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Physical Organic Chemistry on the Brain

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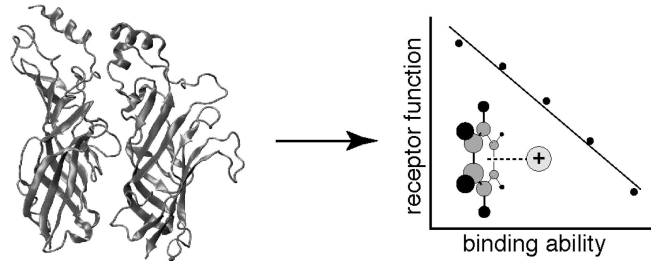

The challenges to obtaining chemical-scale information on the molecules of neuroscience are considerable. Most targets are complex integral membrane proteins that are not amenable to direct structural characterization. However, by combining the tools of organic synthesis, molecular biology, and electrophysiology, rational and systematic structure−function studies can be performed in what we have termed physical organic chemistry on the brain. Using these tools, we have probed hydrophobic effects, hydrogen bonding, cation−π interactions, and conformational changes associated with channel gating. The insights gained provide important guidance for drug discovery efforts targeting ion channels and neuroreceptors and mechanistic insights for the complex proteins of neuroscience.

Introduction

The human brain is the most complex object, natural or artificial, known to man.[1] It is the seat of memory, thought, sensory perception, and awareness. Neuropsychiatric disorders such as depression, schizophrenia, bipolar disorder, substance abuse, and suicide define the world's leading health problem for adults, as measured by the Disability Adjusted Life Year (DALY), which can be thought of as one lost year of "healthy" life.[2] As chemists, we seek a molecular understanding of the world, including biological phenomena. The complexity of the brain seen at the anatomical, cellular, and synaptic levels continues to the molecular scale, where a bewildering array of complex proteins and signaling molecules process, store, and retrieve information. A nearly universal feature of the signaling proteins of neuroscience is that they are integral membrane proteins. This presents a special problem for chemists—a dearth of structural information. Even though membrane proteins represent ~30% of genetically encoded proteins and ~60% of pharmaceuticals targets, they comprise less than 1% of the structures in the Protein Data Bank.

While younger chemists may find it hard to imagine, one can actually learn a great deal about a chemical system without a crystal structure. The mechanistic insights and knowledge of reactive intermediates developed in the 20th century were not gleaned from crystal structures of transition states! Instead, these groundbreaking discoveries derived from the tools of physical organic chemistry—kinetics, substituent effects, linear free energy relationships, stereochemical probes, and so on.

In the present Perspective, I will describe how a chemist can address the daunting complexity of the nervous system. To do so requires the adoption of some methodologies that are uncommon in physical organic chemistry: molecular biology, electrophysiology, and heterologous expression in vertebrate cells. But the intellectual approach—the mindset—is the same

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as the classical studies of small molecules. The essential concept is the structure−function study. We modify the structure of the target protein in rational and systematic ways. We then evaluate how the function of the protein has been altered by the structural change. In this way, important insights into the function and, yes, structure of the proteins of neuroscience can be obtained. We hasten to add that, in recent years, important advances in the structural biology of membrane proteins have been made, and these efforts greatly influence and assist efforts to apply physical organic chemistry to the complex proteins of neuroscience. However, for the foreseeable future, our direct knowledge of the structures of membrane proteins will lag far behind that for soluble proteins, and chemical approaches will be essential.

