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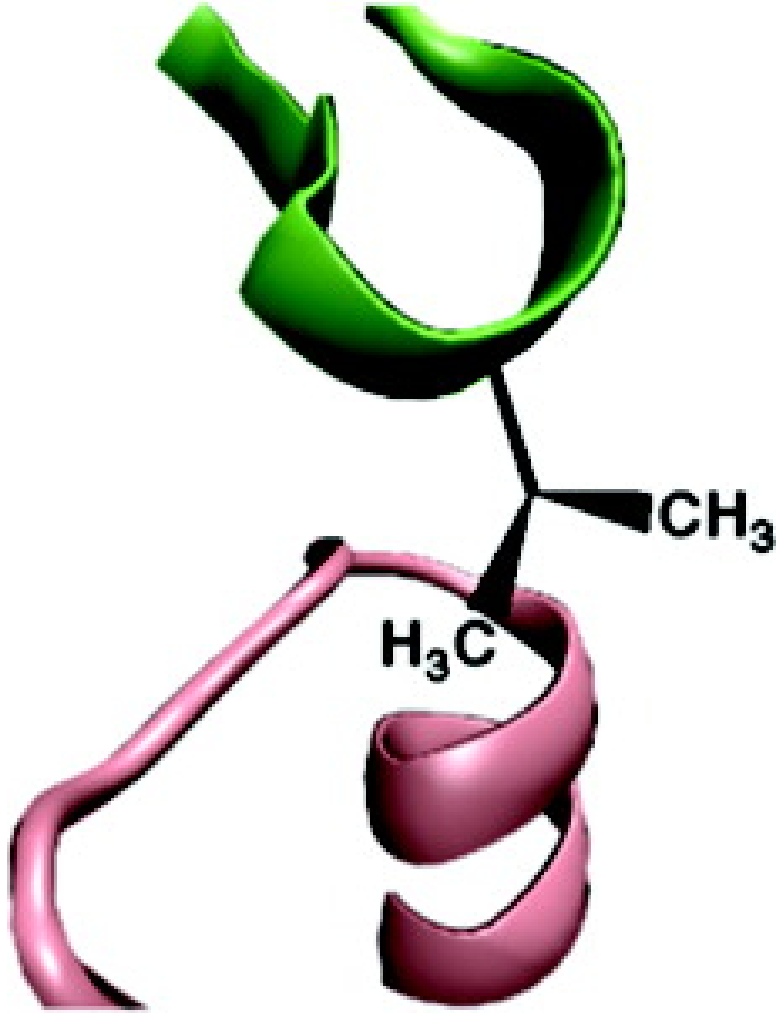

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A Stereochemical Test of a Proposed Structural Feature of the Nicotinic Acetylcholine Receptor

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Understanding the gating mechanism of neurotransmitter-gated ion channels constitutes one of the most significant mechanistic challenges in chemical neurobiology. For some time we have been applying the tools of physical organic chemistry to this important problem, emphasizing strategies that can produce linear free energy relationships.[1,2] In the present work we apply another classical tool of physical organic chemistry—the stereochemical probe[3]—to address a specific proposal concerning the mechanism of ion channel gating.

The nicotinic acetylcholine receptor (nAChR), like all members of the Cys-loop neurotransmitter-gated ion channel superfamily to which it belongs, mediates rapid synaptic transmission in the mammalian nervous system. At rest the nAChR is in a closed, nonconducting conformation. Upon binding of the neurotransmitter acetylcholine, the protein undergoes a conformational change to an open, ion-conducting state, thereby converting the chemical signal to an electrical one.

The muscle-type nAChR comprises five homologous subunits ($\alpha-\gamma-\alpha-\delta-\beta$) arranged pseudosymmetrically around a central, ion-conducting pore. Each individual subunit has a large, extracellular N-terminal domain, four membrane-spanning helices (M1−M4), and a short extracellular carboxy-terminal tail (Figure 1). The extracellular domain is composed largely of β strands connected by short loops, with the agonist binding sites located at select subunit interfaces (α/γ and α/δ). The channel gate is located in the second pore-lining helix (M2) some 40−60 Å from the ligand binding site. The nature of the communication between the binding site and channel gate is incompletely known.

