Chapter 1

Introduction

1.1 A Chemical Understanding of the Brain

The brain is the most complex organ in the human body. On average, the adult human brain contains 10^{11} neurons, each of which makes 10^3 to 10^4 interactions with other neurons through junctions called synapses. Communication from one neuron to another takes place through synaptic transmission. Each synapse involves the communication of two neurons (Figure 1.1), the one producing the signal, called the pre-synaptic neuron, and the one receiving it, called the post-synaptic neuron. The pre-synaptic neuron receives information from other neurons through its dendrites. After processing this information, the neuron fires an electrical impulse, the action potential, which travels down the axon of the pre-synaptic neuron. Upon reaching the axon terminus, the electrical impulse causes vesicles containing neurotransmitter to fuse to the cell membrane, thereby releasing neurotransmitter into the synaptic cleft, the small space between neurons. This effectively turns the electrical signal, the action potential, into a chemical signal, the neurotransmitter. Receptors within the cell membrane of the post-synaptic neuron recognize and bind the neurotransmitters. Activation of the neuroreceptors directly or indirectly leads to the flow of ions across the membrane of the post-synaptic cell, thereby converting the chemical signal back to an electrical one.

Brain function is much more complex than simply understanding synaptic transmission. Neuroreceptors are located in both the pre-synaptic and post-synaptic densities and recognize a variety of ligands. Their activation leads to a myriad of down-

stream effects resulting from the flow of ions across the membrane. Neurotransmitters are most often small organic molecules

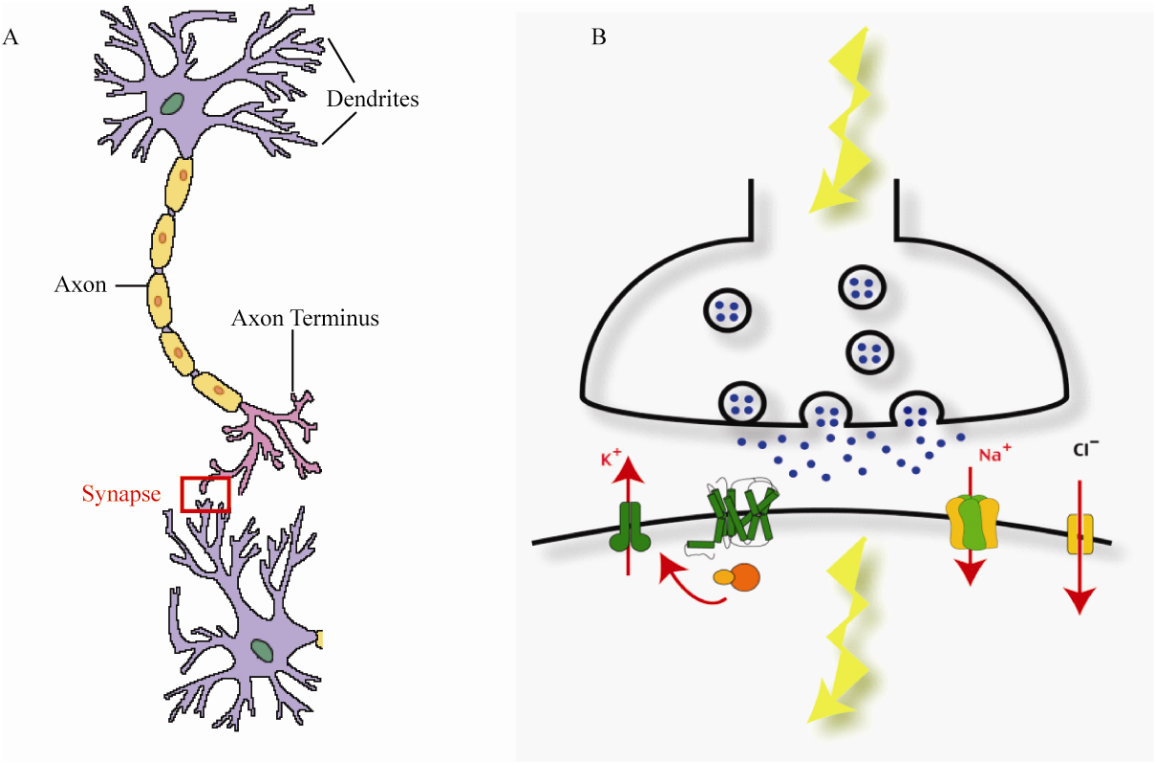

Figure 1.1 Synaptic Transmission. **A**, The interaction of two neurons takes place between the axon terminus (pink) of the pre-synaptic cell and the dendrites (purple) of the post-synaptic cell. The synapse is highlighted (red box). **B**, A cartoon of synaptic transmission. Neurotransmitter is depicted as blue dots.

