Chapter 1: *Chemical-Scale Neuroscience*

1.1 Introduction to Neurochemistry

1.1.1 Chemical Signal Transduction

The entire human experience is derived from four pounds of Nature's most intriguing and complex creation, the brain. The human brain (Figure 1.1a) is comprised of more than 100 billion (>10^{11}) neurons, and each one can connect with 1,000-10,000 (10^3-10^4) other neurons through specialized connections, and synapses (*1*) (Figure 1.1b). In four pounds of tissue, an unimaginable network of a quadrillion (10^{15}) connections regulates communication and unconscious activities and structures our imagination. Yet this complex communication can be traced back to synapses, or chemical junctions, between neurons. In fact, Nature has cleverly devised a way to utilize chemicals, or neurotransmitters, to facilitate communication along this complex pathway of neurons. One of the most fascinating questions we can ask is how does the brain use these chemicals to create and regulate the human experience? Is it possible to learn how the human brain functions by understanding the chemical-scale communication between neurons?

To study the chemical-scale communication of the brain, we turn to synapses. Synapses are the junctures between neurons where synaptic transmission, or chemical communication occurs (Figure 1.1c). Synaptic transmission is initiated by an electrical signal, an action potential, which travels down a neuron to its axon terminal. This axon terminal must form a synapse with the dendrite from a neighboring neuron (Figure 1.1c) in order to generate communication between neurons. Once the action potential reaches the pre-synaptic terminal, vesicles containing neurotransmitters, such as glutamate or acetylcholine (ACh), are released into the synaptic cleft. The neurotransmitters diffuse across the cleft and bind to neurotransmitter receptors on the post-synaptic dendrite (Figure 1.1c). Neurotransmitter receptors then regenerate, either directly or indirectly, the electrical signal, which can activate or inhibit the generation of another action potential. The overall flow of communication begins with an electrical signal in the pre-

synaptic cell, which turns into a chemical signal in between neurons. The electrical signal is regenerated in the following neuron, and the cascade continues throughout thousands of cells and occurs very quickly, on the millisecond timescale (*2*).

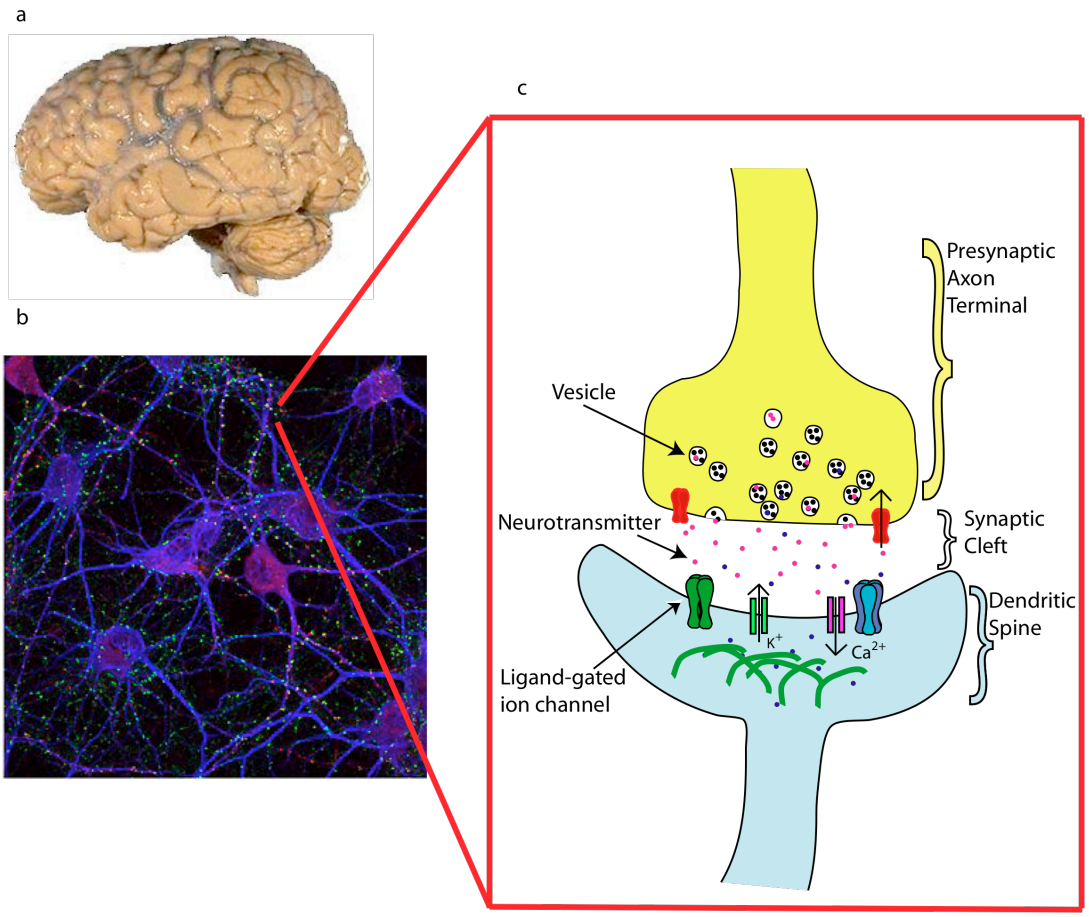

Figure 1.1 Synaptic Transmission. a) The human brain. (http://www.healcentral.org/healapp/showMetadata?metadataId=40566 (John A Beal, PhD. Dept. of Cellular Biology & Anatomy, Louisiana State University Health Sciences Center Shreveport)) b) Stained neurons and receptors. Yellow dots correspond to active synapses, adapted from (*3*). c) Representation of the synapse where the pre-synaptic axon terminal meets with the post-synaptic dendritic spine. An electrical signal in the pre-synaptic cell releases neurotransmitters from vesicles into the synaptic cleft. Neurotransmitters flow across the synaptic cleft and bind to neuroreceptors in the dendrtic membrane where an electrical signal is regenerated.

