

Topical Review

Gating Mechanisms in Cys-loop Receptors

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Abstract

The Cys-loop receptor superfamily of ligand-gated ion channels has a prominent role in neuronal signalling. These receptors are pentamers, each subunit containing 10 β -strands in the extracellular domain and four α -helical transmembrane domains (M1-M4). The M2 domain of each subunit lines the intrinsic ion channel pore and residues within the extracellular domain form ligand binding sites. Ligand binding initiates a conformational change that opens the ion-selective pore. The coupling between ligand binding in the extracellular domain and opening of the intrinsic ion channel pore located in the membrane is not fully understood. Several loop structures, such as loop 2, the Cys-loop, the pre-M1 region and the M2-M3 loop have been implicated in receptor activation. The current “conformational change wave” hypothesis suggests that binding of a ligand initiates a rotation of the β -sheets around an axis that passes through the Cys-loop. Due to this rotation, the Cys-loop and loop 2 are displaced. Movement of the M2-M3 loop then twists the M2 domain leading to a separation of the helices and opening of the pore. The publication of a crystal structure of an acetylcholine binding protein and the refined structure of the *Torpedo marmorata* acetylcholine receptor have improved the understanding of the mechanisms

and structures involved in coupling ligand binding to channel gating. In this review, the most recent findings on some of these loop structures will be reported and discussed in view of their role in the gating mechanism.

Key words: ligand-gated ion channels, Cys-loop receptor, gating, coupling, review

Abbreviations

AChBP	Acetylcholine binding protein
Cu:Phen	Copper phenanthroline
ECD	Extracellular domain
GABA _A R	γ -aminobutyric acid type A receptor
GlyR	Glycine receptor
5-HT ₃ R	Serotonin type 3 receptor
LBS	Ligand binding site
LGIC	Ligand-gated ion channel
MTS	Methanethiosulfonate
nAChR	Nicotinic acetylcholine receptor
NT	Neurotransmitter
P4S	Piperidine 4-sulfonate
REFER	Rate-equilibrium free energy relationship
SCAM method	Substituted cysteine accessibility method
TMD	Transmembrane domain
WT	Wild type

Introduction

Signalling between neurons occurs through the release of neurotransmitters (NT) from pre-synaptic neurons into the synaptic cleft. These NTs can then bind and activate ligand-gated ion channels (LGIC) found in the membrane of post-synaptic neurons. Ligand binding to the extracellular domain (ECD) of LGICs initiates a conformational change that opens an ion-selective pore located within the transmembrane domain (TMD). This generates an excitatory or inhibitory post-synaptic current depending on the ion selectivity of the LGIC in the membrane. The most prominent family of LGICs is the Cys-loop family, so called because of a characteristic loop formed by a disulfide bond between two cysteine residues in the ECD. The Cys-loop family comprises the glycine receptor (GlyR), the γ -aminobutyric acid type A receptor (GABA_AR), the nicotinic acetylcholine receptor (nAChR) and the serotonin type 3 receptor (5-HT₃R). A cartoon

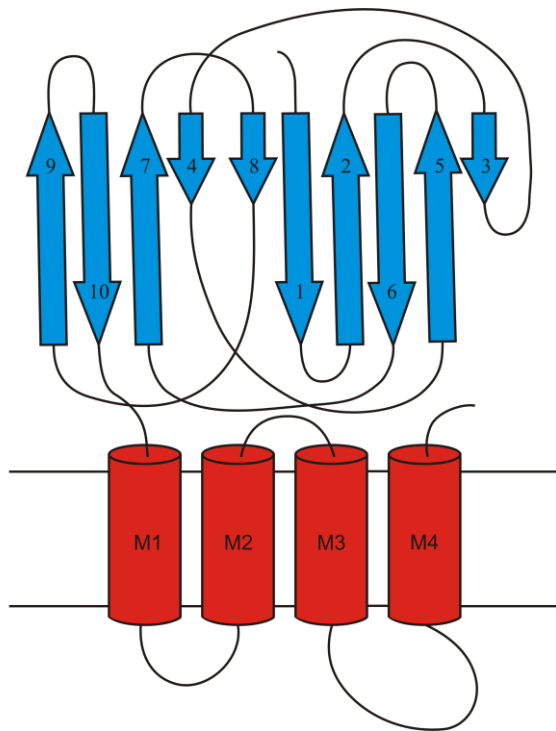


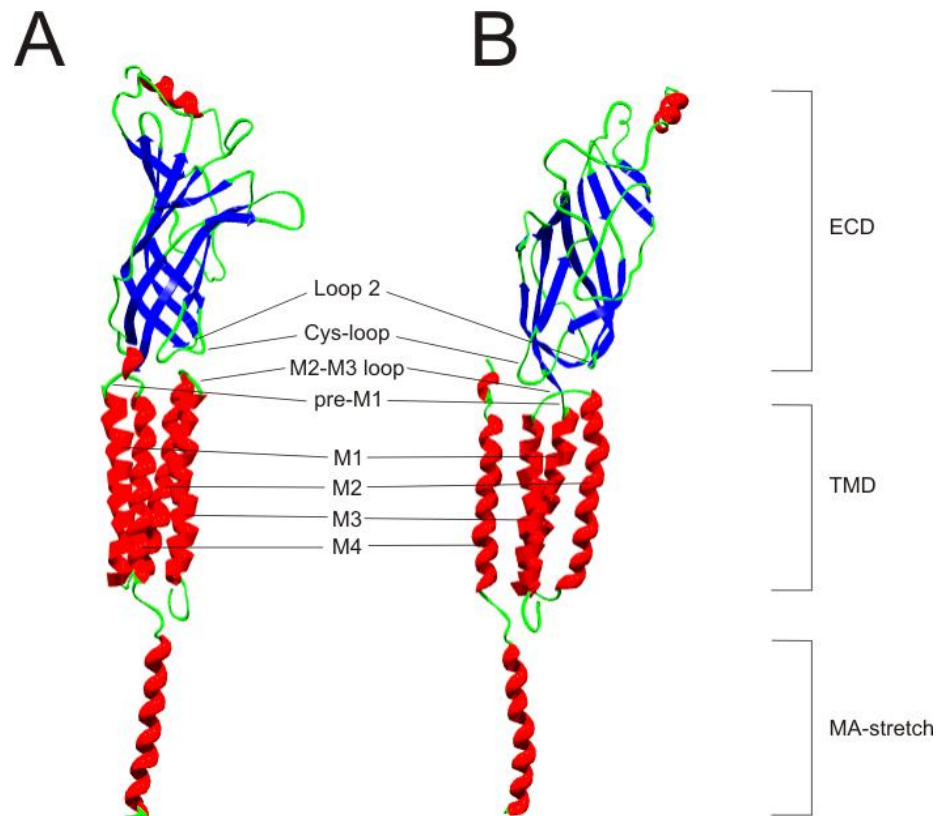
Figure 1 A cartoon of a Cys-loop receptor showing the extracellular domain (ECD), with its β -strands, and the transmembrane domain (TMD) with its four α -helices, M1-M4. The M2 helices from each subunit contribute to forming the channel pore. The inner β -sheet (β -strands 1, 2, 3, 5, 6 and 8) interacts with the outer β -sheet (β -strands 4, 7, 9 and 10), forming a β -sandwich.

