Review

Cys-Loop Neuroreceptors: Structure to the Rescue?

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Cys-Loop Neuroreceptors: Structure to the Rescue?

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1. Introduction

We have entered the age of structural neurobiology. After decades of envying our colleagues who work on soluble proteins, we have our first structural insights into the complex, often multisubunit, integral membrane proteins of the nervous system. Structural information of some sort is available for many of the major classes, including K^+ channels, mechanosensitive channels, several varieties of transporters, G protein-coupled receptors (GPCRs), glutamate receptors, aquaporins, and others.[1] But we still have a long way to go. Even though ~30% of genetically encoded proteins are membrane proteins and ~60% of pharmaceuticals target membrane proteins, less than 1% of the structures in the Protein Data Bank are membrane proteins. Also, in almost all cases, the structural information is not for an intact receptor/channel of the mammalian CNS but rather for a prokaryotic protein or a fragment of a eukaryotic protein. Nevertheless, the hard won structures of membrane proteins provide invaluable guidance to efforts to apply chemical approaches to neurobiology.

A major emphasis of our research has been on a large family of receptors, the Cys-loop neuroreceptors, which are described in detail below. In recent years, highly relevant structural information has appeared for this critical class of receptors. We certainly do not have a high resolution structure of a mammalian Cys-loop neuroreceptor, but we now have the first clues, derived from garden snails and electric rays. These images raise critical questions. How relevant is the model structure to actual receptors of the mammalian central nervous system (CNS)? What state of the receptor does the model structure correspond to, and what do other receptor states look like? How confidently can we extrapolate a single image of a model system across a broad family of related receptors? While the emphasis here is on Cys-loop receptors, we feel the kinds of issues addressed in this review will recur in studies of many classes of the proteins of neuroscience.

For the past 15 years, our group has been using chemical tools to evaluate neuroreceptors and ion channels. With the advent of the more recent structural information, a major focus has been to address the kinds of questions posed above. The present work describes these efforts, emphasizing recent studies from our laboratories and the interplay between functional studies performed on the real mammalian receptors and structural studies of model systems. The recurring theme is that, while beautiful and certainly informative, the static images provided from structural work on model systems must be viewed with caution when considering integral membrane receptors. As such, chemical and biochemical tools, like those

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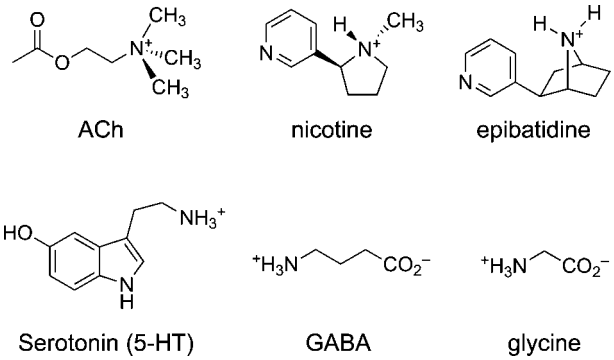

Figure 1. Structures of the agonists considered here.

described below, will continue to provide critical information on the structure and function of these important molecules.

2. Cys-Loop Neuroreceptors

The Cys-loop family of neuroreceptors mediates fast synaptic transmission throughout the central and peripheral nervous systems.[2–5] They are ligand-gated ion channels, integral membrane proteins that contain a binding site for the agonist—the neurotransmitter—and an ion channel that spans the membrane and is typically closed. Binding of neurotransmitter triggers a conformational change in the receptor that opens the ion channel—the metaphor is that a gate swings open. These remarkable molecules thus contain a binding site, a channel, a gate, and, most intriguingly, a mechanism to couple the binding event to the gating of the channel. The Cys-loop receptors are fascinating molecular machines, and we have been probing all aspects of their chemistry for some time.

The longest known, best studied neuroreceptor is a Cys-loop receptor: the nicotinic acetylcholine receptor (nAChR), so named because ACh is the natural neurotransmitter agonist, but nicotine is also a potent agonist (Figure 1). The nAChR holds its special place in receptor biology because of the electric organs of *Torpedo* rays and electric eels. The organ that generates the electric shock, the electroplax, is extraordinarily rich in nAChRs. For over 35 years the *Torpedo* electroplax has been a plentiful source of nAChRs, and all early biochemical studies of Cys-loop receptors employed the nAChR from this source.

Early cryoelectron microscopy studies of the *Torpedo* nAChR revealed the overall layout of the receptors: pentameric, with a symmetrical or pseudosymmetrical array of five equivalent or homologous subunits surrounding a central pore that is the ion channel.[6] Also, biochemical studies established the basic layout (topology) of each subunit: an extracellular N-terminal domain of ~200 amino acids followed by a transmembrane domain with four membrane-spanning segments. Biochemical studies of the *Torpedo* receptor and subsequent site-directed mutagenesis studies of cloned receptors established that the agonist binding site is associated with the extracellular, N-terminal domain and lies at the interface of two subunits. For the *Torpedo* receptor there are two agonist binding sites, and efficient channel opening requires occupation of both by agonist. The extracellular domain also contains the eponymous Cys-loop, formed from a disulfide and containing 13 intervening residues, many of which are fairly well conserved. Concerning the transmembrane region, the second transmembrane segment, M2, lines most or all of the channel pore, and so the ion channel is formed from a confluence of five M2 segments.

The advent of molecular biology greatly expanded understanding of nAChRs and Cys-loop receptors in general. There is in fact a family of nAChRs. At least 17 human genes code for nAChR subunits, and these are termed $\alpha1-\alpha10$, $\beta1-\beta4$, γ, δ, and ϵ. If all possible combinations of 5 nAChR subunits could form functional receptors, hundreds of thousands of nAChR subtypes would exist. This is certainly not the case, but there is definitely a diverse collection of viable nAChRs, with at least 20 functionally distinct subtypes established to be important in humans.

One global distinction among nAChRs is important. The nAChR controls the neuromuscular junction. Every time you move a muscle voluntarily, it is because ACh exited a presynaptic neuron, traversed the synapse, and then activated a nAChR in the innervated muscle. The nAChR of the neuromuscular junction is unique. It has a precise stoichiometry of $(\alpha1)_2\beta1\gamma\delta$ (fetal form; the adult form is $(\alpha1)_2\beta1\delta\epsilon$), and it has some unique chemical and biophysical properties. The nAChR from *Torpedo* is essentially identical to the nAChR of the neuromuscular junction, and so all the biochemical studies of the *Torpedo* receptor are highly relevant to the muscle-type receptor. In both receptors, the two agonist binding sites have been localized to the α/γ and α/δ interfaces. Dysfunctions of this "muscle-type" receptor have been associated with a number of myasthenic syndromes.

The remaining nAChRs of the sort found in the central nervous system (CNS), the "neuronal" nAChRs, are less well defined than the muscle-type. They are comprised of various pentameric combinations of the $\alpha2-\alpha10$ and $\beta2-\beta4$ subunits.[7,8] There are considerable efforts across the pharmaceutical industry to target neuronal nAChRs, with clinical implications for cognition, Alzheimer's disease, Parkinson's disease, schizophrenia, pain, ADHD, epilepsy, depression, and smoking cessation.

