Molecular Structure in Ligand-Gated Ion Channel Function

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The Cys-loop superfamily of ligand-gated ion channels: the impact of receptor structure on function

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Abstract

The Cys-loop receptors constitute an important superfamily of LGICs (ligand-gated ion channels) comprising receptors for acetylcholine, 5-HT_3 (5-hydroxytryptamine; 5-HT_3 receptors), glycine and GABA (γ-aminobutyric acid; $GABA_A$ receptors). A vast knowledge of the structure of the Cys-loop superfamily and its impact on channel function have been accrued over the last few years, leading to exciting new proposals on how ion channels open and close in response to agonist binding. Channel opening is initiated by the extracellular association of agonists to discrete binding pockets, leading to dramatic conformational changes, culminating in the opening of a central ion pore. The importance of channel structure is exemplified in the allosteric modulation of channel function by the binding of other molecules to distinct sites on the channel, which exerts an additional level of control on their function. The subsequent conformational changes (gating) lead to channel opening and ion transport. Following channel pore opening, ion selectivity is determined by receptor structure in, and around, the ion pore. As a final level of control, cytoplasmic determinants control the magnitude (conductance) of ion flow into the cell. Thus the Cys-loop receptors are complex molecular motors, with moving parts, which can transduce extracellular signals across the plasma membrane. Once the full mechanical motions involved are understood, it may be possible to design sophisticated therapeutic agents to modulate their activity, or at least be able to throw a molecular spanner into the works!

Despite the large number of membrane proteins, the high-resolution structure of only a handful of mammalian membrane proteins has been elucidated. The major barriers, to what has been a determined effort, lie in the difficulties of obtaining sufficient amounts of pure protein and crystallization for X-ray diffraction studies. The first problem has been side-stepped by the investigation of bacterial proteins or atypical circumstances in which the protein is naturally concentrated, such as for the acetylcholine receptor in the electric organ of the *Torpedo* ray [1], or by the recombinant expression of a soluble extracellular domain [2]. Subsequent extrapolation from such structures by homology modelling has catalysed progress in the study of related molecules by providing models to generate testable hypotheses [3,4]. Thus the pioneering efforts of the relatively few have provided blueprints to the many 'hungry' biochemists, armed with powerful technologies, capable of stripping structures to the bone within years. Certainly, it is breathtaking the speed and quality at which our understanding of the molecular structure of the Cys-loop superfamily of LGICs (ligand-gated ion channels) has developed over the last few years.

In the absence of an agonist, LGICs have a very low probability of opening. Upon activation by the binding of a specific ligand (neurotransmitter), these receptors increase

Key words: conductance, gating, ion channel molecular structure, ligand-gated ion channel, modulation.

Abbreviations used: LGIC, ligand-gated ion channel; 5-HT, 5-hydroxytryptamine; GABA, γ-aminobutyric acid; (n)ACh, (nicotinic) acetylcholine; ACBP, ACh-binding protein; TM, transmembrane.

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their likelihood of opening to permit ion transfer between the external and internal milieu. However, this is not a generalized floodgate, as ion channels possess a selectivity filter, permitting the transport of defined ions only. Whether an action potential is fired by a neuron is determined by the combined, antagonistic activity of both cationic depolarizing (excitatory) and anionic hyperpolarizing (inhibitory) ion channels. Thus the physiological activity of LGICs shape information flow in the brain, and so control behaviour. Perturbations in the control of this balance lead to abnormal activity, resulting in aberrant activity (e.g. epilepsy), altered behaviour (e.g. anxiety) or even neuronal cell death (excitotoxicity) [5].

The LGICs activated by extracellular ligands may be divided into four superfamilies: the Cys-loop superfamily, the glutamate receptors [NMDA (*N*-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and kainate], the TRP (transient receptor potential) channels and the ATP-gated channels. The glutamate, TRP and ATP-gated superfamilies will not be discussed here. Further details regarding these superfamilies (and the Cys-loop superfamily) may be obtained at the 'Ligand-gated ion channel database' (http://www.pasteur.fr/recherche/banques/LGIC/LGIC.html).