illustrating the general topology of Cys-loop receptors is shown in Figure 1. GlyR and GABA_AR are anion-selective channels and allow for chloride ions to pass through the pore, leading to an inhibitory response in the postsynaptic neuron. In contrast, the nAChR and the 5-HT₃R are cation-selective channels and allow the passage of sodium and potassium ions, generating an excitatory postsynaptic response. Given that the distance between the ligand binding site (LBS) and the ion channel pore is about 40 Å (Unwin 2005), many studies have concentrated on identifying the structures that link these two separate domains to facilitate channel gating. Four loop structures found at the interface between the ECD and the TMD, loop 2, the Cys-loop, the pre-M1 region and the M2-M3 loop, have been the focus of numerous studies. The specific roles of these loops in linking agonist binding to gating have been investigated using a variety of experimental techniques.

The task of identifying the specific pathway of Cys-loop receptor activation has not been easy, mainly due to the lack of a high-resolution crystal structure of a Cys-loop receptor in both the open and closed states. However, a major breakthrough came when the crystal structure of the snail *Lymnaea stagnalis* acetylcholine binding protein (AChBP) was published in 2001 (Brejc et al. 2001). It is a homologue to the ECD of the nAChR, with the ability to bind nAChR ligands and therefore is a potential tool to provide insight into the mechanism and structures involved in ligand binding. The next milestone was the publications of the refined structure of the *Torpedo marmorata* nAChR by Unwin and coworkers (Miyazawa et al. 2003; Unwin 2005). The structure published in 2003 confirmed much of the existing data on ligand binding and greatly helped our understanding of the mechanisms involved in coupling ligand binding to channel gating. The structure published in 2005 allowed for a more comprehensive description of the whole receptor in the closed-channel form.

In this review we will report on and discuss the most recent findings into the molecular mechanisms of channel gating.

Figure 2. Ribbon diagrams of a single nAChR α -subunit (PDB entry 2GB9) viewed parallel with the membrane plane. In a) the M2 pore-lining domain is at the back and b) the subunit has been rotated clockwise so that the M2 is towards the side. The β -strands composing the β -sandwich are in blue; the membrane spanning α -helices are in red. The M3-M4 loop is missing.



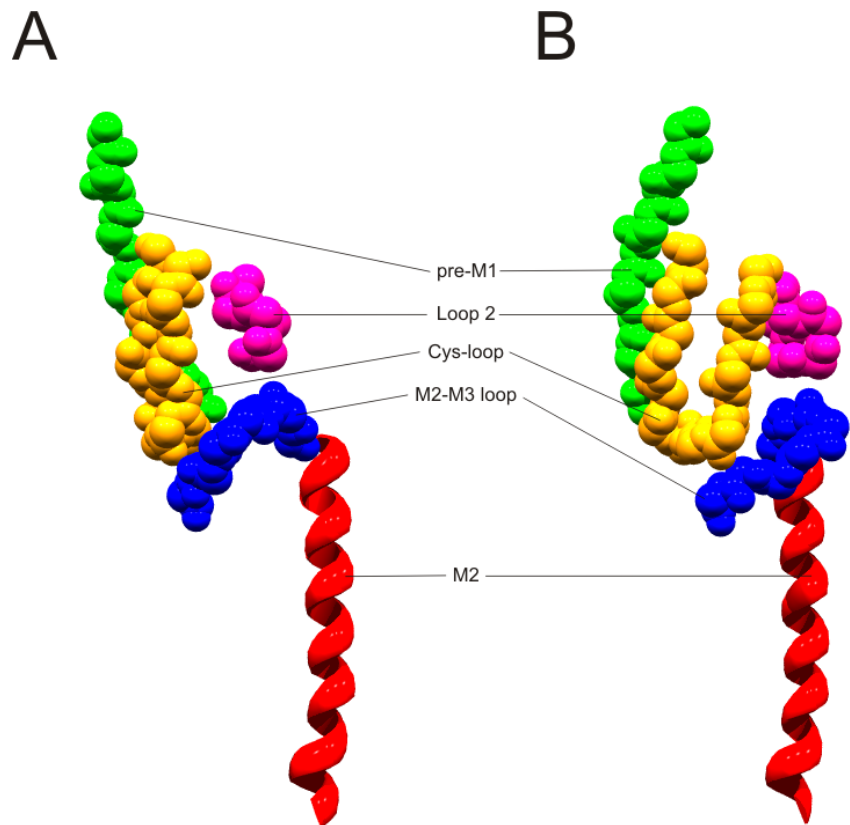
Structure of Cys-loop receptors

Cys-loop receptors consist of five subunits that are arranged around a central ion-channel pore within the membrane. Each subunit contains a large ECD that is made up of 10 β -strands. β -strands 1, 2, 3, 5, 6 and 8 make up the inner β -sheet. The outer β -sheet (β -strands 4, 7, 9 and 10) and the inner β -sheet interact and are organised as a β -sandwich. Each subunit also contains four α -helical membrane spanning domains (M1-M4) (Fig. 2). The pore is shaped by the M2 helices from each subunit and conformational changes of this region upon ligand binding allow for ion flow through the pore. A large intracellular loop is located between domains M3 and M4, and includes an amphipathic α -helix known as the membrane-associated (MA) stretch. The MA-stretch has been shown to be important for single-channel conductance in cation-selective (Deeb et al. 2007; Gee et al. 2007; Hales et al. 2006; Jansen et al. 2008; Kelley et al. 2003), as well as anion-selective ion channels (Carland et al. 2009). The ECD and the TMD interact through the connection at the start of M1 (pre-M1), loop 2, the conserved Cys-loop (loop 7) and the M2-M3 loop (Unwin 2005) (Fig. 3).

Gating of Cys-loop receptors

The conformational changes that occur upon ligand binding are transferred away from the binding site through the ECD towards the interface with the TMD and then to the M2 channel gate thought to be formed, in nAChRs at least, by a girdle of hydrophobic residues. This is referred to as a “conformational change wave” (Grosman et al. 2000b). This conformational wave has been extensively studied in the nAChR by Auerbach and co-workers (Chakrapani et al. 2004; Grosman et al. 2000b; Jha et al. 2007; Purohit and Auerbach 2007a, b; Purohit et al. 2007) and provides a dynamic description of nAChR gating, which builds upon the static information that crystal structures can offer. According to this hypothesis, ligand binding initiates a rotation of the inner β -sheet around an axis that passes through the Cys-loop (Lester et al. 2004; Miyazawa et al. 2003; Unwin et al. 2002). At the interface, the Cys-loop and loop 2 are displaced due to the rotation of the β -sheets. Movement of the M2-M3 loop then twists the M2 domain leading to a separation of the helices and widening of the hydrophobic girdle.