There are many types of neurotransmitters and neuroreceptors that intertwine to produce a plethora of signals. Neurotransmitters come in many flavors, from small molecules to fatty acids and peptides (*4*). There are two main types of neuroreceptors. The first is the ligand-gated ion channel (LGICs), which directly binds a neurotransmitter. Ligand binding triggers a conformational change in the protein, opening the ion channel and allowing ion flux across the dendritic membrane (Figure 1.2). Ion flux across a membrane changes the voltage across the membrane, which can result in the generation of an electrical signal if a sufficiently large change occurs. The second type of neuroreceptor is a G-protein coupled receptor (GPCR), which causes alterations that are indirectly related to neurotransmitter-receptor interactions. Upon neurotransmitter binding, a cascade of second messengers are activated triggering downstream changes in the neuron such as activation of other ion channels, signaling pathways, and regulation of gene transcription (*5*).

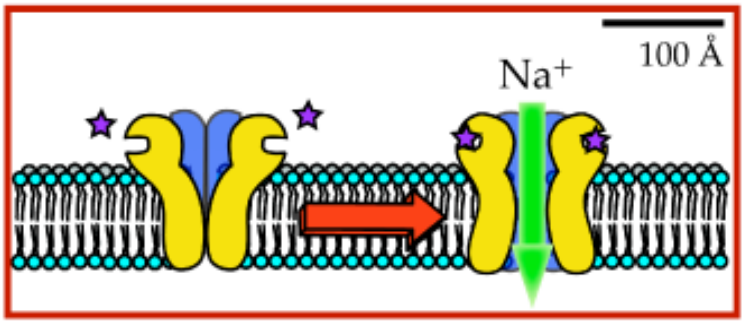

Figure 1.2 Ligand-gated ion channels are closed prior to neurotransmitter binding. Once a neurotransmitter binds, the ion channel opens and allows ion flux across the cell membrane.

The essential goal of chemical-scale neuroscience is to study these neurotransmitter/ neuroreceptor systems. To understand these systems, one must examine at a molecular level how different neurotransmitters are recognized by different neuroreceptors. In addition, one must analyze the structures of these complex, multi-subunit proteins through their gating and activation mechanisms to determine how the structure contributes to the specific cellular functions.

1.1.2 Ion Channel Functional Studies

The first ion channel characterization began in the early 1970's by Hille (*6*). Since the early studies, the development of molecular biology techniques, cloning, and manipulation of DNA has evolved a typical method for analyzing ion channels. This method generally involves a structure/function study where mutagenesis of amino acids at the DNA level is followed by the production of the ion channel in a heterologous expression system, typically a *Xenopus laevis* ooctye. Then using electrophysiological techniques, primarily the two-electrode voltage clamp (TEVC), the functional channels are analyzed. The principles behind TEVC electrophysiology utilize one electrode as a voltage "clamp" of the cell membrane to maintain a constant potential, which is achieved by injecting current into the membrane (Figure 1.3a). The second electrode measures the voltage difference across the membrane in response to the opening of ion channels and ion flux across the membrane (relative to a ground electrode). Typically a neurotransmitter is applied to the oocyte to generate a current and the "clamp" electrode must then inject more voltage into the membrane in order to maintain a constant voltage (Figure 1.3b). Thus, a direct measurement of the ion flow through the channel is recorded and provides information about the ion channel gating mechanism (*7, 8*).

The *Xenopus* expression system is used to express either DNA or RNA of mammalian ion channels. After an appropriate amount of time, the ion channels are produced and trafficked to the oocyte membrane. A solution resembling the fluid at the synapse is perfused around the oocyte followed by the application of varying neurotransmitter concentrations. Many types of ligands are studied, such as, agonists (molecules that activate the ion channel, e.g., ACh), antagonists (inactivate), blockers, and potentiators. Electrophysiological recordings measure the response of the ion channel to varying concentrations of ligand and dose-response curves are generated to analyze the functional channels.