The Synapse and Ion Channels. The synapse is the junction point between a neuron and another excitable cell (another neuron or, for example, a muscle). It is an extraordinarily complicated structure, with many receptors and ion channels, "architectural" molecules that control spatial arrangements, and downstream signaling pathways that display their own complex relationships. An image of such a structure is simply overwhelming to a chemist.[3] In order to study the *molecules* of the synapse, we must get out of the synapse. Fortunately, molecular biology provides a way to do so. Most of the major signaling molecules of neuroscience were cloned in the 1980s and 1990s. It then became possible to express these proteins independently in select cells. An especially convenient vehicle is the *Xenopus* oocyte, an egg cell precursor from an African frog. It is large (∼1 mm diameter) and relatively dormant. However, when the mRNA corresponding to essentially any protein is injected into an oocyte, its protein synthesis machinery springs into action and makes the corresponding protein. Most remarkably, the mRNA injected could code for a complex, integral membrane protein of the human brain that contains multiple subunits (multiple mRNAs injected). Yet, the frog oocyte obligingly synthesizes all the components, folds them properly, assembles the subunits, and transports the intact receptor to the surface of the cell. Importantly, the pharmacology and physiology of the receptor on the surface of the *Xenopus* oocyte are identical to what would be seen in a native environment. This heterologous expression (synthesizing a human protein in a frog cell) has, in effect, allowed us to extract a single receptor from the complex milieu of the synapse and place it in a benign, controlled environment, where it is now amenable to detailed study. The next challenge is characterizing the receptor.

The membrane of a living cell is a highly effective capacitor; it holds a voltage difference between the cell interior and the extracellular medium that is typically on the order of −60 mV (by convention, the extracellular medium is assigned a potential of 0, and the cell interior is typically negative). Embedded in the membrane are proteins that contain pores that allow ions to flow down their electrochemical gradient. These ion channels are typically "gated"—switched between open and closed states—by external stimuli, such as a change in membrane voltage or membrane tension or the binding of a small-molecule ligand. Since the flow of ions across a membrane is identically equivalent to an electrical current, highly sensitive electrical recording approaches can be used to monitor ion channel function. With fluxes as high as 10^8 ions/s, simple calculations reveal that a *single* ion channel could produce tens of picoamps of current at a typical membrane potential. Indeed, for over 30 years it has been possible to monitor ion channels at the single molecule level, using the patch clamp methodology pioneered by Neher and Sakmann.[4] Electrophysiology is thus an extraordinarily sensitive and informative probe of ion channel function. In fact, even absent structural information, many physiologists consider ion channels to be the best characterized class of proteins because of the high information content of electrophysiological measurements. To a physical organic chemist, ion channels provide a natural solution to one aspect of the structure−function challenge—we have a sensitive and highly informative probe of protein function. A large fraction of the signaling molecules of neuroscience are ion channels—or can be made to act through ion channels—and so we have the ability to probe hundreds of structures.

Recall that our goal is to perform structure−function studies on the molecules of neuroscience. With heterologous expression and electrophysiology, we can make the proteins and we can characterize them extensively. The final requisite for a structure−function study is the ability to rationally and systematically modify the structure of the target protein. Of course, with small molecules organic synthesis plays this role. For proteins, the obvious tool is site-directed mutagenesis, and thousands of informative studies of ion channels and neuroreceptors have been performed with this powerful tool, often using the *Xenopus* oocyte heterologous expression system. However, to a physical organic chemist, site-directed mutagenesis is unsatisfactory. The structural variation available with the 20 natural amino acids is neither broad enough nor systematic enough to allow real structure−function studies. As such, we were very much intrigued when, in 1989, a general methodology to introduce unnatural amino acids site-specifically into proteins was described.[5−7]

As originally developed, the unnatural amino acid methodology had two limitations. First, it was developed for in vitro (test tube) protein synthesis. This is fine for soluble proteins, but not for the ion channels and neuroreceptors of the mammalian central nervous system (CNS). Generally, these proteins can only be expressed and assembled in higher level (eg., vertebrate) cells, and they must be probed in a living cell with an intact membrane potential. So, we would have to move the methodology into the *Xenopus* oocyte.