In mammals, the Cys-loop superfamily comprises both cationic [nicotine and 5-HT (5-hydroxytryptamine) receptors (i.e. 5-HT_3 receptors)] and anionic (GABA (γ-aminobutyric acid) receptors (i.e. $GABA_A$ and $GABA_C$ receptors) and glycine receptors] ion channels. The members of this superfamily exhibit important physiological functions, and mutations may lead to a range of pathological states [5]. As such, these receptors provide important therapeutic targets. Nicotinic acetylcholine (nACh) receptors are expressed in both muscle and nerve cells, playing a critical role in fast synaptic transmission. Their importance is exemplified by the existence of pathological mutations leading to epilepsy, and as the target for autoimmunity in myasthenia gravis [5]. 5-HT_3 receptor antagonists are routinely used as anti-emetics post-operatively and during chemotherapy [6]. Furthermore, a polymorphism (C178T) leading to the severe truncation (following residue 7 within the signal sequence) of 5-HT_{3A} has been associated with human harm avoidance [7]. Glycine receptors are the major inhibitory receptors within the spinal chord. Mutations found within the TM (transmembrane) I–II and TMII–III regions of the glycine receptor highlights their physiological importance by causing startle disease (hyperekplexia) in humans [8] and myoclonus in horses and cattle [5]. Several mouse mutations that mimic this disease have been identified in mice (*spastic*, *spasmodic* and *oscillator* mutations) [5]. Developing therapeutic agents against glycine receptors may have significant utility as muscle relaxants and analgesic agents [9]. The $GABA_A$ receptors are the major inhibitory receptors in the central nervous system, and mutations in these receptors have been implicated in Angelman's syndrome [10] and epilepsy [11–14]. More notable is the association of $GABA_A$ receptors with anxiety. Benzodiazepines are used routinely to treat human anxiety by enhancing $GABA_A$ receptor activity [15]. Furthermore, $GABA_A$ receptors are important targets in anaesthesia [16]. A recent addition to this superfamily of receptors is the zinc-activated chloride channel [17]. This receptor is absent from rats and mice, and its significance in normal physiology is unclear at this stage.

The use of benzodiazepines to target $GABA_A$ receptors and treat human anxiety epitomizes the major problems in the therapeutic targeting of the Cys-loop receptors. Benzodiazepines cause side effects by the non-discriminate activation of a wide range of different $GABA_A$ receptor subtypes. Furthermore, prolonged treatment with benzodiazepines causes tolerance and dependence by unknown mechanisms. To date, all drugs in use/development have been based on original serendipitous findings, rather than on a rational basis. This has been necessary due to our ignorance of the way in which these receptors operate. It is therefore of paramount importance to study how these receptors are activated, and how this activation is transduced into ion flow. To achieve this goal, we need to understand receptor molecular structure at rest, and how this is altered following ligand binding, to cause the opening of the ion channel.

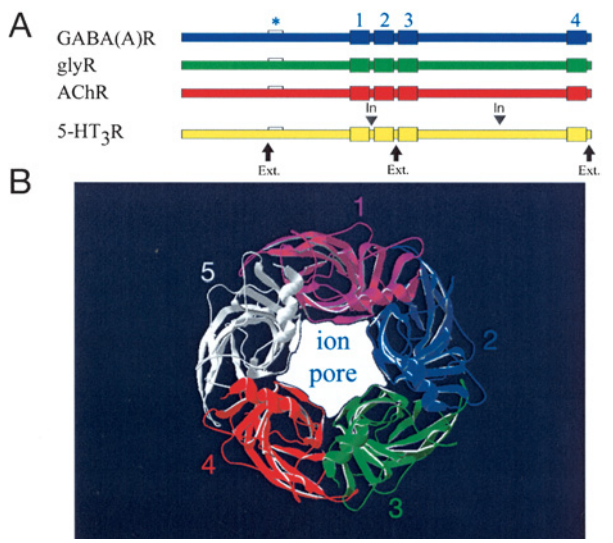
All the members of the Cys-loop superfamily are thought to possess a similar architecture [18]. All members have the Cys-loop signature within their N-terminal extracellular domains, four transmembrane regions and are arranged as a pentameric structure surrounding the ion pore (Figure 1). Sequence alignment of the extracellular regions of subunits from different receptor classes reveal limited homology at the primary structural level (Figure 2). Despite this apparent divergence, several residues are completely conserved within all members of the superfamily, implying some structural conservation [19–21]. Indeed, a structural analysis reveals strikingly similar secondary and tertiary structure. In fact, following the identification of the crystal structure of the ACBP (ACh-binding protein) [18], several members of the superfamily have been modelled on to this structure to generate a prediction of their quaternary structures [3,4]. In support of the validity of these models, the ligand-binding domains formed at the interfaces between subunits are correctly predicted. Furthermore, significant conservation of the location of these ligand-binding domains is observed (Figure 2). Likewise, residues implicated in the gating of these receptors also show significant overlap. Thus, at least at the quaternary structural level, all members of this superfamily may exhibit major conservation.

