Unnatural amino acid mutagenesis in mapping ion channel function

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Unnatural amino acid mutagenesis makes possible the site-specific incorporation of synthetic amino acids, enabling detailed structure–function studies as well as the incorporation of biophysical probes. This method has been adapted for use with heterologous expression in *Xenopus* oocytes, allowing experiments on ion channels.

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Abbreviations

5-HT	5-hydroxytryptamine
5-HT$_{3A}$R	5-hydroxytryptamine-3A receptor
ACh	acetylcholine
nAChR	nicotinic acetylcholine receptor
Npg	2-(nitrophenyl)glycine

Introduction

A powerful extension of site-directed mutagenesis has been the development of nonsense-suppression methods for the incorporation of unnatural amino acids into proteins expressed in living cells [1]. This method is especially appropriate for the study of ion channels. The ability to incorporate synthetic amino acids allows systematic structure–function studies, furnishing a chemical-scale precision at the level of single atoms and bonds. The methodology permits study in a cellular system, allowing direct and relevant functional analysis of the mutated channels. These experiments can provide a more detailed understanding of drug-channel interactions; indeed, unnatural amino-acid mutagenesis, coupled with functional analysis, is capable of analyzing all the major ligand–protein interactions that govern the design of low-molecular-weight drugs. This capability may help to provide useful therapeutics, both for disorders that derive directly from ion-channel malfunction and for other disorders that can be alleviated by targeting ion channels. Finally, although the nonsense-suppression method (and in particular *in vivo* nonsense suppression) produces limited quantities of proteins, this disadvantage is minimized in the study of ion channels because of the sensitivity of modern electrophysiology. We focus here on key work illustrating the use of unnatural mutagenesis in mapping ion channel function, particularly our work on the muscle nicotinic acetylcholine receptor (nAChR) over the past decade.

The method

In 1989 Peter Schultz and co-workers, building on earlier work in the biology of nonsense suppression, reported the first general method for the biosynthetic incorporation of unnatural amino acids [2–6]. More recently, our labs adapted the protocol for use with heterologous expression in *Xenopus* oocytes [1,7].

The basic method for *in vivo* nonsense suppression (Figure 1) first entails mutating a codon of interest to the amber stop codon, TAG. 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 coinjected into a *Xenopus* oocyte. Protein synthesis and surface expression are carried out by the oocyte, allowing electrophysiological study 24–72 hours later.

