Chapter 1. Introduction

1.1 Chemistry and the brain

In terms of speed and complexity, the nervous systems of higher organisms likely represent the pinnacle of intercellular communication. The human brain alone has roughly 10^{12} neurons and each neuron typically makes 10^{3}-10^{4} connections or synapses with other neurons. This means that there are approximately 10^{15}-10^{16} synapses in the human brain, with each synapse representing an intercellular connection for communication between two neurons. Thus, on one level, the brain is essentially a vast network for cell-to-cell communication. Astonishingly, signaling in this massive synaptic network operates on the *millisecond* timescale. It is this speed and complexity that gives rise to human thought and consciousness. An amazing feature of intercellular communication in the nervous system, and ultimately thought and consciousness, is that the events governing their function occur at the molecular level.

The primary mode of synaptic transmission (interneuronal communication) in the nervous system is chemical. In other words, the information conveyed between neurons is encoded in a small molecule, the neurotransmitter. This is recognized and bound by specific receptor molecules that translate the chemical information into cellular processes. The presynaptic neuron initiates synaptic transmission by releasing a neurotransmitter into the synaptic cleft. The neurotransmitter diffuses across the cleft where it binds to a neuroreceptor embedded in the membrane of the postsynaptic neuron. Binding of the neurotransmitter activates the receptor, conveying the synaptic signal across the cell membrane and resulting in the modulation of the postsynaptic neuron.

There are a wide variety of neurotransmitters and many different neuroreceptors. The majority of neuroreceptors can be divided between two main classes: the G-protein coupled receptors which mediate slow synaptic transmission and act through second messenger pathways, and the ligand-gated ion channels (LGIC) which mediate fast synaptic transmission. In LGICs, binding of the neurotransmitter induces a conformational change in the protein, opening an ion permeant channel that spans the cell membrane. The ion channel is fully contained within the LGIC, and once open, ion flow through the channel alters the postsynaptic neuron's membrane potential. This then either encourages or discourages the firing of an action potential in the postsynaptic neuron, depending on whether the LGIC is excitatory (cation selective) or inhibitory (anion selective).

The primary goal of the work presented in this thesis is the chemical-scale elucidation of the structure and function of LGICs. In particular, the studies focus on elucidation of a serotonin-gated ion channel, the 5-hydroxytryptamine-3 receptor ($5\text{-}HT_3R$)[1], a member of the Cys loop family of LGICs. This family of receptors mediates fast synaptic transmission throughout the peripheral and central nervous systems. They are also the targets for therapeutic treatments of Alzheimer's disease, Parkinson's, disease, schizophrenia, stroke, learning and attention deficit disorder, and drug addiction. The motivations for these studies are twofold. The Cys loop receptors are biologically and therapeutically significant, and they are of fundamental interest from a chemical perspective. Their study addresses important concerns in drug-receptor interactions and molecular recognition, and the structural rearrangement associated with activation of the ion channel poses an intriguing chemical question: How does the

noncovalent binding of a small molecule induce a large conformational change in a multisubunit protein?