Figure 3. Diagrams of a single nAChR α -subunit (PDB entry 2GB9) viewed parallel with the membrane plane. In a) the M2 pore-lining domain is at the side and b) the subunit has been rotated anti-clockwise so that the M2 is towards the back. The diagrams show the location of the M2 domain (red), loop 2 (pink), the Cys-loop (yellow) and the pre-M1 region (green) in relation to each other.



Thus, the channel is opened and ions can pass from one side of the membrane to the other.

Involvement of the ligand binding site in gating

The LBS of Cys-loop receptors is formed at the interface between two subunits in the ECD. Before any crystal structure was available, the key residues involved in ligand binding were proposed to be located in six “loops” that come together to form the LBS. These loops are referred to as loops A-F. However, it is now evident that some of the key binding residues are in fact located on β -strands, suggesting that a change in terminology is needed. Until a new terminology has been widely accepted, and to avoid misunderstandings, this review will still refer to the binding sites as loops A-F.

Ligand binding is best understood in nAChRs. Each binding site is formed between the principal subunit and the adjacent complementary subunit (in the anticlockwise direction when looking at the receptor from the synaptic cleft). Here, loops A, B and C from the principal subunit interact with loops D, E and F from the complementary subunit (Gay and Yakel 2007). These loops contain aromatic and

hydrophobic residues in the nAChR (Gay and Yakel 2007; Unwin et al. 2002), but these residues are not conserved across all Cys-loop members.

Of the loops contributing to the LBS, loop C shows the greatest movement upon agonist binding, appearing to close over the LBS (Celie et al. 2004; Gao et al. 2005). This loop, between β -strands 9 and 10, is continuous with the M1 domain via β -strand 10. Thus, a conformational change in loop C is expected to be propagated along β -strand 10 to M1, which is known to contribute to channel gating (see below). Molecular dynamics simulations also predict coupling between loop C and rotation of the M1 and M2 domains (Liu et al. 2008). In addition, β -strand 10 is close to the Cys-loop, which has been shown to interact with the M2-M3 loop in GABA_ARs (Kash et al. 2003). The M2-M3 loop is another of the key structures that contribute to opening of the pore (see below). Thus, it appears that a conformational change in loop C is one of the early steps that contribute to the gating process.

Single-channel kinetic analysis and structural modelling of the muscle nAChR show that three

conserved residues of the α -subunit, Lys145 in the Cys-loop, Asp200 and Tyr190 in loop C, play a significant role in the initial conformational changes involved with gating ([Mukhtasimova et al. 2005](#)). Mutating each of these residues impairs gating and double mutant studies demonstrate that these residues are interdependent. It is suggested that in the resting state a salt bridge exists between Lys145 from β -strand 7 (Cys-loop) and Asp200 from β -strand 10 (loop C). When an agonist binds, Lys145 can be pulled away from Asp200 by electrostatic force from Tyr190 in the highly mobile loop C. These local changes might be transmitted to the channel via β -strand 7 and 10. A salt bridge between residues Glu153 and Lys196 in the GABA_AR β 2-subunit (equivalent to Lys145 and Tyr190 in muscle nAChR) has also been found ([Venkatachalan and Czajkowski 2008](#)). This salt bridge is critical for GABA receptor activation and crosslinking experiments confirm that these two residues (Glu153 and Lys196) are in close proximity. Interestingly, data also suggest that loop C is highly mobile in the resting state and that Glu153 and Lys196 serve to stabilise the ligand-bound state of the receptor upon activation ([Venkatachalan and Czajkowski 2008](#)). Furthermore, residue Arg207 in loop C (equivalent to Asp200 in the muscle nAChR) is believed to play an important role in the resting, unliganded state of the GABA_AR by interacting with Glu153 in the Cys-loop.

A second “triggering structure” adjacent to the LBS has also been observed in the muscle nAChR. Conserved residues Tyr127, Asn39 and Asn41 (in the α -, ϵ - and δ -subunits, respectively) all contribute to channel gating and it seems they are interdependent ([Mukhtasimova and Sine 2007](#)). However, these two pairs (Asn39/Tyr127 and Asn41/Tyr127) of residues are intersubunit, rather than intrasubunit, and their role is only conserved in muscle nAChRs. This suggests a specialised function for the muscle receptor. The authors do suggest the possibility of similar “triggering structures” in the other Cys-loop receptors based solely upon sequence homology. This has yet to be tested experimentally.

The important role of Tyr127 and Lys145 in the mouse nAChR α 1-subunit has also been highlighted in a rate-equilibrium free energy relationship (REFER) study ([Purohit and Auerbach 2007b](#)). REFER employs a simplified kinetic scheme of a single open and closed state to generate a statistical value (Φ) that estimates whether the effect of a number of mutations at a particular site is temporally closer to the open state ($\Phi=1$) or the closed state ($\Phi=0$) transition. From this analysis, three conformational blocks have been described for the gating of the nAChR α 1-subunit. Φ values in the LBS are around 0.93, decreasing to 0.78 in the Cys-loop and loop 2 and to 0.64 in the M2-M3 loop ([Purohit and Auerbach 2007b](#)). The REFER study demonstrates that Tyr127 and Lys145 belong to two different conformational blocks, with Lys145 moving with the ligand binding (Φ of 0.96) followed by Tyr127 (Φ of 0.77), which occurs almost synchronously with residues in the Cys-loop and loop 2. This indicates that if there is a rotation of the β -strands of the α -subunit, it will be of a more flexible nature since Lys145 from the outer β -sheet and Tyr127 from the inner β -sheet belong to two different conformational blocks. Furthermore, this study also found that Tyr127 is energetically coupled to residues in the δ or ϵ subunits, and less so to those in the α -subunits. Thus, their results support the idea proposed by [Mukhtasimova and Sine \(2007\)](#), that there is a link between the α -subunits and the δ - or ϵ -subunits (described above) that is important for the gating conformational cascade.

