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Review

Crystallographic studies of pharmacological sites in pentameric ligand-gated ion channels☆

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ABSTRACT

Background: Pentameric ligand-gated ion channels (pLGICs) mediate fast chemical transmission of nerve signals in the central and peripheral nervous system. *On the functional side*, these molecules respond to the binding of a neurotransmitter (glycine, GABA, acetylcholine or 5HT3) in the extracellular domain (ECD) by opening their ionotropic pore in the transmembrane domain (TMD). The response to the neurotransmitter binding can be modulated by several chemical compounds acting at topographically distinct sites, as documented by a large body of literature. Notably, these receptors are the target of several classes of world-wide prescribed drugs, including general anesthetics, smoking cessation aids, anxiolytics, anticonvulsants, muscle relaxants, hypnotics and anti-emetics. *On the structural side* recent progress has been made on the crystallization of pLGICs in its different allosteric states, especially pLGICs of bacterial origin. Therefore, *structure–function relationships* can now be discussed at the atomic level for pLGICs.
Scope of Review: This review focuses on the crystallographic structure of complexes of pLGICs with a number of ligands of pharmacological interest. First, we review structural data on two key functional aspects of these receptors: the agonist-induced activation and ion transport itself. The molecular understanding of both these functional aspects is important, as they are those that most pharmacological compounds target. Next, we describe modulation sites that have recently been documented by X-ray crystallography. Finally, we propose a simple geometric classification of all these pharmacological sites in pLGICs, based on icosahedrons.
Major Conclusions: This review illustrates the wealth of structural insight gained by comparing all available structures of members of the pLGIC family to rationalize the pharmacology of structurally diverse drugs acting at topographically distinct sites. It will be highlighted how sites that had been described earlier using biochemical techniques can be rationalized using structural data. Surprisingly, the use of icosahedral symmetry allows to link together several modulation sites, in a way that was totally unanticipated.
General Significance: Overall, understanding the interplay between the different modulation sites at the structural level should help the design of future drugs targeting pLGICs. This article is part of a Special Issue entitled structural biochemistry and biophysics of membrane proteins.

1. Introduction

Pentameric ligand-gated ion channels (pLGICs) constitute a large family of receptors widely expressed in multicellular animals from invertebrates to mammalian, including human, as well as in a few bacterial and archaeal species [1]. They allosterically convert the binding of a neurotransmitter in their extracellular domain (ECD) to the opening of an ionotropic pore in their transmembrane domain (TMD) (Fig. 1). First discovered in vertebrates, pLGICs mediate fast chemical transmission of nerve signals in the central and peripheral nervous system. The family encompasses the anionic glycine (Gly), γ-amino butyric type A ($GABA_A$) and γ-amino butyric type B ($GABA_B$) receptors on one side and the cationic nicotinic acetylcholine (nACh) and serotonine ($5\text{-}HT_3$) receptors on the other side. Dysfunction of pLGICs is associated to several disorders of the central nervous system, including hyperplexia, myasthenia gravis, epilepsy, nicotine and alcohol addiction, schizophrenia, as well as Alzheimer's and Parkinson's diseases. Over the past decades, pLGICs have been used as targets for the development of novel therapeutics against nervous-system disorders by the pharmaceutical industry [2]. Indeed, these receptors are the target for several classes of world-wide prescribed drugs, including general anesthetics, smoking cessation aids, anxiolytics, anticonvulsants, muscle relaxants, hypnotics and anti-emetics. These drugs allosterically modulate pLGIC function by acting at several topographically distinct sites, distributed from the ECD to the TMD (Fig. 2). Structural variety of these drugs and differences

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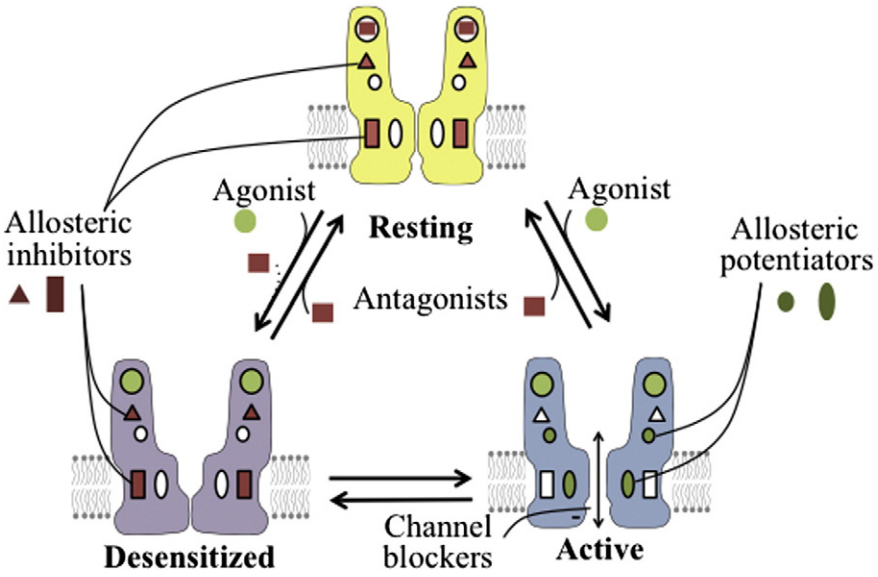

Fig. 1. Schematic allosteric scheme for pLGICs. The resting, active and desensitized states are shown in yellow, blue and purple respectively. Allosteric modulatory sites are white when empty and colored when bound to the ligand. The dashed line indicates that in some particular cases, antagonists stabilize the desensitized state of the receptor.

of their biological effects illustrate the wealth of these receptors pharmacology.

This review focuses on the recent progress made on the crystallization of pLGICs bound to a number of ligands of pharmacological interest. These receptors are not fixed structural entities but are able to undergo discrete allosteric transitions between multiple states, including basal, active and desensitized states. Agonists, antagonists, as well as allosteric modulators, all select and stabilize structurally different conformations, suggesting that allosteric binding sites display multiple modes [3] (Figs. 1 and 2). This review aims to outline how recent progress on the molecular understanding of their allosteric transitions sheds light on the modulation mechanisms of existing drugs and should overall benefit the design of future drugs.

2. Structure and function of pLGICs: general considerations

2.1. Overall structure: conservation of a common core

Recently reported full-length structures of several members of the family provided significant insights into the architecture of these receptors. Integral pLGIC structures encompass the electron microscopy structure of the *Torpedo marmorata* nAchR [4], the X-ray structures of two prokaryotic channels, derived from *Erwinia chrysanthemi* (ELIC) [5] and *Gloeobacter violaceus* (GLIC) [6,7], and the eukaryotic glutamate-activated pLGIC from *Caenorhabditis elegans* (Glu-Cl) [8]. All pLGICs share a conserved organization with five identical or homologous subunits symmetrically arranged around a central ionic pore