There are also Cys-loop receptors that respond to other neurotransmitters, including serotonin (5-hydroxytryptamine, 5-HT), which activates the 5-HT_3 receptor, and also γ-aminobutyric acid (GABA) and glycine, which also activate specific classes of Cys-loop receptors. All these plus all the nAChRs are highly homologous and have evolved from a common ancestral gene.[9,10] As such, the biochemical and structural information obtained on the nAChR is considered to be highly relevant to the other members of the Cys-loop family. The receptors are further classified as excitatory or inhibitory, with the former being cation-conducting channels that promote the firing of an action potential and the latter being anion-conducting channels that discourage the firing of an action potential. These channels are thus not as exquisitely selective as, for example, K^+ channels. The selectivity filters are less precisely defined structurally and seem to involve rings of ionic residues near the termini of the transmembrane regions.[11] The nAChR and 5-HT_3 receptors are excitatory; glycine receptors are inhibitory, while GABA are predominantly inhibitory but can be excitatory.

3. Structure to the Rescue

This is not a chronological tale; decades of beautiful biochemistry and electrophysiology preceded the structural results we will now describe.[12,13] However, the goal of the present work is to show how chemistry can provide rigorous tests of the relevance of structural models to the structure

and function of real receptors, and so we begin by laying out the structural data.

The *Torpedo* electroplax has provided a plentiful source of nAChR for over 35 years, yet still no crystals that diffract to high resolution in three dimensions have been obtained (not for lack of trying!). However, Nigel Unwin mastered a methodology to produce tubes that diffract in two dimensions, and decades of painstaking analysis of cryoelectron microscopy (cryoEM) images of these tubes have provided valuable insights. Early studies produced images of the *Torpedo* receptor at 9 Å resolution, establishing the pentameric layout.[6] Speculative models of ligand binding and channel gating were developed based on these low-resolution images.

A breakthrough came in 2001 when Sixma, Smit, and coworkers reported that the glial cells of garden snails secrete a small, soluble protein that binds ACh—the acetylcholine binding protein, AChBP.[14,15] The structure shows 20−25% sequence identity to the extracellular domains of nAChRs and 15−20% identity to other Cys-loop receptors. Remarkably, this small soluble protein assembles as a cylindrical pentamer, clearly mimicking the extracellular domain of the Cys-loop receptors. The structure is comprised mainly of β-sheets, with ten segments termed $\beta1-\beta10$ and connecting loops L1−L10.[15]

It is especially important with membrane receptors and channels to show that the imagery of the model structure is backed up by chemical and biochemical studies. As described below, studies throughout the 1980s and early 1990s suggested that the nAChR binding site was rich in aromatic amino acids. Importantly, the AChBP binding site is indeed formed by five aromatic amino acids, all of which had been implicated by biochemical experiments as being important in binding.[12,16,17] Thus, the AChBP structure is clearly highly relevant to the nAChR.

Very recently, a high resolution structure of a mutant version of the extracellular domain of the mouse α1 subunit complexed to the snake toxin α-bungarotoxin has appeared. It confirms the β-sheet topology seen in AChBP and includes for the first time images of sugars associated with a conserved glycosylation site.[18]

Ongoing refinement of the *Torpedo* cryoEM work led, in 2003, to images of the transmembrane region at 4 Å resolution.[19] The images established that the four transmembrane segments are α-helical, and a packing arrangement was clear. Finally, guided by the AChBP structure, a 4 Å map of the entire *Torpedo* receptor emerged in 2005 (Figure 2).[20] There is now a pdb file for the nAChR of the *Torpedo* electroplax, and with minimal extrapolation, we can view this as an image of the nAChR of the mammalian neuromuscular junction.

But what is this image, exactly? It is not an X-ray crystal structure, and 4 Å resolution is generally considered inadequate for high precision interpretation. Also, while there is little risk in assuming great similarity between the *Torpedo* receptor and that of the mammalian neuromuscular junction, what about the neuronal nAChRs, the molecules directly responsible for the cognitive and addictive effects of nicotine? It is a further leap still to the 5-HT_3, GABA, and glycine receptors.

In addition, even if the image of Figure 2 was a perfect mapping for all mammalian Cys-loop receptors (which it is not), we need to consider what form of the receptor is being imaged. The whole point of these proteins is that they are

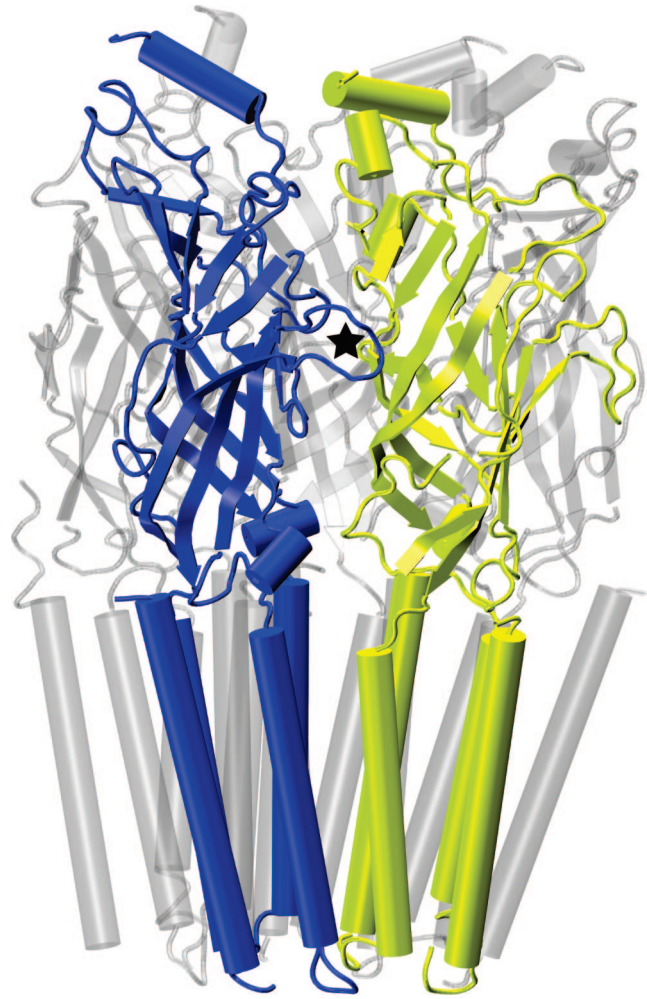

Figure 2. Image of the nAChR from *Torpedo marmorata*, as determined by Unwin[20] (PDB file 2BG9). The membrane would lie roughly aligned with the α-helical segments. A large intracellular segment that is less well defined has been deleted from the bottom of the structure. The two front-most subunits are α1 (blue) and γ (yellow); the remaining three subunits are in the background. One binding site would be at the interface of these two subunits, as denoted by the black star.

dynamic, signaling molecules. Signaling is accomplished by interconversion among a number of conformations, often referred to as states in the neurobiology literature. Minimally, we must consider the following: the receptor with the channel closed; the closed receptor with agonist bound; the open receptor with agonist bound; and the desensitized receptor with agonist bound. The real situation is likely more complicated, with some kinetic schemes invoking dozens of different states. To understand receptor function, we must understand all the accessible states and how they interconvert. It seems likely that the *Torpedo* image generated by Unwin is of the closed receptor, with no ligand bound. Images of AChBP with various ligands bound have been generated. It can be argued that these more likely reflect a desensitized state.