1.2 The Cys loop family of ligand-gated ion channels

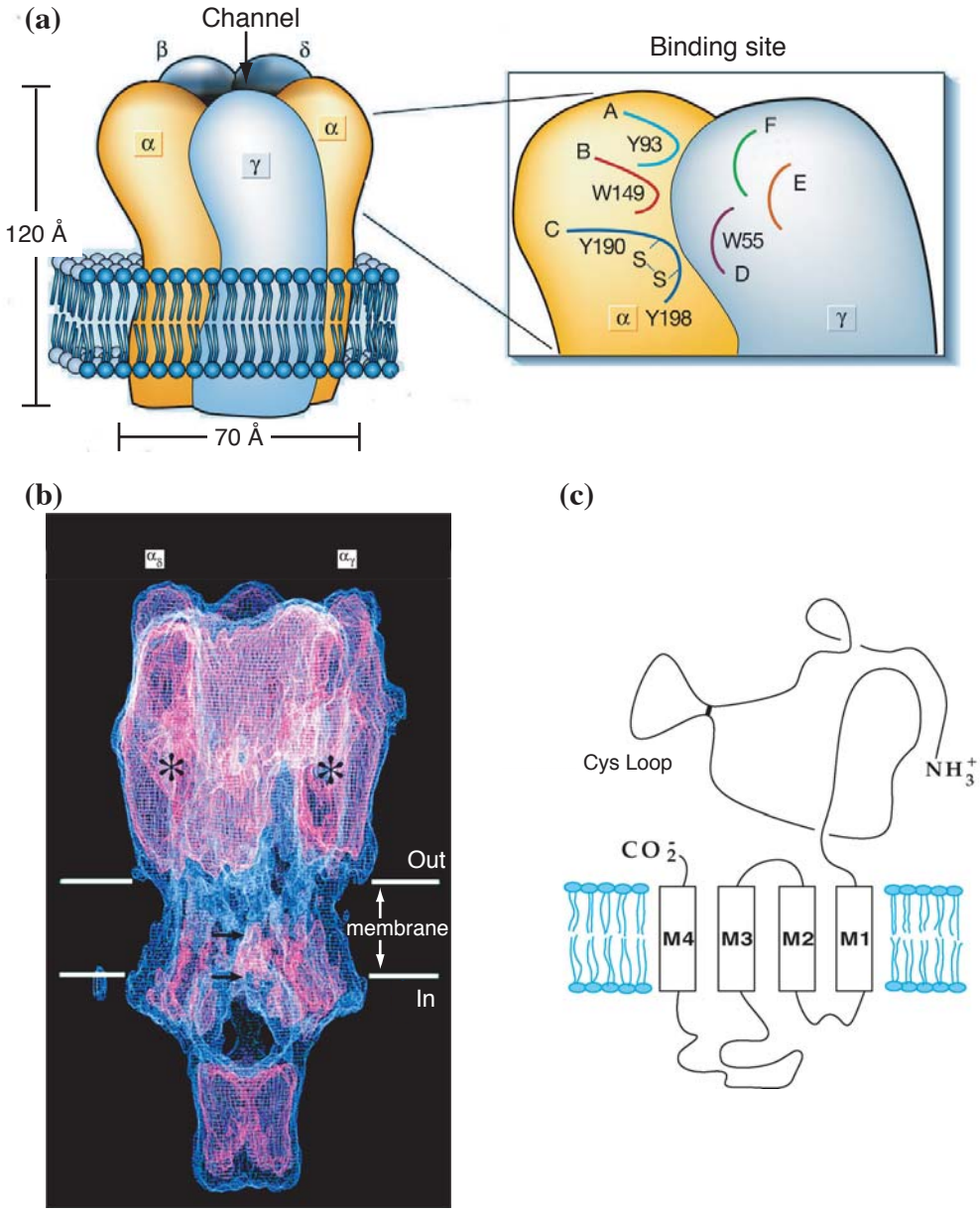
In addition to the 5-HT_3R, the Cys loop family of LGICs also includes the nicotinic acetylcholine (nAChR), gamma-amino butyric acid A ($GABA_A$), $GABA_C$, and glycine (GlyR) receptors.[2-8] These receptors can be separated into two subclasses based on their ion permeation properties: the cation-selective receptors including the nAChR and 5-HT_3R, and the anion-selective receptors including the $GABA_A$, $GABA_C$, and GlyR. The best-studied member of this family is the nAChR. These receptors are quite homologous with 25-30% conservation in the amino acid sequence identity among the various members. Thus, much of what is known about the structure and function of these receptors is inferred from studies of the nAChR.

