogs and cation-π binding energies. Electrostatic potential images show more negative charge density as red and more positive charge density as blue. Binding energies (kcal/mol) are from gas-phase calculations between fluorinated indole ring and sodium cation[2]

1.2.2 Incorporation of Unnatural Amino Acids into Neuroreceptors

To provide researchers with the ability to specifically incorporate unnatural amino acids, such as a fluorinated Trp, into proteins for structure-function studies, the nonsense suppression methodology was developed by Schultz and co-workers in 1989[6–15]. In this

method, one of the cell's stop codons (UAG, the amber codon) serves as the *de facto* codon for the unnatural amino acid (Figure 1.3). A suppressor tRNA with an anticodon (CUA) that can recognize the stop codon is chemically appended with the unnatural amino acid of choice. Instead of terminating protein translation when the UAG codon is encountered, the ribosome incorporates the unnatural amino acid at the site of the stop codon as it would incorporate any standard amino acid. The protein is thus synthesized normally, with the unnatural amino acid incorporated at the site of interest.

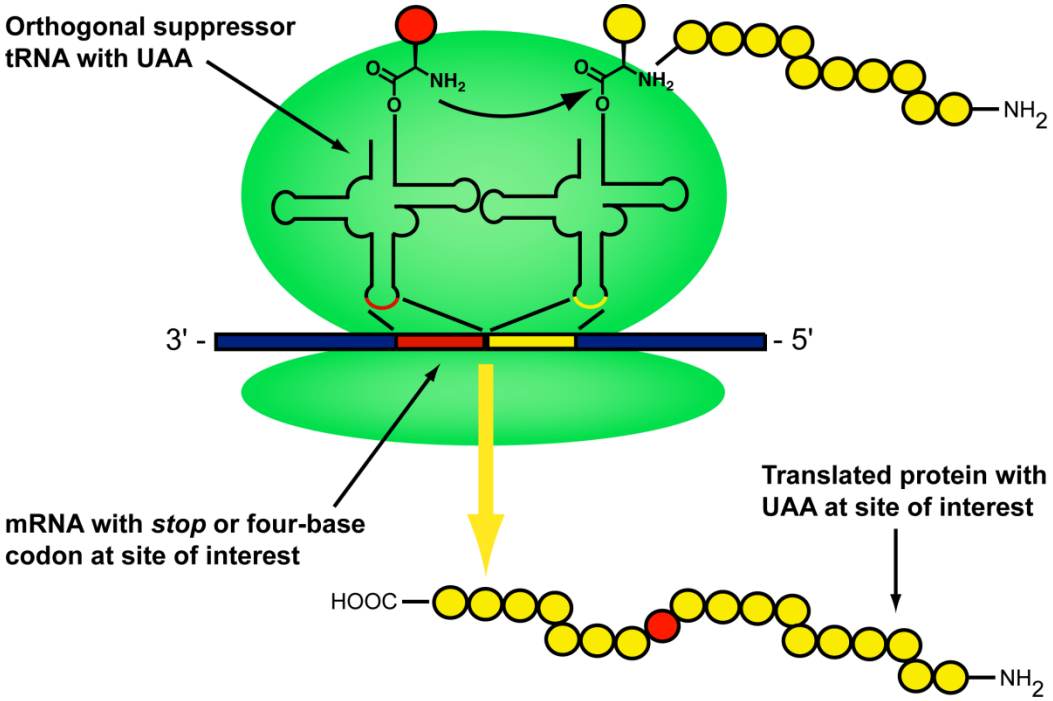

Figure 1.3. Overview of unnatural amino acid (UAA) incorporation using nonsense or frameshift suppression methodologies

Recently, an alternative method for incorporating unnatural amino acids has been developed, called frameshift suppression [16–18]. This methodology is similar to the nonsense suppression methodology, but codes for the unnatural amino acid through a four-base codon (GGGU) instead of a stop codon. A four-base codon normally shifts the ribosome out of the proper reading frame and produces mistranslated proteins. A

suppressor tRNA with the appropriate four-base anticodon (ACCC) that recognizes the four-base codon is used to suppress this shift in reading frame. By chemically appending an unnatural amino acid to this tRNA molecule, the ribosome incorporates the amino acid at the site of interest.