but can also be peptides or fatty acids. The electrical signals that result from neuroreceptor activation can be either inhibitory or excitatory. A given neuron receives multiple signals at different synapses, further complicating matters. The interplay of various inputs at specific regions of the brain give rise to higher brain function, including learning and memory, addiction, and sensory input. Given the complexity of the brain, how can we hope to use chemical-scale studies to elucidate brain function?

We focus our efforts on developing an in-depth understanding of a specific type of neuroreceptor – the Cys-loop ligand gated ion channels. Neurotransmitter binding and

activation of the receptor are chemical-scale events that can be studied by site-specific incorporation of unnatural amino acids.

1.2 Cys-loop ligand gated ion channels

The Cys-loop ligand gated ion channels constitute a superfamily of receptors that mediate rapid synaptic transmission in the mammalian central and peripheral nervous systems through the binding of a neurotransmitter agonist. At rest, the receptor is in an unliganded, closed, non conducting state. Upon binding of agonist, the receptor undergoes a conformational change to an open ion-conducting state, thereby allowing ions to cross the cell membrane. The conformational change removes a hydrophobic barrier that prevents ion permeation, analogous to a gate swinging open; therefore the change from the closed state to the open state is often referred to as channel gating.

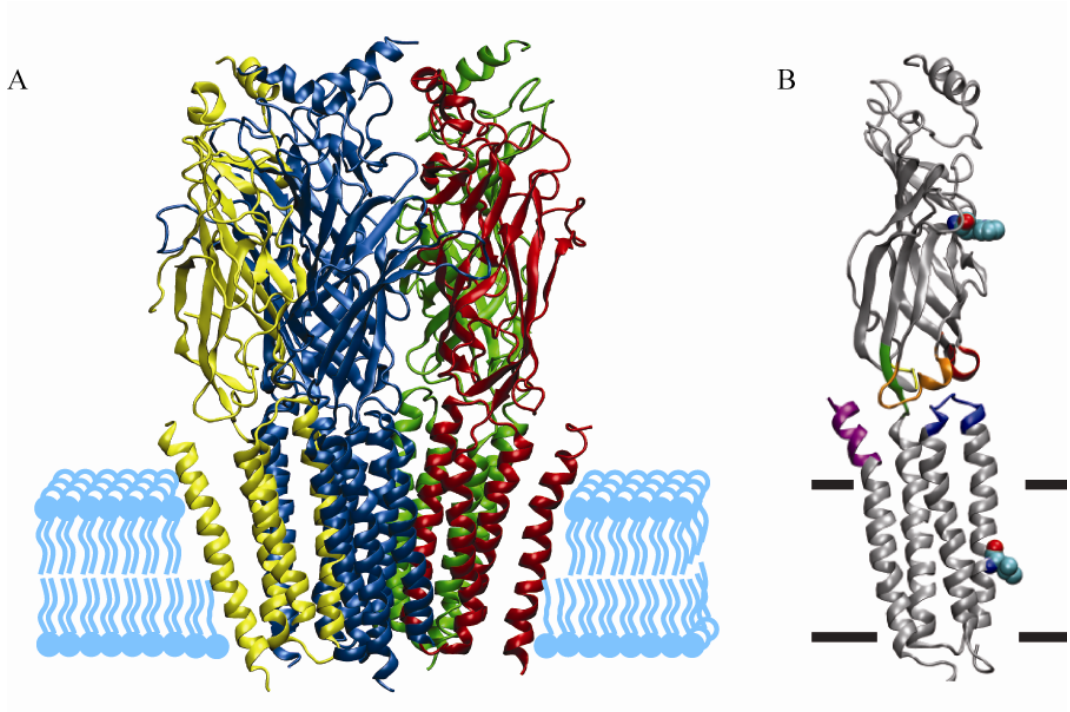

Figure 1.2 General topology of Cys-loop receptors. **A**, The nAChR, a prototypical Cys-loop receptor, has two α_1 (blue), one β_1 (green), one δ (red), and one γ or ε (yellow) subunit. **B**, Each subunit has a predominantly beta sheet, N-terminal extracellular domain and four membrane-spanning helices. The ligand binding site and channel gate are highlighted as VDW residues.