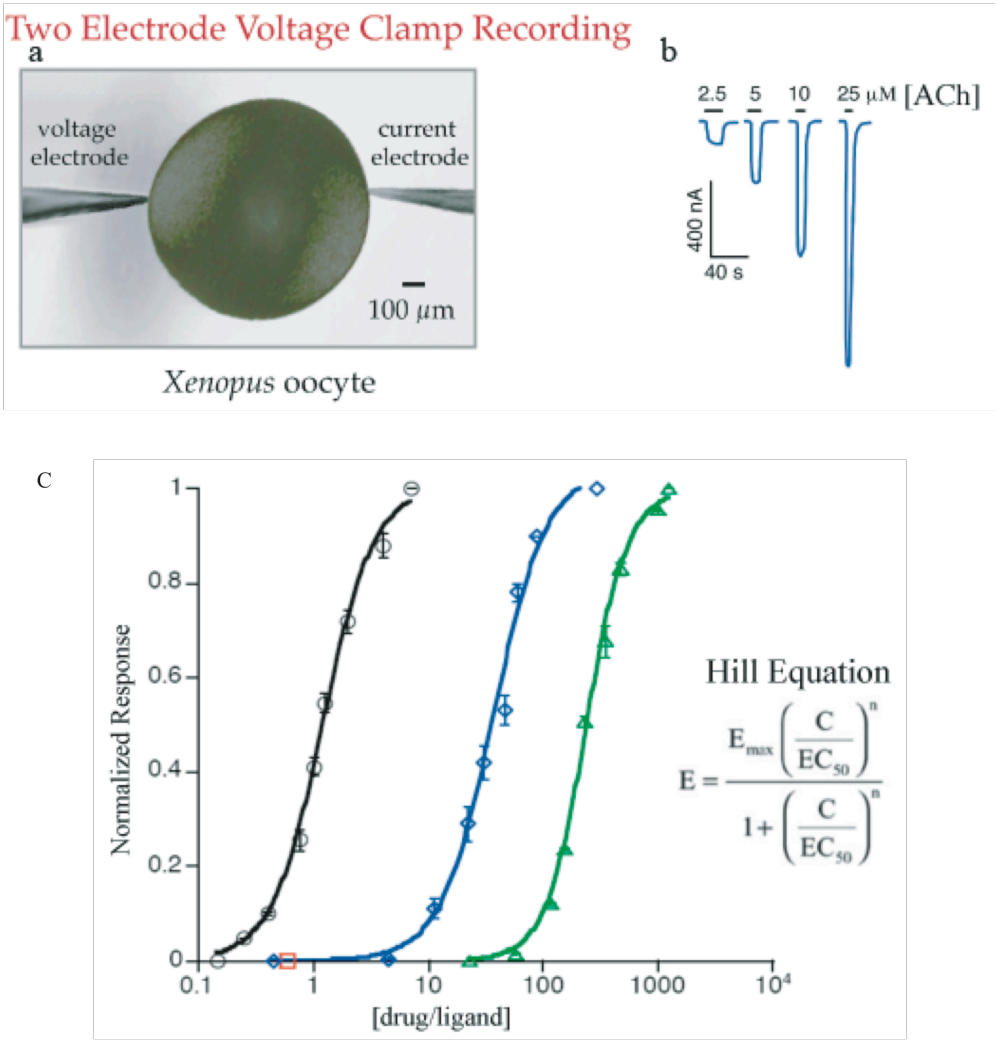

Figure 1.3 Two-electrode voltage clamp. a) The *Xenopus laevis* oocyte with two electrodes, a voltage "clamp" electrode and a second electrode that measures the current across the oocyte membrane. b) An example of an electrophysiological trace. Application of a neurotransmitter (ACh) produces a downward current, measured in nanoamperes (nA). Larger concentrations of neurotransmitters produce larger currents. c) The Hill equation is used to fit dose-response relationships producing EC_{50}, a measure of the concentration of ligand required to produce half-maximal current responses.

The Hill equation (*9*) is used to fit dose-response data. The equation produces a value for EC_{50}, which is defined as the amount of ligand required to produce a half-maximal response from the neuroreceptors (Figure 1.3c) and is a quantitative measure of the potency of a ligand. Potency (EC_{50}) describes the combined effects of the

neuroreceptor's ability to bind a drug or ligand (affinity) and the ability of the ligand to activate the channel (efficacy). Comparing shifts in EC_{50} values is a convenient method for analyzing different ligands as well as mutations to the neuroreceptors. For example, a rightward shift in EC_{50} demonstrates the decreased potency of a ligand whereas a leftward shift would indicate an increase in ligand potency (Figure 1.3c). General assumptions are made when interpreting EC_{50} data. For example, mutations made to a receptor's binding site that shift EC_{50} are believed to mostly affect affinity while mutations outside of the direct binding site that shift EC_{50} are treated as gating mutations affecting efficacy. These assumptions are generally useful, although there are always exceptions to the rule.

Measuring ligand potency is a useful way to characterize the interaction between drugs and receptors. However, different drugs do not activate receptors equally. Many drugs only partially activate or gate a receptor and are termed partial agonists. One can think of partial agonists as binding with full affinity to a receptor, but not fully gating the receptor, resulting in lower efficacy. The most common method to determine the efficacy of an agonist is to compare the amount of current generated by the receptor in response to a saturating dose of each agonist. Full agonists will produce larger amounts of current compared to partial agonists (Figure 1.4). Efficacy measurements are usually reported as a fraction of one (1) determined from the ratio of the current from the partial agonist divided by that generated by the full agonist.

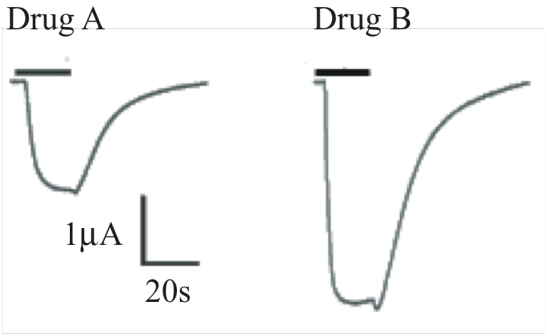

Figure 1.4 Electrophysiological traces representing differences in agonist efficacy. Saturating doses of both Drug A and Drug B generate different levels of current from a receptor. Drug A demonstrates a partial agonist and Drug B demonstrates the response of a full agonist.