Pioneering work using freeze-trapping by Nigel Unwin in 1995 [22] provided the first direct evidence for the existence of a dynamic structure in the acetylcholine receptor by comparing the structure of open and closed channels. Such studies were only possible by electron microscopy, permitting rapid and transient changes in structure to be observed in its native lipid and ionic environment. This model has now been resolved to ≈4.6 Å (0.46 nm), and has identified rotational movements of the α subunits [1,23].

Initial efforts to fine tune this structure and determine the residues involved in ligand binding used mutagenesis

Figure 1 | The molecular structure of the Cys-loop members of ligand-gated ion channels

(**A**) The superfamily comprises nAch, 5-HT_3, glycine and $GABA_A$ receptors. The receptors possess a large extracellular N-terminal domain containing the Cys-loop signature (*), followed by four transmembrane domains (shown by boxes). Intracellular (In) and extracellular (Ext.) domains are identified. (**B**) Receptors are constructed as pentameric ion channels from the assembly of five subunits (numbered 1–5) in a ring structure, creating an ion pore within. This model (courtesy of T.G. Smart, University College London) represents the N-terminal domain of a $GABA_A$ receptor modelled on the structure of ACBP [18].



and photoaffinity labelling. For the $GABA_A$ receptor, these approaches defined some of the specific residues involved [24,25]. However, these methods cannot identify all residues involved in ligand binding or other surrounding residues that create critical structural requirements. The use of the substituted cysteine accessibility method, to regions suspected by photoaffinity labelling, or homology modelling to the ACBP [18], has permitted a more detailed characterization of the binding sites. Using this approach, a more detailed local structure is developing for both the GABA (at α–β interfaces) and benzodiazepine (α–γ interfaces) binding sites [26–32]. Moreover, structures linking these two binding sites may provide critical information on how they couple functionally. In support of a conserved mechanism of ligand binding, the mutation of a single residue (**F**GY → **Y**GY) in the ligand-binding domain of the glycine receptor is sufficient to convert the receptor to being sensitive to GABA [33]; see Figure 2).