The superfamily includes the excitatory, cation conducting, nicotinic acetylcholine receptors (nAChR) and serotonin type 3A receptors ($5HT_{3A}R$), as well as the inhibitory, anion conducting, γ-aminobutyric acid (GABA) type A and type C receptors ($GABA_AR$ and $GABA_CR$, respectively) and the glycine receptors (GlyR). Each receptor is comprised of five homologous subunits arranged pseudosymmetrically around a central, ion-conducting pore (Figure 1.2). The large N-terminal domain of each subunit has a predominantly beta sheet structure with short loops connecting the beta strands. The primary sequence leads directly from beta strand 10 to four membrane-spanning helices, termed M1-M4, followed by a short, extracellular, carboxy terminus. The M2 helix of each subunit lines the channel pore. Neurotransmitter binding sites are located at select subunit interfaces in the extracellular domain, while the channel gate is located ~60Å away in the transmembrane region.

These large, membrane-spanning proteins do not readily lend themselves to crystallization techniques. As such, there are no crystal structures of mammalian Cys-loop neurotransmitter gated ion channels. Instead, structural information about these receptors relies heavily on biochemical studies as well as X-ray crystallography data from the acetylcholine binding protein (AChBP) and cryo-EM images of the nAChRs found in the electroplaques of the *Torpedo californica* ray. AChBP is a soluble pentameric protein isolated from snails which is highly homologous to the extracellular portion of Cys-loop neurotransmitter gated ion channels. Unlike the Cys-loop receptors, the role of AChBP is to bind acetylcholine, not to open an ion pore. Thus, while structural information from AChBP is useful in determining residues involved in neurotransmitter binding, the structure provides no information about the activation or gating pathway of the

mammalian ion channels. Cryo-EM is an inherently lower resolution methodology than X-ray crystallography, thus the amino acid side chains cannot be resolved. However, the cryo-EM image is the only structure of a full mammalian Cys-loop ligand gated ion channel, and has a higher sequence homology to nAChRs than AChBP. As such, the cryo-EM structure (Protein Data Bank code 2BG9) can guide studies relating to the gating pathway of these receptors more readily than AChBP structures.

1.3 Unnatural Amino Acid Methodology

1.3.1 *Advantage of Unnatural Amino Acids*

To study neuroreceptors on the chemical-scale, we need a precise methodology that allows us to investigate these proteins as we would small organic molecules on a bench top. The analogous experiments for neuroreceptors and their ligands are structure-function studies. Manipulation of the neurotransmitter can be carried out synthetically and has been done so extensively by medicinal chemists and pharmacologists. Derivatizing and making analogues of the neurotransmitters provides information regarding the action of neurotransmitter on neurons, and can elucidate the neurotransmitter pharmacaphore, but does not provide data on the structural rearrangements within the neuroreceptor. Conducting similar studies where the structural perturbations are restricted to the neuroreceptor allows the nature of the interactions with the receptor to be illuminated.

Cys-loop neurotransmitter-gated ion channels are multimeric membrane proteins of over two-thousand amino acids, and thus cannot be made synthetically. Furthermore, accurate evaluation of receptor function requires the proteins to be membrane bound. Mutagenesis of the receptor and expression in a heterologous system provide a means to

both perturb protein structure and preserve an *in vivo* context for channel function. However, mutagenesis restricts the structural perturbations to those contained within the twenty naturally occurring amino acids (Figure 1.3).