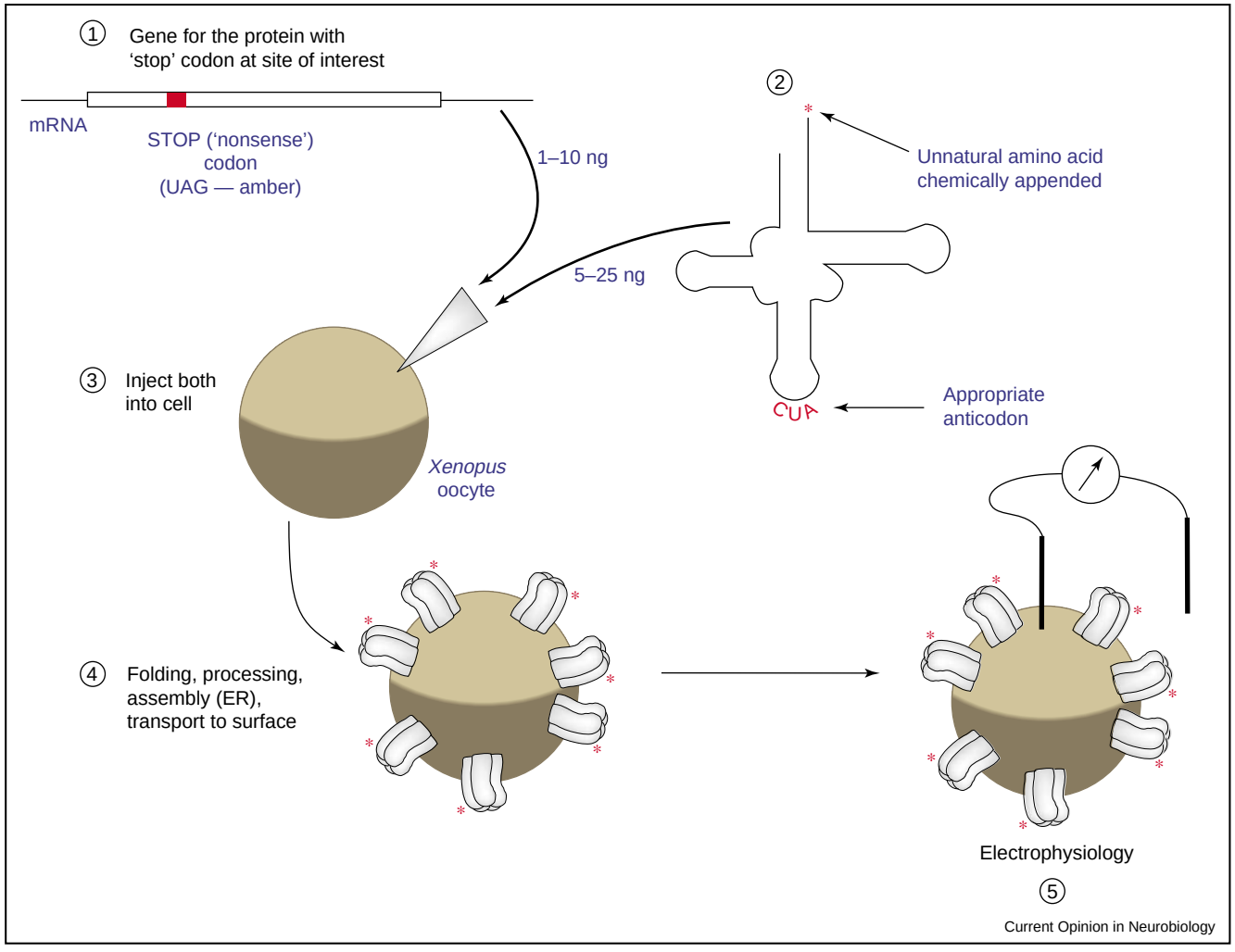
To date >60 amino acids have been incorporated into ion channels (Figure 2). In our experience, the method is amenable to two sorts of studies: first, detailed structure–function studies involving rational perturbation of the residue of interest, and second, the introduction of unnatural side chains with novel chemical and biophysical properties, such as fluorescent probes, 'caged' residues and tethered agonists. The limitations on the types of unnatural amino acids that can be incorporated have not been systematically studied. There is, however, anecdotal evidence that hydrophobic residues suppress better than polar ones, that D-amino acids cannot be incorporated, and that β-amino acids are rarely viable. Within these constraints, the method can accommodate a wide variety of side chains, including several backbone mutations.

Structure–function studies

Conserved tyrosines at the nAChR ligand binding site

One of the first unnatural mutagenesis studies of ion channels probed the role of several conserved Tyr residues at the ligand binding site in the nAChR [8]. The

Figure 1



Schematic outline for nonsense suppression in *Xenopus* oocytes.