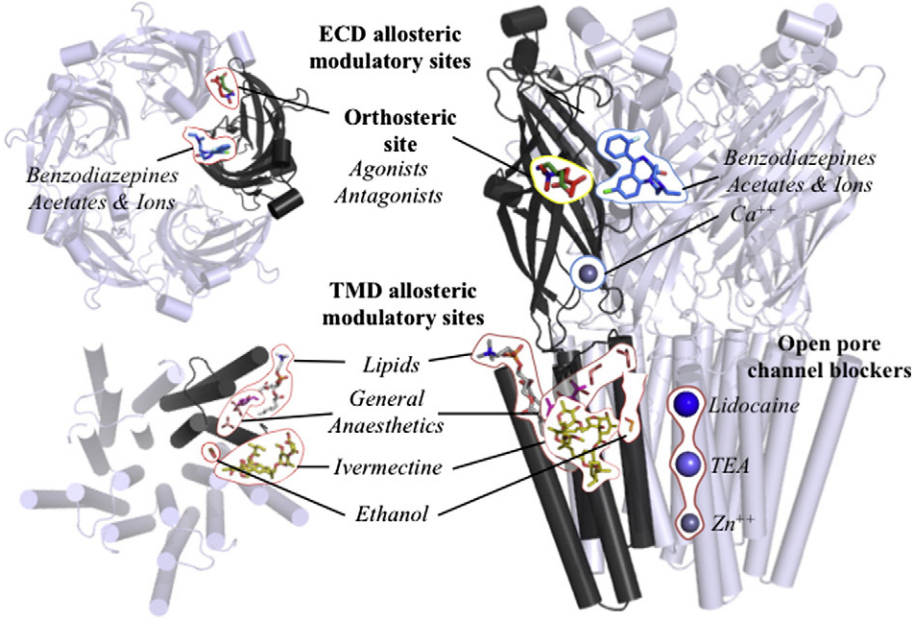

Fig. 2. Allosteric modulatory sites in pLGICs. Cartoon representation of the GLIC receptor viewed from the side (right panel) and from the top (ECD: top left panel and TMD: bottom left panel) superimposed onto a selection of ligand-binding sites. Several topographically distinct modulation sites, distributed either in the ECD or the in TMD, are represented in the stick mode.

(Fig. 2). Each of these subunits is composed of an N-terminal extracellular ligand-binding domain (ECD) and an ionotropic transmembrane domain (TMD). Eukaryotic pLGICs display an additional intracellular domain. Beside integral pLGICs structures, several isolated ECD and TMD structures have been solved: the water-soluble pentameric AChBP homolog [9], the ECD of α1 nAChR in complex with α-bungarotoxin [10], α7nAChR/AChBP chimeras [11,12], GLIC ECD [13,14], as well as isolated TMD of the α1nAChR [15] and α1GlyR [16]. Nevertheless, the functional implications concerning ligand-activation and modulation that can be deduced from isolated domains or monomeric structures are limited because the vast majority of allosteric modulatory sites are located at the interface between adjacent subunits or close to the ECD/TMD interface.

ECDs of all pLGICs are folded in a highly conserved immunoglobulin-like β-sandwich fold, composed of an inner and an outer β-sheet, stabilized through conserved hydrophobic residues [1]. The connecting loops, whose length and structure vary among pLGICs, are critical for the quaternary assembly of the receptor, binding the agonist and transducing its signal to the TMD. The TMD of each protomer that composes the receptor is made of four membrane-spanning helices named M1 to M4. The M2 helices form an ionotropic transmembrane pore and are thus the most important segments of the ion conduction pathway. The M1, M3 and M4 helices face the lipid bilayer and were shown to host lipid-binding sites in GLIC [7]. Similarly to the ECD, the connecting loops play a determinant role for channel function, such as the M2–3 loop that participates actively to signal transduction [1].

2.2. Crystallography of pLGICs binding sites: methodological aspects

Ligand-bound pLGIC structures have provided insight on the binding-sites of several important classes of agonists, allosteric modulators and inhibitors (Table 1). These structures were obtained either by growing crystals in the presence of the molecule of interest (co-crystallization) or by soaking *apo* crystals in a solution containing the molecule of interest. Other drugs, like ivermectine were added during detergent solubilization and remained stably bound to receptor after purification and crystallization [8]. Similarly, endogenous tightly bound native lipids that remained bound throughout the sample preparation process from the original source were also observed in the GLIC receptor [7]. However, high-throughput crystallography for drug discovery is not easily transferable to pLGICs because of the relatively low resolution at which their structures are usually solved. Furthermore, co-crystallization and soaking into a ligand-containing solution usually require additional manipulations of the crystals that can affect their diffraction limit. Indeed, ligand-bound structures of pLGICs were solved at resolutions ranging between 2.7 and 3.9 Å (Table 1). Assigning confidently a small ligand into the electron density is difficult at such resolutions and might lead to spurious results. This limitation can be overcome by making use of the anomalous scattering of certain atoms at or near their X-ray absorption edges to localize specifically these atoms in the electron density (Fig. 3A). This approach has been readily used in pLGICs; bromine and iodide have thus been used to identify monovalent anion-binding sites [8,17], cesium and rubidium to identify monovalent cation-binding sites [17,18], and barium to identify divalent cation-binding sites [19]. This method can also help to localize and orientate a ligand in the electron density, when substituted with derivatized-analogues that contain an anomalous scatterer. This approach proved to be particularly useful when localizing channel blockers [18], benzodiazepines [20], as well as more labile molecules for which, the binding energy is low, like alcohols and general anesthetics. Bromo-ethanol and bromoform have thus been used as surrogate to ethanol [21] and chloroform [21,22] (Fig. 3B).

2.3. Function

2.3.1. Gating (closed to open transition)

Available full-length structures of pLGICs solved in different conformations offered new opportunities to examine channel opening and closure at the atomic level. Indeed, GLIC [6,7] and Glu-Cl [8] display a very similar open-pore conformation, while ELIC displays a closed pore conformation [5,23]. Previous attempts to model the gating transition compared the GLIC or the GluCl open structures to the ELIC closed structure, assuming that ELIC is a good model for the resting form [24,25]. However, the low sequence identity between these structures does not allow disentangling sequence effects from functionally relevant conformational changes. In addition, it is not certain that ELIC represents the resting form, as there are other closed forms of the receptors known to exist, such as the desensitized form for instance. Actually the fact that some residual and unexplained electron density is present in ELIC orthosteric binding site might indicate that ELIC adopts a desensitized form in the crystal state. Among the currently existing X-ray structures, the GLIC prokaryotic proton-gated ion channel is probably the best structural model to study allosteric transitions in pLGICs, as its structure has been determined in 3 distinct forms. GLIC structure was initially solved at acidic pH in its open conformation [6,7], and later in a locally closed (LC) conformation displayed by different mutants [26,27].

Table 1
A selection of ligand-bound pLGIC X-ray structures, indicating the resolution of the diffraction data and the PDB code of the corresponding structure.

	PDBid	Protein	Ligand	Resolution	Reference
Agonists	2YOE	ELIC	GABA	3.9 Å	[20]
	3RIF	Glu-Cl	Glutamate	3.35 Å	[8]
Antagonists	3RQW	ELIC	Acetylcholine	2.9 Å	[43]
Benzodiazepines	2YOE	ELIC	Flurazepam	3.9 Å	[20]
	4A98	ELIC	Br-Flurazepam	3.6 Å	[20]
	4A97	ELIC	Zopiclone	3.34 Å	[20]
General anesthetics	3P50	GLIC	Propofol	3.3 Å	[58]
	3P4W	GLIC	Desflurane	3.1 Å	[58]
	4HFD	GLIC F14′A	Bromoform	3.1 Å	[21]
	4HFH	GLIC	Bromoform	2.65 Å	[21]
	3ZKR	ELIC	Bromoform	3.65 Å	[22]
Alcohols	4HFE	GLIC F14′A	Ethanol	2.8 Å	[21]
	4HFC	GLIC F14′A	2-bromo-ethanol	3.05 Å	[21]
Ivermectine	3RHW	Glu-Cl	ivermectine	3.1 Å	[8]
Channel blockers	2XQ5	GLIC	Tetra-ethyl-arsonium	3.5 Å	[18]
	2XQA	GLIC	Tetra-butyl-antimony	3.7 Å	[18]
	2XQ4	GLIC	Tetra-methyl-arsonium	3.6 Å	[18]
	2XQ3	GLIC	Bromo-lidocaine	3.5 Å	[18]
	3RI5	Glu-Cl	Picrotoxine	3.4 Å	[8]
Divalent	2YN6	ELIC	Ba++	3.3 Å	[19]
ions	2XQ8	GLIC	Zn++	3.6 Å	[18]