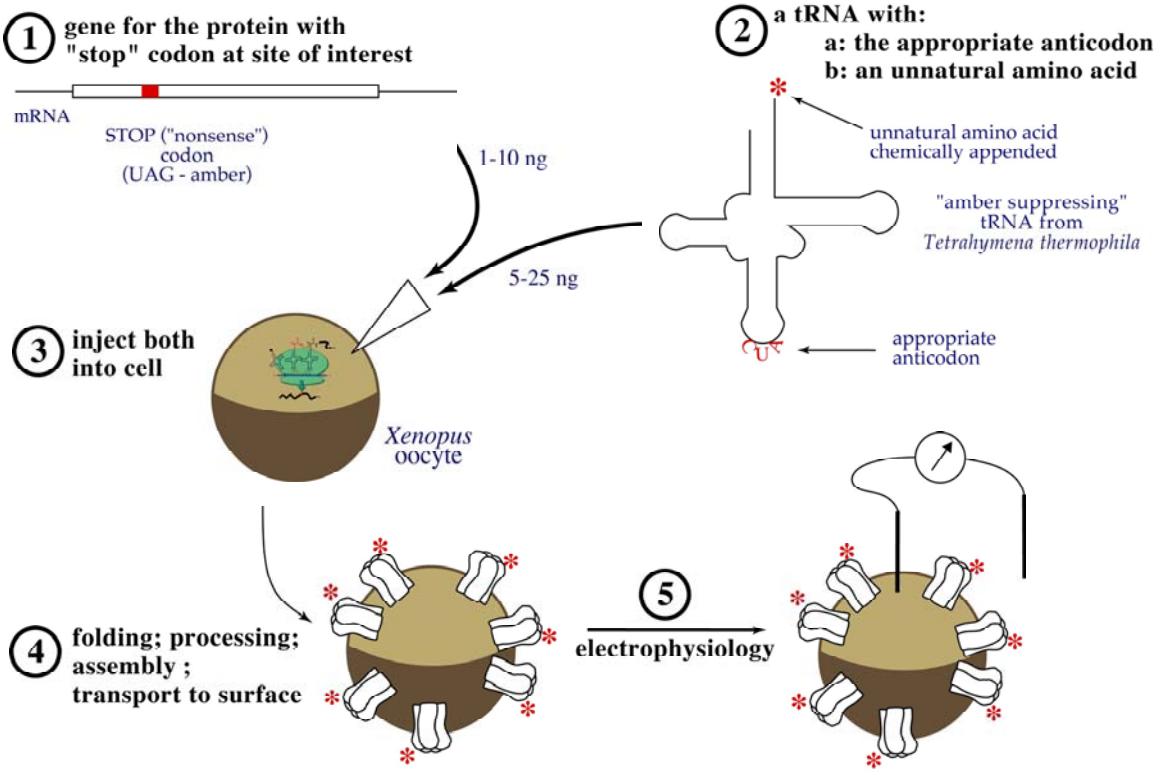

Figure 1.4. Implementation of nonsense suppression methodology for incorporating unnatural amino acids into membrane proteins in *Xenopus laevis* oocytes

In practice, both of these methodologies require a combination of chemical synthesis and simple molecular biology (Figure 1.4)[14,19–22]. The gene for the protein to be studied is mutated at the site of interest to either a stop or a four-base codon through standard mutagenesis protocols. Suppressor tRNA is transcribed without the last two nucleotides of the acceptor stem (C and A). A dinucleotide consisting of deoxy-C and A (dCA) is synthesized chemically and used as a chemical handle for the unnatural amino acid. Unnatural amino acids are prepared for use in these methodologies through the

addition of a photo- or I_2-labile amino protecting group (NVOC or 4-PO, respectively). Formation of a cyanomethyl ester from the free carboxylate activates the unnatural amino acid for acylation of the dCA molecule (Figure 1.5). Once acylated, the dCA molecule is ligated onto the truncated suppressor tRNA body with T4 RNA ligase to yield amino-acylated tRNA. Protection of the amino group of the unnatural amino acid provides stability to the amino-acylated tRNA.