The Cys loop receptors are multisubunit integral membrane proteins. Their quaternary structure is pentameric with five homologous subunits arranged in a pentagonal array around a central pore (Figure 1.1). Each subunit has a large extracellular N-terminal domain and four transmembrane domains, M1-M4. The M2 domains of each subunit line the interior of the channel lumen. The extracellular domain contains the ligand-binding site, as well as the Cys loop. The latter is a loop formed by two disulfide-bonded cysteines and is a common structural motif in all members of the family. Each receptor in the family has a variety of subunit isoforms. These combine in different compositions to form distinct receptor subtypes. For most of these receptors the exact subunit composition is unknown. The muscle receptor of the nAChR, however, has a well-understood composition of $(\alpha 1)_2\beta 1\gamma\delta$ (Figure 1.1). In this receptor, the ligand-

binding sites are located primarily in the α-subunits, and it is known both must be occupied to optimally activate the receptor (i.e., open the ion channel). For the 5-HT_3R, three subunits have been identified; *A*, *B*, and *C*.[1, 8, 9] The subunit stoichiometry for native receptors has yet to be determined, however, *A* subunits can form functional homomeric receptors, indicating that as with the muscle nAChR, the *A* subunit contains the majority of the ligand-binding determinants.

Being integral membrane proteins, the Cys loop receptors have been resistant to characterization by high-resolution structural techniques such as NMR and x-ray crystallography. A significant source of structural information concerning these receptors has come from cryo-electron microscopy (cryo-EM) studies on the *Torpedo* electric ray. The electric organ in these rays is highly enriched in nAChR-containing membranes. The high density of nAChR in this tissue is amenable to structural analysis by cryo-EM.[10-13] Recently, cryo-EM studies by Nigel Unwin have produced an image of the transmembrane domains at 4-Å resolution (Figure 1.2a).[14] This represents the highest resolution structure of this region of the protein, and is supported by much of the earlier structure-function studies in the Cys loop receptors. The structure revealed that each transmembrane domain forms a membrane-spanning α-helix. In addition, it provided strong evidence that the gate of the receptor is formed by the hydrophobic side chains of Leu 9' and Val 13' in M2 (numbering convention for residues in M2 starts with 1' at the C-terminal or cytoplasmic end of M2), confirming results from previous studies.[15, 16] A novel feature of this structure was the clear indication that the M2 domain extends two helical turns above the extracellular membrane. This contradicts the predictions based on

the hydropathy profile of the nAChR subunits, but results from earlier substituted cysteine accessibility studies support this conclusion.[17]



Unwin, *Phil T Soc B,* (2000) **335**.

Figure 1.1. Structure of Cys loop LGICs. (a) Cartoon of the nAChR showing general structural features of the receptor. Inset shows the location of the binding site at the subunit interface and the loops that contribute important aromatic residues to the primary binding site. (b) 4.6 Å cryo-EM of the nAChR. (c) Single subunit showing subunit topology.

While as yet, no x-ray crystal structure exists for these receptors, there is a crystal structure of an acetylcholine binding protein (AChBP) (Figure 1.2b).[18-20] This is a soluble protein from the snail, *Lymnaea helicalis*, and is highly homologous to the extracellular domain of the nAChR. The crystal structure of this protein has been a watershed event in the study of Cys loop LGICs, providing the first high-resolution structural data on the ligand-binding site. This structure confirmed much of the previous biochemical and biophysical studies on the nAChR, including the location of the ligand-binding site at subunit interfaces. In the muscle nAChR, the two binding sites are located at the α/γ and α/δ interfaces (Figure 1.1a and Figure 1.2b). Furthermore, the AChBP structure revealed that the binding site is composed of five noncontiguous loops (loops A-E), each of which contributes one or more aromatic residues to form the primary binding site (see chapters 2 and 3 for more details).