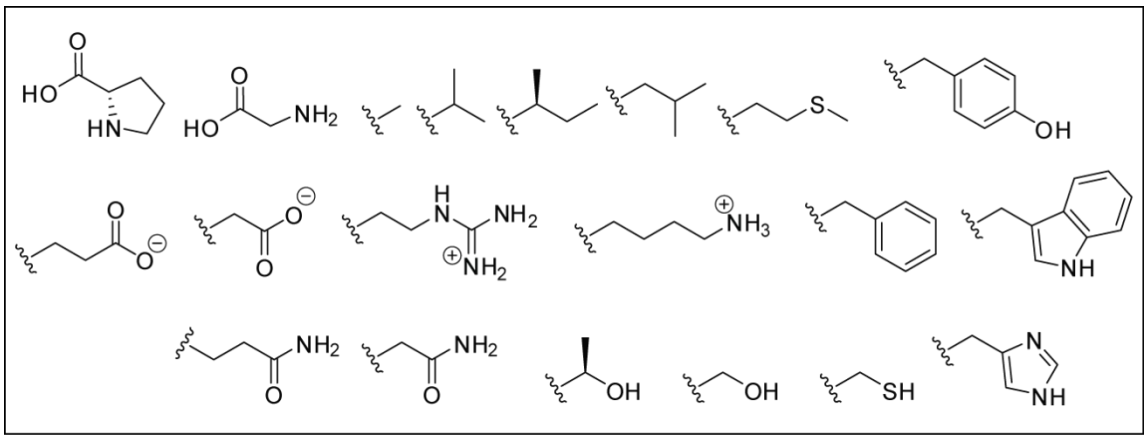

Figure 1.3 Side chains of the natural amino acids. From left to right: top row: proline, glycine (shown as full amino acid), alanine, valine, isoleucine, leucine, methioninine, tyrosine. Middle row: glutamate, aspartate, arginine, lysine, phenylalanine, tryptophan. Bottom row: glutamine, asparagine, threonine, serine, cysteine, histidine

The twenty naturally occurring amino acids have limited chemical functionality in their side chains. For example, there are no side chains with ketones, alkenes, or nitro groups. Even within the chemical functionality provided by these twenty amino acids, the overall shape, size, and length of the side chain is predetermined for a given functionality. For example, mutation from serine to cysteine replaces a hydroxyl group with a sulfhydryl group, yet there is no corresponding mutation for a threonine residue. Additionally, the interaction of two amino acids within the neuroreceptor may require augmenting side chain length or polarity or conversion of the amide peptide bond to an ester linkage. None of these objectives are accurately achieved using conventional mutagenesis. Incorporation of unnatural amino acids, however, allows the introduction of novel side chains to precisely determine the role of a specific amino acid.

While unnatural amino acids provide a virtually limitless pool of structural perturbations (Figure 1.4), how can we incorporate them into our proteins? Synthetic incorporation is not feasible for large, transmembrane bound proteins such as Cys-loop neurotransmitter gated ion channels. Instead we rely on the site-specific nonsense suppression methodology.