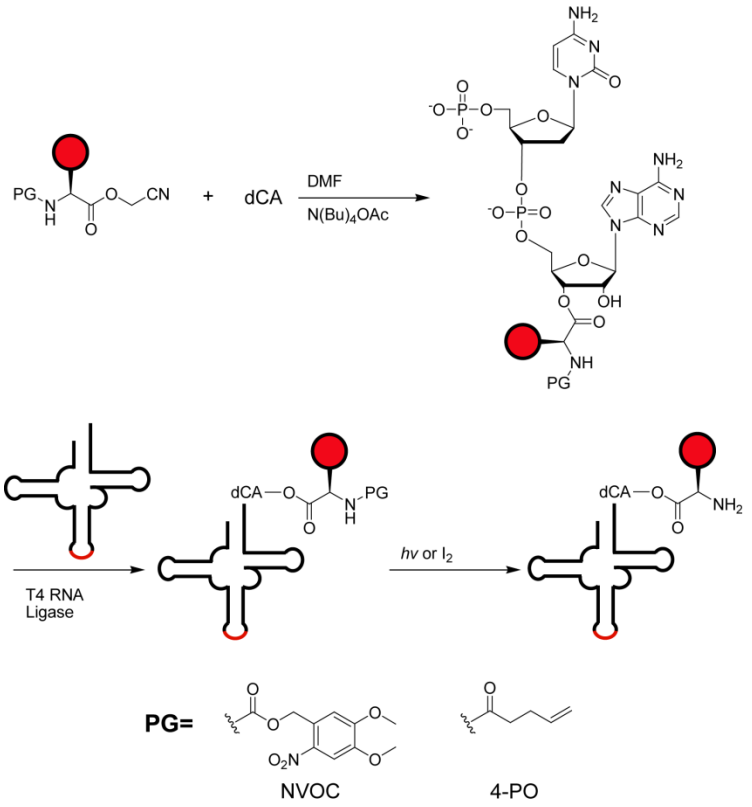

Figure 1.5. Method for chemically acylating unnatural amino acids (red circle) to the acceptor stem of suppressor tRNA

The mutated mRNA and amino-acylated tRNA are then injected into the cell type of choice, which in previous studies of neuroreceptors has been the *Xenopus laevis* oocyte[14,20,23–40]. An incubation period allows the proteins to be translated with the unnatural amino acid, processed, and transported to the surface of the cell. Several control experiments are performed along with the mutation experiment to ensure that the

unnatural amino acid has been properly incorporated into the protein. Injection of the mutant mRNA alone tests for readthrough of the stop or four-base codon by the ribosome. Injection of suppressor tRNA without an amino acid appended to the acceptor stem controls for misacylation—the phenomenon in which the cell's synthetases append a natural amino acid onto the tRNA body. Misacylation produces proteins that do not homogeneously contain the unnatural amino acid at the site of interest. Finally, wild-type recovery experiments, in which the suppressor tRNA is amino-acylated with the wild-type amino acid, ensure that proper protein function can be recovered through the nonsense or frameshift suppression methodology.