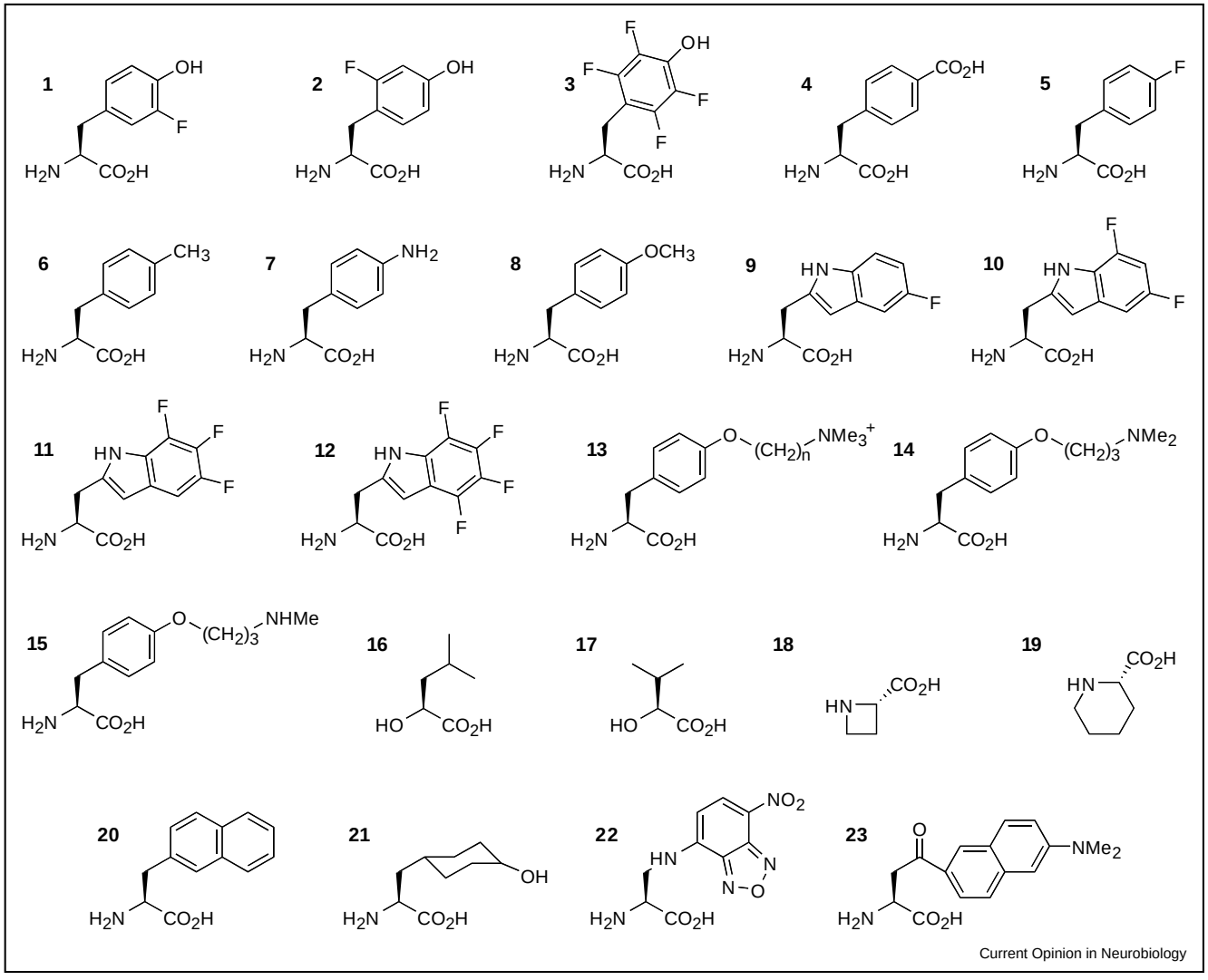
unnatural amino acids used in this study involved rational steric and electronic perturbations to the phenol side chain of tyrosine. In all, 16 different side chains were incorporated at Tyr residues α93, α190 and α198. The incorporation of residues 1–3 from Figure 2 at α93 yielded wild-type EC_{50} values and demonstrated that electronic perturbation of the aromatic side chain at this position has no functional effect. In contrast, the series of residues 6–8 from Figure 2 caused seven-fold increases in EC_{50} values, suggesting a special role for the hydroxyl group. Substitution at α198 produced quite different results. Here residues 1–3 and 5–7 from Figure 2 all lead to a three-fold increase in EC_{50} values, indicating there is no stringent requirement for the hydroxyl group. Rather, it seems the hydroxyl group serves as a steric placeholder.

