

**Site-specific incorporation of
synthetic amino acids into
functioning ion channels**

Thesis by

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in memory of Teresa Hsu and Norman Davidson

Acknowledgments

The life of a man is a dubious experiment.
C. G. Jung

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Abstract

The ability to introduce a synthetic amino acid into a fully folded protein allows the full power of organic chemistry to be applied to protein biochemistry. Any functionalized amino acid which can be incorporated by the ribosomal machinery may be site-specifically introduced. Through the application of chemical creativity, mimicry of the natural behavior of protein side chains and the introduction of novel function may both be attained.

As a class, integral membrane proteins require advanced biochemical tools for their characterization, since many of the classical methods of biochemistry are not applicable. These molecules represent an opportunity for the acquisition of unique information through the use of unnatural amino acid mutagenesis.

Cell-cell communication is fundamental to neurobiology, and leads ultimately to the phenomenon of consciousness. The receipt of extracellular stimuli relies on integral membrane proteins, and membrane-bound ion channels and receptors are the central proteins of molecular biology. Just as integral membrane proteins are well-suited to investigation by unnatural amino acid mutagenesis, molecular neurobiology is an excellent area for the application of this technology.

In the work presented here, tools for the measurement of physical organic parameters associated with molecular recognition events and conformational changes of proteins are developed and implemented in functioning neuroreceptors. In addition, analytical tools are introduced and deployed to investigate the real-time modulation of ion channel function in living cells.

After the work is introduced in Chapter 1, experiments on the use of fluorinated tryptophan analogs to serially modulate the electrostatics of particular amino acid side chains are presented in Chapter 2. The goal of these investigations is to understand an interaction between nicotine and tryptophan residues in the nicotinic acetylcholine receptor. Nicotine is shown not to experience a cation- π interaction with the side chain that mediates this interaction between the receptor and its natural agonist, acetylcholine. Additional studies on analogs of both nicotine and acetylcholine are presented, along with attempts to extend the fluorinated tryptophan methodology to neuronal receptors of the same class.

A series of dynamic amino acids are presented in Chapter 3. The overarching goal of these studies is to obtain information on ion channel conformation, both *in situ* and subsequent to isolation. A photoactive amino acid which induces proteolysis at the site of its incorporation is shown to have significant effects on the nicotinic acetylcholine receptor. Efforts to extend this methodology to biochemically detect backbone cleavage in the functionally affected receptors are also presented in this chapter. Also, the hydrolysis of an ester linkage introduced by the incorporation of a hydroxy acid in place of a natural amino acid is attempted to identify the disulfide connectivity of rat P2X₂ receptors. Finally, attempts to utilize photoreactive amino acid side chains to both crosslink adjacent subunits of the nicotinic acetylcholine receptor and to induce local conformational perturbations in the transmembrane regions of this receptor are detailed.

In Chapter 4, tyrosine containing a photo-removable protecting group is introduced in place of a particular tyrosine residue on which the modulation of Kir2.1 channel function depends. By this means, experimental control is gained over the chemical identity of this

side chain. As introduced, it is neither a substrate for tyrosine kinases nor for protein-protein interactions. However, once photolysis has revealed the wild-type residue, these interactions may occur. Co-injection of the tyrosine kinase v-Src along with the irradiation of cells expressing Kir2.1 containing this caged tyrosine residue at position 242 produces a 50% current reduction over a time course of approximately ten minutes. The roles of phosphorylation and endocytosis in causing this reduction were extensively investigated.

The final chapter presents progress toward controlling the *in situ* phosphorylation state of particular residues in a protein. A general method for synthesis of caged phosphoamino acids is developed and applied to the synthesis of analogs of serine, threonine, and tyrosine. A variety of routes toward caged non-hydrolyzable phosphoamino acid analogs are shown, along with the preparation of important synthetic intermediates.

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