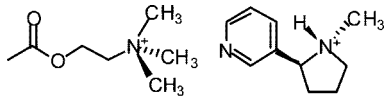
A second issue involved the quantity of unnatural amino acid-containing protein that can be prepared. The unnatural amino acid is incorporated at a stop codon that is inserted into the gene at the position where substitution is desired. The methodology also requires a fairly arduous synthesis of an aminoacyl-tRNA that recognizes the stop codon and has the unnatural amino acid chemically appended. Since the aminoacyl-tRNA is a stoichiometric reagent in the process, the amount of protein that can be prepared is limited. This is where the power of electrophysiology is revealed. As noted above, we can detect ion channels at the single molecule level. Realistically, you have to synthesize much more than a single molecule to do electrophysiology, but in a *Xenopus* oocyte experiment we evaluate on the order of 10 attomol of protein, still an extraordinarily small amount. Thus, we have finessed the quantity issue by applying an ultrasensitive assay. New approaches have been developed that minimize the quantity limitation of the unnatural amino acid methodology, but at present the approaches have primarily been employed in *E. coli*.[8−10]

The details of the unnatural amino acid methodology as applied to ion channels and receptors expressed in *Xenopus*

oocytes have been presented elsewhere.[11,12] Briefly, we had to develop tRNAs that are compatible with the *Xenopus* oocyte expression system.[13–16] Then, coinjection of the mutant mRNA and the aminoacyl-tRNA into the oocyte leads to functional channels with the unnatural amino acid incorporated. The method is adequately efficient and broadly applicable. We have incorporated over 100 different residues at more than 150 different sites in more than 20 different proteins.[17] It is quite remarkable how tolerant the ribosome is to variations in amino acid structure, as long as the amino acid is properly appended to a competent tRNA. With this methodology, we have everything in place to allow us to think and act like a physical organic chemist while operating on the complex molecules of neuroscience.

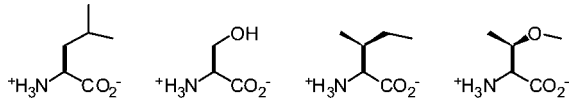
Probing Noncovalent Interactions. When a small molecule binds to a protein or when two proteins or protein subunits interact, a number of noncovalent interactions determine the strength and stereochemistry of the interaction. Chief among these are the hydrophobic effect, the hydrogen bond, the ion pair, and the cation−π interaction. We have been able to provide definitive probes of all these effects using the unnatural amino acid methodology.

The hydrophobic effect is arguably the dominant noncovalent force in biology, and while it is often easy to see when a hydrophobic interaction might be important, it is often difficult to establish with certainty that hydrophobic effects are dominating an interaction. An example occurs in the putative gate of a family of neurotransmitter-gated ion channels known as Cys-loop receptors.[18] These receptors control fast synaptic transmission throughout the nervous system. They are formed from five identical or homologous subunits that form a symmetric, pentagonal array with a channel down the middle. Binding of a neurotransmitter such as acetylcholine (ACh) or serotonin induces a structural change that opens the channel. The prototype member of the Cys-loop family is the nicotinic acetylcholine receptor (nAChR), which is endogenously gated by ACh, but which is also activated by nicotine. The initial chemical event of nicotine addiction is binding to and activation of the nAChR by nicotine.



ACh nicotine

The gate of the channel is thought to be formed from the side chains of leucine (Leu) residues, creating a "hydrophobic gate" that prevents passage of ions. Since the channels is a pentamer, there are five Leu that define the gate. An interesting feature of this constriction—which has been proposed to be relevant to several types of channels—is that the side chains do not need to physically block the channel in the closed state.[19–21] Even if there is a continuous pore through the protein that spans the membrane, if one region of the channel is a bit narrower and highly hydrophobic, the energetic cost of passing an ion such as Na^+ or Cl^- will be too severe.