Because the suppressor tRNA cannot be amino-acylated within the cell with more unnatural amino acid, the suppressor tRNA is a stoichiometric reagent; protein yields cannot exceed the amount of tRNA injected into the cell. Fortunately, studies of neuroreceptors can be assayed through methods that do not require large amounts of protein. Electrophysiology is an extremely sensitive assay for ion channel function that detects currents through whole cells or patches of cell membranes. In fact, single ion channels can be monitored through these assays. When a drug is applied to the cell, ion channels on the cell's surface gate and pass ions into or out of the cell (Figure 1.6a). The aggregate passage of ions by these ion channels produces a cell current that can be measured using the two-electrode voltage clamp method[41,42]. When the concentration of drug increases, these currents also increase as more receptors become active (Figure 1.6bc).

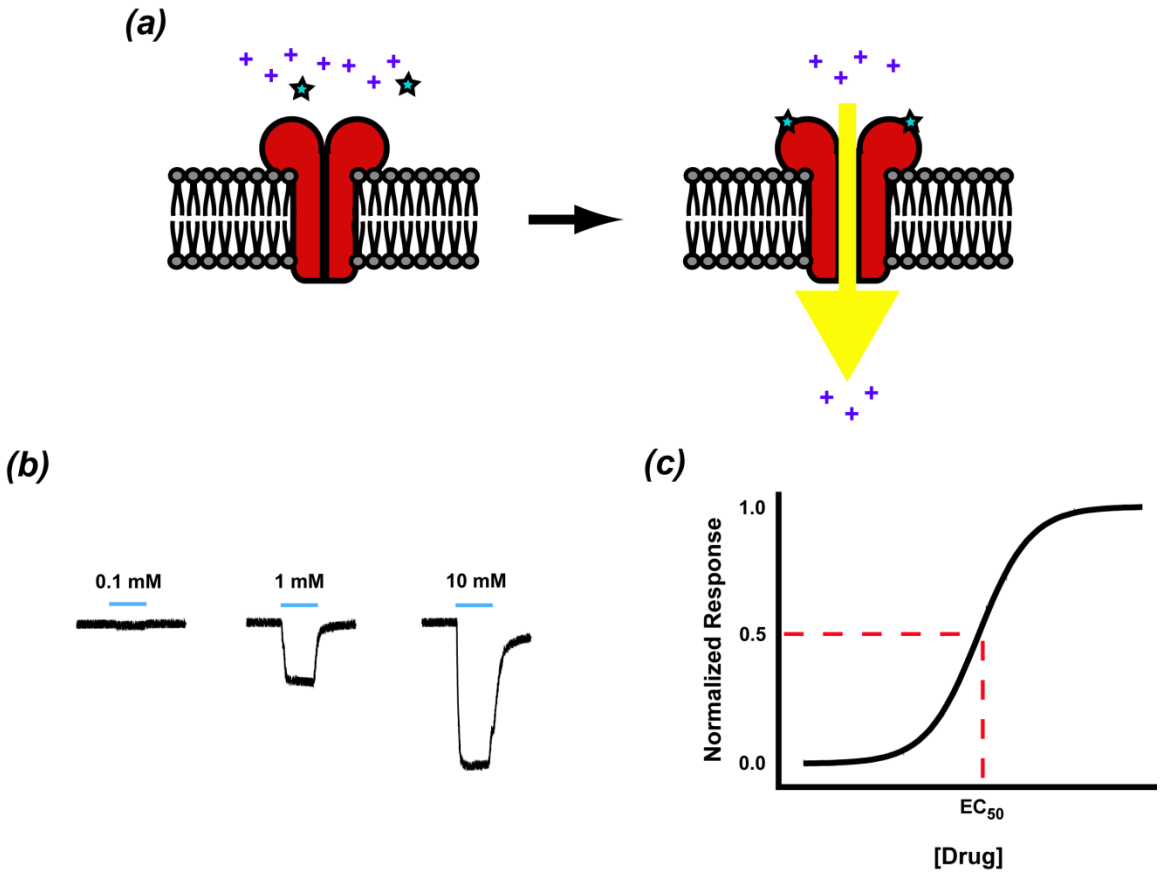

Figure 1.6. Basics of electrophysiology assay. ***(a)*** Drug (stars) binding to LGIC promotes receptor gating. Once open, the channel allows current to pass in or out of the cell producing current signals. ***(b)*** Examples of current response to varying concentrations of drug. From *left* to *right*, low, EC_{50}, and saturating drug concentrations. ***(c)*** Example of dose-response relationship. EC_{50} definition shown in relation to the rest of the curve