In the 5-HT_{3A}R several tyrosine residues from, or around, the LBS loops have been implicated in triggering channel gating upon ligand binding ([Price and Lummis 2004](#); [Venkataraman et al. 2002](#)). Incorporating both natural and unnatural amino acids into several tyrosine positions of the mouse 5-HT_{3A}R allowed [Beene and colleagues \(2004\)](#) to define the roles of these residues. Removal of the aromatic group and/or displacement of the hydroxyl group of Tyr143 in loop E led to an increase of the measured EC₅₀ compared to wild-type (WT) ([Beene et al. 2004](#)), but did not have an effect on binding of competitive

antagonist. This suggests that aromatic and/or hydroxyl groups at Tyr143 are not required for ligand binding, but are important for efficient gating. Furthermore, modelling data suggest that the hydroxyl group of Tyr143 can form a hydrogen bond with the backbone carbonyl of Trp183 from the opposite side of the binding site (Beene et al. 2004). Removal of the hydroxyl group, and hence the hydrogen bond, by introduction of the unnatural amino acid may then be the cause for inefficient gating. Another tyrosine in loop E, Tyr153, was shown to be important for both gating and binding. Mutation of this Tyr153 resulted in large increases in measured EC_{50} , and changes in both receptor kinetics and antagonist binding affinity. Similar to Tyr143, both an aromatic ring and a hydroxyl group at position Tyr153 seem to be critical for proper receptor function. The location of the hydroxyl group in Tyr153 suggested to the authors that it is critical for the binding of 5-HT (Beene et al. 2004). Furthermore, removal of the aromatic ring of the conserved Tyr234 in loop C resulted in non-functional receptors showing that it is essential for receptor function. Such data, when considered in the light of the *Torpedo marmorata* nAChR work (2002), led to the suggestion of a gating model in which 5-HT enters the binding site and forms a cation- π bond with Trp183, leading to the displacement of Tyr143 and Tyr153. These two residues are located on separate β -sheets linked by a turn, thus their movements might lead to a rotation of the 5-HT_{3A}R structure.

Long before the crystal structure of the AChBP was available, a study of the *Torpedo Californica* nAChR showed that the loop F residue, Asp174/Asp180 (in the γ - and δ -subunit, respectively), can come within 9Å of the disulfide on loop C (Czajkowski and Karlin 1991). By introducing unnatural amino acids, it has since been confirmed that loop F interacts with loop C in muscle nAChRs (Gleitsman et al. 2008). Mutating the backbone of the α -subunit residue Ser191 in loop C to α -hydroxyserine (Sah) (giving it more flexibility and also replacing the hydrogen bond-donating N-H group with a non-hydrogen bond-donating O), lead to a 40-fold increase in EC_{50} . In addition, alanine and α -hydroxyalanine (Aah)

mutations were also made at this site. In contrast to the Ser191Ala mutation, but similar to Ser191Sah, the Ser191Aah mutation significantly increased the EC_{50} . Thus, it is clear that it is the backbone of Ser191, and not the side-chain, that is important for receptor function. Mutant cycle analysis was then used to look at possible interactions between loops C and F of the binding site. Results show that there is a large energetic coupling between Ser191Sah (loop C) and the previously studied Asp174/Asp180 in loop F (Gleitsman et al. 2008). All mutations at Asp174/Asp180 that have a major impact on function also show strong coupling to the Ser191Sah backbone mutation (Gleitsman et al. 2008). Thus, the movement of loop F closer to loop C during agonist binding very likely contributes to the gating pathway.

Pre-M1 region

The pre-M1 region connects β -strand 10 of loop C to the M1 domain. The involvement of this region in coupling ligand binding to channel gating has been studied in the rat GABA_AR α 1- and β 2-subunits using the substituted cysteine accessibility method (SCAM) (Mercado and Czajkowski 2006). This region contains several cationic residues including an arginine that is conserved in several Cys-loop receptors. An alignment of the pre-M1 residues is shown in Figure 4. Mutating this Arg216 in the GABA_AR β 2-subunit to a cysteine (Arg216Cys) abolished channel gating without modifying the binding of the GABA agonist [³H]muscimol. Substitution of a cysteine at position Arg220 in the α 1-subunit of this receptor was not tolerated since there was no [³H]muscimol binding nor channel activation by GABA or pentobarbital. Thus, this shows that this residue plays a critical role in coupling GABA binding to gating (Mercado and Czajkowski 2006). Furthermore, by observing the effects of methanethiosulfonate (MTS) reagents to several cysteine substituted mutations in both the closed and open states it was shown that the pre-M1 region moves during channel gating.

<Fig.4 near here>

The Arg216/Arg220 residues in rat GABA_AR subunits are highly conserved among other receptors of the Cys-loop family. The equivalent residue in the GlyR α 1-subunit, Arg218, has been found to be mutated in some cases of hyperekplexia (Arg218Gln). The effect of this mutation has been studied in whole-cell patch clamp experiments and the results explain much of the observed phenotype (Castaldo et al. 2004). It was shown that the Arg218Gln mutation causes a 200-fold decrease in the apparent affinity of glycine and abolishes any channel activation by maximal concentrations of β -alanine and taurine. The observed effects are unlikely to reflect a binding site change as the competitive antagonist strychnine was still effective. Furthermore, at very high glycine concentrations both activation and desensitization rates were faster in WT compared to mutant channels, with no change in deactivation rates. All of these drastic effects support the idea that the mutation has a deleterious effect on channel gating. It is notable that Arg218 lies close to several residues that have been implicated in channel gating, such as Arg271 (Rajendra et al. 1994) and Lys276 (Lewis et al. 1998) in the M2-M3 loop and Asp148 in the Cys-loop (Schofield et al. 2003).

Arg222, in the pre-M1 region of the 5-HT_{3A}R has also been implicated in channel gating (Hu et al. 2003). This arginine is only found in 5-HT_{3A}Rs and α 7 nAChRs, but is adjacent to the highly conserved Arg218 in the GlyR α 1-subunit and Arg216/Arg220 in the GABA_AR subunits. Thus, these residues might play the same role in the different Cys-loop receptors.

Lys215 in the pre-M1 region of the GABA_AR β 2-subunit is adjacent to the highly conserved Arg216 and also seems to be important for coupling ligand binding to channel gating (Kash et al. 2004a). The sensitivity to GABA and the relative efficacy to the two agonists, piperidine 4-sulfonate (P4S) and taurine, was reduced in receptors containing

the the mutant β 2-subunit Lys215Asp. However, the charge reversal double mutant, Asp146Lys/Lys215Asp, almost completely restored the sensitivity to GABA. By mutating the Asp146 (Cys-loop) and Lys215 to cysteine, the oxidising reagent copper phenanthroline (Cu:Phen) could be used to examine the proximity and mobility of these residues. Results demonstrate that Cu:Phen alone had no effect, whereas Cu:Phen together with GABA significantly inhibited receptor function. This suggests that the Cys-loop is only in close proximity to the pre-M1 region during the open or desensitised state.

An alanine scan of 12 residues in β -strand 10 and the pre-M1 region of human GABA_AR α 1-subunits identified two mutations that reduced the potency of GABA, Val212Ala in β -strand 10 and Lys220Ala in the pre-M1 region (Keramidas et al. 2006). Both mutants had a 4- to 5-fold increase in their respective EC₅₀ value. The close location of Lys220 to loop 2, the Cys-loop and the M2-M3 loop prompted the authors to do further investigations. Results showed that removal or reversal of charge at Lys220 (Lys220Asp) did not have a great effect on the function of the receptor. These results support the hypothesis that as long as the charging pattern within the gating zone is preserved, individual changes of charge are tolerated (Xiu et al. 2005).