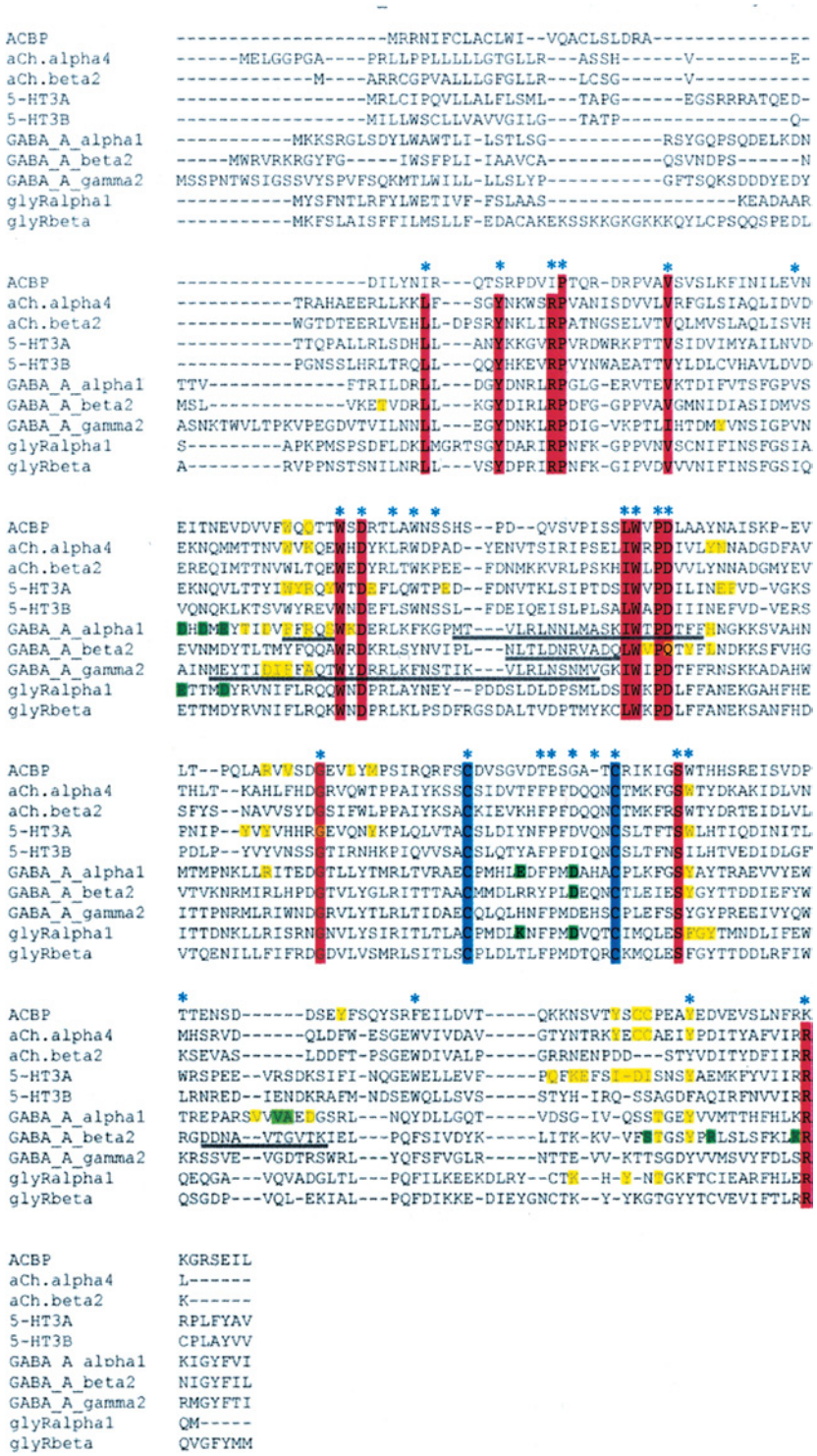
Interestingly, all members of the Cys-loop superfamily are allosterically modulated by zinc ions. Multiple zinc-binding sites may exist in nACh and 5-HT_3A receptors, given their bidirectional modulation, depending upon zinc concentration [34,35]. Indeed, multiple sites have been identified at subunit interfaces in $GABA_A$ [4] and glycine receptors (T. Smart, unpublished work; [36]), causing receptor inhibition, possibly by inhibiting subunit movements during gating. Moreover, zinc sites elsewhere are responsible for the potentiation of glycine receptor activity (T. Smart, unpublished work). Thus extracellular zinc binding plays a complex allosteric role in the modulation of receptor activity. It will be interesting to determine how this binding impinges on receptor structure, and thus function.

As discussed earlier, significant structural movements appear to correlate with receptor activation [1,22]. This movement is termed 'gating', to reflect events downstream of ligand-binding that lead to increased channel opening. Interestingly, some residues lining the GABA-binding site appear to be involved in receptor gating [30,37,38], with particular residues creating spontaneously open channels when mutated [30,37], thus coupling ligand binding to gating. Furthermore, a number of mutations have implicated altered receptor gating in human disease [5]. A number of these sites have been located within the short extracellular region between TMII and TMIII in nAChR (myasthenic syndrome), glycine receptor (hyperekplexia) and $GABA_A$ (epilepsy). Recent studies performed on the $GABA_A$ receptor α_1 subunit have identified that a charged residue within the Cys-loop region is positioned close to the membrane and, therefore, the TMII–III region. Using site-directed mutagenesis and cysteine cross-linking experiments, the proximal location of these two regions has been shown to occur following GABA binding [39] as a consequence of molecular movements (gating). In contrast, asymmetric structural changes occur within the β_2 subunit upon GABA binding, involving possible interactions with the Cys-loop and a pre-TMI segment [40]. Similar asymmetric structural changes have been reported to occur in the glycine receptor [41–43]. Thus major movements within ligand binding and distal regions appear to be responsible for receptor gating, and are consistent with the morphological changes observed by electron microscopy [1,22,23].