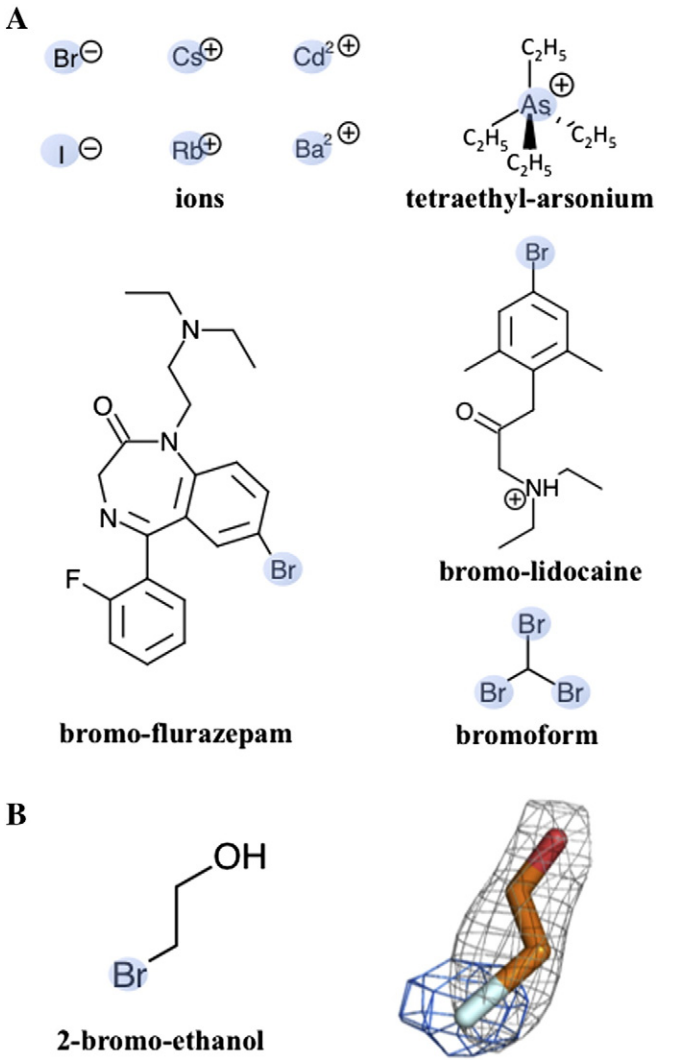

Fig. 3. A selection of ions and ligand analogues displaying anomalous diffusion properties that were used in order to confirm ligand-binding sites in pLGIC structures. (A) Chemical structures of the ligand analogues where the atoms displaying anomalous diffusion properties are highlighted in blue. (B) A representative example of the usefulness of the anomalous diffusion signal. The bromine-specific anomalous signal (shown as a blue mesh contoured at a level of 5σ) helps orient a molecule of 2-bromo-ethanol in the 2mFo-DFc electron density (shown as a gray mesh contoured at a level of 1σ).

The LC conformation shares most structural features with the open form but displays a closed pore as a result of a concerted bending of its M2 helices. Recently, it was demonstrated that the wild-type receptor can adopt the LC form as well and that the open and LC forms co-exist as discrete ones at acidic pH [28]. This is consistent with the possibility that the LC form might represent a late intermediate in the course of activation or a fast-desensitized form. Most importantly, the resting-state structure of GLIC has very recently been solved at neutral pH, thereby providing the two end-points of the gating mechanism in the same pLGIC [28].

Upon activation, a marked quaternary reorganization of the ECD is observed, involving both a twist and an inward tilt motion (the inverse of a blooming motion) of each ECD monomer (Fig. 4A), reshaping the ECD–ECD interface, especially around the agonist binding site. At the tertiary level, tightening of the inner and outer β-sheets profoundly remodels the ECD–TMD interfaces. Extensive interactions of the ECD with the M2–3 loop cause a revolving motion of this loop, accompanied by a concerted unbending of the M2 helices, which opens the pore. The motion of M2 helix and the M2–3 loop is stabilized by a kink of helix M1, both helices now interacting across adjacent subunits (Fig. 4A).

Interestingly, the conformation of the resting-state GLIC TMD is very different from what is observed in ELIC (Fig. 4B). In resting-state GLIC, the pore is closed due to a concerted bending of the upper part of the M2 helices of all five subunits, which detach from M3 and obstruct the pore by forming a packed bundle, along with a revolving motion of the M2–3 loop that is similar to what is observed in the LC conformation. In ELIC, helix M2 and helix M3 do not detach from each other, but remain associated (Fig. 4C). Mutagenesis and electrophysiology studies performed on GLIC are in very good agreement with the idea that the bending/unbending of M2 is a key structural determinant for gating. Crosslinking M2 and M3 in their upper parts as would be allowed in the open form results, as expected, in a marked gain-of-function phenotype [26]. Furthermore, studies that include the surface cysteine accessibility method [29], as well as directed spin labeling and electron paramagnetic resonance spectroscopy [30,31], revealed that activation involves an outward translational movement of the tip of M2 helices whereas the lower part of these helices remains relatively immobile. These observations suggest that the ELIC TMD is not in its resting-state conformation. Because no significant structural change is observed following agonist binding in ELIC structure, this ELIC crystal structure might instead represent a desensitized form [20]. In any case, these data document the inherent important plasticity of the TMD that is often erroneously described as a rigid and optimally packed helical bundle.

2.3.2. Permeation: ion transport

Abundant biochemical, biophysical and mutational data have demonstrated that the ion permeation pathway encompasses the full-length structure of pLGICs but that the transmembrane channel is the dominant determinant part of the molecule for ion permeation and selectivity [32–36]. In contrast to what is observed with the highly selective potassium channels exemplified by KcsA, pLGICs have been shown to screen for charges rather than for a specific radius [37,38]. The open ion-conducting structures of GLIC and GluCl show a highly conserved architecture of the pore. In its open form, the pore is funnel-shaped, characterized by a 12 Å large diameter in the extracellular part and a 5 Å narrow constriction in the cytoplasmic part, that has been shown to host the selectivity filter portion, based on permeation, mutational and computational studies [39,40]. Consistently, in both GLIC and GluCl structures, this hydrophilic region provides favorable binding sites for cations [17,18] and anions [8], respectively. While the molecular determinants of ion selectivity in pLGICs have been actively studied, the molecular mechanism of ion permeation remains poorly understood. Ion permeation implies complex mechanisms that involve protein residues, ions and water molecules that interact together dynamically and transiently as the ions flow down the channel. This kind of description is difficult to derive from existing structures given the limited structural resolution achieved by X-ray crystallography. Recently, the 2.4 Å resolution structure of GLIC allowed a more detailed insight into ion permeation, revealing for the first time the hydration geometry in the pore of a pLGIC [17] (Fig. 5A). Two water pentagons were observed at the level of two rings of hydroxylated residues (Ser 230 and Thr 226, Ser 6′ and Thr 2′ in prime notation, counting from the N-terminus of M2), with one Na^+ ion between them. The pentagon located at position 2′ is part of the hydration shell of a Na^+ ion, which afterwards can be accommodated further down by a ring of negatively charged glutamate residues. In contrast, the water pentagon situated at position 6′ is self-stabilized. This crystallographic description of pore hydration was complemented by molecular dynamics (MD) simulations in the hydrophobic part of the pore, where hydration cannot be studied by crystallography due to the presence of detergent molecules. In this region, the MD simulation-derived water density distribution reveals that water molecules are arranged in five layers, with two of them interacting directly with main-chain carbonyl oxygen atoms of the Ile 9′ and Ala 13′ rings of residues. The three remaining layers of water molecules do not interact directly with the protein but do interact instead