Closer analysis of the results from substitution of the series 1–3 from Figure 2 at α93 reveals that, despite the significant difference in pK_a, the entire fluorinated series displays wild-type behavior. It is unlikely that a neutral side chain and an ionized side chain would produce the same results. The more plausible explanation is that the microenvironment of the binding site produces a perturbed pK_a, yielding the same state of ionization for both Tyr and F_4-Tyr.

Cation-π binding site in nAChR and 5-HT_{3A}R

We also used unnatural mutagenesis to investigate binding-site Trp residues in the nAChR [9]. Early photoaffinity labeling studies identified a number of aromatic residues at the nAChR binding site. One hypothesis for the role of these binding-site aromatics suggests the receptor recognizes the quaternary ammonium of acetylcholine (ACh) via a cation-π interaction (Figure 3a). This is the favorable interaction between the negative electrostatic potential on the face of an aromatic ring and a

Figure 2



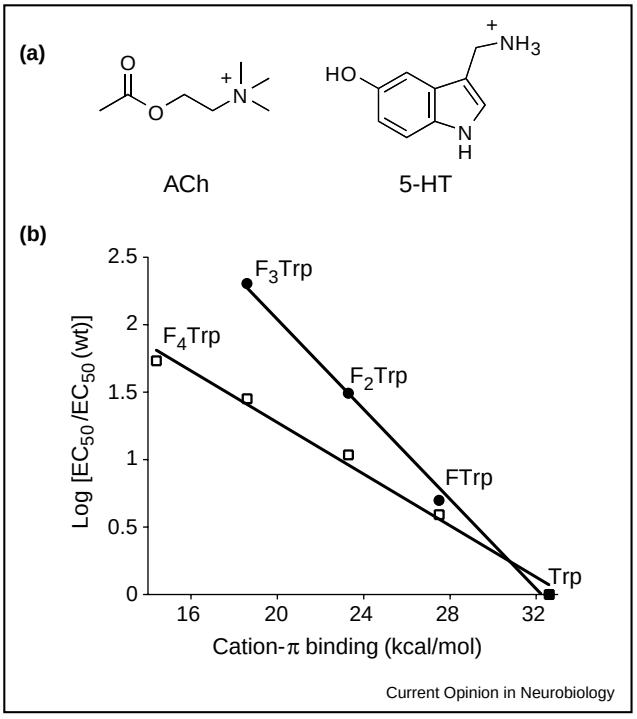
Some of the 60 unnatural amino acids incorporated into ion channels. The numbers in bold are referred to throughout the text.

cation, and represents a common biological strategy for the binding of cations [10,11].

To address this possibility, a series of progressively fluorinated tryptophan derivatives (Figure 2, residues 9–12) was incorporated at various positions in the binding domain of the receptor. Substitution of the aromatic ring with electron-withdrawing groups, such as fluorine, weakens the cation-π interaction. At Trp 149 of the α subunit, the EC_{50} value for receptor activation was strongly correlated with the degree of fluorination. Because the binding energy is expected to vary with log(EC_{50}), this quantity is plotted against the calculated cation affinity of the fluorinated side chains: a linear relationship is seen (Figure 3b). This finding indicates that, upon binding, the quaternary ammonium of ACh makes van der Waals contact with the indole side chain of αTrp-149, which allows us to infer the localization of the binding site to within 0.5 Å. Subsequent results from the crystal structure of an ACh binding protein, which is homologous to the extracellular domain of the nAChR, confirmed these results.

In a follow-up study, incorporation of the fluoro-Trp series at various positions in the serotonin-gated ion channel, 5-hydroxytryptamine-3A receptor (5-HT_{3A}R), identified a cation-π interaction between the primary ammonium of serotonin, 5-hydroxytryptamine (5-HT), and Trp 183, which aligns with αTrp 149 in the nAChR (Figure 3) [12•]. Comparison of the results for ACh and 5-HT revealed that the magnitude of interaction between Trp 183 and the primary ammonium of 5-HT is greater than that for Trp 149 and the quaternary ammonium of ACh (~4 kcal/mol and ~2 kcal/mol, respectively). This is consistent with the large electrostatic component of the

Figure 3



Cation-π binding of neurotransmitters. **(a)** Structures of ACh and 5-HT. **(b)** Linear relationship between the cation binding energy of fluorinated Trps and log(EC_{50}) for fluoro-Trp series in 5-HT_{3A}R (filled circles) and nAChR (open squares).

