Chemical-scale manipulation of ion channels: *in vivo* nonsense suppression and targeted disulfide crosslinking

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Dedicated to my loving parents, George and Mary

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Abstract:

The study of the three-dimensional shape and structure-function relationships of ion channels is a very challenging field of research. Ion channels are integral-membrane proteins that when open allow ions to flux across the cell membrane. The structure and function of ion channels are dependent on the cell membrane that surrounds them. Because an ion channel must be embedded in a cell membrane, many techniques used to probe the structure of soluble proteins cannot be used in the study of ion channels.

One versatile technique that has been shown to be quite valuable in the structure-function studies of ion channels is the *in vivo* nonsense suppression method for unnatural amino acid incorporation. This technique allows one to site-specifically incorporate an unnatural amino acid or hydroxy acid into a protein in a living cell. To date more than 60 amino acids and hydroxy acids have been incorporated into proteins using *in vivo* nonsense suppression. The method has been shown to accommodate a wide variety of unnatural amino acids and hydroxy acids. Chapter One will discusses the *in vivo* nonsense suppression method in greater detail.

A key component of this work is the design and synthesis of new unnatural amino acids that have novel properties. Chapter 2 discusses the synthesis and uses of 5-(*o*-nitrobenzyl)selenyl-2-hydroxypentanoic acid (NBSeOH). NBSeOH is used to site-specifically cleave a peptide backbone. The *o*-nitrobenzyl protecting group is photochemically removed to reveal a selenium anion. The selenium anion then initiates an intramolecular S_N2 displacement that cleaves the backbone of the protein. Preliminary data reveals that NBSeOH can be incorporated into a protein *in vivo* and *in vitro*, and

photolysis of proteins and peptides containing NBSeOH does lead to protein backbone cleavage.

Chapter 4 discusses how the *in vivo* nonsense suppression method was used to incorporate unnatural amino acids containing a quaternary ammonium moiety to mimic the quaternary ammonium on acetylcholine. These unnatural amino acids were used to probe the nicotinic acetylcholine receptor's binding site. These unnatural amino acids are called tethered agonists because when they were incorporated into four different positions on the nicotinic acetylcholine receptor partial opening of the channel occurred even when agonist was not present. These tethered agonists were used to obtain distance information about where acetylcholine binds within the receptor.

Another technique used to probe the structure of ion channels is targeted disulfide crosslinking. In the targeted disulfide crosslinking method, cysteine residues are introduced at various locations throughout a protein and oxidized to see whether disulfide bond formation can occur. Since only cysteine residues close in space will form a disulfide bond, this method can reveal fine structural aspects of a protein. The method was used to study the pore lining structure of the nicotinic acetylcholine receptor. Several cysteine mutants were made using mutagenesis and then studied in functional channels expressed in *Xenopus* oocytes. The channels were then exposed to oxidizing agents, and the ability of these mutant channels to form disulfide bonds was evaluated. Chapter 3 describes the work dealing with the targeted disulfide crosslinking experiments in the nicotinic acetylcholine receptor.

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Chapter 1

Introduction

1.1 Background:

My graduate career has been centered on using different biochemical tools to probe protein structure-function relationships. In the case of the catalytic binding pocket of an enzyme or the ion selectivity of an ion channel, the three-dimensional structure of the protein is essential in carrying out the function of the protein. The work described here is motivated both by the desire to understand the function of nervous system proteins and the desire to develop general methods to determine the molecular basis of protein function.

Nerve impulses are propagated throughout the central nervous system and peripheral nervous system through a vast array of neurons (1). Neurons are excitable cells that can undergo rapid shifts in their electrical potential due to the flux of ions into and out of the cell. A synapse occurs where two neurons meet or a neuron interacts with peripheral organ (eye, muscle, etc.). Signal transduction at the synapse is mediated by G-protein coupled receptors and ligand-gated ion channels that recognize chemical effectors (neuronal or hormonal) and transduce a biological action. Figure 1.1 shows a diagram of a synapse and depicts the proteins that are essential in propagating a signal from one cell to another.