A drug's potency at a receptor can be established through such a dose-response relationship (Figure 1.6c). Potency of a drug for a given neuroreceptor is a combination of the drug's binding affinity and its ability to promote receptor activation (efficacy). Mutations to residues in the binding site of the receptor are assumed mainly to affect affinity, although there are notable exceptions. Typically, those mutations along the gating/activation pathway of the receptor are thought to mainly affect efficacy.

EC_{50}, the dose of drug that elicits a half-maximal response in the receptor (Figure 1.6c), is a means to quantitate drug potency, and thus contains information about both

drug affinity and efficacy. Changes in receptor function are reported through shifts in EC_{50} values: a shift to higher EC_{50} values implies a loss-of-function mutation and, accordingly, shifts to lower EC_{50} values suggest gain-of-function. By combining unnatural amino acid mutagenesis with these electrophysiology experiments, the role of specific chemical interactions in the function of large neuroreceptors can be assayed.

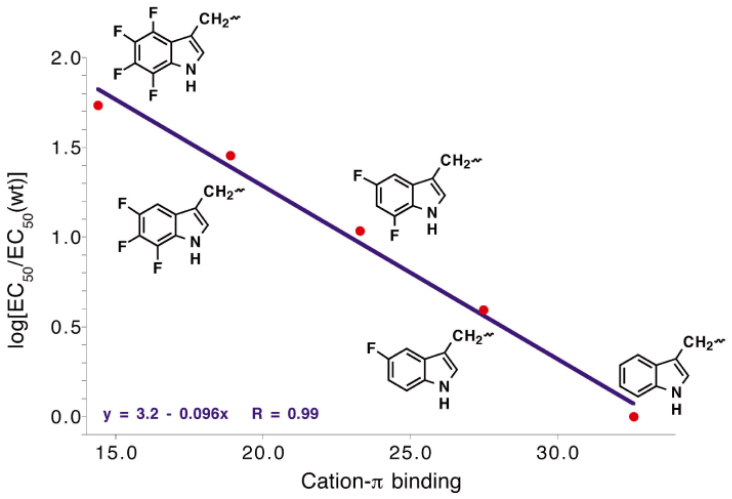

Figure 1.7. Classic Zhong plot for αW149 residue of nAChR. Calculated cation-π binding energy (gas phase) is plotted against the log of the ratio of the EC_{50} of the F_nTrp mutant receptor to the wild-type receptor EC_{50}[40]

A classic early example of this coupling of unnatural amino acid mutagenesis and electrophysiology is the discovery of a cation-π interaction between acetylcholine (ACh) and a binding site residue in the nicotinic ACh receptor (nAChR)[40]. This early study probed a Trp in the α subunit of the nAChR (αW149) for an interaction with the positively charged quaternary amine of ACh. A series of fluorinated Trp amino acids (Figure 1.2) were incorporated at αW149 and the effect on EC_{50} was measured. EC_{50} values progressively shifted upwards with each fluorination (Figure 1.7), and thus suggested that a cation-π interaction existed between the indole ring of αW149 and the quaternary amine of ACh.