Utilizing different agonists, partial agonists, and blockers is very helpful when analyzing these complex receptors. However, generating full pictures to describe the structure and function of these receptors and their interactions with different ligands is difficult. In fact, the atomic-scale details of ion channels, which are large, multi-subunit proteins, is essential to understanding the biological function of these neuroreceptors and their interactions with drugs. For example, agonists for several different ion channels have binding sites that are more than 50 Å away from the ion channel pore where gating occurs. As chemists, we seek to study the atomic-scale interactions that underlie ion channel function. However, there is limited atomic-scale structural data about these receptors due to their complexity. Luckily, we can take advantage of molecular biology tricks such as site-directed mutagenesis to probe chemical-scale interactions by altering specific amino acids in the protein. However, from a chemist's point of view, the 20 natural amino acids are severely limiting when trying to understand how specific bonds and interactions influence the function of an ion channel. Therefore, standard mutagenesis is not always sufficient. As a result, we combine molecular biology with chemistry to utilize unnatural amino acid mutagenesis. Unnatural amino acid mutagenesis allows us to study specific hydrogen bonds, hydrophobic interactions, etc., that influence LGICs. It provides a very clever and precise tool to probe the interactions that underlie LGIC structure and function.

1.2 Introduction to Unnatural Amino Acid Mutagenesis

1.2.1 Unnatural Amino Acid Mutagenesis

Since the late 1980's, site-specific incorporation of unnatural amino acids into proteins *in vivo* has been possible using the nonsense suppression methodology (*10*). This tool provides many opportunities for the chemical biologist to explore. Using chemistry to design unnatural amino acids facilitates the exploration of seemingly endless chemical interactions that structure ion channels, which greatly surpasses conventional mutagenesis techniques. For example, in order to study a phenylalanine residue in protein structure and function our options using conventional site-directed mutagenesis would allow us to incorporate alanine, tryptophan, and tyrosine. Alanine completely

obliterates the side-chain, which is not subtle by any means as it destroys hydrophobic, aromatic, and steric interactions. Tryptophan on the other hand maintains aromaticity, but is sterically bulky and tyrosine is similar, in size and aromaticity, yet introduces a hydrogen-bonding component (Figure 1.5). Each of these options has significant limitations from a chemical perspective.

The use of unnatural amino acid mutagenesis enhances the ability to study precise chemical aspects of an amino acid. In the case of phenylalanine, amino acids like cyclohexylalanine (Cha) can be incorporated to test the aromatic character of Phe without perturbing steric interactions. The importance of the cation-π binding ability of Phe also can be tested using fluorinated phenylalanine derivatives. Organic and inorganic cations can be stabilized through a favorable electrostatic interaction with the π surface of aromatic rings (*11-15*). The π surface of aromatic rings, like those of Phe, Tyr, and Trp, have a build up of negative electrostatic potential due to the quadrupole moment of the ring. The ideal way to study the cation-π interaction in proteins is to sequentially fluorinate the aromatic side chain. Adding fluorines to an aromatic ring decreases the negative charge density of the ring and makes it a weaker cation binder. Unnatural amino acid mutagenesis provides a "series" of fluorinated derivatives that can be used to probe these subtle interactions. Although these mutations are subtle, the chemical interactions they probe are energetically non-trivial. In proteins, it is believed that for approximately every 77 amino acids in the protein data bank there is a cation-π interaction (*13*).

