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Cys-loop receptors: new twists and turns

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New hypotheses and predictions have arisen from recent work revealing atomic-scale or near-atomic-scale structures of receptors in the 'Cys-loop' superfamily. How general is the cation–π interaction between the natural ligand and a tryptophan residue in the aromatic box, and does this interaction extend to other ligands? What is the pathway from the binding site to gating, and what are the conformational changes during gating and desensitization? Is current flow through intracellular 'portals' in the wall of the channel a general feature? This article discusses these and related questions, emphasizing nicotinic ACh receptors and also discussing data from other members of this superfamily.

Fast synapses are exquisitely specialized: they function on a timescale of milliseconds and at sub-micrometer distances. The series of nanomachines in the presynaptic and postsynaptic cell work so efficiently, individually and sequentially, that it took until the mid-1950s to establish that most synapses are not simply electrical junctions but do, in fact, involve a chemical intermediate.

Neurotransmitter receptors are ligand-gated channels, and they play starring roles in fast synaptic transmission. Within a few microseconds after the transmitter is released within the synaptic cleft and binds to an extracellular region of the receptor, the channel opens to excite or inhibit the train of postsynaptic action potentials (or in some cases, to influence the presynaptic cell). Just as importantly, the channel closes within a few milliseconds as the transmitter dissociates to terminate the synaptic event. Many receptors are in the Cys-loop superfamily, which contains some venerable receptors such as the nicotinic ACh receptors, as well as some newly discovered members, such as the zinc-activated channels and the invertebrate GABA-gated cation channel, EXP-1 (Table 1). Table 2 summarizes several diseases that occur because Cys-loop receptor channels open too slowly, or close too quickly or too slowly, along with the therapeutic drugs that now exist or are sought to modify these gating events and the drugs of abuse that act on Cys-loop receptors.

To fulfill their proper roles at synapses, Cys-loop proteins must be assembled and targeted appropriately. Much is known about the general processes that target the muscle receptor to a nerve–muscle synapse, but almost nothing is known about specific interactions between neuronal nicotinic ACh receptors and other proteins. However, there are excellent indications that the function of Cys-loop proteins is not static, but can be modulated by interactions with other proteins (Box 1).

Since 2001, structural studies have described members of the Cys-loop superfamily in general and the nicotinic ACh receptors in particular. For example:

(i) The crystallographic structure of the ACh-binding protein (AChBP) has been reported [1] (Figure 1c,e).

(ii) New research has been published on the interaction between a cationic group on the neurotransmitter and an aromatic side chain within the binding site [2–4]. This cation–π interaction specifies the distance between two groups within 0.5 Å of one another and therefore

Table 1. Currently known members of the Cys-loop superfamily[a]

Ligand	Receptor	Permeant ions	Number of paralogous subunits known
Known in vertebrates			
Ach	Nicotinic ACh receptor	Cations	≥ 16
5-HT	5-HT_3 receptor	Cations	≥ 2
GABA	$GABA_A$ receptor	Anions	≥ 19
Glycine	Glycine receptor	Anions	≥ 5
Zn^{2+}	ZAC	Cations	1
Known only in invertebrates			
Glutamate	GluCl	Anions	≥ 2
5-HT	MOD-1	Anions	1
GABA	EXP-1 [82]	Cations	1

[a]Abbreviations: EXP-1, an excitatory GABA-gated cation channel; GluCl, glutamate-gated Cl^- channel; MOD-1, modulation of locomotor defective; ZAC, zinc-activated ion channel.

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Table 2. Medical aspects of Cys-loop receptor defects[a]

Receptor	Diseases associated with receptor defects	Aims of drugs affecting receptor	Toxin	Abuse
Nicotinic ACh	ADNFLE, slow-channel myasthenic syndrome [55]	Treatment of schizophrenia [83], Alzheimer's disease [84], pain [85], Parkinson's disease [86], ADHD [87,88], Crohn's disease [89], ADNFLE [90] Cognitive enhancement Smoking cessation [91] Insecticidal effects	Conotoxins, snake toxins, lophotoxin epibatidine	Nicotine addiction
5-HT_3	NR	Treatment of emesis, irritable bowel syndrome	NR	NR
$GABA_A$	Epilepsy [92]	Treatment of anxiety, epilepsy, insomnia Anesthesia [93]	Convulsant toxins	Addiction
Glycine	Startle disease [43,44]	NR	Strychnine	NR
GluCl	NR	Anthelmintic and antiparasitic effects	NR	NR

[a]Abbreviations: ADHD, attention deficit–hyperactivity disorder; ADNFLE: Autosomal dominant nocturnal frontal-lobe epilepsy; GluCl, glutamate-gated Cl^- channel; NR, not reported.

does fall within the class of high-resolution structural studies.