1.3 Dissertation Work

This dissertation describes three studies that utilized this combination of unnatural amino acid mutagenesis and electrophysiology. The three studies probed the structure and function of two different ACh receptors, the nAChR and the M_2 muscarinic ACh receptor (M_2AChR). Chapters 2 and 3 outline binding site studies, while Chapter 4 studies gating. In Chapter 2, we descibe an investigation into the role of a highly conserved Asp in the nAChR binding site. Using subtle mutations only available through unnatural amino acids, we determine that this residue is responsible for preorganizing a key region of the nAChR binding site for ligand binding. Chapter 3 discusses our attempts to incorporate unnatural amino acids into a GPCR (M_2AChR), a type of neuroreceptor we had not previously studied through this methodology. We determined optimal conditions that allowed us to obtain robust and reliable data from GPCRs. Initial data on the search for a cation-π interaction between ACh and aromatic residues in the M_2AChR binding site are also discussed. Finally, in Chapter 4, we describe a study of the αM1 transmembrane helix of the nAChR using hydroxy acids. This study sought to determine the nature of structural rearrangements in the helix during channel gating.

1.4 References

(1) Kandel, E. R.; Schwartz, J. H.; Jessel, T. M. *Principles of Neural Science, 4th ed.* McGraw-Hill: New York, NY, 2000.
(2) Ma, J. C.; Dougherty, D. A. *Chem. Rev.* **1997**, *97*, 1303–1324.

(3) Dougherty, D. A. *Science* **1996**, *271*, 163–168.
(4) Gallivan, J. P.; Dougherty, D. A. *Proceedings of the National Academy of Sciences of the United States of America* **1999**, *96*, 9459–9464.
(5) Zacharias, N.; Dougherty, D. A. *Trends in Pharmacological Sciences* **2002**, *23*, 281–287.
(6) Chapman, E.; Thorson, J. S.; Schultz, P. G. *J. Am. Chem. Soc.* **1997**, *119*, 7151–7152.
(7) Chung, H. H.; Benson, D. R.; Schultz, P. G. *Science* **1993**, *259*, 806–809.
(8) Cornish, V. W.; Benson, D. R.; Altenbach, C. A.; Hideg, K.; Hubbell, W. L.; Schultz, P. G. *Proceedings of the National Academy of Sciences of the United States of America* **1994**, *91*, 2910–2914.
(9) Cornish, V. W.; Mendel, D.; Schultz, P. G. *Angewandte Chemie—International Edition in English* **1995**, *34*, 621–633.
(10) Ellman, J. A.; Mendel, D.; Schultz, P. G. *Science* **1992**, *255*, 197–200.
(11) Judice, J. K.; Gamble, T. R.; Murphy, E. C.; de Vos, A. M.; Schultz, P. G. *Science* **1993**, *261*, 1578–1581.
(12) Koh, J. T.; Cornish, V. W.; Schultz, P. G. *Biochemistry* **1997**, *36*, 11314–11322.
(13) Noren, C. J.; Anthonycahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182–188.
(14) Nowak, M. W.; Kearney, P. C.; Sampson, J. R.; Saks, M. E.; Labarca, C. G.; Silverman, S. K.; Zhong, W.; Thorson, J.; Abelson, J. N.; Davidson, N.; Schultz, P. G.; Dougherty, D. A.; Lester, H. A. *Science* **1995**, *268*, 439–442.
(15) Thorson, J. S., Chapman, E., Schultz, P.G. *Journal of the American Chemical Society* **1995**, *117*, 9361–9362.
(16) Hohsaka, T.; Ashizuka, Y.; Murakami, H.; Sisido, M. *Journal of the American Chemical Society* **1996**, *118*, 9778–9779.
(17) Hohsaka, T.; Ashizuka, Y.; Taira, H.; Murakami, H.; Sisido, M. *Biochemistry* **2001**, *40*, 11060–11064.
(18) Rodriguez, E. A.; Lester, H. A.; Dougherty, D. A. *Proc Natl Acad Sci U S A* **2006**, *103*, 8650–5.
(19) Beene, D. L.; Dougherty, D. A.; Lester, H. A. *Current Opinion in Neurobiology* **2003**, *13*, 264–270.
(20) Dougherty, D. A. *Current Opinion in Chemical Biology* **2000**, *4*, 645–652.
(21) Kearney, P. C.; Nowak, M. W.; Zhong, W.; Silverman, S. K.; Lester, H. A.; Dougherty, D. A. *Mol Pharmacol* **1996**, *50*, 1401–1412.
(22) Nowak, M. W.; Gallivan, J. P.; Silverman, S. K.; Labarca, C. G.; Dougherty, D. A.; Lester, H. A.; Conn, P. M. *Methods in Enzymology* **1998**, *293*, 504–529.
(23) Beene, D. L.; Brandt, G. S.; Zhong, W.; Zacharias, N. M.; Lester, H. A.; Dougherty, D. A. *Biochemistry* **2002**, *41*, 10262–10269.
(24) Beene, D. L.; Price, K. L.; Lester, H. A.; Dougherty, D. A.; Lummis, S. C. R. *J. Neurosci.* **2004**, *24*, 9097–9104.
(25) Cashin, A. L.; Petersson, E. J.; Lester, H. A.; Dougherty, D. A. *J. Am. Chem. Soc.* **2005**, *127*, 350–356.
(26) Cashin, A. L.; Torrice, M. M.; McMenimen, K. A.; Lester, H. A.; Dougherty, D. A. *Biochemistry* **2007**, *46*, 630–639.