Loop 2 and the Cys-loop

At the interface between the ECD and the TMD, both loop 2 and the Cys-loop are predicted to be close to the top of the ion channel and the M2-M3 loop (Brejc et al. 2001; Unwin 2005) (Figs. 2 and 3). When a 4Å resolution structure of the *Torpedo marmorata* nAChR was published (Miyazawa et al. 2003), the authors suggested that loop 2 is in a position that allows it to dock into a hydrophobic pocket made by the end residues (Ser269 to Pro272 in the α -subunit) of the M2 domain. They referred to this as a “pin-into-socket” interaction. The “pin” in this structure was proposed to be Val46 of loop 2 in the α -subunit. An alignment of the pre-M1 residues is shown in Figure 4.

Loop 2			Cys-loop		
Tc nAChR δ	46	KETDET 51	130	CPINVL YF FDWQNC	144
h nAChR δ	46	KEVEET 51	130	CPISVT YF FDWQNC	144
Tc nAChR γ	44	NEKEEA 49	128	CPIAVT YF FDWQNC	142
h nAChR ε	44	NEKEET 49	128	CAVEVT YF FDWQNC	142
Tc nAChR β	44	NEKIEE 49	128	CTIKVM YF FDWQNC	142
Tm nAChR α	44	DEVNQI 49	128	CEIIVTH F FDQQNC	142
Tc nAChR α	44	DEVNQI 49	128	CEIIVTH F FDQQNC	142
m nAChR α_1	44	DEVNQI 49	128	CEIIVTH F FDQNC	142
h nAChR α_1	44	DEVNQI 49	128	CEIIVTH F FDQNC	142
h nAChR α_7	44	DEKNQV 49	128	CYIDVR W FDVQHC	142
r nAChR α_7	44	DEKNQV 49	128	CYIDVR W FDVQQC	142
c nAChR α_7	44	DEKNQV 49	128	CYIDVR W FDVQKC	142
h 5-HT _{3A} R	51	DEKNQV 56	134	CSLDIYN F FDVQNC	148
m 5-HT _{3A} R	55	DEKNQV 60	138	CSLDIYN F FDVQNC	152
h GABA _A R β_2	51	SEVNMD 56	136	CMMDLRR Y PLDEQNC	150
r GABA _A R β_2	51	SEVNMD 56	136	CMMDLRR Y PLDEQNC	150
h GlyR α_1	52	AETTMD 57	138	CPMDLK N FMDVQTC	152
h GABA _A R α_1	54	SDHDME 59	139	CPMHLE D FMDAHAC	153
r GABA _A R α_1	53	SDHDME 58	138	CPMHLE D FMDAHAC	152
Ls AChBP	42	NEITNE 47	122	CCVSGVD T ESG-ATC	136
p ELIC	27	NTLEQT 32	112	NDMDFRL F FDQQF	126
p GLIC	31	DDKAET 36	111	SPLDFRR Y FDSQTL	125
pre-M1			M2-M3 loop		
Tc nAChR δ	215	VTFYLI I RRK 224	275	SQRL P ETALAVPLI	288
h nAChR δ	215	ITFYLI I RRK 224	275	SKRL P ATSMAIPLI	288
Tc nAChR γ	209	IIFFLI I QRK 218	270	AQKV P ETSLNVPLI	283
h nAChR ε	210	VIYSLI I RRK 219	271	AQK I PETSLSVPLI	284
Tc nAChR β	207	VTFYLI I QRK 216	267	ADKV P ETSLSVPII	280
Tm nAChR α	201	ITYHFIMQRI 210	261	VELIPSTSSAVPLI	274
Tc nAChR α	201	ITYHFIMQRI 210	261	VELIPSTSSAVPLI	274
m nAChR α_1	201	ITYHFVMQRL 210	261	VELIPSTSSAVPLI	274
h nAChR α_1	201	ITYHFVMQRL 210	261	VELIPSTSSAVPLI	274
h nAChR α_7	198	VTFTVT M RRR 207	258	AEIMPATSDSVPLI	271
r nAChR α_7	198	VTYTVT M RRR 207	258	AEIMPATSDSVPLI	271
c nAChR α_7	198	ITFTVT M RRR 207	258	AEIMPATSDSVPLI	271
h 5-HT _{3A} R	209	MKFYV V IRRR 218	269	SDTL P ATAIGTPLI	282
m 5-HT _{3A} R	213	MKFYV I IRRR 222	273	SDTL P ATIG-TPLI	286
h GABA _A R β_2	208	LSLSFK L KRN 217	269	RET L PKIPYVKAID	282
r GABA _A R β_2	208	LSLSFK L KRN 217	269	RET L PKIPYVKAID	282
h GlyR α_1	210	IEARF H LERQ 219	271	RAS L PKVSIVKAID	284
h GABA _A R α_1	213	MTTHF H LKRK 222	274	RNS L PKVAYATAMD	287
r GABA _A R α_1	212	MTTHF H LKRK 221	273	RNS L PKVAYATAMD	286
Ls AChBP	162	SEYFSQYSRF 171	185	YSCC P EAYEDVEVS	198
p ELIC	190	ITVRIDAVRN 199	249	SNIL P RLPYTTVID	262
p GLIC	183	LDYQLR I SRQ 192	242	ETN L PKTPYMTYTG	255

Figure 4. Aligned amino acid sequences of different subunits from five different Cys-loop receptors with their respective numbering. Alignment is only showing the amino acids that form loop 2, the Cys-loop, the pre-M1 region and the M2-M3 loop. Residues conserved across different subunits are shown in grey background and homologous residues are shown in bold. Ls, *Lymnaea stagnalis*; Tc, *Torpedo californica*; Tm, *Torpedo marmorata*; h, human; m, mouse; r, rat; c, chick; p, prokaryote.

This interaction has also been studied in the GABA_AR and GlyR (Kash et al. 2004b). However, results show that the equivalent “pin” residues (Val53 and His56 in GABA_AR β_2 - and

α_1 -subunit, respectively; Thr54 in GlyR α_1 -subunit) in these receptors are not likely to have a critical impact on the gating process. This suggests that the hydrophobic interaction

observed in the nAChR is not a characteristic shared by GABA_ARs and GlyRs. Indeed, a gating dynamic study of the mouse nAChR α 1-subunit highlighted that more than one loop 2 residue is critical for channel gating ([Chakrapani et al. 2004](#)). Subsequently, cysteine substitutions of the odd-numbered loop 2 residues, Ile51, Glu53, Thr55 and Asp57, of the GlyR α 1-subunit increased glycine EC₅₀, while Thr54 decreased glycine EC₅₀ ([Crawford et al. 2008](#)). The impact of Thr54 on EC₅₀ suggests that this residue is involved in the gating process, which is in contrast to Kash and co-workers (2004b), who found that six Thr54 mutant GlyR α 1-subunits (Thr replaced with Ala, Glu, His, Lys, Phe, or Ser) were not significantly different to WT. Increases of EC₅₀ for the odd-numbered loop 2 residues also demonstrate that in the GlyR α 1-subunit the whole loop structure plays a vital role ([Crawford et al. 2008](#)). This is supported by the spasmodic mutation in the mouse GlyR α 1-subunit, in which Ala52 is replaced with a Ser, and the mutation results in an increase in the GlyR α 1-subunit EC₅₀ ([Ryan et al. 1994](#)). Single channel analysis of the spasmodic mutation suggests that it impairs the transition to a pre-opening conformation of the receptor ([Plested et al. 2007](#)) further supporting the role of the loop 2 as an important intermediate between ligand binding and channel opening. Interestingly, in contrast to the odd-numbered residues of loop 2 in the GlyR α 1-subunit ([Crawford et al. 2008](#)), mutation of loop 2 residues in the chick nAChR α 7-subunit that are equivalent to the even-numbered Ala52, Thr54 and Met56 in the GlyR α 1-subunit, resulted in a significant increase in EC₅₀ ([McLaughlin et al. 2007](#)). This suggests that the loop 2 structure differs between nAChRs and GlyRs.