(iii) The 4-Å-resolution structure of the nicotinic ACh receptor transmembrane domains from *Torpedo* electric organ has also been published [5] (Figure 1e).

All of these 'snapshots' of the nicotinic ACh receptor provide valuable information, but each has raised further questions. Globally, we are very far from translating these static images to a coherent view of the protein dynamics associated with the gating process.

The nature of the 'extended' binding event

Once one has the crystalline structure of a binding domain from a ligand-gated channel, one hopes to rationalize previous data based on biochemistry, site-directed mutagenesis, electrophysiology and cysteine-scanning mutagenesis (SCAM) [6–10]. Indeed, the AChBP structure [1] does correlate well with 40 years' worth of such data. The AChBP structure reproduces previous conclusions that an agonist binds at inter-subunit interfaces [11], near residues from four to six polypeptide 'binding segments', designated A–F (this terminology should replace the former 'loopA' to 'loopF' terminology [12], because some of the key binding residues are in β-strands rather than in the loops between them). In addition, the cation–π binding site identified by unnatural amino acid mutagenesis has been fully confirmed. AChBP continues to serve as the foundation for information about binding site structure [4,13–18]. Lysine-scanning mutagenesis even confirms the residues responsible for intersubunit contacts, which are among the most variable in the N-terminal domain [13].

At most Cys-loop receptors, the dose–response relationship for agonist activation has a Hill coefficient approaching 2, indicating that the open state of the channel is much more likely to be associated with two bound agonist molecules than with only one. Both agonist molecules remain bound to the receptor for the entire duration of the channel opening [19,20]. The AChBP molecule is a fivefold symmetrical homopentamer, and some subunits are occupied by a ligand. In the first reported AChBP structure, the ligand is not an agonist or antagonist, but a molecule of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) that is present in the crystallization buffer used. It is uncertain whether the AChBP structure corresponds to the binding site in the resting, active or desensitized state. Some have argued, on the basis of the relatively high ligand affinity of AChBP and computational modeling, that the structure is in the desensitized state [9]. Two 9-Å-resolution electron microscopic images of the nicotinic ACh receptor were described in 1995, without ACh bound and ~5 ms after ACh was applied [21]. The AChBP best matches the structure of the α-subunit when agonist is bound, and this observation gave rise to the hypothesis that agonist binding to the α-subunit induces the inner β-sheet, consisting of β-strands 1, 2, 3, 5, 6 and 8, to undergo a rotation of ~15° [22] (Figure 1d,e).

Cysteine accessibility data on the $GABA_A$ receptor suggest that a major function of the agonist–receptor contacts is to induce a contraction of the 'aromatic box' (Figure 1c), a feature of the agonist-binding site defined by five conserved aromatic side chains [22,23]. A cation–π interaction was found between an amine-containing agonist and precisely one residue (Trp) in each of three receptors examined (the ACh receptor, the 5-HT_3 receptor

Box 1. Functional modulation of Cys-loop receptors by other proteins

Nicotinic ACh receptors and members of the ATP-binding P2X receptor family display cross-inhibition in neurons [69–73] and heterologous expression systems [73–75]. The interaction depends on the history of activation, on a time scale of several seconds. P2X receptors interact with other members of the Cys-loop superfamily [76], emphasizing the generality and importance of such interactions. There is some evidence that the interaction is direct, mediated by the M3–M4 loop of nicotinic ACh receptors and the C-terminal domain of P2X [76].