To be clear, the AChBP structure and the refinement of the *Torpedo* nAChR cryoEM represent huge advances for the field. All subsequent studies have been influenced by the insights they have provided. But to understand receptor function, and to inform efforts at drug discovery, we need much more. We need to understand what states are being imaged and how the states interconvert. We need to learn which features are universal for the Cys-loop family,

and which are particular to a given receptor. It is our view that only functional studies of the actual mammalian receptors can answer such questions. In addition, we need approaches that are powerful enough to discriminate among many possibilities and general enough to apply to many receptors. We describe here our efforts to develop and apply such a tool.

4. Unnatural Amino Acid Mutagenesis Enables Chemical-Scale Studies

As noted above, the Cys-loop receptors have been the targets of a great number of beautiful functional studies using a vast array of techniques. While initial work focused on nAChRs from the electroplax, in the 1980s and 1990s most of the receptors were cloned, and it became possible to study the whole family. For such studies, the receptors must be expressed in eukaryotic cells that have the necessary machinery to assemble the subunits and transport them to the cell surface. In most cases, the receptors must be embedded in the membrane of a living cell with a resting membrane potential, so that the powerful tools of electrophysiology can be used for characterization. It is beyond the scope of this review to cover all the prior work, but certainly many laboratories have made critical contributions to our understanding of Cys-loop receptor structure and function, and all of our work builds off those efforts.

Perhaps uniquely, our group comes at this problem from the perspective of a physical organic chemist. We view Cys-loop receptors as organic molecules that undergo a remarkable reaction, and we want to discern the mechanism of that reaction. We want to obtain "chemical-scale" insights into the structure and function of these complex proteins. By chemical scale we mean, in effect, the distance scale to which chemists are accustomed: the functional group; the specific bond rotation or local conformational change; the precise noncovalent interaction.

The essential tool of physical organic chemistry is the structure−function study. Monitoring responses in reactivity to systematic changes in structure can provide compelling insights. In small molecule physical organic chemistry, organic synthesis provides the structural modifications. For large membrane proteins, total synthesis is not feasible, and the natural approach to structure modification is site-directed mutagenesis. Indeed, many informative studies of Cys-loop receptors have been performed using site-directed mutagenesis. But, to a physical organic chemist the tool is too crude. The limited structural variation provided by the 20 natural amino acids simply does not allow the sort of subtle, systematic modifications necessary for a convincing structure−function study.

To perform the subtle, controlled structure−function studies associated with small molecule physical organic chemistry on the complex receptors of neuroscience, unnatural amino acid mutagenesis is required. In 1995 we described the first incorporation of an unnatural amino acid into a protein expressed in a living cell.[21] The protein was the nAChR, and the cell was a *Xenopus* oocyte. This was the beginning of a fruitful and ongoing collaboration with Henry Lester, Bren Professor of Biology at Caltech and a renowned authority on Cys-loop receptors and many other aspects of molecular neurobiology. The approach was based on an *in vitro* methodology that had been developed by Schultz and others.[22–25] The *in vivo* methodology has been described in detail elsewhere,[26–28] so we provide only the briefest description here.

Receptors are expressed in oocytes from the frog *Xenopus laevis* and probed using electrophysiology. A dose−response curve for application of neurotransmitter produces EC_{50}, the effective concentration for half-maximal response. EC_{50} is a functional measure, and so it reflects both the binding affinity of the drug and the gating efficiency of the receptor, two factors that are sometimes difficult to deconvolute. For conventional studies, one simply injects the mRNA(s) for the receptor of interest into these large (∼1 mm diameter), cooperative cells, and the subunits are expressed, folded, assembled, and transported to the cell surface. Importantly, the physiology and pharmacology of receptors expressed in *Xenopus* oocytes are identical to what is seen in their native environment. For unnatural amino acid mutagenesis, we introduce a stop (nonsense) codon at the site of interest. Along with this mutant mRNA, we inject a stop codon-recognizing (suppressor) tRNA to which the desired unnatural amino acid has been attached using organic chemistry. The strategy of using synthetic chemistry to acylate the suppressor tRNA with the desired unnatural amino acid[22,29] affords maximal flexibility in terms of the range of unnatural amino acids that can be incorporated. Figure 3 shows some of the over 100 residues we have incorporated using this methodology. In this strategy, the aminoacyl tRNA is a stoichiometric reagent, and this significantly limits the quantities of protein that can be prepared by this approach. However, the incredible sensitivity of electrophysiology overcomes this limitation. Very large currents can be seen from a *Xenopus* oocyte that is expressing as little as 10 attomol of receptor on its surface. And, of course, the patch clamp allows detection of single channel molecules. We really are not limited by the quantity issue. In a recent methodological advance, we have developed several new tRNAs that allow us to incorporate multiple different unnatural amino acids into a single receptor.[30–32]

5. Chemical-Scale Studies: the Binding Site

5.1. ACh Receptors

The AChBP crystal structure[14] revealed a remarkable ACh binding site, an image of which is shown in Figure 4. What has been termed the "aromatic box" is formed by five aromatic amino acids—three tyrosines and two tryptophans. As had been anticipated, the agonist binding site is at an interface between two subunits. Four of the aromatics (A, B, C1, C2) come from the "principal" subunit (α1 in muscle-type nAChR), with the remaining (D) contributed by the "complementary" subunit (γ or δ in muscle-type nAChR).

As noted above, an aromatic-rich binding site was anticipated by biochemical studies, and it is also what initiated our interest in the nAChR. As far back as 1990, we predicted that ACh would bind to its receptors and other proteins through a cation−π interaction.[33] This conclusion was based in part on studies performed in the 1980s on cyclophane model systems, in which we established that, in an aqueous environment, an aromatic binding site could bind ACh and related compounds well. In fact, our cyclophane binding site very much created its own "aromatic box".[34,35]

Our extensive studies of the cation−π interaction[36,37] also established a way to probe for one in a protein. A fluorine substituent is deactivating in the cation−π interaction, and multiple fluorines show an additive effect. Coupled with the