To give a brief understanding of neural signal transduction, the initiation of muscle movement by the muscle-axon synapse is described (1, 2). The transmitter released by the axon terminal is acetylcholine (ACh). Muscle fibers contain nicotinic acetylcholine receptors (nAChR). The nAChR is a ligand-gated cation channel and opens when it binds its agonist (ACh). When an action potential occurs in the pre-

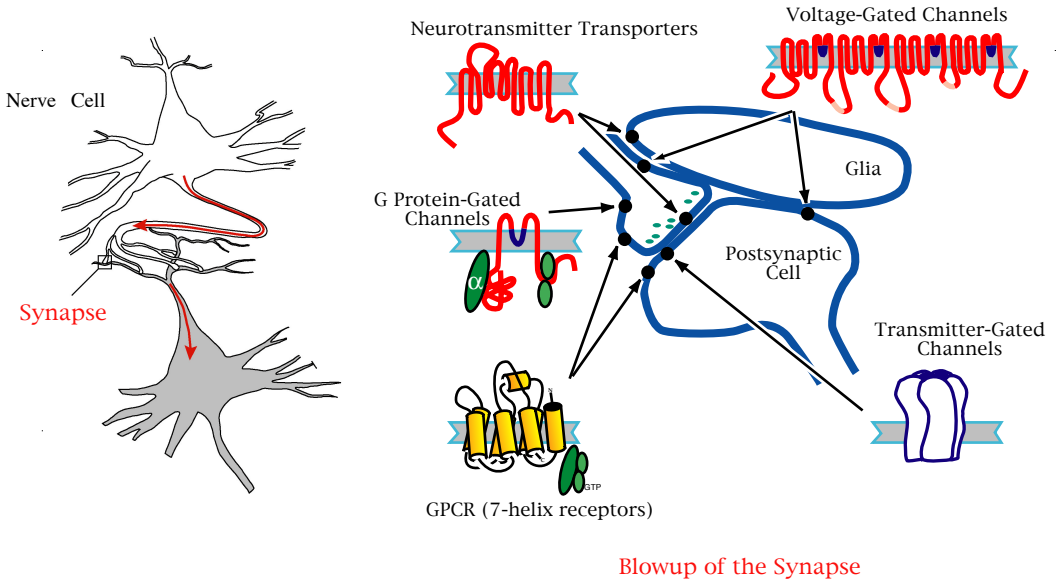


Figure 1.1 Molecules of neurobiology

synaptic motor neuron, ACh is released into the muscle-axon synapse and binds to nAChRs on the surface of the muscle fiber. Binding of ACh by the nAChR leads to a conformational change in the receptor that opens the pore of the channel, allowing positive ions to flow into the cell. When a large number of nAChRs open, the potential of the cell becomes more positive and the cell depolarizes. This depolarization opens voltage-gated Na^+ channels, resulting in an influx of Na^+ ions into the muscle fiber. This converts the pre-synaptic action potential into a post-synaptic action potential in the muscle fiber. Nicotinic acetylcholine receptors convert a chemical signal (ACh release) into an electrical signal by allowing ions to flow through the receptor's pore across the cell membrane (2).

The proteins depicted in Figure 1.1 serve as ≈ 30% of all pharmaceutical drug targets (3). These proteins have been shown to be directly linked to psychological and senility disorders (Parkinson's, Alzheimer's, schizophrenia, depression) (1, 2). All of these proteins are membrane bound proteins. Their structure and function are dependent on the membrane that surrounds them. Because of this, many of the biochemical techniques used to probe the structure of soluble proteins cannot be used in the study of integral membrane proteins.

One of the most useful approaches to study ion channels and GPCRs is the combination of site-directed mutagenesis and electrophysiology, which allows for systematic structure-function studies. This capability was greatly enhanced by the adaptation of the nonsense suppression methodology for unnatural amino acid incorporation (4-6) to the heterologous expression system of *Xenopus* oocytes (7, 8).

This technique allows one to site- specifically incorporate an unnatural amino acid or hydroxy acid into a protein in a living cell. The method incorporates an unnatural amino acid or hydroxy acid into a protein by 'tricking' the ribosome of a cell into inserting an unnatural amino acid at a UAG (stop) codon rather than dissociating from the mRNA template and terminating translation. Combining this method with electrophysiology allows us to probe the structure-function relationships of ion channels and receptors in ways not possible with conventional mutagenesis. The method has been used to incorporate unusual amino acids bearing large side chains like a biotin analog or 'caged' side chains, in which, a chemical moiety on the side chain is protected by a photochemically removable group (9, 10). It has also been used to make very subtle changes to an amino acid's side chain composition like the incorporation of tryptophan whose ring is mono-,di-, or tri-fluorinated (11, 12). The method is described in Figure 1.2.