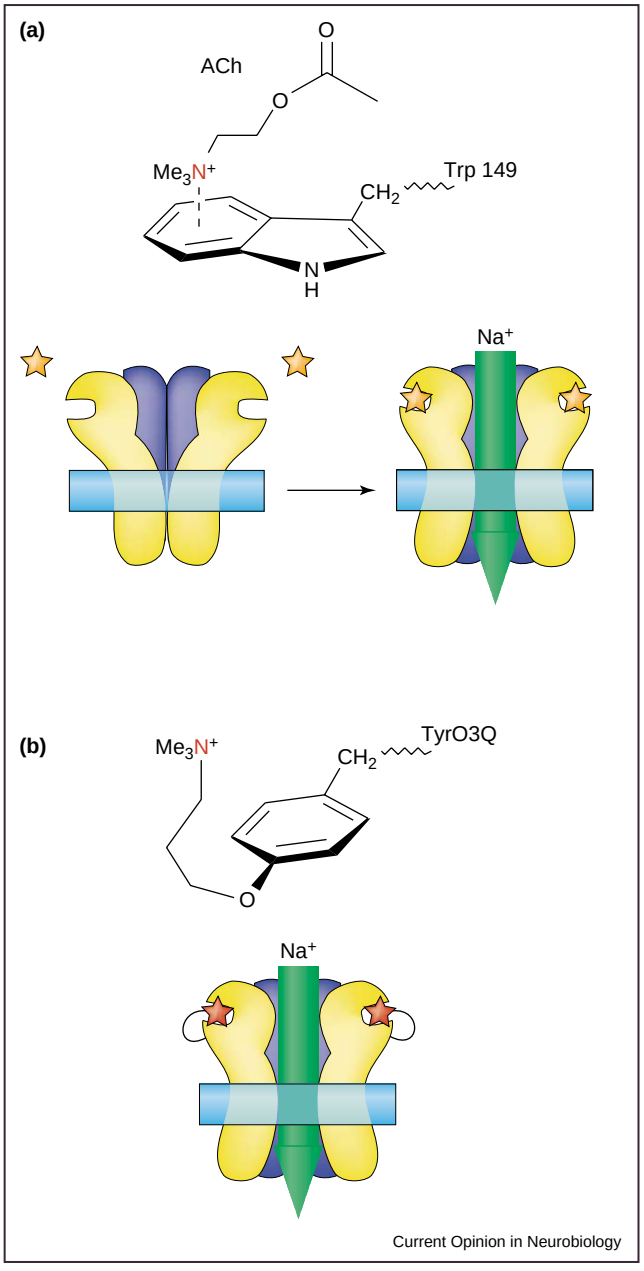
cation-π interaction. In addition, these studies examined the binding mode of nicotine to the nAChR by the same the technique and found that it differs significantly from that of ACh. It will be important to study possible cation-π interactions underlying the considerably higher-affinity binding of nicotine to neuronal AChRs.

General unnatural mutagenesis methods for ion channel study

Tethered agonists

The combination of unnatural amino acid mutagenesis and tethered agonists has proven an effective means of mapping ligand–receptor relationships. In this technique, an analog of the agonist is covalently appended to the side chain of an unnatural amino acid placed near the ligand binding site (Figure 4). The correct positioning of the side chain activates the receptor in the absence of agonist (constitutive activity). The tethered-agonist approach allows for the functional mapping of a ligand binding pocket. By varying the tether length and the position at which the tethered agonist is introduced, one can probe the spatial location of various positions relative to the binding site. Additionally, by varying the chemical characteristics of the agonist, one can both probe the microenvironment of the binding site and identify functionally important ligand–receptor interactions.