It is notable that in the GlyR α 1-subunit and GABA_AR α 1-subunit the Lys276/Lys279 residue located in the M2-M3 loop is conserved, but is absent in the nAChR M2-M3 loop. The GABA_AR α 1-subunit Lys279 has been proposed to interact with Asp57 in loop 2 ([Kash et al. 2003](#)). This suggests that equivalent interactions between the even-numbered loop 2 and M2-M3 loop residues might occur in the nAChR. Perhaps the Arg266 residue in the

nAChR α 7-subunit M2-M3 loop that has been found to be important for coupling (discussed below) somehow interacts with one of these even-numbered loop 2 residues. More experiments are needed to test this proposal.

Many other residues in loop 2 have also been shown to be critical for channel gating. Other charged loop 2 residues in both the GlyR α 1-subunit ([Absalom et al. 2003](#)) and the GABA_AR α 1-subunit ([Kash et al. 2003](#)) have been implicated in receptor activation, and a specific salt bridge between Glu45 in loop 2 and Arg209 in the pre-M1 region of the nAChR α -subunit appear to be critical for proper channel gating ([Lee and Sine 2005](#)). In fact, this salt bridge has been suggested to be the key pathway linking agonist binding to channel gating. The same salt bridge has also been found to be important in the GABA_AR ([Price et al. 2007](#); [Wang et al. 2007](#)), although it does not appear to exist in the 5-HT_{3A}R ([Price et al. 2007](#)). The notion that such specific electrostatic interactions are critical to gating has, however, also been disputed. Xiu and colleagues (2005) propose that it is the global charge of the gating interface and groups of interacting ionic residues that are essential for receptor function.

The role of Glu45 and Arg209 in gating of nAChR has also been challenged by Purohit and Auerbach (2007a). In their study they mutated all five residues of the pre-M1 region and Glu45 in loop 2 and examined the effect on gating kinetics. They found that perturbation of a salt bridge between Glu45 and Arg209 was unlikely to be the main event in the gating of nAChRs. Several pieces of evidence point toward this conclusion. Firstly, there is no significant difference between the open- and closed-state energy for the different Arg209 mutations. Secondly, there is a very weak energetic coupling between Glu45 and Arg209. Thirdly, several double mutant constructs that are not able to form a salt bridge are still functional. Compared to the Cys-loop, loop 2 and the M2-M3 loop, it appears that the pre-M1 region has only a minor role in the energy transfer between the ECD and the TMD. Considering all of this, the authors suggest that

a combination of side-chain interactions at several positions between loop 2 and the M2 domain, and the Cys-loop and the M2-M3 loop allows for energy to be transferred from the ECD to the TMD (Purohit and Auerbach 2007a). Overall, it is evident that different members of the Cys-loop receptor family use different molecular interactions within the same structures during gating. More work is needed to clarify these differences.

The involvement and importance of the Cys-loop in GlyR gating was first examined by Schofield et al. (2003). A highly conserved aspartate residue within the GlyR α 1-subunit Cys-loop, Asp148, was reported to be critical for receptor activation. The equivalent residue in the GABA_AR α 1-subunit, Asp149, is also important (Kash et al. 2003) and mutant cycle analysis of double reverse charge mutations show that a direct electrostatic interaction exists between Asp149 and Lys279 (equivalent to Lys276 in the GlyR α 1-subunit) in the M2-M3 loop. However, the same group found no evidence for coupling between the equivalent residues (Asp146 and Lys274) in the GABA_A β 2 subunit (Kash et al. 2004a). Instead they found that Asp146 interacts with Lys215 in the pre-M1 region. Single mutations of these residues significantly reduced GABA sensitivity, but it was almost completely restored in the double reverse charge mutant, Asp146Lys/Lys215Asp. Thus, not only do these findings demonstrate the importance of the Cys-loop they also show that the same structure in different subunits has different molecular interactions that are critical to channel gating.

Possible interactions between Asp148 and Lys276 have also been tested in the GlyR α 1-subunit (Absalom et al. 2003; Schofield et al. 2003), but the lack of a full recovery from double reverse charge mutation experiments suggest that while these residues are involved in the gating process, they do not form a direct salt bridge in the GlyR. Further studies using different chimeras, molecular modelling and resolving the crystal structures of a Cys-loop receptor in an open versus closed state might

provide some of the answers to these differences in mechanism between the LGICs.

A chimeric receptor composed of the AChBP and the pore domain from the 5-HT_{3A}R (Bouzat et al. 2004) revealed that even though binding and pore domains were folded correctly and the receptors could be expressed on the cell surface, the receptor was not functional as the correct molecular interactions were absent. Mutating residues in the Cys-loop, loop 2 and loop 9 of the AChBP to the 5-HT_{3A}R counterparts restored functional coupling, highlighting the importance of the specific interactions between these loops in the transduction mechanism between the ECD and TMD. Changing only one or two of the loops to the 5-HT_{3A} sequence produced receptors with impaired function, indicating that correctly matching loops 2, 9 and the Cys-loop with the M2-M3 loop are all required.