Several small proteins are members of the Ly-6/ urokinase-type plasminogen activator receptor (Ly-6/uPAR) superfamily, including secreted mammalian Ly-6/uPAR-related protein 1 (SLURP-1) and lynx1 [77–79]. These receptors and secreted proteins contain a C-terminal consensus sequence motif CCXXXXCN, one or more repeats of a distinct disulfide bonding pattern of 8–10 cysteine residues, and a glycosylphosphatidylinositol (GPI) membrane anchoring linkage. Similar to the homologous three-fingered members of the elapid neurotoxin family, the Ly-6/uPAR superfamily members interact functionally with nicotinic ACh receptors subunits, especially α7 and α4β2. Recent data suggest that lynx1 binds directly to α4β2 [78]. However, the general importance of these interactions is not yet appreciated [80].

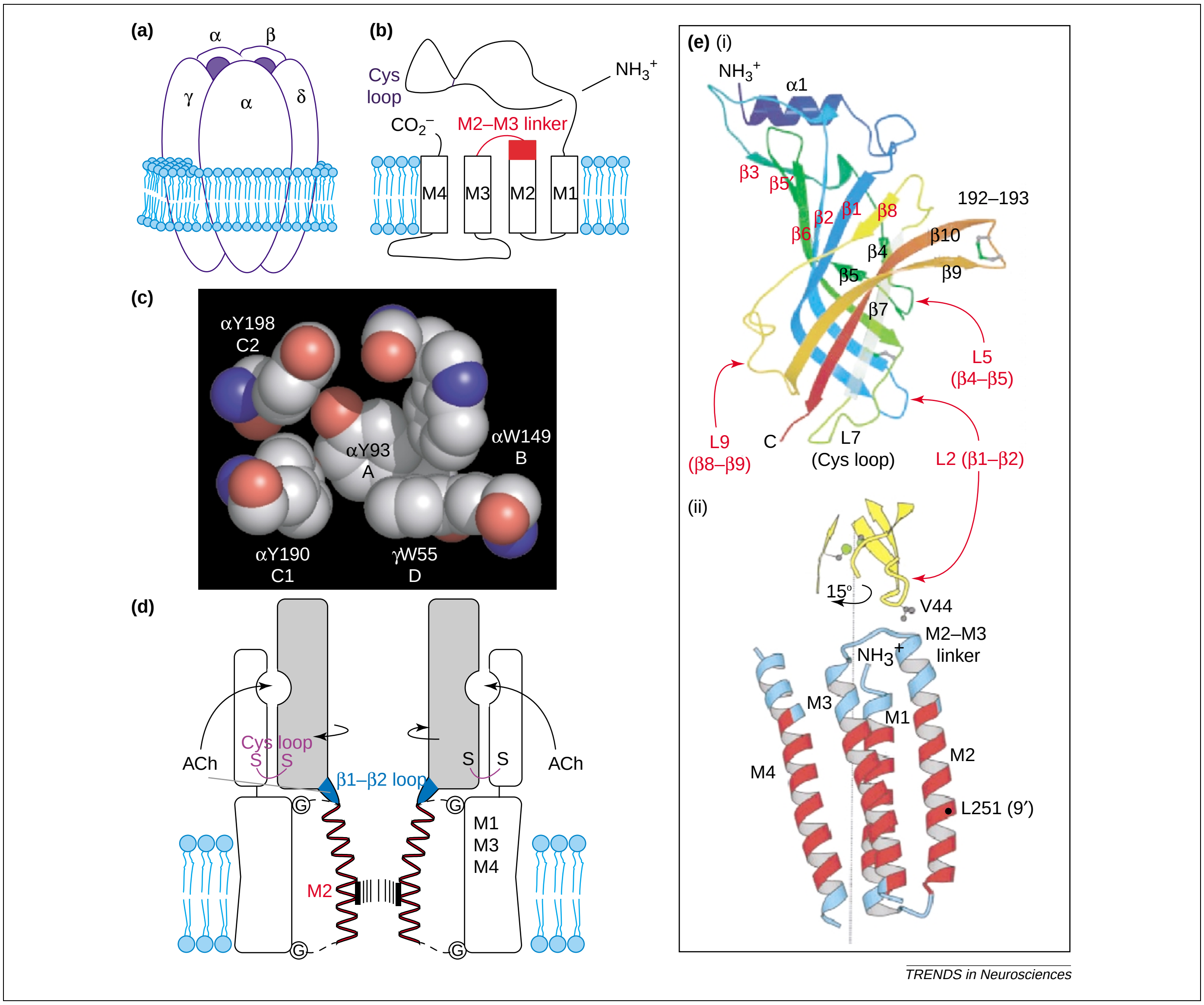

Figure 1. Images of the nicotinic ACh receptor. **(a)** Overall layout of the five subunits of the muscle receptor. **(b)** The topology of each subunit. Note the M2–M3 linker (red), consisting of the top two turns of the M2 helix plus the M2–M3 loop. **(c)** The 'aromatic box' that forms the agonist-binding site of the ACh-binding protein (AChBP). Residues are numbered for the muscle receptor α and γ subunits, and are also labeled by binding segment (in the terminology introduced by Changeux's laboratory, these were called 'loopA' to 'loopD'). As usual, oxygen and nitrogen atoms are colored red and blue, respectively. **(d)** Unwin's 2003 model of structure and gating, showing only the two α subunits [5]. Binding of agonist (e.g. ACh) causes a rotation of the shaded region around an axis that passes through the Cys loop (L7 loop) disulfide. The β1-β2 loop (L2 loop) contacts the M2–M3 linker (the nonhelical region is shown as a series of vertical lines), and the rotation torques the M2 region, opening the channel [5]. The M2 helix contains the gate of the channel. **(e)** AChBP. (i) One molecule of the pentameric structure is shown. All β-sheets are labeled, along with the L5 loop [81], L7 Cys loop, and the β1-β2 L2 loop. L2 is proposed to play a key role in gating [5]. The red labels indicate the β-strands comprising a β-sheet that might rotate as a result of