Figure 4



Outline of tethered agonist approach. **(a)** Top: schematic of the quaternary ammonium of ACh positioned over αTrp 149 in the nAChR binding site. Bottom: binding of ACh (orange stars) to the nAChR results in channel gating and ion conduction. **(b)** Top: structure of Tyr-O3Q (13 in Figure 2), showing that the tethered quaternary ammonium can be positioned in roughly the same location as the quaternary ammonium of ACh. Bottom: incorporation of a Tyr-O3Q (red stars) at α149 mimics the effects of ACh, inducing channel gating.

In studies of the nAChR, unnatural amino acid mutagenesis was used to incorporate a series of tyrosine derivatives with tethered quaternary ammonium groups (see Figure 2, residue 13; [13•]). Introduction of the series at three separate sites — α93, α149 and γ55/δ57 — yielded

constitutively active receptors, and the pattern of activation for each differed with the tether length. At position α149, there was a clear preference for a three-carbon tether, whereas at position α93 tethers of two to five carbons were comparably effective. At γ55/δ57, all tethers except the two-carbon tether activated the receptor. These data provided valuable geometric information: all three sites are near the binding site, with α149 being the closest, followed by α93. These results, too, were supported by the recent crystal structure of the ACh binding protein.

In a more recent study, a series of tyrosine derivatives with tethered amines (Figure 2, residues 14 and 15) was incorporated at position α149 [14•]. Here the tethered amines differed in the degree of methylation at the amino center. This series probed both the local pK_a at the binding site and the protonation state for the active form of a non-quaternized amine. Titration of tethered agonists with secondary and tertiary amines showed significant increases in constitutive activity at low pH, providing evidence that the local pK_a of the binding site is substantially shifted. The pK_a for the tethered tertiary amine was <6 when incorporated at α149 versus a value of ~9 in free solution.

α-Hydroxy acids

The nonsense-suppression method can incorporate backbone mutations [15–18]. A well studied and useful backbone mutation arises from the replacement of an α-amino acid with an α-hydroxy acid. This substitution leads to an amide-to-ester mutation in the protein backbone and removes a hydrogen bond donor at this position. Both β-sheets and α-helices depend on backbone hydrogen bonding, rendering ester incorporation an effective probe of secondary structure.

We used the unique properties of α-hydroxy acids to probe the role of a conserved transmembrane proline in both the nAChR and the 5-HT_{3A}R [19,20]. Proline is unique among the natural amino acids in that its α-nitrogen is part of a pyrrolidine ring. Such a structure imposes unique constraints on the peptide backbone and prevents the nitrogen from serving as a hydrogen-bond donor. Backbone ester mutations were used to examine which of these properties were functionally significant.

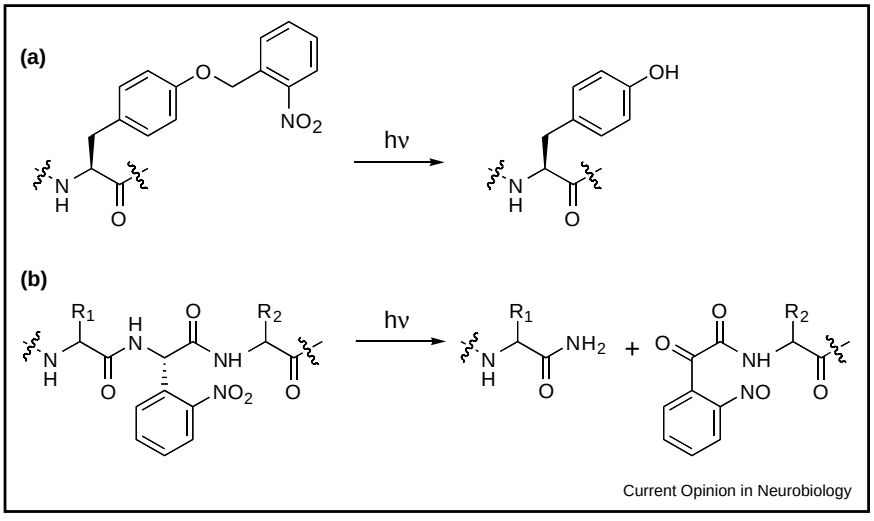
Incorporation of α-hydroxy acids at Pro 221 in the nAChR and Pro 256 in the 5-HT_{3A}R yielded functional receptors with near-wild-type properties. In contrast, substitution at these sites with Gly, Ala, or Leu yielded non-functional receptors. We concluded from these studies that the functional importance of this conserved proline in the nAChR and 5-HT_{3A}R was the removal of backbone hydrogen bonding at this position.