The story of receptor function does not end with the opening of ion channel floodgates, there is still much to be achieved. Ion channels are not indiscriminate pores permitting the transport of all ions small enough to enter, but exhibit an incredible ability for selectivity. Logically, the ion selectivity filter has been assumed to be at the narrowest point of the pore. The pore region is constructed by the alignment of five TMII regions that extend through the membrane as α-helices that bend outwards [44]. Both anions and cations appear to be able to access the extracellular end of the pore [45–47]. In fact, the selectivity filter begins on the cytoplasmic side of the transmembrane pore and extends beyond into the short TMI–II cytoplasmic loop. Again, this feature is conserved among all members of the Cys-loop superfamily [48–51]. Furthermore, these regions are not static features, but appear to exist in three different structural states representing resting, open and desensitized receptors [49]. In support of a conserved structural domain, certain mutations within this region are capable of switching the ion selectivity from cationic to anionic [48,52], and vice versa [51]. Intriguingly, a naturally occurring cationic GABA receptor in *Caenorhabditis elegans* has been identified recently [53]

Figure 2 | Sequence alignment of selected subunits from the different receptor classes in the Cys-loop superfamily

Red highlighting indicates residues conserved within members of the superfamily. Blue highlights identify the cysteines involved in the formation of the Cys-loop. Yellow residues are ligand-binding; green represents gating residues, blue asterisks indicate essential residues, and underlined residues are assembly signals. A lack of identification does not imply an absence of an involvement, only that no data are available, to our knowledge. Information in this Figure was gathered from [3,18,19,26–41,55,56,63–77].



that possesses features consistent with a cationic selectivity filter.

In addition to this selectivity filter within the first intracellular loop between TMI–II, a second molecular barrier has now been determined to exist within the second intracellular loop between TMIII–IV of 5-HT_3 receptors [54]. This second (downstream) filter controls the magnitude of ion flow (conductance) into the cytoplasm. Structurally, this filter is thought to exist as a cytoplasmic vestibule that is perforated by small openings through which ions are transported [55]. Charged residues framing these ion portals appear to determine the efficiency with which ions may be transported [54].

The biogenesis of integral membrane proteins requires a co-ordinated sequence of events, including membrane targeting, translocation and the proper formation of tertiary and quaternary structure. This process must have the fidelity of protein function at its heart. In this respect, it is encouraging that a region important in the assembly of the α_1 subunit of the $GABA_A$ receptor overlaps the GABA-binding site [56,57]. Similarly, an endoplasmic retention signal (retains unassembled receptors within the endoplasmic reticulum), found within the cytoplasmic domain of the 5-HT_{3B} subunit [58], overlaps the ion selectivity filter region within the 5-HT_{3A} subunit [48]. Finally, the cytoplasmic stretch controlling ion conductance and receptor gating also forms a critical structural element, whose formation is a prerequisite for the biogenesis of nACh receptors [59]. Thus receptor structure appears to be highly policed by quality control mechanisms to ensure reliable ion channel function.

The development of new pharmaceuticals to modulate members of the Cys-loop superfamily has focused on subunit-selective agents capable of improving the risk/benefits index of such treatments. Nowhere is this more pressing than for the hunt to find alternatives to benzodiazepines. Benzodiazepines are used widely to treat anxiety, but side effects such as sedation, and the long-term development of tolerance and dependence, severely limit their usefulness. Recent advances using transgenic knock-ins have identified a subunit dependent contribution to the behavioural responses to benzodiazepine agonists. In particular, specific $GABA_A$ receptor subunits have been implicated in sedation and narcosis (α_1), anxiolysis (α_2) and trace fear/cognition (α_5) [15,60–62]. Similar specificity has recently been observed for etomidate-induced anaesthesia, with sedation (β_2) and anaesthesia (β_3) being performed by different receptor subtypes [16].

In summary, following receptor activation by ligand binding, the changes in molecular structure of the Cys-loop receptors that occur at multiple (interconnected) levels are a prerequisite for receptor function. Based on these studies (and future refinements), it may be possible to design novel therapeutic 'spanners' to manipulate the molecular structure and function of this clinically important group of ion channels. Prior to the onslaught of such sophisticated approaches, there is still a significant utility for oiling the LGIC machine, or just being able to throw a spanner into the works! But where?

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