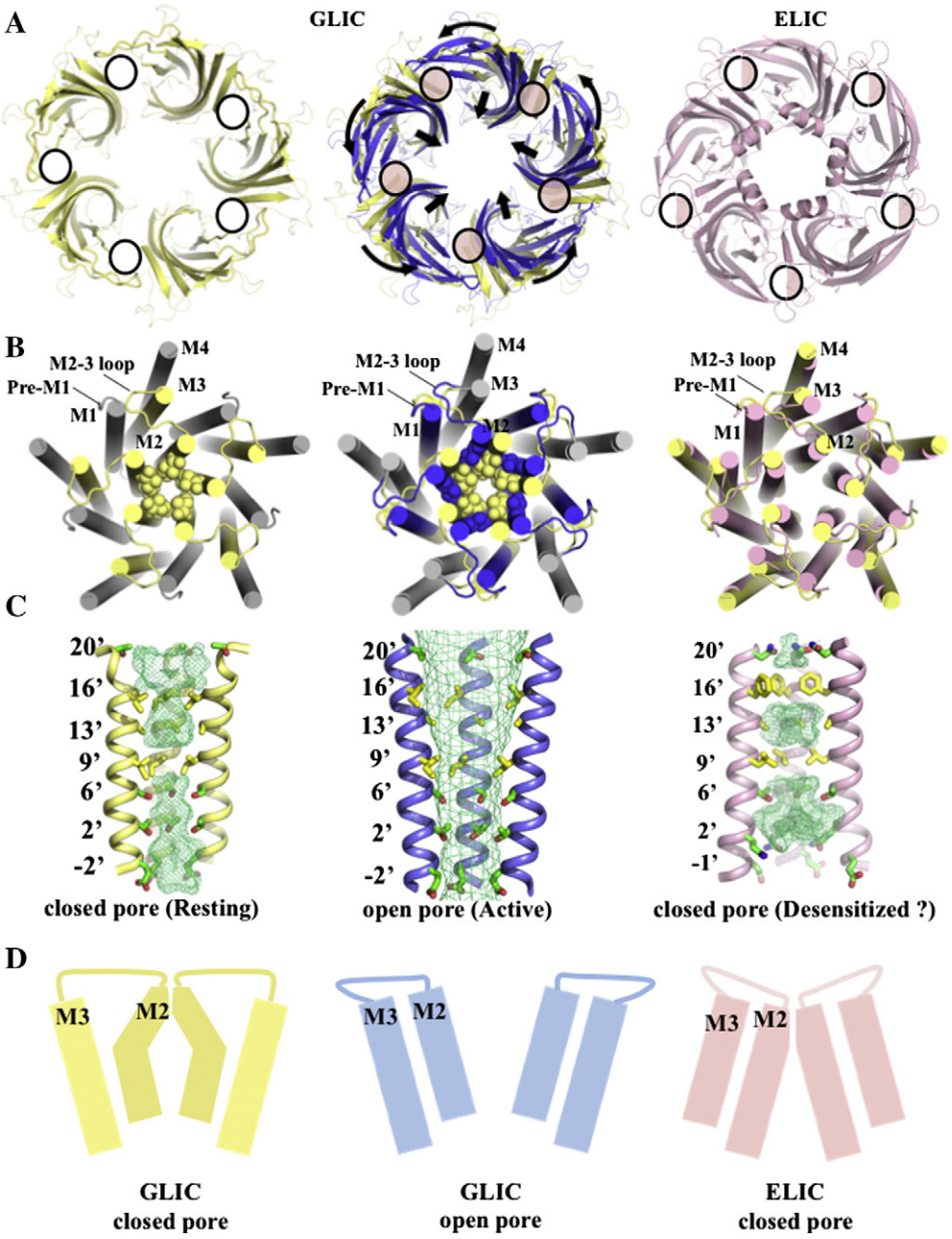

Fig. 4. Allosteric transitions in pLGICs. (A) Comparison of the GLIC resting-state (yellow), GLIC open (blue), and ELIC (pink) ECD structures. This cartoon representation is viewed from the top of ECD. A circle schematically represents the location of the interfacial orthosteric agonist-binding site. The agonist-binding sites are white when unbound and colored pink when bound. The quaternary twist and shrink motions are indicated with black arrows. (B) Superimposition of the GLIC resting-state (yellow), GLIC open (blue) and ELIC (pink) TMD structures. This cartoon representation is viewed from the top of TMD. The side chains of the 9′ and 13′ pore-ling residues are shown as spheres. (C) Enlarged views of the pore. The solvent-accessible region is shown by a green mesh. The side chains of the pore-lining residues are shown as sticks. Polar and hydrophobic residues are respectively colored in green and yellow. (D) The M2 and M3 helices in different conformations of GLIC open, GLIC closed and ELIC structures. The top panels display a schematic representation of the M2–M3 helices viewed from the side.

with the two principal layers of water molecules. This network of water molecules covers the edge of the hydrophobic half of the pore thus rendering it polar (Fig. 5A). Simulations that pulled a cation through the pore revealed that the water pentagons observed in the crystal actively contribute to ion translocation. Mutations of residue Ser 6′ to Val or Gly severely impaired channel conductance, due to a local dehydration of the 2′–9′ region [17]. Altogether, these data suggest that ordered water molecules contribute to lower the energy barriers encountered by the permeant ion when it crosses hydrophobic constrictions that are located along the selectivity filter. Electrostatic calculations also documented the roles of residues Ser 6′ and Thr 2′ during permeation, suggesting that their dynamical properties could facilitate ion transport by reducing electrostatic free energy barriers encountered by the ion during its translocation. Due to the strong sequence conservation of M2 pore-lining residues, these observations might be transferable to all the pLGIC channels.

3. The neurotransmitter orthosteric binding site

3.1. Conserved ligand recognition in AChBPs and pLGICs

The ligand-binding site is located at the ECD–ECD interface of the pentamer. For GLIC, the unique proton-gated channel of the family, it is clear that the proton-sensing domain is located in the ECD since a chimera construct made of the ECD of GLIC and the TMD of GlyR is functional and can be activated by protons [41]. Within the whole family,

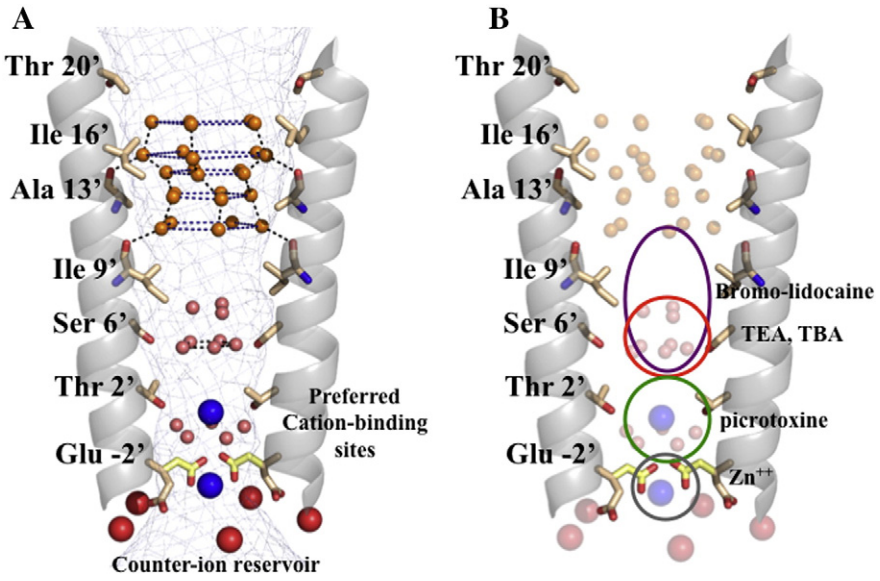

Fig. 5. The interior of GLIC open-pore structure. (A) Enlarged representation of the pore with the M2 helices shown as a cartoon. The side chains of the pore-lining residues are shown as sticks. Monovalent cation (blue) and anion (red) preferred binding sites in the GLIC open-pore structure are shown as spheres. Structurally ordered water molecules observed experimentally are shown in pink. Water binding-sites predicted by MD simulations are shown in orange. The alternative conformation that is adopted by Glu-2′ is also represented (yellow). (B) Distribution of channel blockers in the pore of pLGICs.