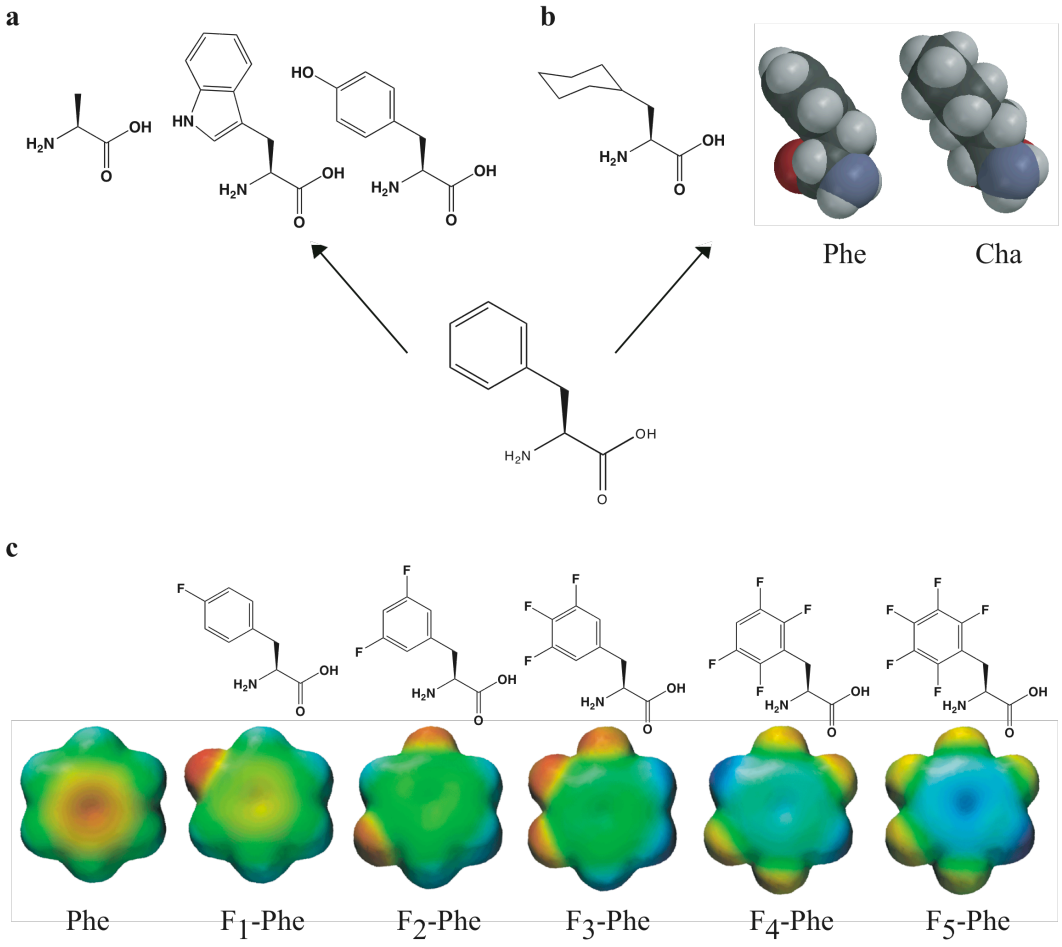

Figure 1.5. The power of unnatural amino acid mutagenesis. a) Conventional mutagenesis allows for alanine, tryptophan, and tyrosine mutations. b) The importance of side chain aromaticity can be explored with the cyclohexylalanine (Cha) mutation without altering side chain sterics. c) The cation-π interaction can be tested with a series of fluorinated Phe derivatives.

1.2.2 Nonsense Suppression

Unnatural amino acids provide a powerful tool for chemical biologists to study proteins. There are several different methodologies for incorporating unnatural amino acids site-specifically into proteins. We utilize the nonsense suppression methodology (*16-21*). The basic procedure is outlined below in Figure 1.6, and makes use of one of the cell's stop codons, UAG (Amber stop codon), as the codon for the unnatural amino acid. The UAG stop codon is incorporated into the ion channel mRNA at the site of interest using mutagenesis. Additionally, a suppressor $tRNA_{CUA}$ (containing a CUA anticodon) that

recognizes the amber stop codon, is chemically prepared and appended with the unnatural amino acid of choice (*22, 23*). During translation, the ribosome "reads" the UAG stop codon and instead of terminating protein translation, it is suppressed by the $tRNA_{CUA}$, and the unnatural amino acid is inserted into the protein in the same manner as any naturally occurring amino acid. Protein translation continues and produces a full-length protein with the unnatural amino acid inserted at a specified site.

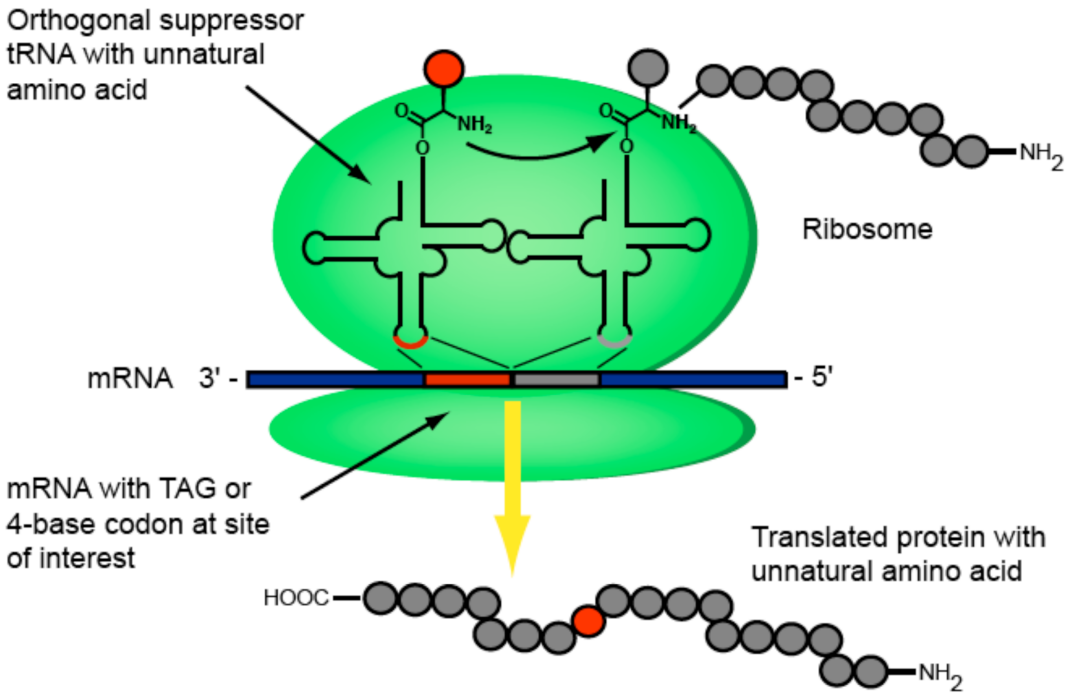

Figure 1.6 Outline of the nonsense suppression methodology. The ribosome of the expression system incorporates an unnatural amino acid at the site of interest (red dot).

A similar approach utilizes a four-base codon instead of the UAG stop codon. This methodology, called frameshift suppression (*24*), uses the four-base GGGU codon. The four-base codon normally would shift the ribosome out of the proper reading frame, resulting in the production of mistranslated proteins. However, a specialized tRNA containing the appropriate anticodon, ACCC, suppresses the mistranslation by inserting an unnatural amino acid (chemically appended to the $tRNA_{ACCC}$) at the site of the frameshift mutation. This methodology has also been extended to another stop codon,

the opal suppressor (UGA), which has allowed for the incorporation of multiple unnatural amino acids into a single protein (*25*).