agonist binding [5]. Unwin's 2003 model of gating in more detail (ii), showing only one α subunit. The postulated axis of rotation is shown (central vertical line). The highly conserved Leu9′ residue thought to lie at the gate in the M2 helix is emphasized by vertical shading on the channel axis in (d) and named (e)(ii). In (e), the C terminus of the structure in (i) actually lies near the N terminus of the M1 helix in (ii). Panels (d) and (e)(ii) are reproduced, with permission, from Ref. [5]; panel (e)(i) is reproduced, with permission, from Ref. [1] © (2001) Nature Publishing Group (http://www.nature.com/).

and the invertebrate 5-HT-gated Cl^- channel, MOD-1) [3,4,24]. In these experiments, unnatural Trp analogues were incorporated at several Trp sites, and changes in $\log(EC_{50})$ showed a remarkable correlation with established cation–π binding energies of the modified side chains.

The recently described MOD-1 receptor was of particular interest because a tyrosine residue aligns with the cation–π-binding Trpα149 and Trp183 of the nicotinic ACh receptor and the 5-HT_3 receptor, respectively, affording the opportunity to test the hypothesis that a cation–π interaction also occurs at tyrosine residues. Results were surprising: there is no cation–π interaction at tyrosine; but instead a cation–π interaction occurs at a tryptophan residue some 7.5 Å across the box at position C2 (Figure 1c) [4]. Thus, the cation–π interaction occupies different faces of the aromatic box in two homologous 5-HT receptors [4], suggesting that the role of the agonist is to serve less as a 'key in a lock' and more like a 'wedge'. As another indication that the aromatic box is a general 'capture' area, activation becomes constitutive if appropriate groups are tethered at any of several positions in the box [2,24–28].

The aromatic box might provide a 'special' environment. Primary, secondary and tertiary amines were incorporated to generate a pH-activated receptor when the tethered amine is protonated. The pK_a of the tertiary tethered agonist in the binding site appears to be 6 or lower, differing substantially from its pK_a in solution (~9.3) [29].

How does nicotine bind to the nicotinic ACh receptor?