the best functionally and biochemically characterized orthosteric binding site is that of nAChRs. Early affinity labeling experiments followed by extensive mutagenesis experiments have located the acetylcholine (ACh) binding site at the interface between two neighboring subunits, with the contribution of three regions from a principal subunit, named loops A, B, and C and four regions of a complementary subunit named loops D, E, F and G [42] (Fig. 6A). Detailed insights into structural determinants of ligand-recognition have initially been provided by the high-resolution structures of soluble AChBPs that have been widely adopted as surrogates of the ECD of pLGICs. More than 60 X-ray structures of AChBPs have been determined in complex with a wide variety of ligands. More recently, two full-length structures of pLGICs, glutamate-bound GluCl and GABA-bound ELIC, have been solved in complex with neurotransmitters (Fig. 6A) [8,20,43]. Overall, an amazing similarity of structures emerges from comparison of the various modes of neurotransmitter binding. The primary/tertiary/quaternary amine moieties of the neurotransmitters interact with a conserved aromatic box of the orthosteric agonist binding site. For a recent review and a structural view of the conserved sequence motifs in the family, see [44].

3.2. Ligand activation in pLGICs

While AChBP provides a successful model to decipher the molecular determinants of ligand recognition in pLGICs, it is not a suitable model for tracking the conformational transitions that couple ligand-binding to channel activation. Indeed, all AChBP structures display the same conformation, with the notable exception of the “clamping” loop C that adapts to the size of the ligands. Loop C thus alternates between a fully contracted conformation observed for agonists, a fully extended conformation for peptide inhibitors and an intermediate conformation for non-peptide antagonists [45] (Fig. 6B). In contrast, the GLIC X-ray structures, solved in their open and resting-state closed conformations, provide a direct molecular basis for understanding the agonist-induced opening of the channel [28]. Comparing the unliganded and ligand-bound GLIC structures reveals that the orthosteric agonist-binding site is much more profoundly remodeled than in the AChBP agonist/antagonist bound structures (Fig. 6B). Rearrangement of the interface of the orthosteric pocket involves the sliding (translation) of the complementary subunit by about one interstrand distance resulting in a more extensive surface interaction between neighboring subunits in the open form. Consequently, activation of the receptor results in a marked contraction of the orthosteric pocket. Both the amplitude and the mechanism of this expansion/contraction in GLIC differ from what is usually described in AChBP structures, especially because there is no quaternary rearrangement in the latter structures, whereas there is a profound one in GLIC (Fig. 6B).

In addition, this study also revealed a functionally important feature of the unliganded resting-state form of the receptor that displays a much larger conformational basin than the open form does [28]. In contrast to the open form, the GLIC resting state structure fluctuates between an ensemble of closed conformations that differ from each other mainly at the level of the ECD. This population of receptors in a resting state could thus adapt to much more structurally diverse antagonists than the open state can bind agonists, in line with the fact that antagonists cover a much wider chemical space than agonists.

4. Modulation through allosteric sites in the ECD

Agonist-response is modulated by structurally diverse molecules targeting topographically distinct sites in the ECD. First we will describe how the ELIC structural model has permitted to identify several binding sites for benzodiazepines and divalent cations that allosterically modulate agonist-response. Finally, we will compare recently identified ion binding sites in GLIC with those identified in ELIC.

4.1. Benzodiazepines

pLGICs are allosterically modulated by benzodiazepines, a class of widely prescribed clinical drugs that display anxiolytic, anticonvulsant, muscle relaxant and sedative–hypnotic effects [46]. Benzodiazepines have been shown to modulate ELIC with effects comparable to those seen on $GABA_A$ receptors, which are the principal target for benzodiazepines among the pLGIC family [20]. Co-crystal structures of ELIC with flurazepam indicate that benzodiazepines, depending on their concentration, occupy two distinct sites in ELIC (Fig. 7A). Indeed, benzodiazepine has been shown to occupy an intersubunit site that partially overlaps agonist-binding orthosteric site, as well as an intrasubunit