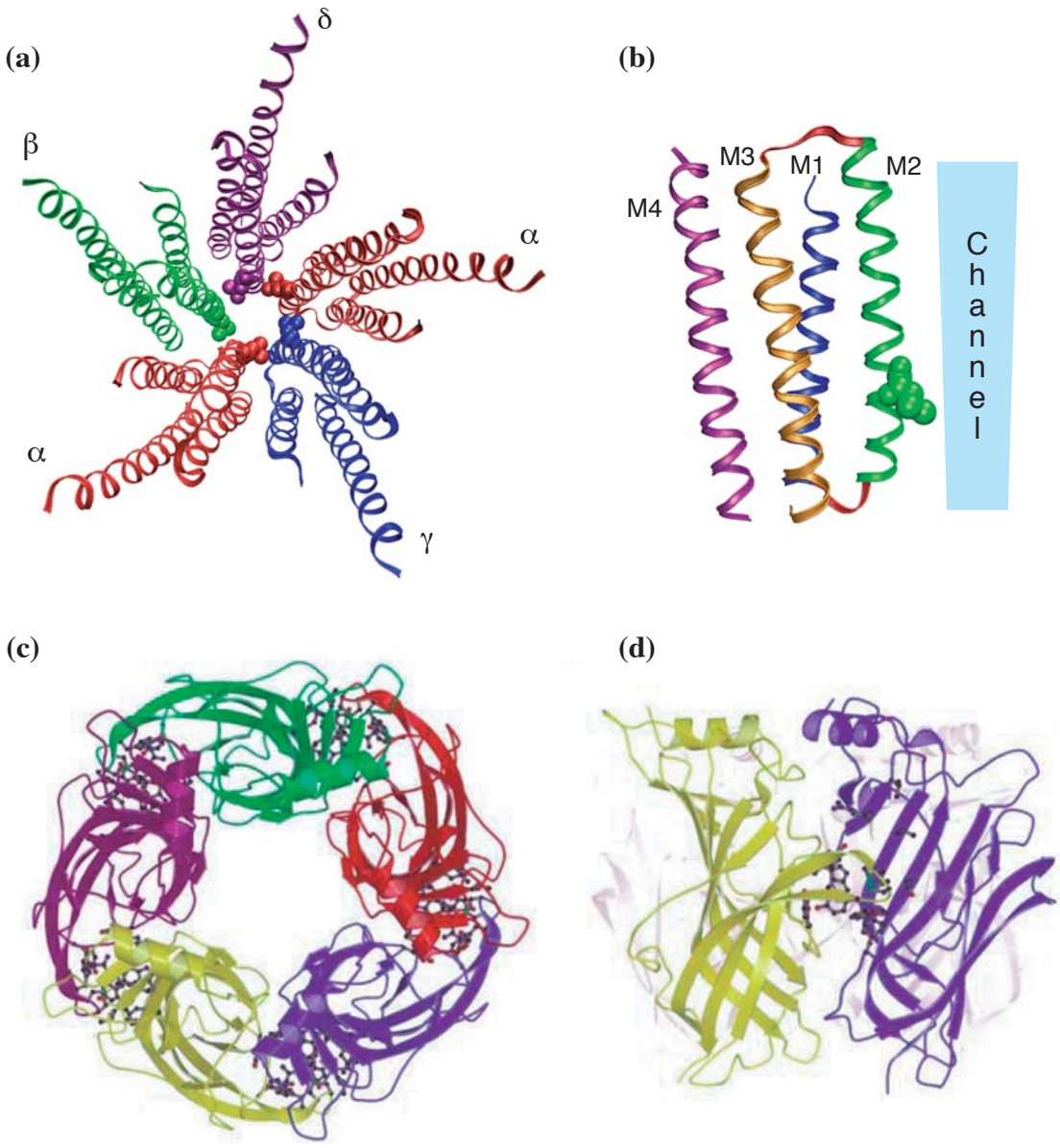


Figure 1.2. Cryo-EM structure of nAChR transmembrane domains and AChBP structure. (a) Top view (looking down from the extracellular side) of the transmembrane domains based on Unwin's 4.0Å images. Leu 9' for each subunit is shown in CPK representation. (b) Transmembrane domains of a single subunit. (c) Top view of the AChBP structure. (d) Side view of AChBP with two subunits highlighted and the binding site residues shown in ball-and-stick representation.