Recent cryo-EM images[4−8] of nAChRs from the *Torpedo* ray show that loop 2, a short loop connecting β strands 1 and 2, lies directly over the extracellular terminus of M2 and to one side of the M2−M3 linker (Figure 1). Furthermore, the structure shows that the side chain of a specific loop 2 residue in the α subunit, valine 46, points toward the top of the M2 helix.[8] αVal46 is part of the "gating interface" between the extracellular and transmembrane domains, and we and others have argued that this interface plays an important role in communicating neurotransmitter binding to the gating region.[9−17] Specifically, Unwin et al.[4−8] propose that αVal46 makes a key "pin-into-socket"[6] interaction with the side chain of αVal46 tucked into a hydrophobic pocket formed by the top of M2. This interaction allows αVal46 to communicate changes in the structure of the extracellular domain to the M2 helix, releasing the channel gate. While it was immediately recognized that αVal46 of the nAChR is not conserved among Cys-loop receptors,[12,16] and therefore that this proposed mechanism of gating cannot be conserved across the superfamily, it has also been shown that the gating mechanisms of these receptors are quite varied.[10,12,15,16]

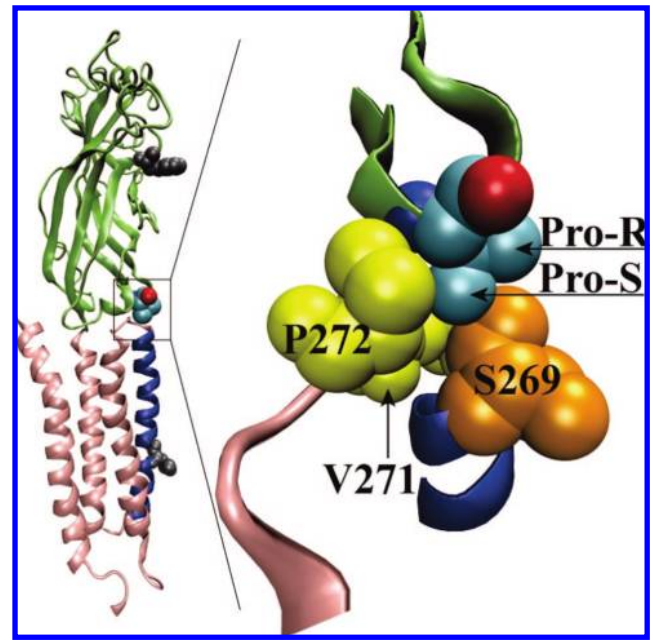

Figure 1. *(*Left) Topology of a single α subunit (chain A of PDB 2BG9). The extracellular domain (green) contains the ligand binding site located at αW149 (dark gray). There are four membrane spanning helices (M1, M3, M4 in pink) including the pore-lining M2 (blue) helix containing the channel gate at αL251 (gray). (Right) αVal46 (side chain in cyan, backbone carbonyl in red) is oriented such that the pro-S methyl points into a pocket formed by residues 269 (orange), 270, 271, and 272 (yellow), while the pro-R methyl points away from the pocket.

Therefore the proposed pin-into-socket mechanism for the nAChR merits further scrutiny.

To probe the hypothesis that αVal46 makes a key pin-into-socket interaction, we use nonsense suppression methodology to incorporate unnatural amino acids in place of αVal46. In contrast to conventional mutagenesis, the use of unnatural amino acids allows us to perturb side chain hydrophobicity while retaining the overall size and shape of the side chain. Therefore, we can attribute changes in receptor function to the alterations in the polarity of the side chain with minimal concern that these subtle mutations have altered the nature of communication between the binding site and channel gate.