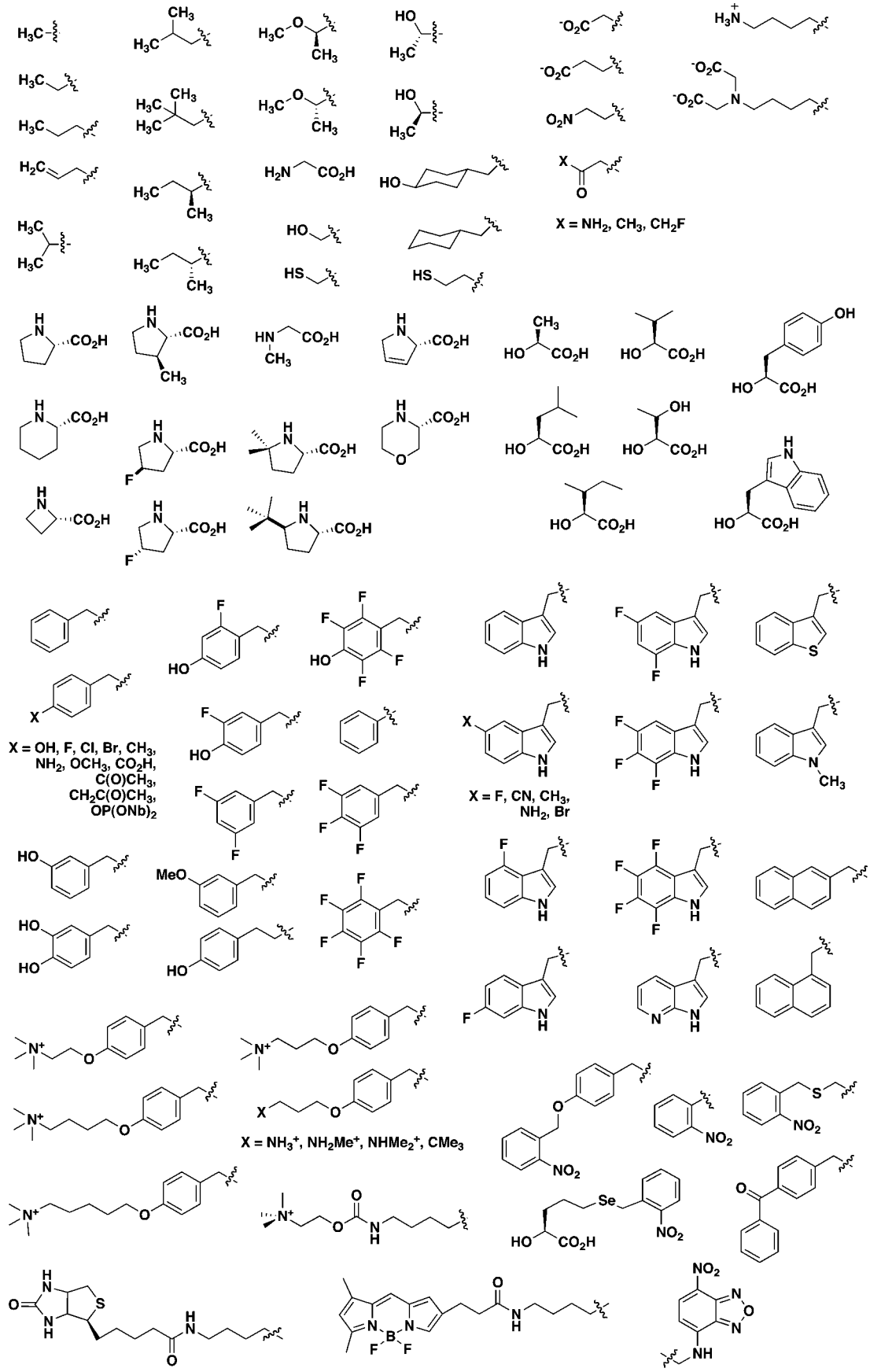

Figure 3. Selection of residues that have been incorporated into functional receptors/channels expressed in *Xenopus* oocytes. Both natural and unnatural amino acids are shown, along with several α-hydroxy acids.

minimal steric perturbation introduced by fluorine substitution, this suggested a fluorination probe of potential cation−π binding sites. One takes the aromatic amino acid of interest and progressively replaces it with the monofluoro-, difluoro-,

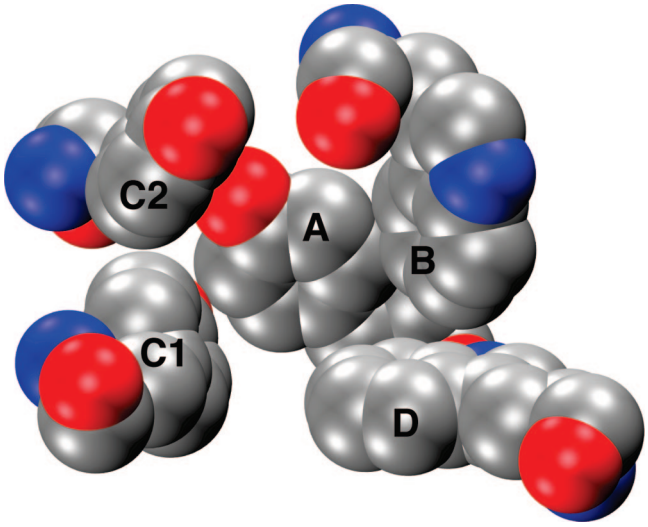

Figure 4. The "aromatic box" of AChBP (PDB file 1I9B)[14]. Residues are labeled by the accepted system, in which letters designate the "loop" of the receptor structure on which the residue resides, as first described by Changeux.[12] Residue identities in AChBP (and the nAChR) are as follows: A, Tyr; B, Trp; C1, Tyr; C2, Tyr; D, Trp.

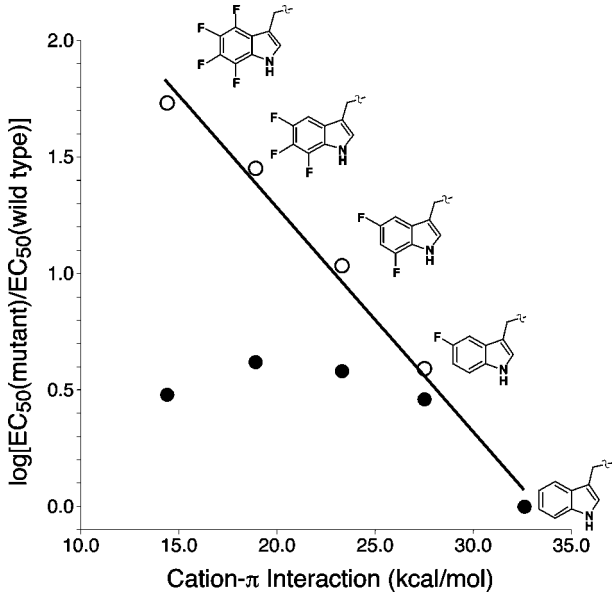

Figure 5. Fluorination plot for TrpB of the muscle-type nAChR for ACh (open circles) and nicotine (closed circles) as agonists. Note that the plots for both agonists share the point for the wild type Trp residue.

trifluoro, etc. derivatives. If a clear correlation between agonist affinity and degree of fluorination is seen, a cation−π interaction is established.

Using this approach, we evaluated in the muscle-type nAChR several tyrosines and tryptophans that had been proposed to contribute to the agonist binding site.[21,38] A clear effect of fluorination was seen at one and only one site—TrpB.[38] Figure 5 shows a "fluorination plot" for TrpB of the muscle-type nAChR. We use a quantitative measure of cation−π binding ability—the binding of a Na^+ to the ring—for the *x*-axis.[39,40] To relate function to this energy scale, we plot log(EC_{50}). A cation−π interaction is established not only by the linearity of the fluorination plot but also by the magnitude of the effect—EC_{50} changes more than 50-fold on going from Trp to F_4-Trp. Note that the full Trp side chain is present in all mutants; we are simply modulating the electrostatic potential of the ring so as to diminish the cation−π interaction.

It was gratifying to see three years later that TrpB was prominently positioned in the aromatic box of AChBP. Subsequent AChBP structures with an ACh analogue or nicotine bound support a cation−π interaction to TrpB in AChBP.[15]

At this point we might conclude that the problem is solved. The cation−π interaction provides an absolute anchor point for ACh. The trimethylammonium of ACh must make van der Waals contact with the side chain of TrpB. The structural model and the functional studies are in complete agreement. However, as a chemist who has been involved with neuroscience for almost 20 years, one truism I have observed is that the nervous system is always more complicated than you think.

While it is nice to know where ACh binds, this is the *nicotinic* receptor. For drug discovery targeting the many pathologies mentioned above, nicotine is the lead compound, not ACh. Certainly, the nicotinic pharmacophore is one of the oldest known. It is widely accepted that, when comparing ACh and nicotine, the two cationic centers line up and the two "polar" regions (acetyl of ACh; pyridine N of nicotine) line up. A nicotinic agonist must have an appropriate separation between these two features.