Other moieties on the agonist contribute to binding at other functional groups in and near the aromatic box; were this not so, tetramethylammonium would act precisely like ACh. Furthermore, the cation–π interaction might not be a dominant feature of all agonist binding events at nicotinic ACh receptors. For example, nicotine itself, the prototypical agonist and the addictive compound in tobacco, does not produce a strong cation–π interaction at the muscle nicotinic ACh receptor [3]. Whether nicotine and related compounds make important cation–π interactions at neuronal receptors such as α4β2, where nicotine has one fiftieth the EC_{50} value of that at muscle receptors, is unknown and deserves further study. In addition, nicotine does not experience the same perturbation of phenomenological pK_a in the aromatic box as do the tethered amine agonists [29]. Thus, it might be possible to describe two types of interactions at the 'aromatic box' as ACh-like or nicotine-like, with 5-HT falling in the ACh-like category.

The first reported AChBP structure had no information about actual binding contacts for real agonists at the aromatic box; but recently published data are more informative [30]. Several homology models of the nicotinic ACh receptor and 5-HT_3 receptor with ligands bound have been built [9,13,31,32]. Some suggest that there is a hydrogen bond between an agonist such as nicotine and the backbone carbonyl of Trpα149, the cation–π residue. This hypothesis is being tested with experiments that place an ester link [33,34] in the backbone at the 149–150 linkage.

Now we need to understand how the known interactions propagate away from the binding site. Such basic mechanistic information about the link from binding to gating might help in understanding the pathophysiology and therapy for the diseases listed in Table 2.

Structure of the transmembrane domains in the closed state

Nearly all researchers believe that the latest electronic microscopic structure for closed-state transmembrane domains [5] is an excellent model for all members of the Cys-loop superfamily, because it correlates so well with 15 years' worth of other data. This structure confirmed the prescient observation that the transmembrane domain M2 continues on for two additional turns above the membrane [35] and the concept, especially familiar in the $GABA_A$- and glycine-receptor families, that there is a water-filled space between the M2 and M3 helices [36].

The link between binding domain and channel domain: the M2–M3 linker region?

In their report on the electron microscopic structure of the closed-state transmembrane domains, Unwin and colleagues speculated about the structural reorganizations in gating [22] (Figure 1d,e). In the suggested chain of events, binding of agonist causes a rotation of part of the extracellular binding region (the shaded region in Figure 1d) around an axis that passes through the Cys loop (L7 loop) disulfide. This produces contact between the β1-β2 loop (L2 loop) (Figure 1e) and the M2–M3 linker, which projects above the membrane. The movement of the M2–M3 linker torques the M2 region, opening the channel [5]. These theories for the conformational transitions that produce the open state and for the structure of the open state (Figure 1d,e) are as yet unproven, although some data are consistent with the rotation mechanism. For example, a recent study shows that a fluorescent group attached near the top of the M2 helix does move into a more hydrophobic environment when the channel opens (Figure 2). This is consistent with the theory that a rotation of the M2 region, in the direction indicated, opens the channel [5], because this rotation would place the side chain at position 19′ in contact with several hydrophobic side chains (labeled in Figure 2). The data are also consistent with earlier ideas that a water-filled M2–M3 pocket changes shape during Cys-loop activation [36]. The proline residues in the M2–M3 linker at position 23′ (also