(27) Dang, H.; England, P. M.; Farivar, S. S.; Dougherty, D. A.; Lester, H. A. *Mol Pharmacol* **2000**, *57*, 1114–1122.
(28) Dougherty, D. A. *J. Org. Chem.* **2008**, *73*, 3667–3673.
(29) Dougherty, D. A. *Chem. Rev.* **2008**, *108*, 1642–1653.
(30) England, P. M.; Lester, H. A.; Dougherty, D. A. *Tetrahedron Letters* **1999**, *40*, 6189–6192.
(31) England, P. M.; Zhang, Y.; Dougherty, D. A.; Lester, H. A. *Cell* **1999**, *96M1 nAChR*, 89–98.
(32) Li, L. T.; Zhong, W. G.; Zacharias, N.; Gibbs, C.; Lester, H. A.; Dougherty, D. A. *Chemistry & Biology* **2001**, *8*, 47–58.
(33) Lummis, S. C. R.; Beene, D. L.; Lee, L. W.; Lester, H. A.; Broadhurst, R. W.; Dougherty, D. A. *Nature* **2005**, *438*, 248–252.
(34) Lummis, S. C. R.; L. Beene, D.; Harrison, N. J.; Lester, H. A.; Dougherty, D. A. *Chemistry & Biology* **2005**, *12*, 993–997.
(35) McMenimen, K. A.; Petersson, E. J.; Lester, H. A.; Dougherty, D. A. *ACS Chem. Biol.* **2006**, *1*, 227–234.
(36) Mu, T. W.; Lester, H. A.; Dougherty, D. A. *J. Am. Chem. Soc.* **2003**, *125*, 6850–6851.
(37) Padgett, C. L.; Hanek, A. P.; Lester, H. A.; Dougherty, D. A.; Lummis, S. C. R. *J. Neurosci.* **2007**, *27*, 886–892.
(38) Petersson, E. J.; Choi, A.; Dahan, D. S.; Lester, H. A.; Dougherty, D. A. *J. Am. Chem. Soc.* **2002**, *124*, 12662–12663.
(39) Xiu, X.; Hanek, A. P.; Wang, J.; Lester, H. A.; Dougherty, D. A. *J. Biol. Chem.* **2005**, *280*, 41655–41666.
(40) Zhong, W.; Gallivan, J. P.; Zhang, Y.; Li, L.; Lester, H. A.; Dougherty, D. A. *PNAS* **1998**, *95*, 12088–12093.
(41) Hodgkin, A. L.; Huxley, A. F. *Cold Spring Harbor symposia on quantitative biology* **1952**, *17*, 43–52.
(42) Hodgkin, A. L.; Huxley, A. F. *J. Physiol.* **1952**, *117*, 500–544.