M2-M3 loop

One of the first structures implicated in the channel gating mechanism of Cys-loop receptors was the M2-M3 loop of the GlyR α 1-subunit (Lynch et al. 1997). Several naturally occurring mutations that result in human hereditary hyperekplexia (or startle disease) have been located in this region (Elmslie et al. 1996; Shiang et al. 1995; Shiang et al. 1993). Large increases in the EC₅₀ value of two M2-M3 loop mutations (Lys276Glu and Tyr279Cys), and the conversion of β -alanine and taurine from full agonists to competitive antagonists, with little disruption to their binding affinities, suggested that these startle disease mutations alter the coupling of ligand binding to channel gating (Lynch et al. 1997). A complete alanine scan of the GlyR α 1-subunit M2-M3 loop revealed that several residues in this loop are important for proper channel gating (Lynch et al. 1997). The importance of residues in the M2-M3 loop for linking agonist binding to Cys-loop receptor gating has been confirmed by numerous studies over the last decade (Bera et al. 2002; Campos-Caro et al. 1996; Grosman et al. 2000a; Lewis et al. 1998; Lynch et al. 1995; Rajendra et al. 1995; Rovira et al. 1999). As indicated above, chimera

studies have highlighted the need for compatible residues in the ECD loops and the M2-M3 loop for functional gating (Bouzat et al. 2004; Castillo et al. 2006). Asp266 in the nAChR α 7-subunit M2-M3 loop (Campos-Caro et al. 1996) and an equivalent residue in other neuronal nAChRs (Rovira et al. 1999) is involved in coupling binding to gating. An alignment of the pre-M1 residues is shown in Figure 4. Mutating individual residues throughout the M2-M3 loop of the rat α 7 nAChR/mouse 5-HT_{3A}R chimera to aspartate increased receptor function. The Thr266Asp mutation had the most improved function, showing the smallest EC₅₀ value (Castillo et al. 2006). Thus, charged residues are critical for the “correct” interaction of loop structures to enable channel gating. These results support the hypothesis of Xiu et al. (2005) that it is the overall electrostatic environment rather than specific interactions that is key to correct channel gating.

A proline residue in the M2-M3 loop, which is only found in the 5-HT_{3A}R and the nAChR, has been shown to be critical to the gating mechanism through a *cis-trans* isomerisation of the protein backbone (Lummiss et al. 2005). A gating model for the 5-HT_{3A}R was proposed in which Pro308 (Pro272 in the nAChR α -subunit) is in the *trans* conformation when the channel is closed. When the ligand binds to the receptor, and a conformational change is initiated (possibly via loop 2), Pro308 in the M2-M3 loop undergoes a *cis-trans* isomerisation leading to a rotation of the M2 domain and, thus opening the channel pore. In the nAChR α -subunit Pro272 has been shown to be part of two energetically coupled triads, the first one with Val46 and Glu45 (both in loop 2) and the second with Val46 and Ser269 (in the M2-M3 loop) (Lee and Sine 2005). Coupling energies between residues were estimated from changes in the gating equilibrium constants (from single channel analysis) measured with individual mutations and combinations of two or three mutations of the residues that interact. Individually mutating Val46Ala and P272Gly reduced the gating equilibrium constant and the combination of these mutations reduced the gating constant further, although it was 20-fold

more efficient than expected from the sum of the two mutations. This indicates a significant coupling energy between these residues. Analysis of introducing a third mutation, Glu45Ala, revealed a further increase of the coupling energy between Val46 and Pro272. The authors speculate that the increase in coupling energy is made possible by the release of Glu45 from Arg209, which then gives Val46 the freedom to interact more strongly with Pro272. In a second triad (Val46, Ser269 and Pro272) the gating equilibrium constant was reduced by Val46Ala, but increased by Ser269Leu. The double mutant revealed a significant coupling energy, and this decreased with the introduction of the third mutation Pro272Gly. Interestingly, this coupled triad involves residues that were proposed to form a “pin-into-socket” interaction in nAChRs (Miyazawa et al. 2003). Since these findings, an additional “pin-into-socket” assembly linking the ECD to the TMD has been found in the nAChR α -subunit (Lee et al. 2008). It comprises Val46, Val132 (in the Cys-loop) and Pro272, with Val46 serving as the pin and the other two residues as the socket. Using the REFER method, all three of these residues were shown to move almost simultaneously (Φ ranging from 0.87 to 0.93). Findings also show that these three residues contribute to channel gating in an interdependent manner. Thus, several lines of evidence point to the importance of a proline in the M2-M3 loop for coupling ligand binding to gating in 5-HT_{3A}Rs and nAChRs. Given that this proline is not conserved among other members of the Cys-loop family, it yet again shows that although the overall gating mechanism of these receptors might be similar, individual interactions contributing to this mechanism differ.

Recent structures of prokaryotic ligand-gated ion channels

Very recently X-ray structures of prokaryotic LGICs from *Erwinia chrysanthemi* (ELIC) (Hilf and Dutzler 2008) and *Gloeobacter violaceus* (GLIC) (Bocquet et al. 2009; Hilf and Dutzler 2009) were published. The major difference between these two structures is in the pore

geometry. While the ELIC structure represents a non-conducting conformation, the GLIC structure is likely to represent an open conformation. Superimposing these two structures reveal that although they only show moderate sequence conservation (20% identical residues), they are structurally very similar (Hilf and Dutzler 2009). Furthermore, the superimposed structure reveals that GLIC has undergone structural rearrangements compared to ELIC. The most prominent movements are of the inner β -sheet, with smaller changes observed for loop 2 and the Cys-loop (Hilf and Dutzler 2009). Compared to the ELIC structure, loop 2 appears to have moved downward (towards the pore) in the GLIC structure, while a small movement away from the pore axis is seen for the Cys-loop (Bocquet et al. 2009; Hilf and Dutzler 2009). Surprisingly, the pre-M1 region does not seem to have moved much at all. In contrast, the M2-M3 loop appears to move in the same plane as the membrane, swivelling away from the M2 domain and the pore axis. In addition to these movements, the β -sandwich of the ECD has rotated by approximately 5-8° (Bocquet et al. 2009; Hilf and Dutzler 2009). A similar degree of rotation of the inner β -sheet has also been determined in the nAChR (Unwin 2005).

Global conformational changes are also suggested by comparison of the ELIC and GLIC structures. The receptor complex appears to undergo a twisting motion between the closed state (ELIC structure) and the open state (GLIC); the ECD rotates in a clockwise direction, while the TMD rotates in an anticlockwise direction (Bocquet et al. 2009). Similar concerted twisting motions, described to be like wringing out a washcloth (Samson and Levitt 2008), have been observed with normal mode analysis (Bahar and Rader 2005) of a number of different models of Cys-loop LGICs. Analysis of both homomeric $\alpha 7$ nAChR (Cheng et al. 2006; Taly et al. 2005) and $\alpha 1$ GlyR (Bertaccini et al. 2007), and heteromeric AChR (Liu et al. 2008; Samson and Levitt 2008) all reveal a similar concerted twisting motion that generally occurs as the lowest normal mode. This suggests that the twisting motion is not simply a consequence of a

homomeric (symmetrical) receptor complex. Further, this twisting motion mode does not occur within the lowest 25 modes of the AChR model when α -bungarotoxin (a competitive inhibitor at AChRs) is docked into the LBS (Samson and Levitt 2008). Results from molecular dynamics simulations also support the rotations of the ECD and TMD seen with normal mode analysis (Cheng et al. 2007; Liu et al. 2008). These predicted conformational changes now need to be experimentally tested.