One important feature of the nAChR of the neuromuscular junction—the form we have been discussing thus far—is that it is significantly less sensitive to nicotine than the high affinity nicotine sites in the brain associated with nicotine addiction (see below). In fact, if the nAChR of the neuromuscular junction were as sensitive to nicotine as the neuronal receptors, cigarette smoking would be impossible; the levels of nicotine inhaled would produce widespread paralysis. When we performed the fluorination at TrpB of the neuromuscular junction receptor, but using nicotine instead of ACh as the agonist, no strong effect was seen[41] (Figure 5). EC_{50} shifts less than 10-fold, and no correlation with degree of fluorination is apparent. Thus, chemical-scale studies established that the long-standing pharmacophore for the nAChR is incorrect, at least at the neuromuscular junction.

We also studied the agonist epibatidine[42] (Figure 1). This alkaloid isolated from the skin of a South American frog is clearly a nicotine analogue, and it is very potent at the neuromuscular junction. Indeed, we find that epibatidine gives a strong cation−π interaction at TrpB, with a fluorination plot that is superimposable on that for ACh. Clearly, the cation−π interaction is a discriminator between potent and impotent agonists of the nAChR.

Another distinction between ACh and nicotine/epibatidine is that the latter pair can act as a hydrogen bond donor. In looking at the AChBP structure, we and others noticed that the backbone carbonyl associated with TrpB pointed into the aromatic box. We wondered whether a hydrogen bond between agonist and this carbonyl oxygen could be another feature that distinguishes ACh-type agonists from nicotine-type agonists. One of the remarkable aspects of the nonsense suppression methodology is that it can be used to incorporate not only unnatural amino acids but also α-hydroxy acids. When an α-hydroxy acid is incorporated, the backbone amide (peptide) unit is replaced by an ester. The ester carbonyl is a much poorer hydrogen bond acceptor than an amide carbonyl, and so this can provide a subtle probe of hydrogen bonding.[43] Once again, we are not destroying an interaction,

we are simply modulating it. When the appropriate substitution was made, a clear dichotomy was revealed.[44] Nicotine and epibatidine became less effective, with the more potent agonist epibatidine showing the larger effect. In contrast, ACh actually became more potent when the ester was incorporated. We considered this good evidence that the nicotine-type agonists make a hydrogen bond to the carbonyl of TrpB.

While the nAChR of the neuromuscular junction is intrinsically interesting, the cognitive and addictive effects of nicotine result from activation of the neuronal nAChRs, and the neuronal nAChRs are certainly the primary targets of the pharmaceutical industry.[7,8] Of course, it would be very valuable to understand how nicotine and ACh bind to and activate the neuronal nAChR. However, despite the fact that we have successfully applied the nonsense suppression methodology to over 20 proteins expressed in *Xenopus* oocytes, usually with minimal method development required on moving to another receptor, for almost a decade we were frustrated by features of the neuronal nAChRs that made application of the unnatural amino acid methodology to them problematical. Only over the past two years have we been able to overcome those issues and finally probe the neuronal nAChRs.

Of the perhaps 20 different neuronal nAChRs known to be active in humans, two appear to play especially significant roles. The receptor that is most prominently associated with nicotine addiction is comprised of α4 and β2 subunits.[45] The smoking cessation drug Chantix was designed to target this receptor.[46] Both the 2:3 and 3:2 stoichiometries are viable and appear to be active in man.[47] The $(\alpha4)_2(\beta2)_3$ receptor shows the higher affinity for nicotine, and it has been the target of our investigations. Fluorination at TrpB produces a plot very similar to that of Figure 5 for *both* ACh and nicotine![48] At least to some extent, nicotine is active in the brain—but not at the neuromuscular junction—because the cation−π interaction is viable in the former but not the latter.

The other important neuronal nAChR we have considered is the homopentamer derived from α7. The α7 receptors are implicated in schizophrenia and have been suggested as viable targets for Alzheimer's treatments. Remarkably, when TrpB of the α7 receptor is fluorinated, *nothing changes*.[48] For ACh, nicotine, and epibatidine, the TrpB cation−π interaction is completely gone. Further probing of the aromatic box reveals that ACh makes a strong cation−π interaction with TyrA. In contrast, epibatidine makes a strong cation−π interaction with TyrC2 and perhaps a weak cation−π interaction with TyrA. Compared to α4β2 and the muscle-type, in α7 the cation−π interaction has moved to a new location.

We now have three different binding models for nAChRs: a cation−π interaction to TrpB for ACh but not nicotine (muscle-type); a cation−π interaction to TrpB for ACh and nicotine (α4β2); and a cation−π interaction to TyrA for ACh and TyrC2 for nicotinic agonists (α7). Remarkably, the aromatic box of AChBP is 100% conserved in all nAChRs, both neuronal and muscle-type; there are always three tyrosines and two tryptophans as shown in Figure 4. Clearly, one must think outside the box to explain these variations.

The perfect preservation of the aromatic box seen in the nAChR family is not retained when one considers all Cys-loop receptors, but aromatics are conserved at all but the A site. We have looked for cation−π interactions in several other Cys-loop receptors, with intriguing results.

5.2. Serotonin Receptors

The 5-HT_3 receptor responds to serotonin, and it is the target of a number of pharmaceuticals, including drugs that treat chemotherapy-induced emesis.[49–51] We find a compelling fluorination plot at TrpB.[41] The slope of a given fluorination plot is not directly interpretable, because the *x*-axis does not in any way relate to a neuroreceptor. It is simply a measure of intrinsic cation−π binding ability. However, we believe that the *relative* slopes of fluorination plots are meaningful, because the *x*-axis is the same for all plots. Interestingly, the slope of the fluorination plot is significantly steeper in the 5-HT_3 receptor than in any of the nAChRs. We interpret this to mean that the cation−π interaction is stronger in the 5-HT_3 receptor. This is consistent with well-established observations concerning the cation−π interaction. The more focused positive charge of the RNH_3^+ group of serotonin is expected to make a stronger cation−π interaction than the more diffuse $RNMe_3^+$ group of ACh, given the essentially electrostatic nature of the cation−π interaction.

Another serotonin-gated Cys-loop receptor termed MOD-1 has been found in *C. elegans*. It is homologous to the 5-HT_3 receptor and responds to the same agonist. Interestingly, the B site of the aromatic box is now a Tyr rather than a Trp. It is well established that all the aromatic amino acids—Phe, Tyr, and Trp—can participate in a cation−π interaction, so this was not a concern. However, a fluorination study at TyrB of MOD-1 revealed no cation−π interaction.[52] Another subtle change is at the C2 site, which is Tyr in 5-HT_3 but Trp in MOD-1. When we fluorinate TrpC2 of MOD-1, an excellent correlation is seen, and the slope is the same as seen at TrpB of 5-HT_3. On going from 5-HT_3 to MOD-1, the cation−π site has moved completely across the box, a distance of ∼9 Å.