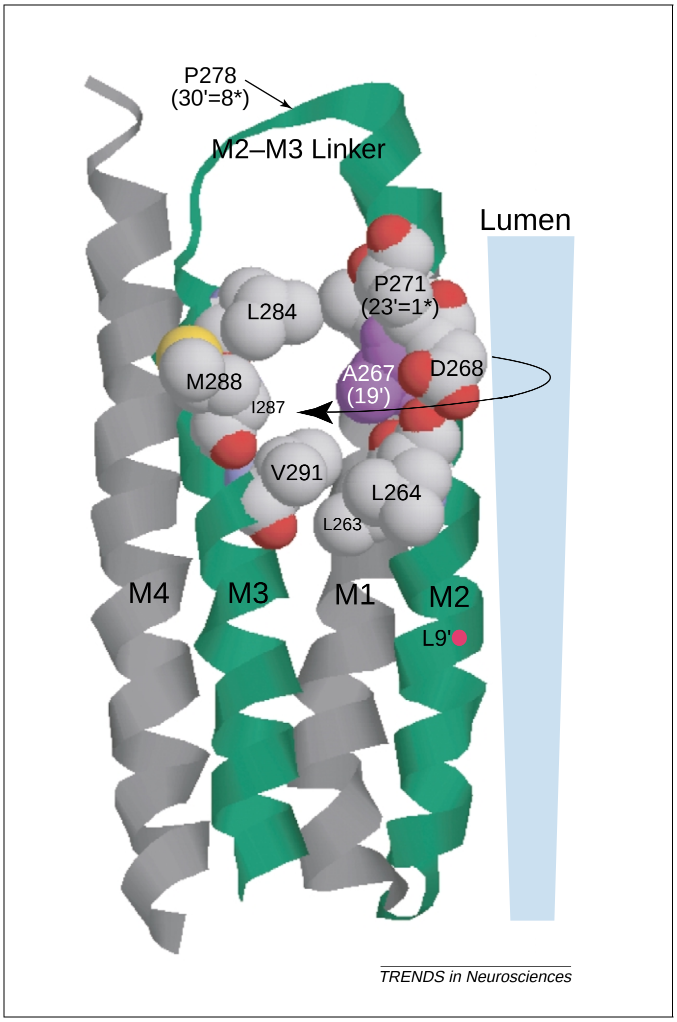

Figure 2. Structure of the β-subunit of the nicotinic ACh receptor, showing the postulated rotation of the M2 helix (Figure 1d) during activation of the channel [5]. A fluorophore (tetramethylrhodamine) was attached at the position corresponding to Ala267 (19′; purple). During agonist application, there was a fluorescence increase and a shift in the emission peak to lower wavelengths, indicating that the fluorophore enters a more hydrophobic environment [64]. This changed environment could be caused by the postulated rotation. For clarity, the M1 and M4 helices are shown in gray, and the M2 and M3 helices are shown in green

known as position 1*) are absolutely conserved, and those at position 30′ (also known as position 8*) are present in the nicotinic ACh receptor and the 5-HT_3 receptor (Figure 3).

Nevertheless, the search for conformational changes during gating has been controversial and frustrating [37–39], for at least two reasons.

(i) There is no high-resolution structure of a Cys-loop receptor in both closed and open states. The highest-resolution open-state data at present are limited to 9 Å [21].

(ii) There are many other informative techniques to study changes in protein conformation during gating, but no global structural change is implicated by these observations. Thus, single channels provide open–closed information. Fluorescence resonance energy transfer (FRET) and lanthanide-based resonance energy transfer (LRET) provide distance, and FRET sometimes provides information about rotations of chromophores. Selective accessibility to covalent modification, such as SCAM [40], disulfide trapping [41] and electrophysiology-coordinated mass spectrometry [42], indicates which side chains are accessible in functionally defined states. Conventional mutagenesis, and unnatural amino acid mutagenesis, allow one to correlate functional measurements with the chemistry of side chains. Backbone mutagenesis can probe interactions with, and conformational changes of, the backbone.

The Unwin model (Figure 1d,e) emphasizes the role of the M2–M3 linker region (comprising the top of M2 and the M2–M3 loop; Figure 3) as a mechanical ‘receiver’ for binding site conformational changes initiated by productive occupation of the agonist- binding site. Presumably the forces received by this linker region would then pull, push or torque the gate-containing M2 helix. Functional changes have been observed as a result of M2–M3 linker region mutations in glycine receptors [43–45], nicotinic α1, α3, α7 and β4 receptors [46–48], 5-HT_3 receptors [49] and $GABA_A$ receptors [14,35,50–53]. Some of these involve surface accessibility during agonist exposure [54]. One elegant study combined charge reversal, charge exchange and cysteine crosslinking approaches [14]. Other observations concern the effects of naturally occurring pathological mutations in the M2–M3 linker region of the glycine receptor α1 subunit [43,44] and the nicotinic ACh receptor α1 subunit [55].