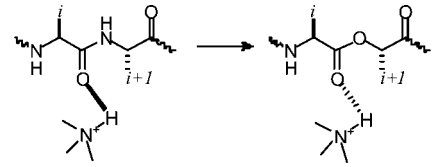


Leu Ser Ile Omt

Evidence for a hydrophobic gate formed by Leu side chains came, in part, from conventional mutagenesis.[22] Replacement of one or more Leu with serine (Ser), a much more polar residue, made the channel much easier to open. However, to a chemist, a Leu-Ser mutation is hardly subtle. Along with increasing polarity, a large "hole" is produced and a hydrogen-bonding group is introduced.

We sought a more subtle probe that would prove that hydrophobicity really is the determining factor. The most convincing pairing begins with isoleucine (Ile), a natural amino acid that in this context performs essentially identically to Leu.[23] We then replaced Ile with *O*-methylthreonine (Omt), an unnatural amino acid that is isosteric to Ile. Now, we are not introducing a hole, and we are not introducing a hydrogen bond donor (there is a hydrogen bond acceptor; nothing's perfect). We found that, indeed, the channel with Omt was significantly easier to open than the channel with Ile, confirming a prominent role for hydrophobicity in the channel occlusion.

Hydrogen bonding is a very important force contributing to protein secondary structure and ligand recognition. Several side chains can contribute to hydrogen bonding, and it is not difficult to imagine unnatural amino acids that can probe such interactions. A more challenging hydrogen bond is one involving the protein backbone. The peptide bond is an excellent hydrogen bond donor and acceptor, and of course, such hydrogen bonding defines the protein α-helix and β-sheet. In addition, protein−ligand interactions often involve hydrogen bonding between the ligand and the protein backbone. To probe such an interaction, another remarkable feature of the protein synthesis machinery can be exploited. Not only can the ribosome incorporate unnatural amino acids, it can also incorporate α-hydroxy acids. This converts the backbone amide to a backbone ester, destroying the hydrogen bond donor and significantly weakening the hydrogen bond acceptor. Other workers have exploited this approach to probe the contributions of backbone hydrogen bonds to α-helix and β-sheet stabilities.[24–26]



We used this strategy to probe a proposed hydrogen bond between nicotine and the nAChR.[27] As noted above, the nicotinic acetylcholine receptor (nAChR) is a paradigmatic neuroreceptor. Interestingly, glial cells from some snails secrete a small, soluble protein that binds ACh. This acetylcholine binding protein (AChBP) crystallizes as a pentamer and is significantly homologous to the binding region of the nAChR, making it a valuable model for some aspects of the full receptor.[28,29] Based on the AChBP structure, it was proposed that a backbone carbonyl makes a hydrogen bond to select agonists of the receptor. In the nAChR, we converted this amide carbonyl to an ester carbonyl by introducing an α-hydroxy acid at the $i + 1$ position. We find that nicotine and related agonists are indeed less potent when the backbone ester is incorporated, supporting the hydrogen bonding hypothesis. Importantly, ACh, which cannot make an analogous hydrogen bond, does not respond in this way to the ester substitution. This indicates that the backbone perturbation did not globally alter the receptor, but rather is specific to agonists that are hydrogen bond donors.

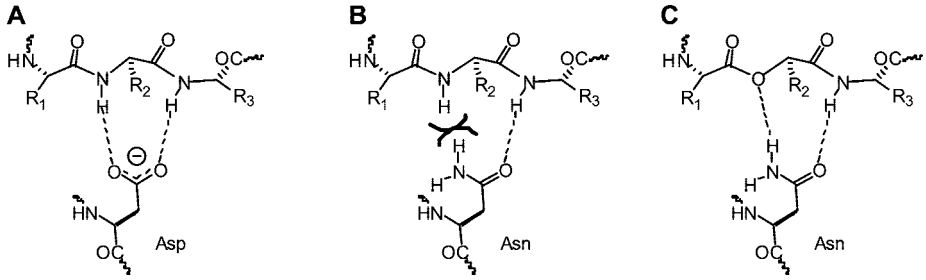


FIGURE 1. (A) Proposed network of hydrogen bonds in the nAChR. The Asp side chain hydrogen bonds to two backbone NHs. In addition, side chains R_1 and R_3 contain OH groups that hydrogen bond to the aspartate. (B) Conventional mutant Asp-Asn removes a negative charge but also introduces a steric clash between NH groups. (C) The steric clash can be removed by backbone ester substitution, reconstituting a hydrogen bonding network that is similar to that of the wild type (A), but lacking the negative charge.