5.3. GABA Receptors

The $GABA_A$ receptor is the famous benzodiazepine receptor, and it remains a target of ongoing pharmaceutical efforts. The $GABA_C$ receptor—also known as the ρ receptor—is a close homologue found primarily in the retina.[53] The aromatic box of GABA receptors has no tryptophans, being comprised entirely of tyrosines and phenylalanines. As part of an ongoing collaboration with Dr. Sarah Lummis of Cambridge University, we have studied both the $GABA_A$ and $GABA_C$ receptors. In the $GABA_C$ receptor, TyrB shows a clear cation−π interaction.[54] However, in the $GABA_A$ receptor, the cation−π interaction has moved to TyrA.[55] The slopes of the two plots are very similar and, again, significantly steeper than those seen for the nAChR, as expected for a RNH_3^+ agonist.

5.4. Overview of Binding Site Studies

We have probed seven different Cys-loop receptors, and in each one we find a strong cation−π interaction between agonist and receptor. However, the location of the cation−π interaction is variable. In four receptors (nAChR (muscle-type and α4β2), 5-HT_3, and $GABA_C$), it is at site B. In other receptors, the cation−π interaction can be at site A (nAChR (α7/ACh) and $GABA_A$) or at site C2 (MOD-1 and nAChR (α7/epibatidine)). Despite significant homology and a conservation of overall layout and function, when probed at a

chemical scale, there is considerable variation among the Cys-loop receptors.

This presents a serious challenge for homology modeling and drug discovery efforts. Most large pharmaceutical companies have drug discovery efforts targeting one or more members of the Cys-loop family of receptors. No doubt when the AChBP structure appeared, homology models of the extracellular domains of the real human receptors were built. And, it had already been established at that time that ACh made a cation−π interaction to TrpB in the muscle-type receptor, proving a potential anchor point for docking studies. It is now clear, however, that it is very risky to extrapolate from one receptor to another and even from one drug to another targeting the same receptor. It is clear that nature has conserved the overall layout and the functional properties of Cys-loop receptors. However, when probed at a chemical scale, there is considerable variability across the family.

6. Chemical-Scale Studies: The Gating Interface

The interaction of small molecule agonists and antagonists with the binding site of Cys-loop receptors may be the question of most direct relevance to drug discovery efforts. However, the most scientifically challenging and intellectually intriguing question is the nature of the gating mechanism. Unwin's image of the complete receptor from *Torpedo* electroplax (Figure 2)[20] inspired many groups to search for conserved features and structurally interesting elements that could define the gating mechanism for the family.

Figure 6 shows several structural landmarks that are relevant to the gating issue. The agonist binding site is located by residue B of the aromatic box, shown in space-filling (on the scale of this image, residues A and C2 are very close by). The location and nature of the "gate" are less certain. Certainly, the second transmembrane region, M2, is essential, and studies using a variety of approaches suggest that the major occlusion of the channel is more toward the intracellular half of this helix. One residue that seems to play an essential role in channel function is a completely conserved leucine in M2 that is generally referred to as Leu9', and it too is shown in space-filling in Figure 6. The channel is fairly narrow in the region of Leu9', and mutations at this site strongly impact channel function. Consider the mechanistic challenge presented by this system. TrpB lies a full 60 Å from Leu9′. How is it that a relatively weak (μM) binding interaction with a small molecule is able to throw a switch 60 Å away in a pentameric, molecular weight 300,000, integral membrane protein?

In the nAChR, there is a clear structural demarcation that coincides with the differing functional domains. The extracellular domain—containing the agonist binding site—is primarily comprised of β-sheets; the transmembrane domain—containing the ion channel and the channel gate—is comprised of α-helices. We and others reasoned that the interface between these two structural domains must play a pivotal role in communicating changes at the agonist binding site to the channel gate. This interfacial region contains a number of charged residues (Arg, Lys, Asp, Glu). Several workers used the *Torpedo* structure, or homology models based on it, to propose potential ion pairing interactions that might play a key role in the gating mechanism.[56–58] While convincing studies were performed on particular ion pairs in specific Cys-loop receptors, we were struck by the fact that none of these ion pairs were conserved across the collection of Cys-loop receptors.

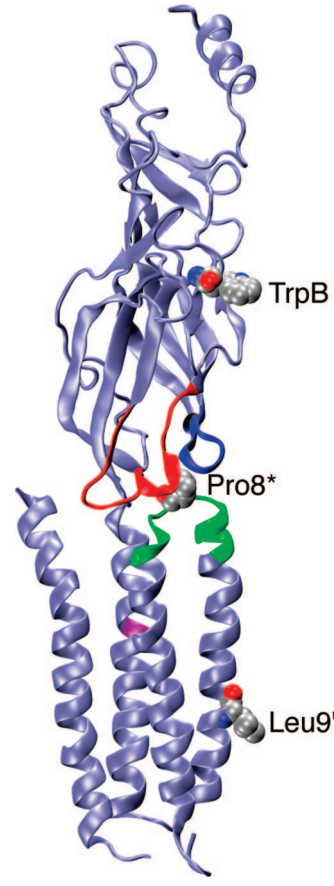

Figure 6. The α subunit of the *Torpedo* receptor as described in Figure 2. Key residues discussed in text are drawn as filling and labeled. Also highlighted are: the Cys-loop (red); the M2-M2 loop (green); loop 2 (blue); and a conserved MI proline (purple).

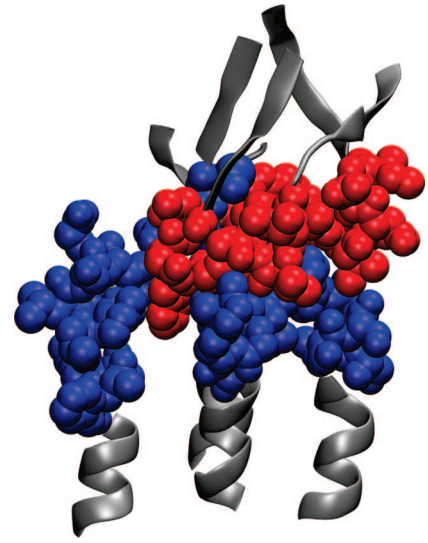

Figure 7. The gating interface of the nAChR α subunit (only one subunit is shown). Orientation is roughly as in Figure 6. Residues that contribute to the gating interface are shown in space-filling; red from the extracellular component, blue from the transmembrane component.

To probe this issue further, we defined a region of the receptor that we termed the gating interface (Figure 7).[59] Our selection criterion was geometric, considering residues that could reasonably be considered to contribute to communication between the extracellular domain and the transmembrane domain. Within the gating interface, residues can

come from either the extracellular component or the transmembrane component. We next analyzed the gating interfaces of 124 Cys-loop receptor subunits, and several clear trends emerged.

We noted above the large number of charged residues in the gating interface, and indeed, of the 47 residues present in a typical gating interface, ~11 are charged. These are divided evenly between the extracellular component and the transmembrane component. However, the extracellular component (red in Figure 7) always has a net negative charge (average: −3.9), while the transmembrane component (blue) always has a net positive charge (average: +2.3). There is a *global* electrostatic attraction that holds the gating interface together. There is considerable variability among different receptors as to the locations and exact numbers of charges, but this global charging pattern holds. Importantly, of the 11 charged residues in the gating interface, only 2 are universally conserved across the Cys-loop family. We conclude that the critical feature for proper receptor gating is the overall pattern of charges—extracellular component negative, transmembrane component positive. No particular pairwise interaction between residues is essential.