There is some indication that hydrophobic residues at the 5* position bias the conformational equilibrium towards open channels; side-chain volume is not important [56] (Figure 3). However, in the words of Grosman *et al.* [56], ‘It is remarkable that similar modifications to adjacent residues in the M2–M3 loop have such diverse effects on gating. As opposed to the effect of Ser → Ile and Ser → Ala mutations at position 5*, similar substitutions at positions 3* (Thr → Ile and Thr → Ala) or 4* (Ser → Ile) impair gating…making the interpretation of the structure–function results less straightforward’. Therefore, one can raise the straightforward hypothesis [35] that the M2–M3 linker undergoes changes in backbone structure during gating; such changes would be probed only indirectly by conventional side-chain mutations, but directly by backbone mutations. There are already examples of insights from backbone mutagenesis: (i) a systematic series of amide-to-ester mutations suggests that the upper part of M2 (residues 13′ to 19′) undergoes changes in main chain structure as the channel opens [33]; and (ii) a highly conserved proline residue at position 221 in M1 of the nicotinic ACh receptor β subunit, or in the aligning position in α7 or 5-HT_{3A} receptors, cannot be replaced by another side chain, although receptors function if esters, or the proline analogs pipecolic acid or 3-methylproline, are incorporated [33,34,49,57].

Assuming (for the moment) that the M2–M3 linker region is the mechanical ‘receiver’ for the binding event, which part of the binding region is the mechanical ‘actuator’ that engages the M2–M3 linker region? The constancy of the signature Cys loop L7 (Figure 1e) has led many workers to believe that it is the actuator. Considerable evidence has accumulated over the past 15 years, implicating L7 in gating [14,42,58]. However, Unwin postulates that L2 is the actuator [5]. These two loops are close to each other in the AChBP (Figure 1e); therefore,

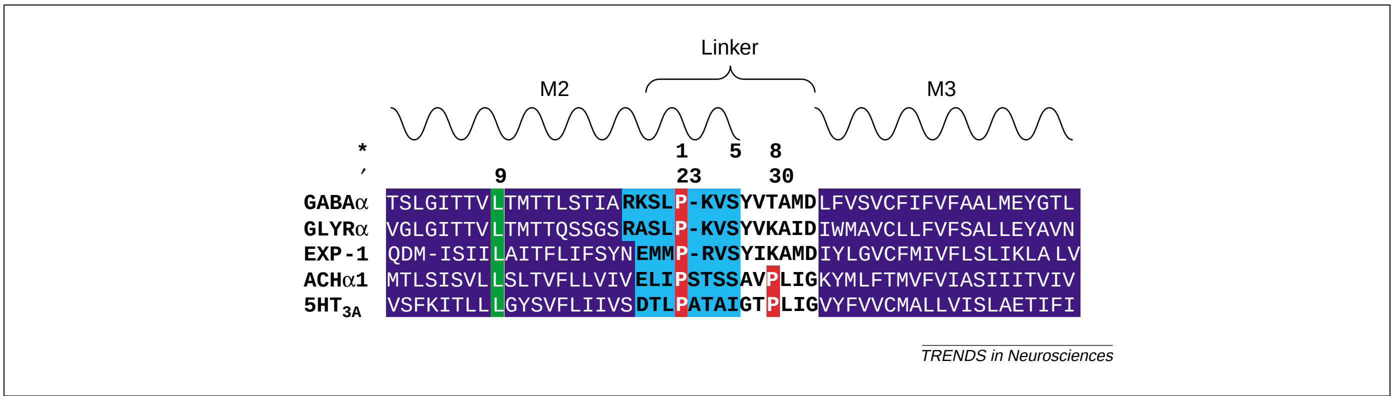

Figure 3. The M2, M2–M3 linker and M3 regions of several Cys-loop superfamily members. Both the common ‘primed’ numbering system and the newly introduced asterisk numbering system are shown; the latter is anchored at the conserved proline residue in the M2–M3 linker (red shading) [56]. Another red-shaded proline residue is present at position 30′ (also called 8*) in many cation channel subunits. The green shading shows the conserved 9′Leu residue thought to lie at the gate (Figure 1(e)ii). The dark blue shading denotes the conventional α-helical regions. The pale-blue shading shows residues recently revealed to be part of the M2 helix, which extends two turns above the membrane surface in the closed state [5,35]. $GABA_A$ receptor α subunit, glycine receptor α subunit, and α1 nicotinic ACh receptor sequences are from mouse; the 5-HT_{3A} receptor sequence is from rat (out of all reported 5-HT_3 receptor sequences, only the mouse sequence lacks the second alanine residue between the two proline residues); and EXP-1 is a GABA-gated cation channel from *Caenorhabditis elegans* [82].