In the method, the codon representing the amino acid where the unnatural will be incorporated into the protein of interest is mutated to an amber (TAG) stop codon. The amber stop codon is used because it is the least used of the three stop codons in *Xenopus* oocytes, which is the expression system used in our experiments. This construct is then transcribed *in vitro* to mRNA. A suppressor tRNA is then synthesized that contains the corresponding anti-codon CUA and is chemically acylated at the 3' end with the unnatural amino acid/hydroxy acid of interest (13). Both the mRNA for the protein of interest and the suppressor tRNA are injected into *Xenopus* oocytes. After 24 to 48 hours electrophysiology experiments are conducted.

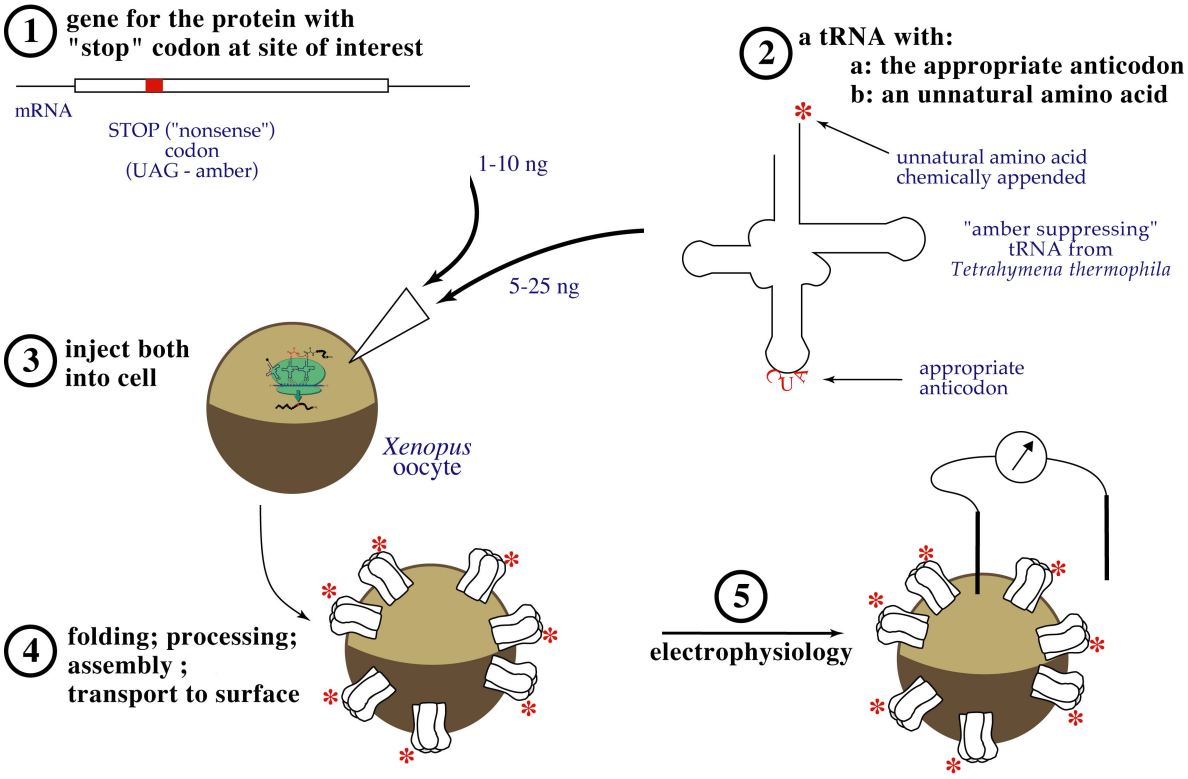
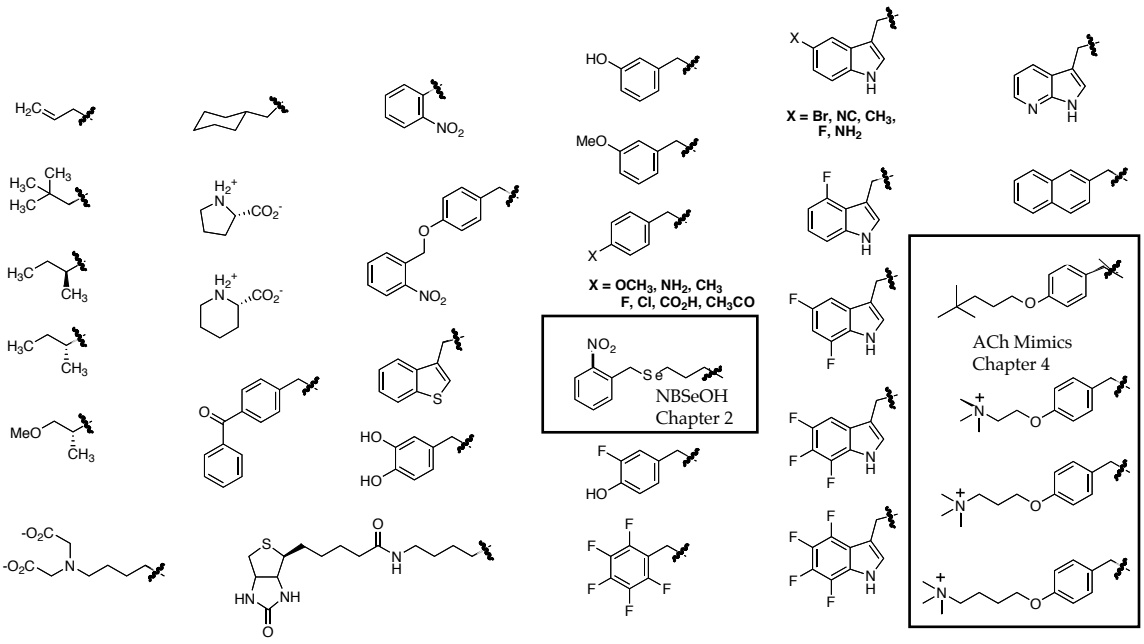