1.3 Unnatural amino acid mutagenesis

Although the recent structural breakthroughs detailed above have been invaluable to the study of Cys loop receptors, there are still many outstanding questions concerning

their function. By their very nature, these receptors are dynamic proteins undergoing large conformational changes and interconverting among multiple states (i.e., non-conducting closed state to conducting open state). X-ray crystal structures provide a starting point for evaluating these processes, but are only static snapshots. Structure-function studies are still necessary to determine what features revealed by the crystal structure are functionally significant. The central approach of the research presented here is the use of *in vivo* nonsense suppression for structure-function studies. This method allows for the site-specific incorporation of a synthetic amino acid into a protein expressed in a living cell. In many ways, this method is ideal for the study of ion channels. The ability to incorporate unnatural amino acids permits systematic structure-function studies, furnishing a chemical-scale precision unique among biochemical methods. In addition, the methodology allows for study in a cellular system, obviating the need for functional isolation of these multisubunit integral membrane proteins. One of the drawbacks of the nonsense suppression method (and in particular *in vivo* nonsense suppression) is the limited quantity of protein produced. This limitation is minimized, however, in the study of ion channels due to the extraordinary sensitivity of modern electrophysiology. Thus, the functional consequences of the introduced mutations can be readily assayed.

In 1989, Peter Schultz reported the first general method for the biosynthetic incorporation of unnatural amino acids using nonsense suppression.[21-24] The method works by commandeering a non-coding codon or 'nonsense codon' (Figure 1.3a). The commandeered codon– usually a stop codon– is placed in the gene at the site of interest. Typically, this would result in the premature termination of the protein's biosynthesis.

However, if the translation system is supplied with a suppressor tRNA (a tRNA whose anticodon recognizes the nonsense codon) that is charged with a synthetic amino acid, then translation proceeds with the unnatural amino acid incorporated into the protein at the site of interest. The *in vivo* nonsense suppression method extends this methodology for *in vivo* usage with the *Xenopus* oocyte expression system.[25-27]