In another region of the nAChR, we probed an intriguing network of hydrogen bonds (Figure 1) that was proposed to form when the side chain carboxylate from a highly conserved aspartic acid (Asp) residue hydrogen bonds to backbone NHs and side-chain OHs (the OH groups are not shown in the figure).[30] We were able to make a novel use of the backbone ester strategy to probe this system. Earlier work had shown that converting the Asp to the amide analogue asparagine (Asn) is highly deleterious. It was proposed that this observation established a critical role for the negative charge of the Asp residue. However, the Asp-Asn mutation also sets up a severe steric/electrostatic clash between the backbone $NH^{\delta+}$ and the Asn side chain $NH^{\delta+}$ (Figure 1B). By converting the backbone NH to an O (Figure 1C) we could remove the clash and rescue the deleted hydrogen bond. We found that this double mutant (Asn on the side chain plus backbone ester) had near wild type behavior. This established that maintaining the network of hydrogen bonds was most critical to receptor function, with the charge on the Asp playing a secondary role.

Stereochemical Probes. Stereochemistry provides a classic probe of reaction mechanism. The subtle changes associated with stereoisomerism can often create telling insights into structural issues. The receptors and ion channels considered here are large molecules, with molecular weights typically in the hundreds of thousands. It might be imagined that simply altering the stereochemistry of an amino acid side chain would have little effect on the function of a large, integral membrane protein. Remarkably, though, we have found several cases where just the opposite is true.

Recall the leucine-based hydrophobic gate discussed above. We used Ile as a Leu surrogate, allowing a subtle probe of hydrophobicity with Omt, and the results were consistent with the hydrophobic plug model. Another feature of Ile is that it is one of two natural amino acids (the other being threonine) for which the side chain contains a stereocenter. The side chain epimer is termed *allo*-isoleucine (*a*Ile) and it is, of course, an unnatural amino acid. We wondered if something more subtle was going on with the hydrophobic gate, and so we replaced individual Ile residues (recall, the channel is a pentamer) with *a*Ile.[23] In one particular subunit, the receptor was 5.3-fold more difficult to open when *a*Ile was present vs Ile. It is worth contemplating the significance of this experiment. We are probing a pentameric, molecular weight ~300,000, integral membrane protein. At one amino acid side chain, we swap the positions of a methyl group and an ethyl group. Yet, we see an easily measurable change in protein function. Note that this is not at a ligand-binding site or an enzyme-active site, where stereochemical effects might be anticipated, yet it is specific to one side chain in one subunit. The ~1 kcal/mol energy difference implied by this observation is consistent with typical estimates for the energetics of burying one CH_2 group in a hydrophobic environment. Apparently, in some state of the receptor this particular side chain is buried in a stereochemically defined, hydrophobic pocket. We were able to use such information to reach a conclusion about the overall layout of the subunits in the receptor.

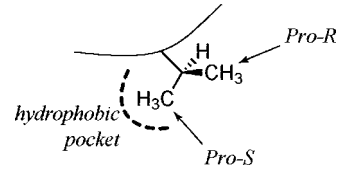
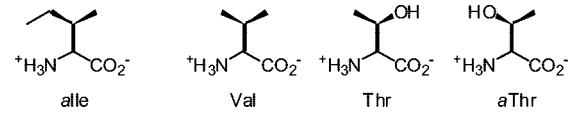


FIGURE 2. "Pin-into-socket" arrangement of a valine side chain and a hydrophobic pocket of the nAChR.