The αVal46Thr mutant was made and channel function was evaluated using whole-cell voltage clamp techniques.[18] Incorporation of Thr proved highly deleterious, causing a >20-fold rightward shift in EC_{50} to >1000 μM (Table 1, Figure 2).[16] Threonine is isosteric to valine, thus the shift in EC_{50} must be attributed to the increase in side-chain polarity. Surprisingly, incorporation of the unnatural amino acid *allo*-threonine (*a*Thr) at the same position

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Table 1. Measured EC_{50} Values (μM) for αVal46 Mutants

amino acid	EC_{50}	Hill	*n*
Val (wt)	50 ± 5	1.4	4
Thr	>1000		8
Ile	58 ± 7	1.4	4
Omt	152 ± 5	1.4	12
*a*Thr	102 ± 5	1.4	19
*a*Ile	48 ± 2	1.7	8
*a*Omt	30 ± 1	1.7	12

caused only a 2-fold increase in EC_{50} (Table 1, Figure 3). Threonine and *allo*-threonine have the same overall side chain polarity and both are isosteric to valine, yet Thr causes a large change in channel function while *a*Thr does not. Since the amino acids differ only in the side chain stereochemistry, the data clearly indicate that changing the polarity of the pro-S methyl of the αVal46 side chain affects channel gating to a much greater extent than changing the pro-R methyl (Figure 3). These results and the cryo-EM image of this region suggest that the pro-R and pro-S methyls are in distinct environments (Figure 1B), consistent with the pin-into-socket proposal.

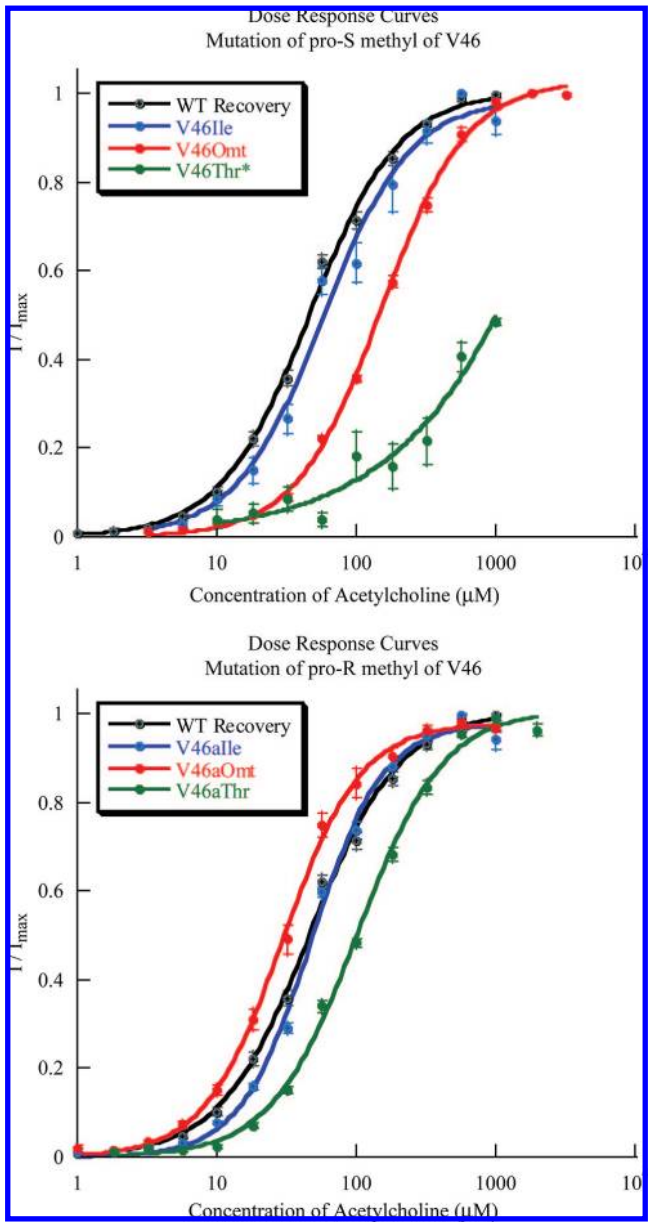

Figure 2. Dose response curves for mutations at αVal46. Mutations to the pro-S methyl (top panel) impact EC_{50} to a greater extent than mutations to the pro-R methyl (bottom panel). (∗) The Thr mutation was deleterious and the EC_{50} could not be measured (further discussion in the Supporting Information).