Nonsense suppression blends together tools of molecular biology and chemistry. Simple molecular biology allows for mutagenesis of the codon of interest. However, the limiting step is chemical production of the aminoacylated tRNA. In the late 1970's, Hecht and coworkers were able to synthesize misacylated, chemically derived tRNAs (*23*). Since then, several different groups have expanded the methodology to include a large variety of unnatural amino acids and several different protein systems (*26-28*).

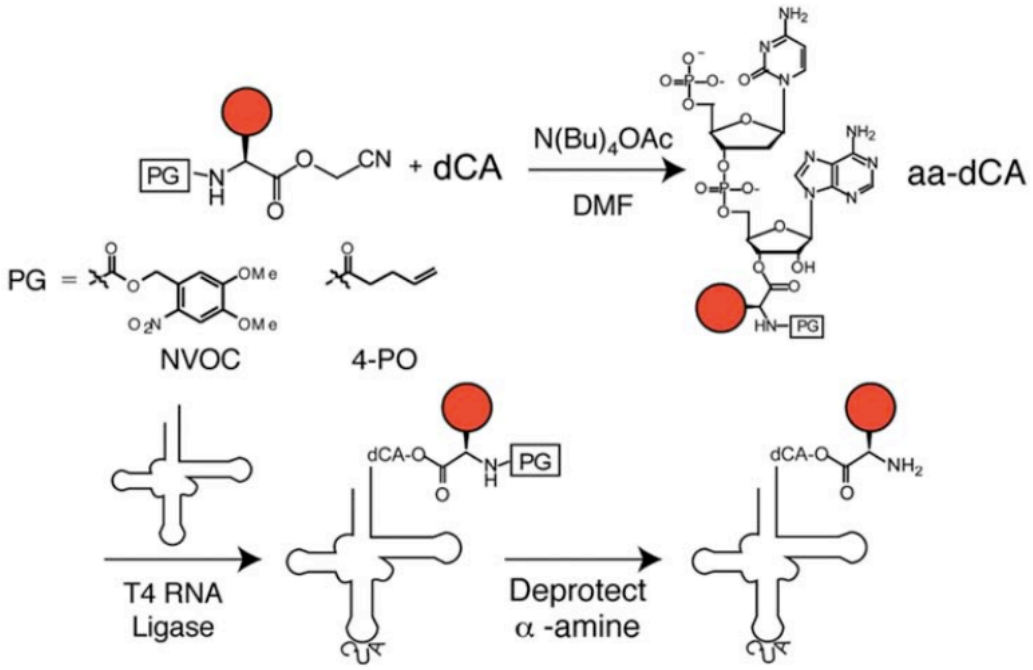

Figure 1.7 The steps involved in the semisynthesis of suppressor aminoacylated tRNA. The unnatural amino acid is protected by either NVOC or 4-PO and coupled to dCA in DMF. Aminoacylated-dCA (aa-dCA) is ligated to a truncated 74-mer $tRNA_{CUA}$. The final step involves deprotection of the α-amine.

The nonsense suppression method begins with the transcription of a truncated 74-mer suppressor tRNA lacking the final two nucleotides, C and A. A separate step requires the chemical synthesis of the final two nucleotides, deoxy-C-A (dCA) (Figure 1.7). The unnatural amino acid is also chemically synthesized and it contains an α-N-protecting group that is either photolabile, 6-nitroveratryloxycarbony (NVOC) or I_2-

labile, 4-pentenoyl (4-PO) groups, chemistry that is orthogonal to tRNA functional groups (*29*). The carboxyl group is synthesized with an activating group, a cyanomethyl ester, for coupling to dCA, aminoacyl tRNA (aa-tRNA). The dCA is used as a handle for acylation of the suppressor tRNA. Then, aa-dCA is enzymatically ligated with T4 RNA ligase to the 74-mer $tRNA_{CUA}$ to produce a full-length 76-mer $tRNA_{CUA}$ (Figure 1.7) (*18, 21, 22, 30-32*). Additionally, the α-N-protecting group remains on the amino acid through all of the coupling and ligation reactions to stabilize the bond between the terminal adenosine and the amino acid.