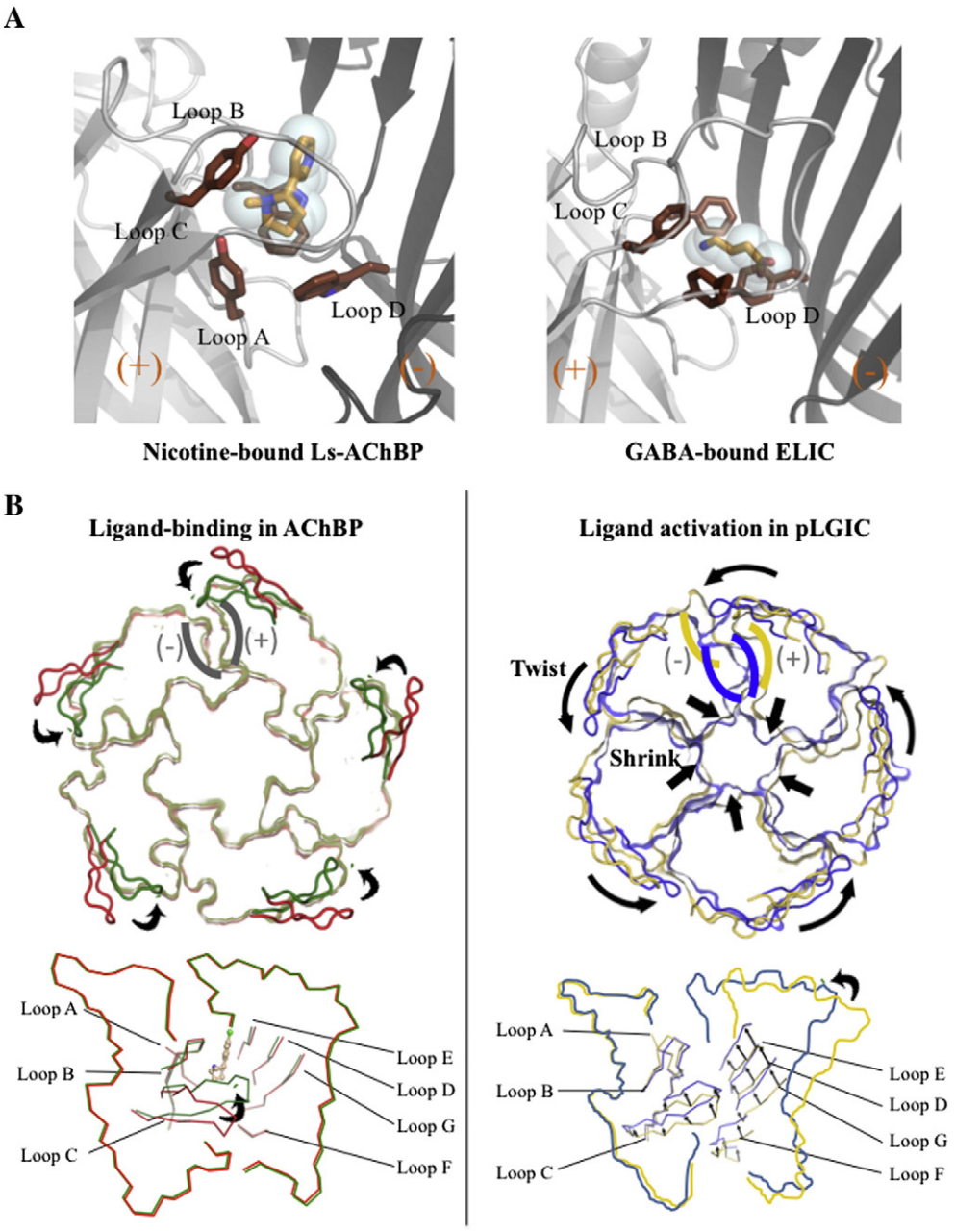

Fig. 6. Structural basis for ligand binding in AChBP versus ligand-activation in pLGICs. (A) Conserved cation–π interactions in AChBPs and pLGICs. (Left panel) Nicotine in agonist binding pocket of Ls AChBP (1UW6). (Right panel) GABA in the agonist binding pocket of ELIC (2YOE). The principal side of the ligand binding site is displayed in light gray; the complementary side in dark gray. The side chains of the residues that contribute to cation–π interactions are indicated in brown. (B) (Left panel) Comparison of the epibatidine-bound (agonist; 2BYQ) and α-conotoxin bound (antagonist; 2BYP) structures of the *Aplysia californica* AChBP viewed from the top (colored in green and red, respectively). Curved lines illustrate the principal (+) and complementary (−) sides of the orthosteric site. Loop C are shown as tubes. (Right panel) Same view, but showing the comparison between the open and resting state GLIC structures (colored in blue and yellow, respectively). The quaternary twist and shrinking motions are indicated with black arrows. Bottom panels show enlarged views of the orthosteric agonist-binding site at the interface between neighboring subunits.

site facing the channel vestibule. The latter is localized at the same height as the GABA-recognition site but lies opposite the inner β-sheet of the neighboring agonist-binding site. In consequence, this site is ideally positioned to allosterically modulate GABA response (Fig. 7A and B) [20]. Mutagenesis experiments combined to electrophysiology recordings revealed that these two sites are associated with opposite modulatory effects; the intrasubunit and intersubunit sites are respectively responsible for potentiating effects at low concentration of benzodiazepines, and the inhibitory effects at higher concentrations [20]. While the intrasubunit site appears to be novel, the intersubunit site matches with a previously identified benzodiazepine low-affinity inhibitory site in eukaryotic $GABA_A$ receptor [47].

4.2. Divalent ion modulatory site

The modulation of pLGICs by divalent cations, such as Ca^{++} and Zn^{++}, is thought to play an important regulation role in a physiological context [2]. For instance, millimolar concentrations of Ca^{++} potentiate channel activation of nAChRs [48,49] and inhibit those of 5HT3Rs [50], while Zn^{++} can either inhibit or potentiate the agonist response, depending on its concentration and the type of pLGIC [51,52]. The successive occupation of binding sites of different affinities is thought to cause these opposing effects. Recently, divalent ions like Ca^{++} were shown to inhibit channel activation on ELIC [19]. Combining a crystal structure of the receptor solved in complex with Ba^{++} and mutagenesis

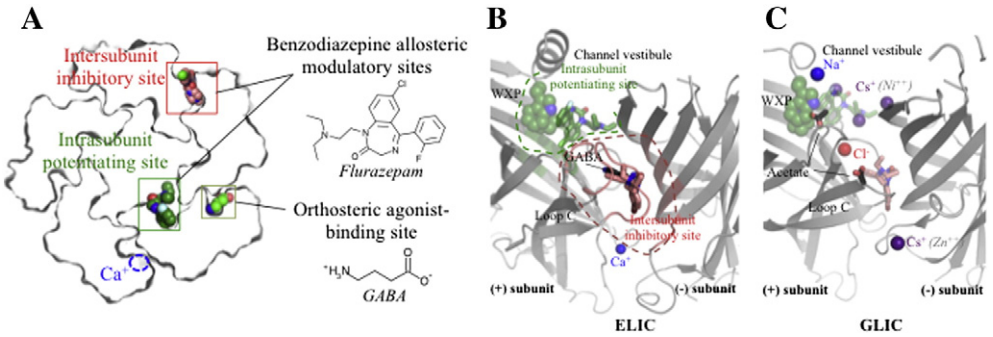

Fig. 7. Allosteric modulatory sites in the ECD. (A) Surface representation of ELIC ECD viewed from the top showing benzodiazepine-binding at the intrasubunit (green) and intersubunit (pink) modulatory sites. Ligands are shown as spheres. A blue circle schematically represents the divalent cation modulatory site located lower than the orthosteric site, at the level of Loop F. (B) Cartoon representation showing an enlarged picture of the same ECD modulatory sites in ELIC but viewed from the side. Ligands and ions are shown as sticks and spheres, respectively. (C) Same view than in (B) but illustrating the multiple ion-binding sites seen in GLIC that partially overlap with the benzodiazepine-binding sites observed in ELIC.

experiments has identified the site responsible for divalent inhibition in ELIC [19]. It is located at the outer rim of the ECD, at the level of the interface between subunits, but at a site topographically distinct from the agonist-binding site (Fig. 7B).

4.3. Other ion binding sites in GLIC

A recent 2.4 Å structure of GLIC, combined with the systematic use of anomalous diffraction data collected on crystals soaked with Br^-, Cs^+ and Rb^+ allowed the assignment of several distinct ion-binding sites in the vestibule-edge region of the ECD [17]. These ion-binding sites are located within two highly conserved regions of the ECD that have been shown to host allosteric modulation sites in other pLGICs.

The first one is situated at the vestibule edge, next to the Trp-X-Pro canonical motif. A Na^+, a Cl^-, as well as an acetate molecule present in the crystallization solution bind to this region (Fig. 7C). These sites were also observed in the crystal structure of the isolated GLIC ECD [13]. In addition, ten Cs^+ were identified in the lumen of the vestibule encircled by two rings of acidic residues (Asp 86 and Asp 88). These Cs^+ are replaced by one divalent Zn^{++} [18] or Ni^{++} [28] cations in other GLIC crystal structures. These ion binding sites, whose physiological implications remain to be explored, partly overlap the intrasubunit benzodiazepine-binding site seen in ELIC [20] (Fig. 7C), as well as a Zn^{++} modulation site identified in GlyR [52,53]. The second region is located at the interface between adjacent subunits, next to the region that hosts the agonist-orthosteric binding site in other pLGICs. An acetate molecule and a Cs^+ binding-site (that can be replaced by a divalent Zn^{++} [18]) have been observed in the GLIC ECD at the same place (Fig. 7C). The acetate binding site overlaps with the inhibitory intersubunit benzodiazepine site in ELIC and is thought to be an inhibitory allosteric site in GLIC that accounts for the micromolar range inhibition of caffeic acid on GLIC [54]. As a conclusion, pLGIC displays multiple ion modulatory sites, which are highly selective from one subtype to the other. These sites are still largely unexplored and constitute plausible targets for drug design.

5. Modulation through allosteric sites in the TMD

The TMD is the target of a wide variety of allosteric modulators including ethanol and other alcohols, general anesthetics (GAs), lipids and open-channel pore blockers.