In support of this view, we performed extensive mutagenesis studies on the gating interface of the nAChR.[59] Over 100 mutants, involving both natural and unnatural amino acids, were probed, with special emphasis on charged residues. In a great number of instances, charge neutralization and even charge reversal (eg., Glu to Lys) had no substantive effect on receptor function. The implication is that the receptor can tolerate considerable variation in this region, as long as the global charging pattern is retained. As noted above, several workers have proposed that specific ion pair interactions in a particular Cys-loop receptor play a critical role in gating. Evaluating those data in the context of the gating interface model, we find evidence instead for clusters of charges that form a structural unit, rather than a precise ion pairing interaction. Within that cluster, charge variation is possible as long as it is not too extreme. For example, making all three residues of a charge triad cationic is not tolerated, but many combinations that give a net +1 or −1 charge are viable.

Our conclusion is that the detailed image of the gating interface provided by the *Torpedo* structure does not provide universal guidance for all Cys-loop receptors. Across the family, different kinds of interactions are involved, and variability is tolerated, as long as it does not deviate too far from the essential pattern.

In many ways this more diffuse model of electrostatic interactions at the gating interface makes sense. Most workers feel there is some movement in this region of the receptor associated with gating. In the gating process, the receptor rapidly oscillates between (at least) two states, closed and open, that cannot be too far apart in energy. If, for example, the closed state contained a strong, specific ion pair interaction, it would likely have to break on going to the open state. Perhaps a new ion pair forms in the open state, but what happens in the transition state? The mutagenesis data are not consistent with the notion that one specific ion pair is replaced by another, because so many charge neutralization/reversal mutations are well tolerated. On the other hand, a more diffuse, global electrostatic attraction—extracellular component negative, transmembrane component positive—would allow the extracellular and transmembrane domains to slide past one another without severe energetic penalty. Clearly, each receptor has evolved to tune the gating interface to achieve the desired kinetics and thermodynamics of gating. The essential mechanism has been preserved, but at a chemical scale, there is great variation across the Cys-loop family.

7. Chemical-Scale Studies: The Gating Switch

Our analysis of the gating interface provides some idea of the overall strategy for a receptor design that can create a linkage between an extracellular domain and a transmembrane domain, each of which has distinctly different secondary structures. However, a detailed gating mechanism does not emerge from such an analysis. As noted above, this is an extraordinary challenge, and it seems likely that only application of a wide array of mechanistic probes will produce a detailed view of the mechanism. By definition, this is a dynamic process that might be especially difficult to unravel with static structural images.

Many groups have made important contributions to the gating analysis.[60] Mutagenesis studies throughout the transmembrane region, especially at Leu9′, have identified important residues.[61–65] Fluorescence labeling strategies, especially FRET-based studies, seem ideally suited to the problem. Their application to Cys-loop receptors is challenging, but some progress has been made.[66,67] Computer modeling approaches have suggested possible conformational changes associated with the gating mechanism.[68–72] However, full simulations of a receptor (and associated membrane plus water) are generally limited to time scales on the order of tens of nanoseconds, while gating rates approach the millisecond time scale. Still, as computing power increases, it is exciting to contemplate the time when the single molecule observations made by the patch clamp and the MD simulations reach the same time scale.

An especially interesting approach to unraveling the gating mechanism of the nAChR has been developed by Auerbach.[73–76] It is based on one of the most time-honored physical organic chemistry tools, the linear free energy relationship (LFER). Briefly, several (conventional) mutants at a given site of the receptor are subjected to detailed kinetic analysis using the patch clamp. From these studies, a rate-equilibrium free energy relationship (REFER) can be developed, comparing the degree to which a mutation perturbs the channel opening rate constant (termed β) to the perturbation of the open-closed channel equilibrium (termed Θ). If a plot of β vs Θ for a series of mutants at a given site is linear, the slope (Φ) is akin to a Brønsted α or β value; it measures whether the transition state is "late" or "early". More precisely in the present case, it measures whether movement of the probed residue occurs early or late in the gating process. The detailed analysis is complex, and it involves some ideas about the mechanism of channel gating that are a bit different from how chemists usually think about mechanisms of small molecule reactions.

From such studies, a fascinating mechanistic model emerges. Consider the image of one nAChR subunit shown in Figure 6. Beginning at the agonist binding site (near TrpB) and progressing all the way down to Leu9′, Φ values have been determined for a large number of residues. Remarkably, a clear pattern emerges. Residues near the agonist binding site move first (large Φ values), followed by residues in the region we have called the gating interface, followed by a further propagation down the transmembrane helices. The

image of a "conformational wave" emerges, in which a conformational change induced by agonist binding propagates down the receptor to finally reach the "gate" in the vicinity of Leu9′.

Using an alternative strategy, we attempted to find specific conformational changes on the scale of individual amino acids that might play an important role in gating. Within the gating interface, an especially intriguing feature is the connector between transmembrane helices M2 and M3, the M2−M3 loop (Figure 6). It had long been appreciated that this loop within the transmembrane domain must point up toward the extracellular domain, and with M2 lining the channel and containing Leu9′, a role in the gating process seemed likely. In the M2−M3 loops of the nAChR and the 5-HT_3 receptor, there are two conserved proline residues, including one—termed Pro8*—that lies right at the apex of the loop in the Unwin structure (Figure 6). In this position, Pro8* contacts the Cys-loop and also loop 2 of the extracellular domain, both of which have been proposed to play important roles in gating.

Prolines are actually fairly common in transmembrane regions, where they are often proposed to provide a kink in α-helical segments. For example, in the Cys-loop receptors, there is another proline that is completely conserved and lies in the middle of M1 (designated by purple in the M1 ribbon of Figure 6). Replacement with any other conventional amino acid leads to a nonfunctional receptor. We reasoned that if the role of the M1 proline is to disrupt the M1 helix by deleting the backbone NH, perhaps a backbone ester could function at that position. We probed the M1 proline in both the nAChR and the 5-HT_3 receptor, and we found that α-hydroxy acids function well at the site, regardless of side chain.[77,78] That is, α-hydroxy analogues of alanine, valine, or leucine all produce essentially wild type behavior. We concluded that this highly conserved proline is required because it disrupts the α-helix of M1. Interestingly, though, there is no obvious kink in M1 in the images of the *Torpedo* nAChR (Figure 6). Perhaps the kink is important in a state of the receptor other than that imaged by Unwin.

Returning to Pro8* of the M2−M3 loop, we studied this intriguing residue in the homopentameric 5-HT_3 receptor.[79] Incorporating an α-hydroxy residue at Pro8* gave a nonfunctional receptor, so backbone hydrogen bonding is not the issue here. Of course, the other unique feature of proline is that it tolerates a *cis* peptide bond more so than any other natural amino acid. Note that esters prefer a *trans* conformation even more than amides, so an α-hydroxy analogue cannot probe this issue. We replaced Pro8* with a number of proline analogues, including the four shown in Figure 8. In studies of model peptides, this series shows a progressively increasing *cis* bias, starting at 5% for proline and going all the way to 71% *cis* for 5,5-dimethylproline. When this series is incorporated into the 5-HT_3 receptor, it is clear that as the innate *cis* bias of the proline analogue increases, it becomes much easier to open the channel, as evidenced by the progressive drop in EC_{50} for activation by serotonin (Figure 8). Control experiments established that these proline analogues influence the open−closed gating equilibrium of the receptor, not the affinity of the agonist for the binding site (recall EC_{50} is a composite of these two effects). This is as expected for a mutation that is in the gating interface and is quite remote from the agonist binding site.