More recently, we were able to use a similar approach to evaluate another proposed interaction in the nAChR. Based on low-resolution cryo-EM images of the nAChR, it was proposed that the side chain of a particular valine (Val) made a key "pin-into-socket" interaction with a small cavity formed by an adjacent region of the receptor (Figure 2).[31,32] In particular, the Pro-*S* methyl group of the Val side chain was proposed to be tucked into the receptor pocket, while the Pro-*R* methyl was proposed to be exposed to solvent. This interaction is potentially important because it occurs at the crucial interface[33] between the ACh binding domain and the channel-forming region.

With Val as a side chain, a stereochemical test was easily designed.[34] Converting Val to Thr converts the critical Pro-*S* methyl to a hydroxyl, which should have a profound effect if the proposed interaction is important. Indeed, the Val-Thr mutant is ~60-fold more difficult to gate than wild type. By physical organic chemistry standards a Val-Thr mutation is not subtle, and so interpreting this result is risky. It is under such circumstances that the power of unnatural amino acid mutagenesis becomes clear. The epimer of Thr *allo*-Thr (*a*Thr)- replaces the Pro-*R* methyl of Val with hydroxyl. If the model is correct, this should be much less deleterious, and, indeed, we see a much smaller effect for this substitution. This stereochemical probe provides strong support for the notion that this key Val residue does indeed have its side chain tucked into a stereochemically well-defined pocket.

Linear Free Energy Analysis. In the preceding examples, we used subtle substitution studies to evaluate the viability of proposed noncovalent contacts such as hydrophobic interactions or hydrogen bonds. The results provided strong support for the proposed interactions, but are still open to alternative interpretations. In some more favorable cases, we have developed a much more compelling assay, a linear free energy relationship (LFER).

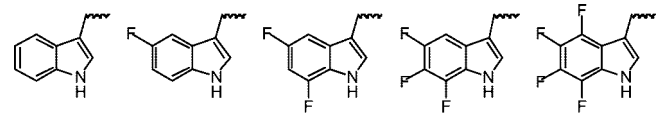
The Hammett plot and similar LFERs have been a workhorse of physical organic chemistry. For proteins, LFER studies of enzymatic reactions are common and informative. Typically, the substrate for the enzyme is modified systematically, and LFER analysis provides valuable insight into the enzymatic mechanism. While there is a great deal of medicinal chemistry and SAR concerning small molecules that bind to ion channels and receptors, LFERs are less common.[35] We have found that the flexibility afforded by unnatural amino acid mutagenesis allows us to reverse this paradigm. Instead of systematically altering the substrate, we vary the protein. In favorable cases we have been able to see clear LFERs even in a complex protein.

This is one of the most powerful implementations of the unnatural amino acid methodology. It is impossible to change a residue in a protein without changing a number of variables—sterics, hydrophobicity, hydrogen bonding, polarity, conformational flexibility, etc. This is why conventional mutagenesis is limited; too many things change when most conventional mutants are made. Even the very subtle unnatural amino acid mutations described above are not perfect. We feel we can be more confident in interpreting the results than with the more intrusive conventional mutations, but still there is always some doubt. However, when a systematic *series* of related mutations can be made, and when the consequences can be directly correlated to a known property that is systematically changing, much greater confidence in interpretation is possible.

Essentially all neurotransmitters and molecules that bind to receptors and ion channels contain a positive charge. Some time ago, we noticed that the emerging sequence and biochemical information on channels and receptors suggested that aromatic amino acids—phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp)—were over-represented at these binding sites. This led us to propose[36] that cation−π interactions[37,38] would be important in drug−receptor interactions in these systems.