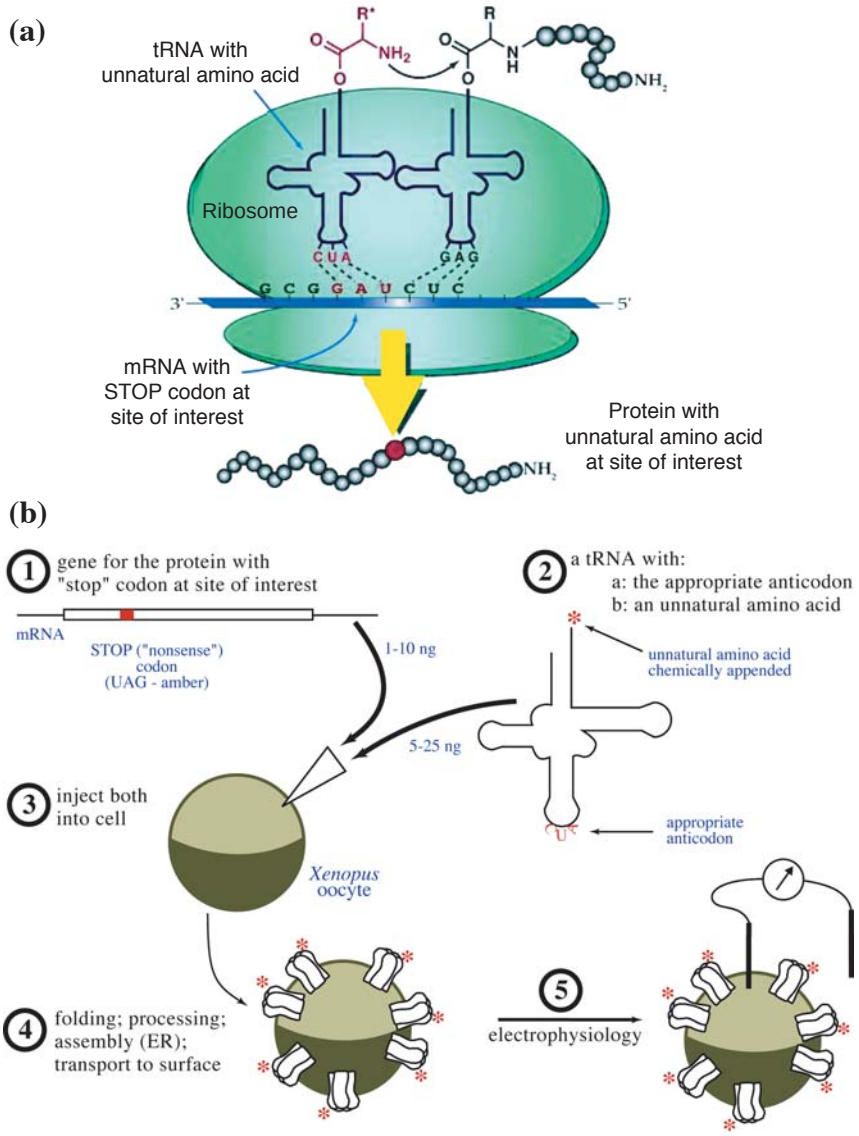


Figure 1.3. Nonsense suppression method. (a) Basic details of how nonsense suppression works. (b) *In vivo* nonsense suppression method.

The basic method for *in vivo* nonsense suppression first entails the placement of the amber stop codon (TAG) at the amino acid position of interest (Figure 1.3b). This is done using conventional site-directed mutagenesis, followed by *in vitro* transcription of UAG containing mRNA. Separately, a suppressor tRNA containing the appropriate anticodon (CUA), is prepared and chemically acylated with an unnatural amino acid. The tRNA and mRNA are then co-injected into a *Xenopus* oocyte. Protein synthesis and surface expression are carried out by the oocyte, allowing for electrophysiological study 24 to 72 hours later.

To date, more than eighty mutations (including both α-amino and α-hyroxy acids) have been incorporated into 15 different integral membrane proteins (Figure 1.4). In addition, the method has been extended for use with mammalian expression systems.[28] In our experience, the method is amenable to two sorts of studies: 1) detailed structure-function studies involving rational perturbation of the residue of interest[29-31] and 2) the introduction of unnatural side chains with novel chemical and biophysical properties such as fluorescent probes, 'caged' residues, and tethered agonists.[32-34] The limitations as to the variety of unnatural amino acids that can be incorporated have never been rigorously studied. There is, however, anecdotal evidence that hydrophobic residues suppress better than polar ones, and both D-amino acids and β-amino acids are poorly incorporated, if at all. Having noted this, the method can accommodate a wide variety of side chains, including several backbone mutations.[35-38]