Ester incorporation not only removes a hydrogen bond donor, but also reduces the electronegativity of the carbonyl oxygen, making it a weaker hydrogen-bond acceptor. A recent study from the Schultz and Jan groups used this fact to examine the interaction of backbone carbonyls in the selectivity filter of potassium channels with permeant ions [21•]. Two conserved Gly residues were substituted with their α-hydroxy analogs. The results demonstrate that K^+ ion selectivity is retained even with a significant reduction in electronegativity at the selectivity filter. Also, conformational changes arising from interactions between the filter and permeant ions contribute directly to channel gating.

Photoreactive amino acids

Photoreactive amino acids have been used in the study of ion channels [22–24]. 'Caged' amino acids have side

Figure 5



Schematic results from photolysis of **(a)** caged Tyr, liberating the free amino acid, and **(b)** Npg, yielding a cleaved backbone.

chains protected by a photochemically removable group (Figure 5a). This approach can provide temporal resolution of both the mechanistic and the physiological function of ion channels.

Caged Tyr, Ser, and Thr show particular promise for probing the role of side-chain phosphorylation in ion-channel function. Kinase-dependent modulation of ion channels and neuroreceptors probably plays a role in synaptic plasticity. In a recent study, caged Tyr was incorporated at a conserved Tyr site in the potassium channel Kir 2.1 [25•]. Photodecaging of this residue led to a ~30% decrease in channel activity, if — and only if — tyrosine kinases were active and tyrosine phosphatases were inhibited. Apparently, decaging initiated two kinase-dependent pathways. One involves direct modulation of the channel, and the other appears to involve kinase-initiated endocytosis. Deconvolution of these competing pathways would have been very challenging with conventional methods.

Another photoreactive strategy is the use of the unnatural amino acid 2-(nitrophenyl)glycine (Npg) [26]. Rather than being a caged side chain, Npg can be viewed as a 'caged' backbone (Figure 5b). Photolysis of this residue severs the protein backbone, which represents a site-specific, nitrobenzyl-induced photochemical proteolysis. The ability to cut the protein backbone can be a valuable tool for mechanistic studies in ion channels.

Fluorescent amino acids

The incorporation of unnatural amino acids with fluorescent side chains is a promising strategy for the study of ion channels [27•,28,29,30•]. Fluorescent probes can serve as sensitive indicators of the microenvironment at a particular residue, as well as reporting directly on protein dynamics. Pioneering work from the Sisido and Hecht laboratories has demonstrated the feasibility of doing fluorescence resonance energy transfer studies between two unnatural amino acids incorporated into a single protein. To date, however, technical challenges such as the limited quantities of protein from *in vivo* nonsense suppression and the exclusion of many standard fluorophores due to ribosomal constraints on side chain size have slowed the application of fluorescent residues and unnatural mutagenesis to the study of ion channels. One successful study incorporated amino acid *22* from Figure 2 into the G-protein-coupled receptor NK2 [31]. Using membrane preparations, this study determined intermolecular distances between a fluorescently labeled antagonist and amino acid *22* from Figure 2 at several positions in the protein by measuring fluorescence resonance energy transfer.

Conclusions and future directions

Unnatural amino acid mutagenesis allows detailed studies of ion channels with admirable molecular precision in a cellular environment. Further application of unnatural amino acid mutagenesis will provide additional insights into ion-channel function. Strategies for future studies include the development of phosphorylated unnatural amino acids for the elucidation of signal transduction pathways in ion channels and the more efficient incorporation of biophysical probes such as fluorescent side chains.

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