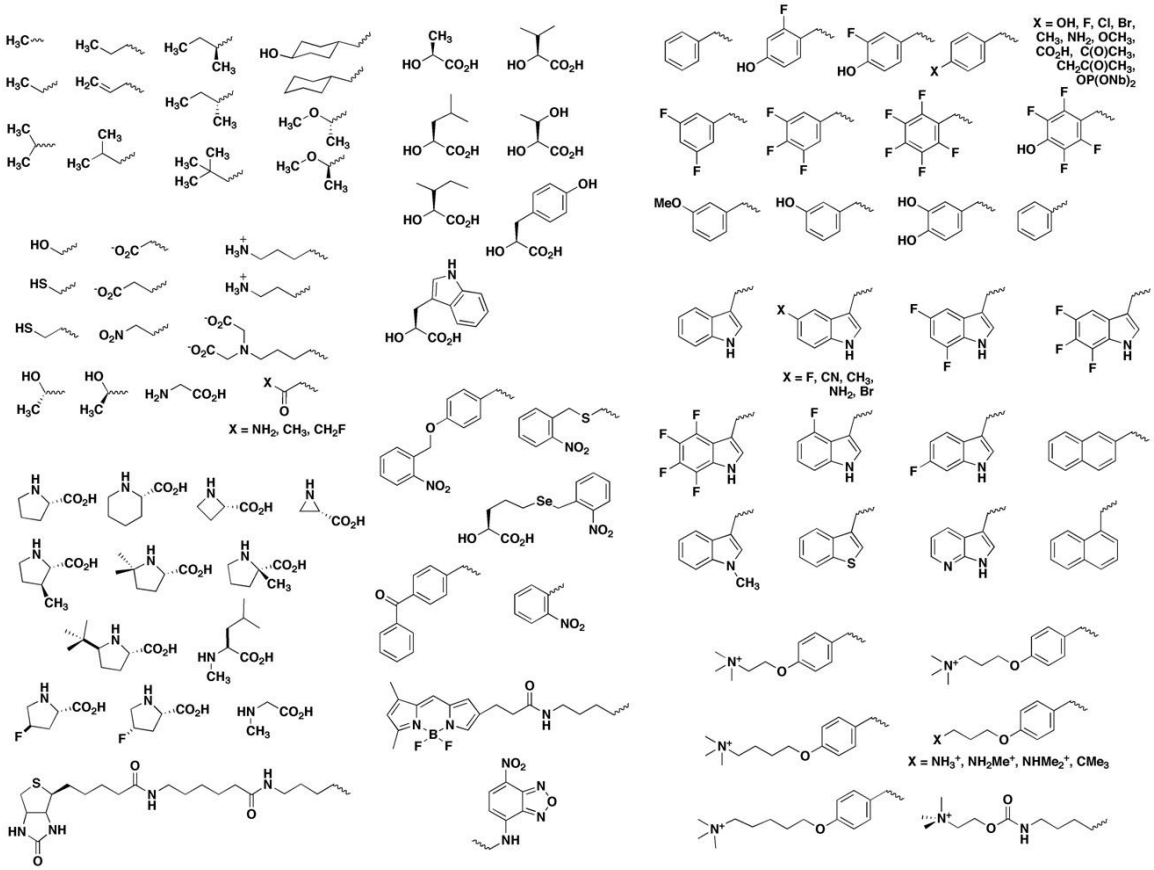

Figure 1.4 A subset of unnatural amino acid side chains and alpha hydroxy acids incorporated via the nonsense suppression methodology[1]

1.3.2 *Nonsense Suppression Methodology*

The ability to site-specifically incorporate unnatural amino acids into proteins was developed by Schultz and co-workers in 1989.[2-11] To achieve incorporation at a specific site, a codon that is not recognized by any of the native tRNAs must be utilized, a tRNA molecule recognizing this codon must be made, and the unnatural amino acid must be

chemically attached to the tRNA molecule. Only three codons, UAG, UAA, and UGA, do not code for one of the twenty naturally occurring amino acids. These three codons are STOP codons and normally mark the termination of translation. We incorporate a STOP codon at the site of interest and engineer a tRNA molecule with the appropriate anticodon. Instead of terminating protein translation when the STOP codon is reached, the ribosome incorporates the unnatural amino acid at this site as it would incorporate any of the naturally occurring amino acids (Figure 1.5).

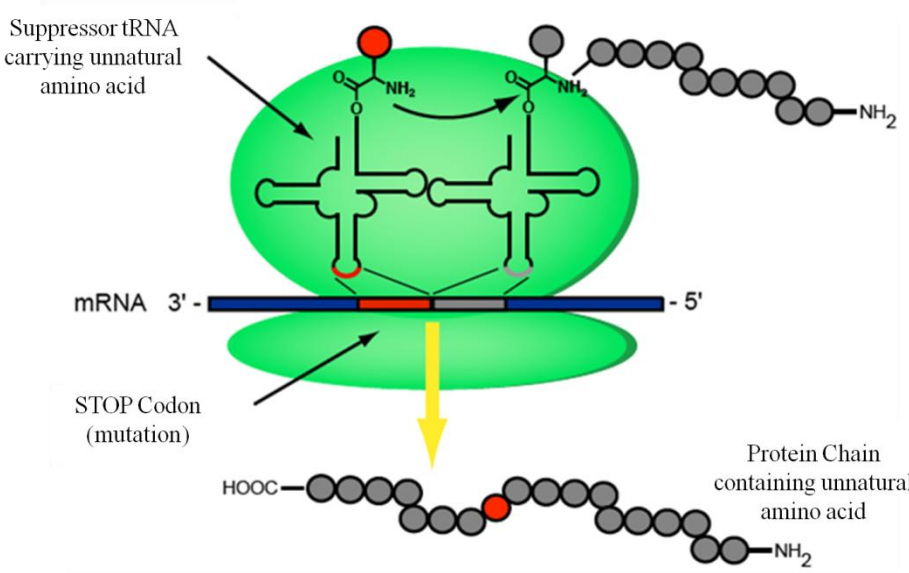

Figure 1.5 Overview of unnatural amino acid incorporation using nonsense suppression

Nonsense suppression requires a combination of molecular biology and chemical synthesis.[9,12-15] Most often, the amber codon, UAG, is used as the STOP codon because it was the first developed[8] and the suppressor tRNA engineered to recognize this codon has been shown to be more efficient.[16,17] The UAG mutation is first incorporated into the gene via quickchange PCR. *In vitro* transcription is used to make mRNA based on the DNA template. The suppressor tRNA without the last two nucleotides (C and A) of the acceptor stem are transcribed *in vitro.* The last two ribonucleotides of all tRNA

molecules are CA. Deoxy-CA (dCA) is chemically synthesized and the unnatural amino acid is attached chemically to give Uaa-dCA. The unnatural amino acids are prepared by addition of a photo- (NVOC) or iodine- (4-PO) labile amino protecting group. The carboxylic acid is activated as the cyanomethyl ester prior to acylation of the dCA molecule (Figure 1.6) to give Uaa-dCA. Once acylated, the dCA is ligated to the truncated suppressor tRNA using T4 RNA ligase to yield the full length amino-acylated tRNA. The amino group of the Uaa is left protected to stabilize the acylated tRNA.