Our extensive studies of the cation−π interaction also suggested a viable way to probe for one in a protein. A fluorine substituent is known to be deactivating in the cation−π interaction, and multiple fluorines show an additive effect.[39,40] If a cation−π interaction to a particular aromatic residue is important, then the monofluoro, difluoro, trifluoro... series should show a clear trend of diminishing ligand affinity as the level of fluorination increases. Another advantage of fluorine substitution is that the steric perturbation introduced by fluorine is minimal. By developing a quantitative measure of the cation−π binding ability of a side chain, we can create a true LFER for such studies. We chose to use the gas phase binding of a Na^+ ion to the ring, as determined by an ab initio calculation, as our measure of intrinsic cation−π binding ability of a given aromatic. The choice of cation is not crucial, as many studies have shown that the *trend* in cation−π binding is not influenced by the identity of the cation, only the magnitude of the effect changes. If we plot the log of the ligand affinity against the Na^+ binding energy of the ring, a true LFER can emerge.

For cation−π binding to receptors and channels, we have used "fluorination plots" to produce LFERs that are quite compelling. For example, in the nAChR we have been discussing, the cation−π interaction involves a Trp residue, and so the fluorination series shown was employed.[41] Similar series were developed for Tyr and Phe residues. To date, we have established cation−π interactions for the neurotransmitters ACh,[41] serotonin (5-HT),[42,43] and γ-aminobutyric acid (GABA)[44,45] binding to their cognate receptors. There are multiple forms of each of these receptors, and so in fact, we have seen seven (three for ACh and two each for 5-HT and GABA) cation−π interactions. We have also shown that a cation−π interaction is involved in the binding of tetraethylammonium (TEA, a common ion channel blocker) to a voltage-gated K^+ channel;[46] in the binding of the local anesthetic lidocaine to a voltage-gated Na^+ channel;[47] and in the binding of $Ca^{2+}(H_2O)_n$ to a voltage-gated Na^+ channel.[48] On the other hand, a proposed cation-π interaction between Mg^{2+} and a Trp near the pore of the NMDA glutamate receptor is actually nonexistent, as fluorination of the suspected Trp does not alter Mg^{2+} affinity.[49]



Another example concerns the toxicity of tetrodotoxin (TTX), the notorious poison associated with the fugu pufferfish. TTX blocks voltage-gated Na^+ channels (Na_V), causing muscle paralysis. Since the toxin cannot cross the blood−brain barrier, the victim is fully conscious and generally dies of asphyxiation. Humans have an array of Na_V channels, and TTX efficiently blocks some (Na_V 1.1, 1.2, 1.3, 1.4, 1.6, and 1.7) but not others (Na_V 1.5, 1.8, and 1.9). For example, Na_V 1.4 is the form found in skeletal muscle, while Na_V 1.5 is found in cardiac muscle. It has been known for some time that at a particular location thought to be near the channel mouth, the Na^+ channel of skeletal muscle and other TTX-sensitive channels contain a Tyr, while TTX-insensitive channels contain a nonaromatic residue. (The fugu fish survives because its skeletal muscle form has an asparagine instead of the tyrosine at this site.[50]) This led us to speculate some time ago that a cation−π interaction might be the feature that enhances TTX binding to the sensitive channels.[37] Recently, we have shown that, indeed, in the skeletal muscle channel Na_V 1.4 the indicated Tyr makes a strong cation−π interaction to TTX.[51] Using unnatural amino acid mutagenesis, we have been able to show that one specific noncovalent interaction can have life-or-death consequences.

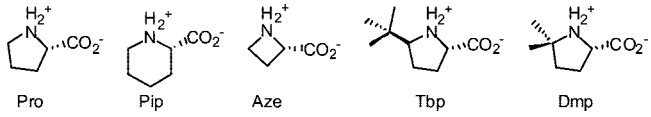
Another LFER was obtained while probing a very different effect. Arguably, the greatest chemical challenge in studying the molecules of neuroscience concerns the gating mechanism of the neurotransmitter-gated ion channels we have emphasized here. As noted above, the protein is pentameric and has a molecular weight of ~300,000. The neurotransmitter has a molecular weight under 200. The agonist binding site and the gate of the ion channel are ~60Å apart. Yet, binding of neurotransmitter launches a conformational change that propagates across that distance and ultimately leads to channel opening.