Figure 1.2 *In vivo* nonsense suppression technique

The suppressor tRNA is created using conventional molecular biology except for the last two nucleotides on the 3' end of the tRNA. The last dinucleotides in all tRNAs are cytosine and adenosine. In the *in vivo* nonsense suppression technique, organic synthesis is used to produce deoxycytosine-adenosine (dCA) dinucleotide (13). Deoxycytosine is used to reduce hydrolysis of the unnatural amino acid. The unnatural amino acid of interest is then chemically linked to dCA. This moiety is then ligated to the rest of the tRNA using RNA ligase. A full detailed procedure for the *in vivo* nonsense suppression technique can be found in reference (7, 8).

To date more than 60 amino acids and hydroxy acids have been incorporated into proteins using *in vivo* nonsense suppression (Figure 1.3) (14). A key component of this work is the design and synthesis of new unnatural amino acids that have novel properties. Chapter 2 discusses the synthesis and uses of 5-(*o*-nitrobenzyl)selenyl-2-hydroxypentanoic acid (NBSeOH). NBSeOH is used to site-specifically cleave a peptide backbone. The *o*-nitrobenzyl protecting group is photochemically removed to reveal a selenium anion (15, 16). The selenium anion then initiates an intramolecular S_N2 displacement that cleaves the backbone ester (17).

Most of my work has been focused on the nicotinic acetylcholine receptor (nAChR), which was discussed earlier in the chapter. Both nicotine and acetylcholine are agonists for the receptor. Chapter 4 deals with work done in collaboration with Lintong Li, Wenge Zhong, and Caroline Gibbs to probe the acetylcholine-binding site in the nAChR (18). Lintong Li used the *in vivo* nonsense suppression methodology to incorporate unnatural amino acids that mimic acetylcholine into the ligand-binding



Unnatural amino acids and hydroxy acids side chains that have been incorporated using *in vivo* nonsense expression

Figure 1.3

domain in the nAChR (Figure 1.3). Unnatural amino acids containing a quaternary ammonium that mimics the analogous functional group on acetylcholine were incorporated into the binding site. These unnatural amino acids are called tethered agonists. It was shown that when these amino acids are incorporated into four different positions on the nAChR, partial opening of the channel occurs even when agonist is not present (11, 18). The mimics were used to obtain distance information about where acetylcholine binds within the receptor.

There are many biochemical techniques that are used specifically to study membrane bound proteins. Targeted disulfide crosslinking is often used to reveal fine structural aspects of membrane bound proteins (19-21). Targeted disulfide crosslinking was used to study the pore lining structure of the nAChR. nAChRs with single cysteine mutations in each of the □ subunits were expressed in *Xenopus* oocytes and treated with Cu(phenanthroline)$_3$ to catalyze the formation of a disulfide bond. Only residues that are close in space will be responsive to such treatment (21). Chapter 3 deals with all of the work done in trying to detect crosslinking between residues in the pore of the nAChR.

All three chapters deal with the uses of a particular technique to the study of a particular ion channel. However, all three techniques (NBSeOH incorporation, tethered agonists, disulfide crosslinking) could be used in theory in the study of any integral membrane bound protein. The knowledge gathered on how to implement these three techniques could be used to further understand membrane bound proteins.

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