While minimally perturbing in a steric sense, converting a methyl to a hydroxyl is still a strong chemical disruption. Thus we considered other mutants at αVal46 (Table 1). Previously, we have employed the *O*-methyl threonine (Omt)/*allo*-O-methyl threonine (*a*Omt) epimeric pair.[3] However, since Omt is isosteric to isoleucine, not to valine, we first incorporated isoleucine (Ile) and *allo*-isoleucine (*a*Ile) mutations as reference points. As expected, replacement of Val with the similarly hydrophobic Ile and *a*Ile does not appreciably impact channel function (Table 1). Mutation to Omt produced a 3-fold increase in EC_{50} to 152 μM, indicating a decrease in channel function, while *a*Omt decreased EC_{50} slightly. These results provide an intermediate point between wild type and threonine that clearly demonstrates the importance of a hydrophobic group at the pro-S methyl of the valine side chain.

On the basis of the location and the subtlety of the changes, it seems unlikely that these mutations could significantly impact ligand binding events, whereas αVal46 is located at the interface of the extracellular and transmembrane domains at an ideal location to influence channel gating.[9−11,14,16,17,19] Additionally, detailed kinetic studies of conventional mutations at αVal46 have demonstrated that changes to this residue primarily affect gating events.[17] Therefore we ascribe the changes in EC_{50} here to reflect changes in the gating equilibrium constant (k_{open}/k_{closed}) for the channel. Interpreted this way (Figure 3), the threonine mutation at αVal46 results in a >7.6 kJ/mol change in the gating equilibrium, with the introduction of a polar substituent either stabilizing the closed state or destabilizing the open state.

We consider the present results to provide strong support for the pin-into-socket mechanism. It is worth noting that the images of Figure 1 are based on cryo-EM data that are at best of 4 Å resolution. Yet, the clear prediction that the pro-R and pro-S methyls of αVal46 are in stereochemically distinct environments, with the pro-S tucked into a pocket, is substantially confirmed by our data. Others have probed Val46 by conventional mutagenesis, and concluded that it plays a key role in gating.[14,17,19] An interesting issue is the nature of the interacting partner(s) for the pro-S methyl of Val46. Based on mutant cycle analysis using much more perturbing, conventional mutants, Lee and Sine suggest that a hydrophobic interaction between αVal46, αSer269, and αPro272 exists.[14] Single-channel analyses by Jha et al. suggest that Ser269 and Ala270 move early in the gating process, contemporaneously with Val46, while Pro272 moves later.[19] This could be interpreted to indicate that Val46 is more likely to interact with Ser269/Ala270. We have made no effort here to identify the interacting partner of Val46. We do note, however, that assuming the image of Figure 1 is of the closed state, the assignment of the pocket as being hydrophobic[7,8] seems inconsistent with our data. Changing the pro-S methyl to a polar group apparently stabilizes the closed state (raising EC_{50}), therefore a polar pocket to receive that polar group is more consistent with our data. This pocket could be formed by backbone carbonyls and/or the side chain of S269.