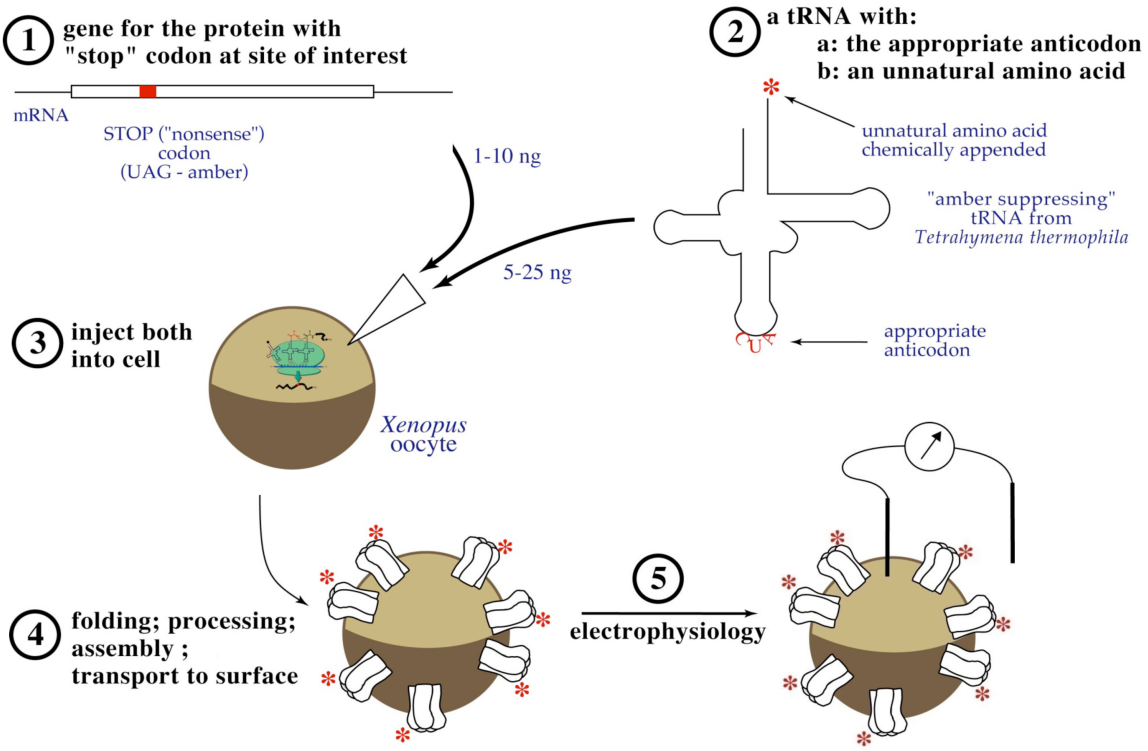

Figure 1.8 *In vivo* nonsense suppression methodology. It begins with mRNA containing a UAG (or 4-base) codon and aa-tRNA that are injected into an oocyte, where the endogenous ribosome synthesizes the protein and traffics it to the surface. Then functional electrophysiological studies are performed on the ion channels.

The deprotected amino-acylated tRNA and the mRNA containing the UAG codon (or four-base codon) are manually injected into a *Xenopus laevis* oocyte (*18-20, 22, 26, 31, 33*). The mRNA and aa-tRNA are incubated in the cell, typically for 1-3 days to

allow for protein synthesis, folding, and trafficking to the surface (Figure 1.8). This methodology allows for an almost limitless incorporation of amino acids into proteins. Over 100 different amino acids have been incorporated into proteins (Figure 1.9) both *in vivo* and *in vitro (21)*. The method is very versatile as this approach allows the incorporation of alpha hydroxy acids, hydrazides, and N-hydroxylamines (*20, 34*), along with many other fluorescent and protonated amino acids, etc. However, this technique has limits, as D-amino acids and β-amino acids are not incorporated (*35*).

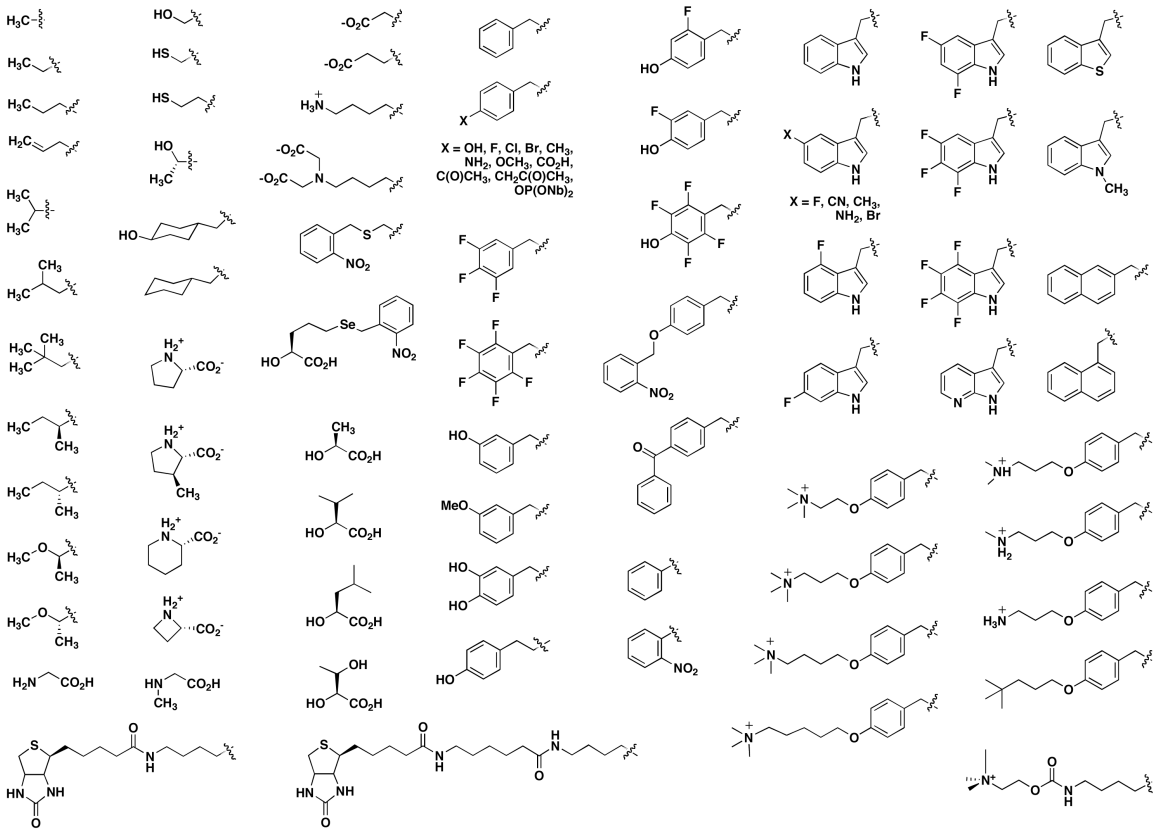

Figure 1.9 Examples of some of the unnatural amino acid side chains that have been incorporated into proteins.