In the pre-M1 region of GLIC, Arg191 forms a pair of salt bridges with Asp31 in loop 2 and Asp121 in the Cys-loop. By aligning 228 full length sequences of cation-selective LGIC subunits from the Uniprot database (S1) we observed that the Arg191 (pre-M1) and Asp121 (Cys-loop) in GLIC are completely conserved. Similarly, alignment of 109 anion-selective LGIC subunits (S1) revealed that homologous aspartate and arginine residues are also completely conserved. These conserved residues are also indicated in Figure 4. As described earlier, the equivalent residues in the GlyR $\alpha 1$ -subunit (Ala52, Asp148 and Arg218), the GABA_AR $\alpha 1$ -subunit (Asp149 and Arg220) and $\beta 2$ -subunit (Asp146 and Arg216) and the nAChR α -subunit (Arg209) have been shown to be critical for channel gating. Some of these residues have experimentally been found to interact with residues in loop 2, the Cys-loop and the pre-M1 region (Castaldo et al. 2004; Kash et al. 2004a; Kash et al. 2003; Lee and Sine 2005; Mercado and Czajkowski 2006; Ryan et al. 1994; Schofield et al. 2003). Considering that the arginines are conserved the effect they have on gating is perhaps not surprising. However, although these residues have been individually characterised as important in Cys-loop receptors, a triad similar to the one found in the GLIC structure, has not yet been reported.

Conclusions

The task of identifying the structures linking ligand binding in the ECD to opening of the channel pore in the membrane was made easier when the crystal structure of the AChBP (Miyazawa et al. 2003) and the refined structure of the *Torpedo marmorata* nAChR (Unwin

2005) were published. Many studies have since shown important roles of structures such as loop 2, the Cys-loop, the M2-M3 loop and the pre-M1 region in the gating mechanism of Cys-loop receptors. However, even though the gating mechanism is now clearly understood many questions remain to be resolved. For example, why do specific residue interactions and mechanisms seem to differ between different receptor subtypes? Future structural and functional studies and perhaps a crystal structure of a Cys-loop receptor in both the open and closed states, will help to answer many of these questions.

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Cation-selective LGICs	P02712	O96760	P49579
	P02718	Q21645	P49580
	P02714	P91197	Q9I8C7
P02709	P22456	Q9GQU9	P09481
Q07263	P09628	Q93329	P49581
P04758	P05376	Q22224	Q9PTS8
P04759	Q7QD62	O16926	P43679
P02715	Q7QD60	P48180	P26153
P13536	Q7QF72	Q6F6K3	P02711
P09479	Q66T32	O17848	P54131
P09480	Q7PHY7	O62083	Q8SPU6
P09482	Q8MUB7	Q7JKI4	Q9XS62
P26152	Q8MUR0	Q9N5U8	Q9GZZ6
P22770	O18394	Q9GP98	Q15822
Q03481	Q9NFR5	O61884	P43681
P09484	Q8T7V5	P48182	Q15825
P02717	Q8T7R9	Q93149	Q9UGM1
P02713	Q86MN7	O76554	Q91X60
P09478	P25162	P54246	Q8R4G9
P17644	O46128	Q9N4M3	O70174
P04755	O96631	P45963	Q2MKA5
P18845	O96632	Q23355	Q9R0W9
P19370	Q9XZI4	Q18556	P49582
P13908	O96633	O44452	Q05941
P18257	O46132	P34271	Q9ERK7
P02708	O46133	P54244	Q8BMN3
P32297	O46134	Q9U298	Q8R493
P30532	O46135	Q19351	Q5IS52
P36544	P91766	O61520	Q5IS77
P17787	O76960	O61519	Q5IS51
P11230	P91765	P54245	Q5IS76
Q05901	P91764	Q9XTE5	Q5IS75
P30926	Q9U941	O61216	Q9JLB5
Q07001	Q9U940	Q27218	P25108
Q04844	Q9U939	Q9XTY8	P43144
P07510	P23414	Q95Y94	P25109
P04756	Q8T9S0	O16426	P25110
P09690	Q8T0Y9	P48181	P18916
P02716	Q8WSF9	Q23022	Q98880
P20782	Q2XWL2	Q9N587	Q7ZZP7
P04760	Q2XWL1	Q9XW95	Q800C7
P12389	Q2XWL0	O18228	Q7ZZP6
P04757	Q2XWK9	Q6BEQ0	Q75XT1
P09483	Q2XWK8	Q9N4R6	Q7T2W5
P20420	Q2XWK7	Q9GUF0	Q7T2W4
P12390	Q2XWK6	Q9NA46	Q7T2S5
P12391	Q2XWK5	P92039	Q7T2S4
P12392	Q2XWK4	P54247	Q7T2S3
P09660	Q2XWK2	Q94789	Q7T2S2
P02710	Q2XWK3	P05377	Q7T2S1

Q7T2U1	P10063	P26714	Q91ZM7
Q7T2U0	P10064	Q16896	Q90288
Q7T2T9	P20237	Q9U9B8	Q9DDD9
Q7T2S0	P08220	Q08832	Q9YGQ4
Q7T2R9	P22300	Q24352	Q9YGQ3
Q7T2R6	P19150	O18471	Q9YGQ5
Q7T2R5	P19019	Q08861	O42157
Q7T2R3	P24045	Q9GYU4	Q9YGQ2
Q7T2R4	P21548	Q17367	P23415
Q7T2R8	P25123	Q25035	P23416
Q7T2R7	P14867	Q7LZ70	P07727
Q7T2T8	P47869	P34904	P22771
Q7T2R2	P34903	P48169	P24524
Q7T2Q8	P31644	Q16445	P20781
Q7T2Q7	P18505	P47870	O75311
Q7T3S4	P28472	O14764	Q5JXX5
Q7T2Y8	P18507	P78334	P48167
Q7T2Y7	P24046	Q8N1C3	Q64018
P23979	P28476	Q99928	Q7TNC8
Q9GQ00	P62812	O00591	Q91XP5
O70212	P26048	A8MPY1	Q61603
P46098	P26049	Q9UN88	P48168
O95264	P16305	Q9D6F4	O93430
Q8WXA8	P22933	P50571	Q9DES9
A5X5Y0	P22723	P63137	Q90W14
Q9JHJ5	P27681	P63080	Q90WT3
Q9N0F4	P62813	Q9R0Y8	Q9DES8
Q9GL84	P23576	Q8QZW7	Q8JIU9
P35563	P20236	P56475	Q94900
Q9JJ16	P28471	P56476	Q9U990
P0C7B7	P19969	B2RXA8	O17793
	P30191	Q9JLF1	Q17328
Anion-selective	P15431	Q9ES14	P91730
LGICs	P23574	P50572	Q9TYG6
	P18508	P47742	Q9VDU9
P08219	P28473	P50573	P41849

S1. Accession numbers from the Uniprot database (<http://www.uniprot.org/>) for the full length protein sequences of 228 cation-selective and 109 anion-selective ligand-gated ion channel subunits. These accession numbers can be copied into the ‘Align’ tool of the Uniprot database thus providing you with an alignment. The sequence for the *Gloeobacter violaceus* ligand-gated ion channel (GLIC) has not yet been assigned a Uniprot accession number, but can be retrieved from the Protein Data Bank (<http://www.rcsb.org/pdb/home/home.do>).