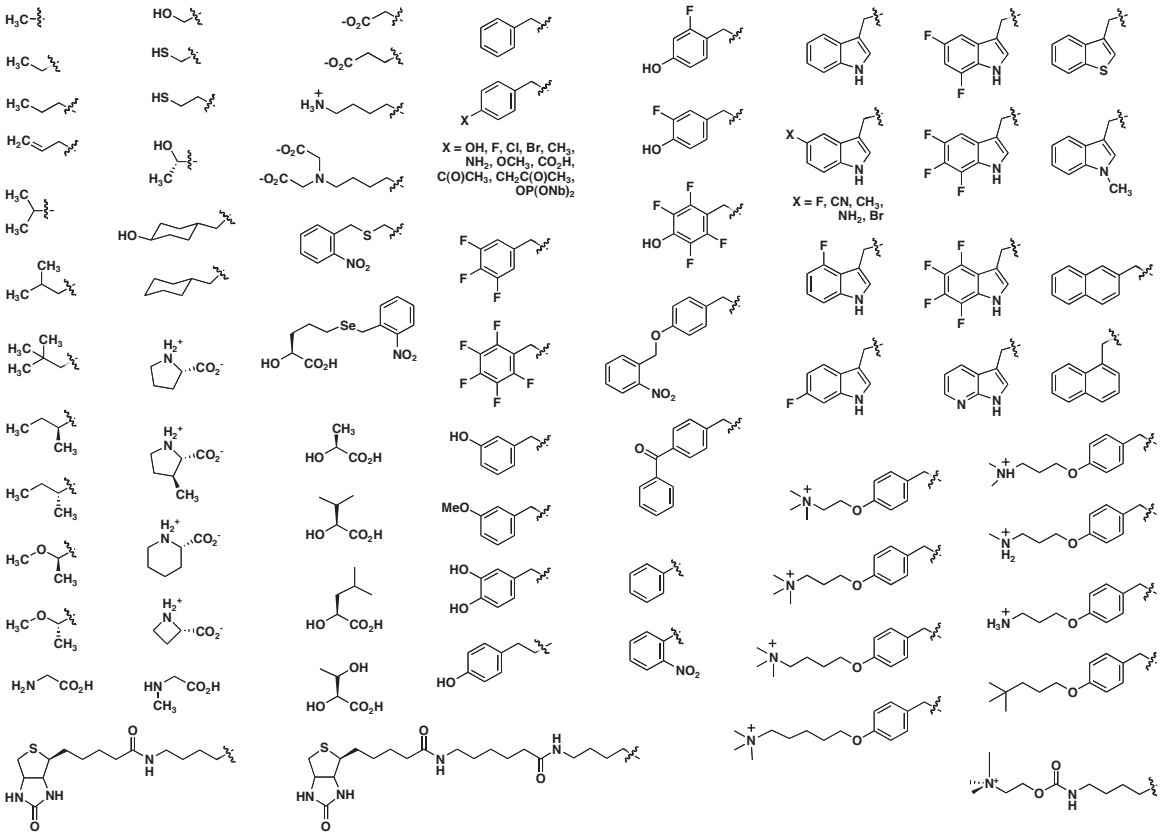


Figure 1.4. Representative α-amino and α-hydroxy acids incorporated into integral membrane proteins using *in vivo* nonsense suppression.

1.4 Structure-function studies

The nonsense suppression method is an excellent tool for structure-function studies. Much like conventional mutagenesis, it allows for site specificity in the mutations to be introduced. However, unlike conventional mutagenesis, it is not limited to the standard 20 amino acids. With the use of synthetic amino acids, one has rational control over the mutations to be introduced. In addition the chemical precision afforded by the technique is unique among biochemical methods. Consequently, it can provide chemical-scale information on the functional role and structural context of a given amino acid.

The majority of the work presented in this thesis centers on structure-function studies of the 5-HT_3R. Chapter 2 describes studies on the ligand-binding site in the

5-HT_3R and nAChR in which a series of progressively fluorinated tryptophans is used to identify the cation-π binding site of the 5-HT_3R and to compare and contrast ligand-recognition properties between the two receptors. This work is largely based on a *Biochemistry* paper written in collaboration with Gabriel Brandt, who performed all of the experiments on the nAChR.[39] The studies presented in Chapter 3 use a series of tyrosine analogs to probe the role of tyrosine residues near the ligand-binding site in the 5-HT_3R. Results from these studies are used to assess several models in which serotonin is computationally docked into the binding site. This chapter is based on a *Journal of Neuroscience* paper written in collaboration with the Sarah Lummis group at the University of Cambridge.[40] They performed all of the computational work, and Kerry Price in particular, contributed significant effort to this project. The work in Chapter 4 investigates the role of two conserved prolines in the M2-M3 loop of the 5-HT_3R. The findings from this work support a recently proposed model for the functional role of this loop in the gating pathway of Cys loop receptors. This work represents as well a collaboration with the Sarah Lummis group. Finally, Chapter 5 includes three miscellaneous studies: investigations of Phe 226 (Tyr 190 in the nAChR) in the 'aromatic box' of the 5-HT_3R; work identifying Tyr 198 (Trp 149 in the nAChR) as a cation-π binding site in the $GABA_C$ receptor; and efforts towards the incorporation of a backbone thioamide via nonsense suppression. The first two studies in Chapter 5 were done in collaboration with Sarah Lummis.

1.5 References

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