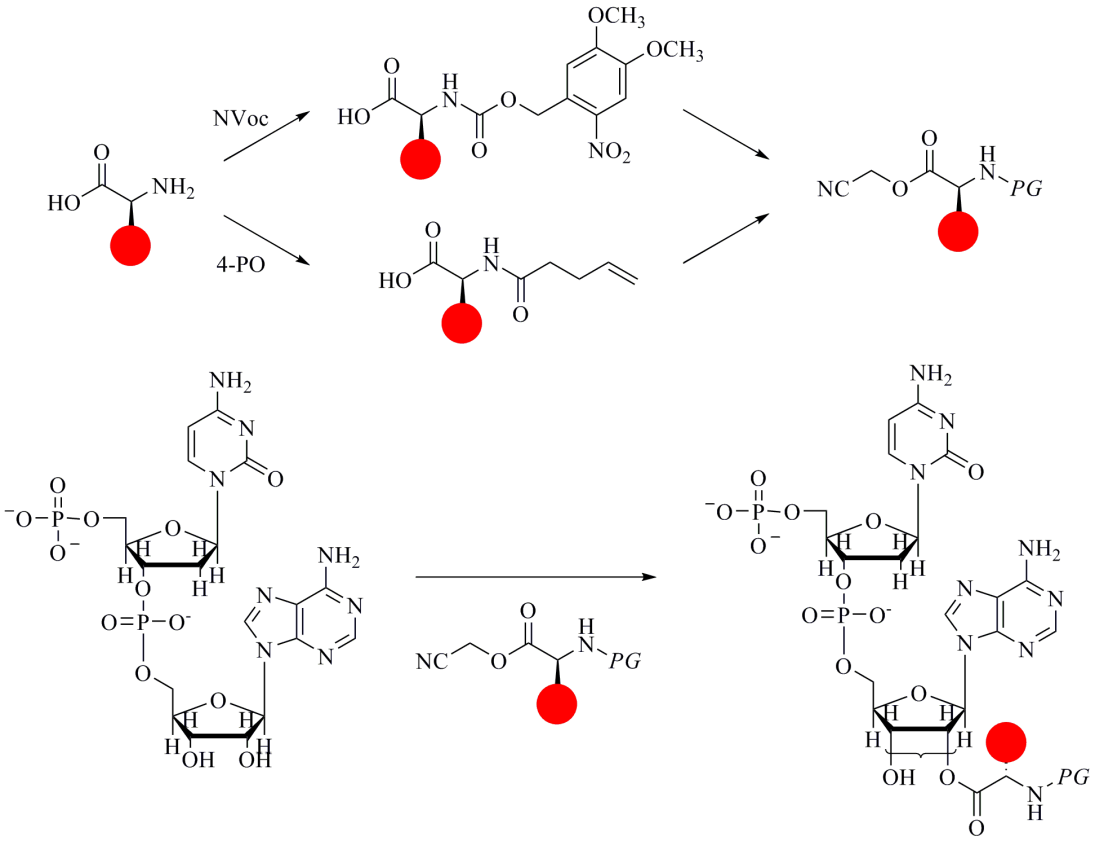

Figure 1.6 Standard preparation of unnatural amino acid (Uaa) coupled to deoxy-Cytosine, Adenine (dCA)

Immediately prior to injection into *Xenopus laevis* oocytes (Figure 1.7), the amino group of the unnatural amino acid is deprotected, and tRNA-Uaa is mixed with mRNA. Each oocyte is injected with 50 nL of RNA mix and then allowed to incubate for twenty-four to forty-eight hours. During this time, the Cys-loop neurotransmitter gated ion

channels are transcribed, folded, processed, assembled, and transported to the cell surface. Once an unnatural amino acid is incorporated into the nascent protein chain, the tRNA is released back into the cell. However, there is not a way to put more Uaa on the tRNA molecule, thus the Uaa-tRNA is used as a stoichiometric reagent, thereby limiting the amount of protein made. Fortunately, these ion channels can be evaluated by electrophysiology, a highly sensitive assay that detects currents through the whole cell.