if one is close to the M2–M3 transition region, so is the other. Another recent experimentally based model literally ‘straddles’ both possibilities: L2 and L7 bracket the M2–M3 loop, mechanically actuating both the opening and closing events [14]. Photolabeling with lipid-soluble probes on ACh receptors in closed, open and desensitized states reveals that L7 does indeed appear to make contact with the membrane, although changes in labeling between open and closed states could not be discerned [42]. In reasonable agreement with these views, the adjacent L9 moves towards a hydrophobic environment during activation [59]. But there is also evidence that the transduction pathway runs from the C terminus of the binding domain, through M1, and thus to the M1–M2 transition region [33,34,38,39,60,61]. In summary, the obvious binding domain to membrane domain actuator to receiver pairings based on the two high-resolution structures [1,5] are all still in play.

Indeed, a simple concerted switch that rotates the M2 region might not describe the gating event. Linear free-energy analysis of single-channel recordings reveals the position of the transition state for the gating event, on a scale from $\Phi = 0$ (‘closed-like’) to $\Phi = 1$ (‘open-like’). There is a gradient of Φ from the agonist-binding site to the intracellular end of the M2 domain, leading Auerbach and co-workers to favor a gradual series of transitions (‘conformational wave’) [62,63].

Symmetry

Cys-loop receptors have five (pseudo)symmetrical subunits but require only two (or in some cases three) bound agonists for activation. For heteromeric receptors such as the muscle nicotinic ACh receptor and 5-$HT_{3A/B}$ receptor, it is necessary to determine whether: (i) just the two binding domains move; (ii) there is a sequence of movements, beginning with the binding domains and leading to movements of all subunits; or (iii) all five extracellular domains move in synchrony. Most researchers assume that, by the time channel opens, all five M2 regions have undergone the same gating movement. Perhaps subunit-specific mutational, optical [64] and state-specific mass spectrometric probes [42] will reveal aspects of this passage from asymmetrical binding to symmetrical gating.

Ion flow near the intracellular regions

Experiments from 1988 onwards show how the M1–M2 loop and M2 region determine the charge selectivity of Cys-loop superfamily members [65]. An additional aspect is reviewed in this article. The intracellular M3–M4 loop is the most variable region of Cys-loop subunits, ranging in length from 70 (the glutamate-gated Cl^- channel GluClβ) to 271 (the α4 nicotinic ACh receptor) residues. In 1999, the 4.6 Å structure of the intracellular aspect of the nicotinic ACh receptor revealed a then-surprising conclusion: there are transverse tunnels framed by α-helices in the channel wall [66]. Similar ‘portals’ have been described for several channels and could help to screen out large cytoplasmic blocking ions. Although it was not possible to trace chains to identify the groups that lined the tunnels, Miyazawa *et al.* [66,67] reminded readers that four acidic side chains occur at $n + 7$ spacing and noted that anion channels contain positively charged groups at aligning positions.

The hypothesis of cytoplasmic tunnels has received some support from site-directed mutagenesis experiments on the 5-HT_3 receptor. Kelley *et al.* [68] noticed that (i) the 5-HT_{3A} receptor subunit contains arginine residues at the aligning $n + 4$ positions and the homopentameric receptor exhibits a rather low (0.4 pS) conductance, and (ii) the 5-HT_{3B} receptor subunit contains at least three neutral or negative residues in these positions and, when part of a heteropentamer with the 5-HT_{3A} subunit, confers a larger (16 pS) conductance. Their experiments show that in 5-HT_{3A} receptors mutated to contain the aligning 5-HT_{3B} receptor residues, single-channel conductance increased to 25 pS.

Concluding remarks

This article, and others in this issue, show that ion channels continue to take the lead as well-understood proteins. For the Cys-loop family, researchers now have access to primary sequences, high-resolution functional data and, at last, high-resolution structural data; more rapid progress can be expected in the near future. But we still lack a definitive structure of any Cys-loop receptor in open, closed and desensitized states, and we have only the most rudimentary of ideas about structural dynamics of the transitions among these states.

Acknowledgements

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