For proline and the four analogues, we now have data concerning their impacts on two equilibria: the *cis*−*trans* equilibrium at the proline in a model peptide, and the open−closed equilibrium for the 5-HT_3 receptor. Referencing everything to proline, we can plot $\Delta\Delta G$ (*cis*−*trans*) vs $\Delta\Delta G$ (open−closed), as shown in Figure 8. The clear linear correlation, with a slope of 1, provides a compelling link between *cis*−*trans* isomerization at Pro8* and gating of the receptor. Note that residues that show a stronger *trans* preference than that of proline, such as methanoproline and the α-hydroxy analogue of valine, gave nonfunctional receptors. Considering a homology model of the 5-HT_3 receptor based on the *Torpedo* nAChR, a detailed model for coupling agonist binding to channel gating could be developed, with *cis*−*trans* isomerization at Pro8* playing a key role.

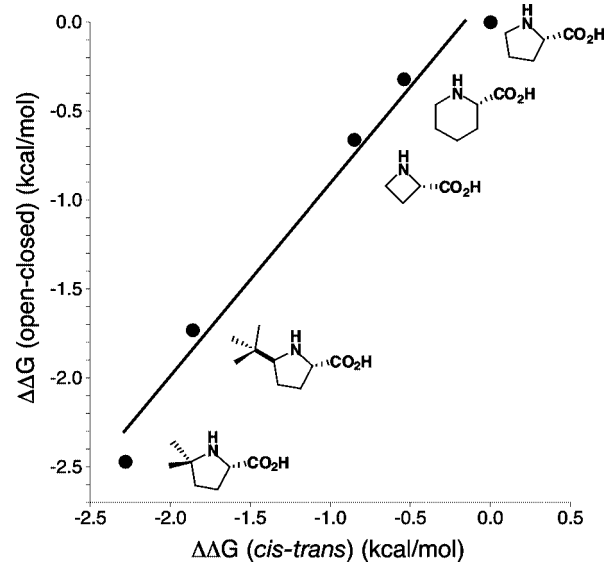

Figure 8. Probing Pro8* in the 5-HT_3 receptor.

There is no doubt that Unwin's structure of the *Torpedo* nAChR was essential in suggesting Pro8* might play a role in gating. Unnatural amino acid mutagenesis then provided key support in the case of the 5-HT_3 receptor. What about other Cys-loop receptors? Pro8* is present in the nAChR, but even before doing any experiments, we can anticipate a possible problem. That potential problem is the kinetics of channel opening. Considering first the 5-HT_3 receptor, the channel opening rate constant has been estimated to be in the 10−100 s^{-1} range. Intrinsic proline *cis*−*trans* isomerization rates are slower by a factor of ∼10, but it is not difficult to imagine the receptor accelerating *cis*−*trans* isomerization. Simply providing a hydrophobic environment can significantly accelerate isomerization, and a single hydrogen bond can provide a ∼300-fold rate increase.[80,81] So, it is not difficult to imagine proline *cis*−*trans* isomerization occurring on a time scale compatible with gating. However, the 5-HT_3 receptor is one of the slowest of the Cys-loop receptors. At the other end of the spectrum is the nAChR of the neuromuscular junction, with channel opening rate constants estimated to be on the order of 50,000 s^{-1}. While there is some evidence that Pro8* plays a role in channel gating of the nAChR,[58] we consider it unlikely that *cis*−*trans* isomerization is involved. Indeed, we find that, in the nAChR, substituting Pro8* with conventional amino acids gives functional receptors, an effect not seen in the 5-HT_3 receptor.

In addition, the two inhibitory Cys-loop receptors (GABA and glycine receptors) do not even have an analogue to

Pro8*; clearly a comparable proline *cis*−*trans* isomerization is not operative in these systems. We feel the correlation of Figure 8 provides compelling evidence for a key role for *cis*−*trans* isomerization of Pro8* in the 5-HT_3 receptor. But, once again, we see a mechanism that is convincingly demonstrated for one member of the Cys-loop family that does not carry over to other members.

8. Structure to the Rescue?

The Cys-loop neuroreceptors form a large, ubiquitous family of signaling molecules that play diverse roles throughout the central and peripheral nervous systems and provide a multitude of pharmaceutical targets. More generally, though, we feel the present state of molecular research on Cys-loop receptors mirrors that for many other receptors and channels. Consider the G protein-coupled receptors (GPCRs), the famous 7-helix receptors that, as a class, form the largest target for marketed pharmaceuticals. For some time, the field has been aided by the structure of bovine rhodopsin, a GPCR that responds to light by isomerizing a retinal molecule that is covalently linked to the receptor.[82,83] This structure has certainly been inspirational, but it is not a GPCR that is a target of the pharmaceutical industry. Very recently, structures of the human β2 adrenoreceptor have appeared.[84–86] This is certainly a landmark in GPCR research. However, it remains to be seen to what extent this structure will allow higher precision insights into the hundreds of other GPCRs that are important targets.

Our studies of Cys-loop receptors began well before detailed structural models appeared, but there is no doubt that they have been greatly aided by the AChBP and *Torpedo* structures. In addition, the structural results have enabled the development of homology models of many Cys-loop receptors. Certainly, these models are very useful. The issue is the level of precision with which one can interpret them.

We would argue that, for those of us who are interested in obtaining chemical-scale insights into the mechanisms of action of receptors and channels, the present structures provide guidance and inspiration for new experiments more than they provide answers. Time and again, we have seen that a key interaction for one Cys-loop receptor does necessarily carry over to another, even though there is high residue conservation. This is true even for receptors that respond to the same ligand. For ACh, serotonin, and GABA, we have shown that different members of the receptor family use different binding interactions to bind the same agonists. The same holds for the mechanism of gating. In one receptor, the 5-HT_3 serotonin receptor, we have compelling evidence that *cis*−*trans* isomerization of a pivotal proline residue plays a key role in gating. However, in another Cys-loop receptor we have evidence that proline is not essential, and in other Cys-loop receptors the proline is not even present. The same is true of ion pairing interactions at the gating interface, which also are not conserved across the family. With the Cys-loop receptors, as with many other aspects of biology, nature has evolved a fundamental scaffold, in this case a pentameric structure with a certain overall layout. Within that framework, however, many functional strategies are viable, and it will be very challenging to predict what is retained and what varies from system to system.

Chemistry is the best tool for revealing the variations in receptor function. We have emphasized one chemical-scale tool here, unnatural amino acid mutagenesis. As described in other articles in this issue, there are other powerful ways to apply chemical tools to the complex proteins of neuroscience. While we all look forward to the next exciting structural insight for a neuroreceptor or ion channel, chemistry will always have a central role in unraveling the mechanisms of the nervous system.

9. Acknowledgments

I thank the extraordinarily talented graduate students and postdocs whose intelligence, hard work, and creativity produced the results described here. Much of this work results from ongoing and enjoyable collaborations with Henry Lester and Sarah Lummis, to whom I am grateful. Our work has been supported by the National Institutes of Health (NS-34407) and, more recently, the California Tobacco-Related Disease Research Program of the University of California, Grant Number 16RT-0160.

10. Note Added in Proof

Recently, a full crystal structure at 3.3 Å resolution of a bacterial channel termed ELIC has been reported. The channel is pentameric and clearly related to the Cys-loop receptors, although there is no Cys-loop and, at present, the natural ligand is not known. Hilf, R. J. C.; Dutzler, R. *Nature*, **2008**, *452*, 375–379.

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