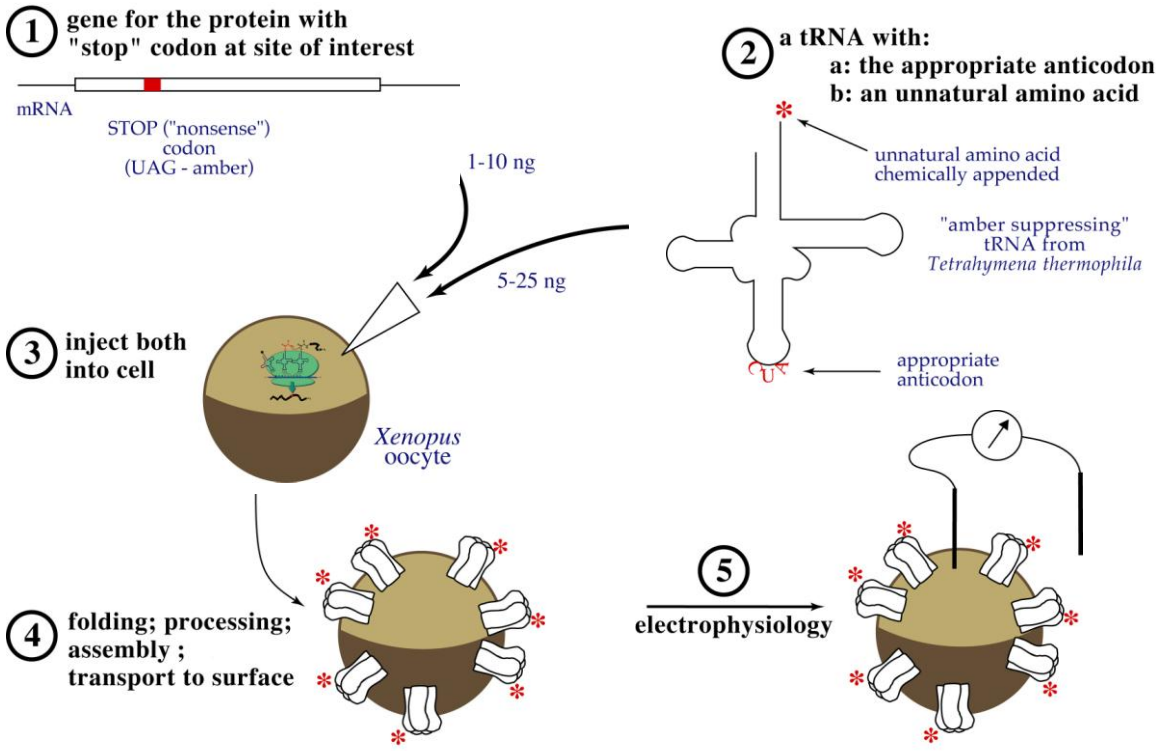

Figure 1.7 Implementation of nonsense suppression methodology using *Xenopus laevis* oocytes

Direct application of neurotransmitter to the oocytes results in neurotransmitter binding to the surface-expressed receptors followed by activation to the open state. The flow of ions across the cell membrane produces a macroscopic whole cell current that can be measured (Figure 1.8) by the two-electrode voltage clamp method. The amount of current is dependent on the number of surface expressed receptors, the proportion of the

receptors binding neurotransmitter, and the ability of the drug to activate the receptor. Applying increasing concentrations of agonist results in larger whole cell currents (Figure 1.8B). The relationship between the concentration of neurotransmitter, or drug, and the current size is the dose response relationship (Figure 1.8C). The EC_{50}, or effective concentration to induce the half-maximal response, is used as a metric of ion channel function. A leftward shift in EC_{50} is termed gain of function because less neurotransmitter is required to achieve the same level of activation. A rightward shift in EC_{50} is a loss of function mutation because more neurotransmitter is required to achieve the same level of activation. The EC_{50} is an equilibrium measurement dependent on both neurotransmitter binding and the potency (ability to activate the neuroreceptor) of the neurotransmitter.