5.1. General anesthetics and alcohols

GA and alcohols exert many of their actions on the central nervous system by binding to and modulating pLGICs. Modulation sites for alcohols and GA have been characterized experimentally by combining photolabeling studies [55], site-directed mutagenesis and electrophysiology [56,57]. Three principal GAs and alcohol binding-sites have thus been identified within the TMD: (i) an intrasubunit site located within the M1–4 helix bundle, (ii) an intersubunit site and (iii) a channel-site situated at the extracellular end of the pore. Consistently, pLGICs crystal structures solved in complex with GA and alcohols have covered all three binding sites (Fig. 8). Propofol, desfluran and bromoform were shown to bind to an intrasubunit site in GLIC [21,58]. Ethanol, 2-bromo-ethanol and bromoform were found to bind to an intersubunit cavity in the structure of a GLIC ethanol-sensitive variant [21]. In ELIC, bromoform was recently shown to bind both in the ion channel and in an intersubunit site [22]. GAs and alcohols binding to these distinct sites produce opposing effects on channel function. In GLIC, the intersubunit cavity hosts an allosteric potentiating site [21], while the intrasubunit hosts an allosteric inhibitory site [58]. Furthermore, a single-mutation in the intersubunit site turns desflurane, chloroform and bromoform from inhibitors to potentiators, suggesting that their hosting sites are competing allosteric sites [21,59]. Modulation is thus the net effect of competitive binding between the intersubunit potentiating site and an intrasubunit inhibitory site. This multiple-site model of allosteric regulation is also relevant when studying mammalian pLGICs, as GA and alcohols produce opposing effects depending on the pLGIC subtype. Indeed, they potentiate function of most inhibitory $GABA_A$Rs and GlyRs, while they inhibit most of the excitatory nAChRs [60]. These distinct binding sites will now be reviewed in light of recent crystal structures and functional experiments.

5.1.1. Intrasubunit GA modulatory site

Similarly to nAChRs, GLIC is inhibited by clinically relevant concentrations of GAs [61], long chain alcohols and is weakly sensitive to potentiation by ethanol [62]. Co-crystal structures of GLIC bound to propofol, desflurane [58] and bromoform [21] reveal that GAs bind to overlapping sites within a cavity located in the upper part of the TMD at the center of the bundle of alpha-helices of each subunit (Fig. 8). This tunnel-shaped cavity is accessible from the lipid bilayer and penetrates in the interior of the subunit towards the ECD–TMD interface. The bromoform-bound GLIC structure revealed that bromoform adopts three distinct poses within the cavity, suggesting that GAs display a relatively high mobility in this cavity [21], a feature consistent with results of MD, which show that Van der Waals interactions are the main contributor to the binding energy [58]. A recent study on the *Torpedo marmorata* nAChR using a photoreactive propofol analogue and radioligand competition assays revealed for the first time a single intrasubunit propofol-binding site and suggests that the cavity of GLIC is a strong candidate for the nAChR allosteric inhibitory site [63]. Yet, the molecular mechanism of inhibition induced by GAs upon binding to this intrasubunit site remains an open question as existing GLIC structures of the receptor bound to GA all display an open conformation while GAs should promote a closed state of the receptor. However, the recent resting-state structure of GLIC reveals that the intrasubunit cavity is essentially unchanged [28]. GAs binding to the TMD thus do not

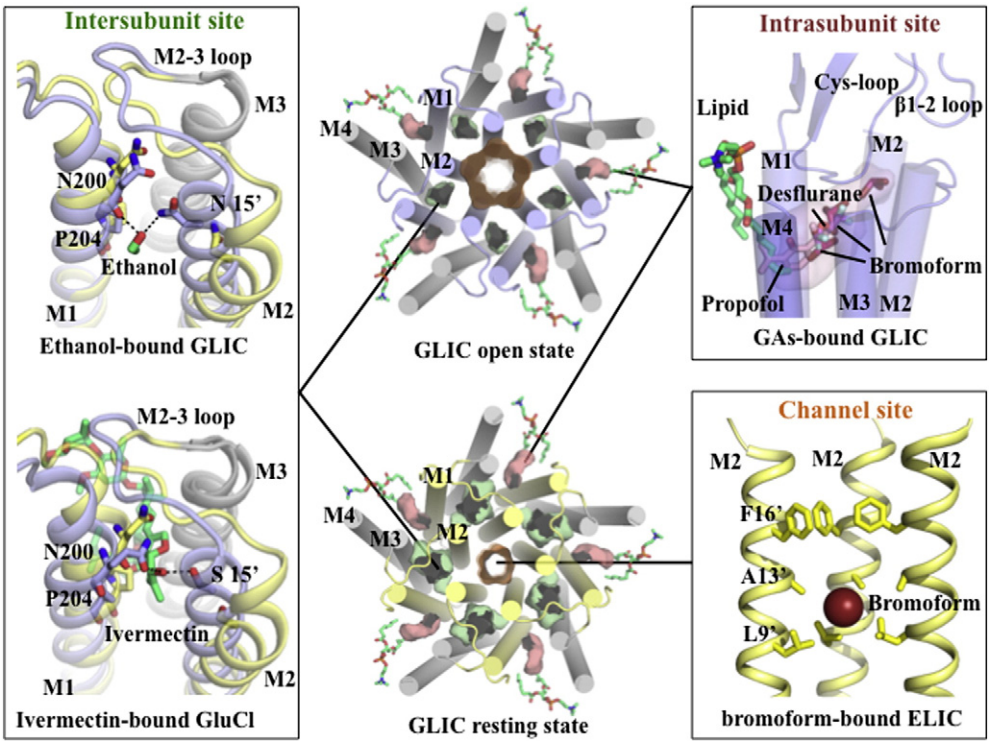

Fig. 8. Transmembrane pharmacology in pLGIC. (Central panels) TMD of the open state and resting state GLIC structures viewed from the top. The inter subunit, intrasubunit and channel modulatory sites are shown in green, pink and orange, respectively. (Left panels) Enlarged view of the ethanol-bound GLIC and ivermectine-bound GluCl structures (in blue) superimposed onto the GLIC resting-state closed structure (yellow). The receptors are represented as cartoons; the side chains and the ligands as sticks. In both cases, ligand binding promotes the open form of the receptor by favoring the quaternary interaction between the tip of M1 and M2. (Top-right panel) Enlarged view of the intrasubunit site showing the superimposition of the propofol (light pink), desflurane (magenta) and bromoform (red) binding sites. The phospholipid that faces the transmembrane entrance of the cavity is also represented (green). (Bottom-right panel) The bromoform pore binding-site as observed in ELIC.

appear to be preferred in the GLIC resting state structure. Consistently, a recent study measuring the rate of modification by sulfhydryl-reactive reagents of cysteines introduced in the intrasubunit cavity in the presence or absence of propofol suggested that propofol stabilizes a distinct closed-state [63,64]. This closed-state could be the desensitized state, as suggested by a recent radiolabeled-ligand competition study on the *Torpedo marmorata* nAChR, where propofol-binding was shown to stabilize the desensitized state of the channel and the mode of binding was found to involve a cavity similar to the intrasubunit one found in GLIC [63].

5.1.2. Intersubunit GA modulatory site

A recent structure–function study on the GLIC F14′A ethanol-sensitized variant shed light on the molecular basis of potentiation by GAs and alcohols [21]. The absence of a phenyl ring in F14′A is sufficient to inverse the pharmacology of GLIC for GAs and alcohols. Indeed, while the pharmacology of the GLIC wild-type receptor for GAs and alcohols is similar to that of the nAChR (see above), the pharmacology of GLIC F14′A variant resembles that of the GlyRs and $GABA_A$Rs; it is potentiated both by GAs [21,59] and by ethanol at pharmacologically relevant concentrations (20 mM) [62]. In the mutant structure, the inter-subunit cavity is expanded, penetrating one to two registers deeper into the membrane spanning region. The F14′A GLIC ethanol-sensitive variant was solved in the presence of ethanol, 2-bromo-ethanol and bromoform, which all bind in the expanded transmembrane cavity thus providing the first experimental description of ethanol bound to a pLGIC target [21] (Fig. 8). Co-crystallization with alcohols and GAs appeared to stabilize the GLIC F14′A structure, resulting in decreased B-factors in the transmembrane domain, consistent with a macroscopic role for these drugs in stabilizing the open state of the channel [21].