As noted above, Val46 is not conserved in the Cys-loop superfamily, and so the key interaction probed here cannot play a critical gating role in all Cys-loop receptors.[12,16] As discussed in detail elsewhere, this is more nearly the norm, rather than the exception, with most pairwise interactions in the crucial gating interface not being conserved across the family.[10−16,19] For example, Lummis et al. found that the cis−trans isomerization of

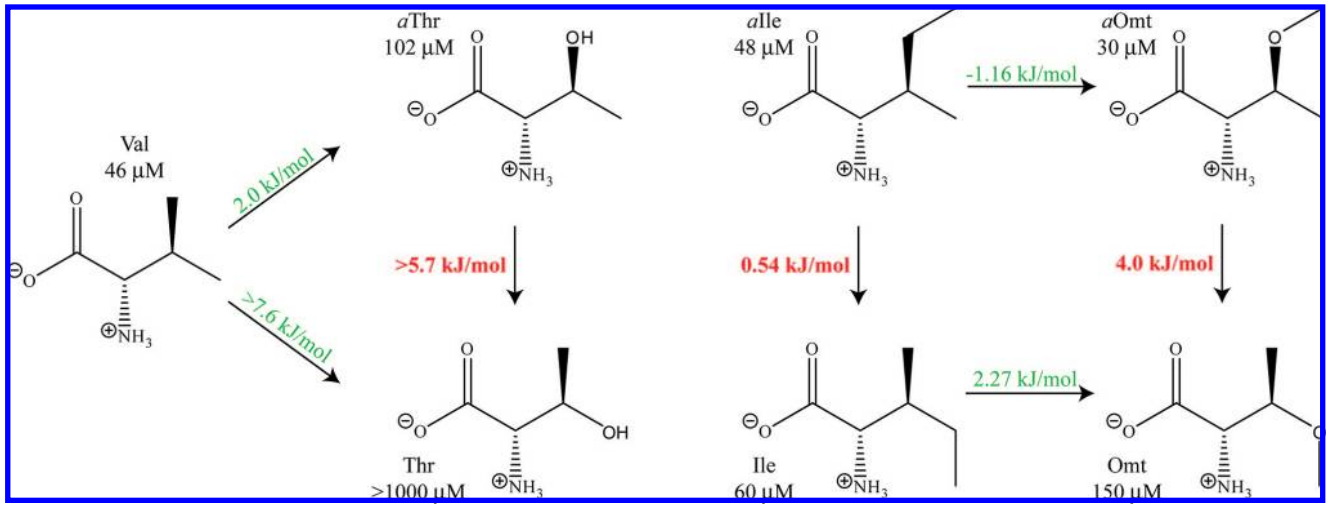

Figure 3. Incorporation of polar groups in the αVal46 side chain inhibits channel function to a greater extent at the pro-S position (lower row). Decrease in channel function is attributed to destabilization of the open state, stabilization of the closed state, or a combination of both by the energies shown.

a semiconserved proline residue in the $5HT_{3A}R$ plays a key role in the gating of this channel.[15] However, in the nAChR, mutation of this same proline to serine, alanine, and glycine results in functional channels, demonstrating that cis−trans isomerization cannot wholly account for the gating of nAChR.[1,15] One candidate for a conserved gating interaction is a proposed salt bridge between the residue adjacent to Val46 on loop 2 (αGlu45) and a conserved Arg residue in the preM1 region. Several studies have evaluated its possible role in nAChR function, but it has not been evaluated in other Cys-loop receptors.[14,16,19]

We conclude that, in the nAChR, αVal46 does play a key role in receptor gating by a pin-into-socket mechanism, whereby the pro-S methyl group is nestled into a pocket at the top of the M2 helix. In addition to validating an intriguing feature of the cryo-EM images of this receptor, the present results further illustrate the power of unnatural amino acid mutagenesis in enabling physical organic chemistry studies on the complex proteins of neuroscience.

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Supporting Information Available: Experimental procedures for incorporation of unnatural amino acids and electrophysiological evaluation of mutants, dose response curves for mutations discussed herein, sample traces of select mutants, discussion of previously reported conventional mutants, as well as the synthesis and characterization of NVOC-aThr cyano-methyl ester. This material is available free of charge via the Internet at http://pubs.acs.org.

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