When a low-resolution cryo-EM image of the nAChR became available, we and others noticed a proline residue

positioned at the boundary between the neurotransmitter binding site and the channel-forming segments, a region we have called the "gating interface".[33] One is always suspicious of a well-positioned proline (Pro) because of the specific chemical properties of this amino acid. Of the natural amino acids, Pro is unique in that the amide N of a proline peptide bond is disubstituted. This has two important consequences. First, the lack of an amide NH removes a potent hydrogen bond donor that plays a key role in defining both α-helix and β-sheet secondary structures. Frequently, Pro is considered to disrupt/kink an α-helix. For example, there is a proline residue in the middle of a transmembrane α-helix of Cys-loop receptors that is completely conserved; substitution with any other natural amino acid produces a nonfunctional receptor. It is generally assumed that this Pro somehow disrupts the transmembrane helix, but direct evidence is lacking. Note that a backbone ester substitution of the sort discussed above also removes this NH. While all conventional amino acid mutations fail, we can produce essentially wild type receptors by putting an α-hydroxy acid at the proline site, regardless of the side chain.[52,53] This establishes that, indeed, it is the lack of the backbone NH that makes this particular Pro essential for receptor function.

The second unique feature of Pro is that *s-cis*−*s-trans* isomerization around the amide bond is perturbed, such that the *cis* form is much more likely with Pro (∼5%) than with any other natural amino acid (<1%). It is this aspect of Pro chemistry that we thought could be relevant to the aforementioned Pro at the gating interface of the receptor. Gating requires a conformational change in the receptor, and *cis*−*trans* isomerization of a Pro peptide bond seemed like a good candidate. To evaluate this possibility, we replaced the suspect Pro with a series of proline analogues.[54] As in a fluorination study, we are using a series of closely related structures and seeking a trend in the data.

The structures we chose to evaluate differ in their innate *cis*−*trans* bias. Beginning with Pro at 5%, we can progress to pipecolic acid (Pip, 12%), azetidine carboxylic acid (Aze, 18%), 5-*tert*-butylproline (Tbp, 55%), and 5,5-dimethylproline (Dmp, 71%). These values represent equilibrium constants, and so we can assign $\Delta\Delta G$ (*c*−*t*) values, which measure the increasing bias toward *cis*, all referenced to Pro. When these Pro analogues are incorporated into a serotonin receptor that is a close analogue of the nAChR (the 5-HT_3 receptor), a clear trend emerges. The larger the % *cis* in the proline analogue, the easier it is to open the channel, producing a ∼60-fold increase in receptor activation for Dmp.



We can show that our substitutions change receptor function by changing in the equilibrium between open and closed states (open and closed are always in equilibrium; binding agonist simply pushes the equilibrium toward the open state). This equilibrium, too, can be put on an energy scale, producing $\Delta\Delta G$ (open-closed), again referenced to Pro. A plot of $\Delta\Delta G$ (*c*−*t*) vs $\Delta\Delta G$ (open-closed) produces an excellent straight line.[54] Remarkably, the slope of this line is 1. This means that for every kcal/mol that we bias the innate *cis*−*trans* equilibrium of the proline analogue, we bias the open-closed equilibrium of the receptor by 1 kcal/mol. We consider these data to provide compelling evidence that *cis*−*trans* isomerization around an amide bond at a key Pro residue represents a critical step in the gating mechanism of the receptor.

Conclusions

By adapting the unnatural amino acid mutagenesis methodology to the *Xenopus* oocyte heterologous expression system and combining it with the remarkable sensitivity and information content of electrophysiology, the complex proteins of neuroscience can be probed with the tools of physical organic chemistry. Key noncovalent interactions such as hydrophobic interactions, hydrogen bonds, and cation−π interactions can be established, and mechanistic insights into the function of ion channels and neuroreceptors can be obtained. Many challenges remain, but it is clear that physical organic chemistry on the brain is possible and that the future for the field is bright.

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