In order to increase the integrity of the nonsense suppression technique, we utilize two controls, both involving the suppressor $tRNA_{CUA}$. The first control experiment tests for "readthrough" of the stop or 4-base codon by endogenous tRNAs. The mutated mRNA containing the UAG or 4-base codon alone is injected; no ion channel production should result, since translation should be blocked in the absence of suppressor tRNA. In

the second control experiment, a full-length suppressor tRNA without an amino acid appended to it is co-injected with the mutated mRNA into an oocyte to control for misacylation. The "orthogonality" of the $tRNA_{CUA}$ (its ability to be unrecognized) by the cell's endogenous tRNA synthetases is extremely important. tRNA synthetases are enzymes that charge tRNAs with the appropriate amino acid during protein synthesis. However, the $tRNA_{CUA}$ must remain orthogonal, otherwise, after it incorporates the desired unnatural amino acid into the protein, it could be "misacylated" with a natural amino acid, producing mixtures of proteins, some with the unnatural amino acid and some without. This would be problematic for our studies since we are unable to separate the proteins. To gain orthogonality, Schultz and co-workers originally used a yeast phenylalanine $tRNA_{CUA}^{Phe}$ with the modified anticodon in an *in vitro Escherichia coli* expression system (*10*). In the *in vivo Xenopus* expression system, we utilized a $tRNA_{CUA}^{Phe}$ from *Tetrahymena thermophila*, which is not recognized by the endogenous *Xenopus* synthetases. This modified tRNA is often called THG73 and is also viable in an *in vitro E. coli* system (*22*).

Since the suppressor $tRNA_{CUA}$ is orthogonal to the expression system, it cannot be reamino-acylated with the unnatural amino acid in the cell, and therefore is a stoichiometric reagent. Theoretically, only one ion channel can be synthesized per aa-tRNA using this methodology. In practice, even fewer ion channels are produced due to complications such as amino acid hydrolysis from the tRNA. Luckily, we can take advantage of a very sensitive assay for studying ion channels, TEVC electrophysiology. Limited amounts of ion channels can be detected on the whole cell level, as low as 10 attomols (10^{-18}) (*21*). In fact, electrophysiology is sensitive enough to detect single ion channels as well.

1.3 Dissertation Work

The work described here can be divided into three sections, which combine the tools of unnatural amino acid mutagenesis and electrophysiology to study several neuroreceptors. The first studies, Chapters 2 and 3 will focus on a type of LGIC that is a member of a large, superfamily of ionotropic glutamate receptors (glutamate-gated receptors), iGluRs. Chapter 4 describes work performed to study a different LGIC, the muscle nicotinic

acetylcholine receptor (nAChR), which is a member of a different superfamily, the Cys-Loop superfamily of LGICs, and is gated instead by the neurotransmitter acetylcholine (ACh).

The first study described will focus on the N-methyl-D-aspartate (NMDA) receptors, which are members of the iGluR superfamily of neuroreceptors. Prior to the work described, the unnatural amino acid methodology had not been used to study any of the iGluR neuroreceptors. Our initial studies incorporating unnatural amino acids into an NMDA receptor were to study a potential cation-π interaction between an external Mg^{2+} ion and a conserved tryptophan in the pore of NMDA receptors and are described in Chapter 2. Since these were the first studies to incorporate unnatural amino acids into any iGluR, we developed optimal conditions necessary for expression of these ion channels and describe their characterization.

In Chapter 3 we continued to use unnatural amino acid mutagenesis to study the NMDA receptors. In these experiments we used conventional and unnatural amino acids to probe the interactions involved in agonist binding to the ligand-binding domain (LBD) of an NMDA receptor. We also used unnatural amino acids to create a functional probe of the mechanism that different agonists use to activate the NMDA receptor. We developed a novel method for determining how a NMDA receptor responds to different partial agonists.

Chapter 4 describes a series of studies designed to understand the mechanism by which the muscle nAChR discriminates between several ligands, including ACh and nicotine. Nicotine is a more potent agonist for neuronal nAChRs than it is for muscle nAChRs, and we are interested in identifying the structural components involved in ligand discrimination. These studies encompass a series of experimental work involving unnatural amino acid mutagenesis, conventional mutagenesis, and computational modeling.

1.4 Conclusion

Every year, millions of people are affected by neurological disease and disorders. Although great strides are being made towards understanding complications of the nervous system, there is still a great deal to be determined. Neuroreceptors lie at the nexus of regulating learning and memory, basic cellular homeostasis, and neurological disorders and diseases, such as Alzheimer's Disease, Parkinson's Disease, and Huntington's Disease, and are implicated in drug addictions and non-degenerative disorders as well. Ion channels in particular are the perfect targets for chemical biologists to study due to their complicated structures that correspond to regulation of very specific functions. Many current drugs target LGICs and their neurotransmitters, such as Namenda© (AD), Xanax© (anxiety disorders), and Aricept© (AD). One overarching goal of our research is to use the chemical-scale structural information we obtain to enable more precise drug design to target and regulate these neuroreceptors.

Our understanding and characterization of ion channels has come a long way since the 1970's however, much is still unknown about their structure. These are trans-membrane proteins that are often composed of multiple, different subunits. As such, ion channels remain elusive targets of the crystallographer. As a result, direct structural information available is very limited. That is why our functional studies to probe the chemical interactions necessary to maintain ion channel structure and influence agonist binding are optimal for determining how these essential receptors function.

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