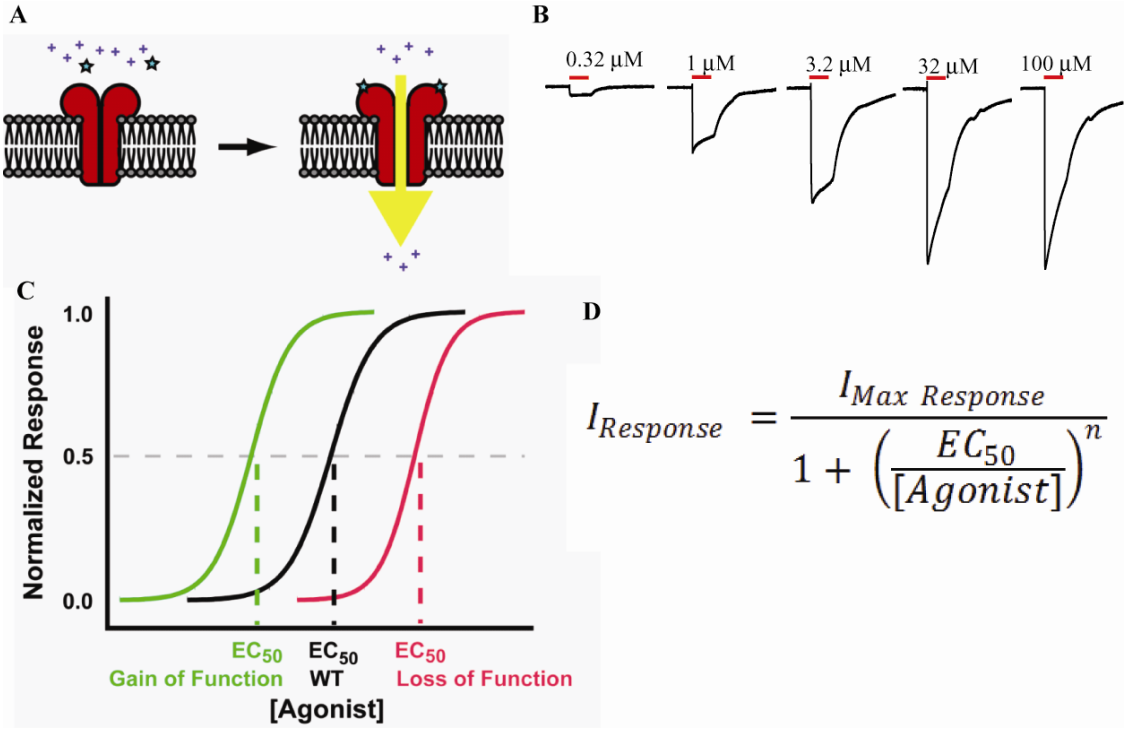

Figure 1.8 Typical electrophysiology assay. (**A**) Neurotransmitter, or agonist, (stars) binds to the neurotransmitter gated ion channels which leads to receptor gating and ion flow. (**B**) The current size increases as the concentration of agonist is increased until all receptors are saturated. (**C**) The dose response relationship is determined and fit to the Hill equation (**D**) to give the EC_{50} and Hill coefficient (n).

1.4 Dissertation Work

This dissertation describes multiple studies that utilize conventional mutagenesis as well as unnatural amino acid incorporation combined with electrophysiology. These studies focused on individual members of the Cys-loop ligand gated ion channel (LGIC) superfamily, specifically the muscle-type nAChR, the $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_2$ $GABA_AR$, and the α_1 GlyR.

Chapters 2 through 4 describe research investigating the role of loop 2 residues in the α_1 subunit of nAChRs during channel activation. The highly charged nature of the loop is investigated through conventional mutagenesis in Chapter 2.[18] Mutation of loop 2 residues to histidine followed by pH experiments to change the protonation state of the loop are the subject of Chapter 3. In Chapter 4, site-specific incorporation of unnatural amino acids is used to evaluate a proposed interaction[19-23] between a loop 2 residue and the transmembrane domain of the receptors. Due to the precision of the nonsense suppression methodology, this study[24] is more conclusive than any previous study[25,26] investigating this interaction.

In Chapter 5, our focus shifts to the binding site of two inhibitory members of the Cys-loop family; the $\alpha_1\beta_2$ $GABA_AR$, and α_1 GlyR. Herein we identify the location and strength of a cation-π interaction formed between the cognate neurotransmitter and neuroreceptor by introduction of fluorinated phenylalanine residues.

Finally, Chapters 6 and 7 discuss our efforts to identify the role of an unstructured linker in the α_1 subunit of $GABA_ARs$ in channel activation and benzodizapine potentiation. We investigate both side chain and backbone mutations which affect the gating pathway of these receptors.

1.5 References

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