Site-directed mutagenesis of residues bordering the ethanol-binding pocket revealed that potentiation by ethanol relies on hydrophilic and hydrophobic contacts, while potentiation by bromoform is essentially mediated by hydrophobic contacts. Interestingly, a wealth of converging experimental evidences show that this cavity is conserved in human ethanol-sensitive pLGICs. The cavity identified in GLIC is consistent with specific amino-acid interactions previously shown to control allosteric modulation of both GlyRs [65] and $GABA_A$Rs [66]. In addition, photo-labeling experiments localized an etomidate [67] and a propofol [68] photo-reactive analogue binding-site at the transmembrane interfaces between subunits, overlapping the crystal structure site. Furthermore, the knowledge of the open and resting state structures of GLIC provides a structural rationale to the potentiating mechanism of ethanol. This potentiating site identified in the GLIC variant structure occupies a pivotal location for gating. It overlaps with a quaternary interaction between the M2 helix of one chain and the tip of the M1 helix of the neighboring chain, a critical interaction for the ligand induced opening of the pore [28]. Consistent with its potentiating effect, ethanol favors this quaternary interaction by simultaneously contacting the side chains of Asn 15′ and Glu 19′ in M2 and the main chain carbonyl oxygen of residue Asn 200 in the tip of M1 (Fig. 6). Disrupting the interaction between ethanol and the side chain of residue Asn 15′ abolishes potentiation by ethanol [21]. Interestingly, this residue aligns with the most critical residue for alcohol potentiation of $GABA_A$Rs and GlyRs [69].

In addition, this potentiating site overlaps with the binding-site of ivermectin, which activates α_1-Glu-Cl receptors [8]. Similarly to ethanol in GLIC, ivermectin makes a critical hydrophilic contact with residue 15′ (a serine in the case of Glu-Cl) (Fig. 8). Disruption of this contact was shown to abolish action of ivermectin in $GABA_A$R [70] and GlyR [71]. However, ivermectin binding also produces a local displacement of the M1 and M3 transmembrane helices, suggesting slightly different mechanisms for stabilizing the open state of pLGICs by the small (46 Da) ethanol and the relatively large (875 Da) ivermectin. In fact,

one could hypothesize that ivermectin, through binding to this site, bypasses the ECD–TMD allosteric coupling regulated by the binding of a neurotransmitter to the orthosteric binding site. Overall, these data suggest that the transmembrane intersubunit interface hosts a potentiating modulatory site conserved from prokaryotic to eukaryotic pLGICs.

5.1.3. Ion channel GA binding site

Electrophysiology, mutagenesis and photoaffinity labeling and simulation have suggested that the hydrophobic region made of the top-half of the transmembrane pore might constitute a pharmacological relevant-site for pore block inhibition by GAs and alcohols. Recently, the co-crystal structure of ELIC with bromoform provided the first experimental evidence that anesthetics such as bromoform and chloroform can bind in the hydrophobic portion of the pore [22] (Fig. 8), in line with computational studies that had previously revealed binding of isoflurane and propofol to the pore of GLIC [72]. GAs could thus inhibit cationic pLGICs, whose pore is more hydrophobic than anionic pLGIC, by binding preferentially the transmembrane pore in its closed conformation rather than its open form.

5.2. Lipids

Lipids, free fatty acids and steroids (cholesterol) are known to allosterically modulate pLGICs [73,74]. The open GLIC structure shows electron density at the protein/lipid bilayer interface consistent with the presence of three lipid molecules per subunit [7]. Because no exogenous lipids were added during the detergent solubilization and protein purification, the observed molecules are most likely endogenous tightly bound native lipids that were carried along throughout the sample preparation process from the original source. The intrasubunit phospholipid-binding site identified in the upper part of the TMD occupies a critical position for gating as it contacts simultaneously the tip of M1 and the Cys-loop via its polar head, two pivotal regions for the receptor's activation [28] (Fig. 8). Structural observations suggested that this phospholipid-binding site could be one of the molecular determinants that stabilize the open form of the channel: it is displaced upon propofol binding [58] and destabilized in the GLIC locally closed form as a consequence of rearrangement of side chain conformations in this region of the receptor [26]. A very recent study has further documented the structural sensitivity of the GLIC receptor to its membrane environment [75]. These binding sites thus constitute strong candidates for allosteric modulation by lipids.

5.3. Channel blockers

Open-channel pore blockers are non-competitive inhibitors that interfere with ion conduction when the channel is open but do not interfere with the agonist-mediated activation [76]. The open-channel pore blockers encompass a structurally diverse class of molecules such as picrotoxine, tetraethylammonium (TEA), local anesthetics (e.g. chlorpromazine, lidocaine and quinacrine), and some divalent transition metals. They often combine a positively charged amino group, corresponding to the net charge of the permeating ions in cationic-selective channels with a hydrophobic aromatic group and are thus too bulky to pass the channel constriction. Extensive affinity labeling with *Torpedo marmorata* nAChRs identified the bundle of M2 segments as constituting the binding sites for channel blockers [77,78]. Recently, X-ray structures of GLIC solved in the presence of a variety of open-pore channel blockers [18] and the picrotoxin-bound structure of Glu-Cl [8] clarify where channel blockers bind within the pore. Channel blockers bind at different locations between positions −2′ and 9′ within the pore and overlap with critical regions identified for ion transport (Fig. 5B). Quaternary amines and lidocaine both bind within the transmembrane field near the 9′ hydrophobic constriction and the 6′ conserved ring of polar residues that was shown to host a water pentagon. In GluCl, picrotoxin binds to the cytosolic part of the pore between the −2′ and 2′ rings of residues that host preferential cation binding sites in Glu-Cl and GLIC. The divalent transition metal ion Cd^{2+} inhibits ions conduction by binding to the narrow intracellular end of the pore at a position that matches a preferred binding site of the permeant cation. Due to the sequence conservation in this region of the pore, these molecular mechanisms of channel block are likely to be conserved within the pLGIC family.

6. An attempt to classify pharmacological sites in pLGICs

Pharmacological sites occupy topographically distinct locations in pLGICs distributed from the ECD to the TMD. In order to provide an integrated picture of how these modulatory sites are related to each other as well as to regions critical for channel function, we propose a simple geometric visualization using icosahedrons. Icosahedrons are regular polyhedrons with 20 equilateral triangular faces and 12 vertices and using them in the context of pLGICs is a natural idea given their deep relationship to five-fold symmetry. It is interesting to note that the communications between each vertex of a regular icosahedron are special as the derived graph is distance-regular and symmetric and is Hamiltonian. This may be favorable for establishing an efficient allosteric communication between the sites located at the vertices. In the context of pLGICs, we propose to call them Nicosahedrons (for Nico-tinic receptor-like icosahedron). When they are aligned on the receptor's 5-fold symmetry axis, they display (from the ECD to the TMD) an upper apical vertex, five upper vertices arranged in a pentagon, five lower vertices arranged in another (lower) pentagon and a lower apical vertex (Fig. 9A).

In the ECD, aligning the five vertices of the upper-pentagon onto the agonist binding-sites and adjusting its size generates a first icosahedron, named Nicosahedron-ECD-inter. Obviously, the distance between neighboring agonist binding-sites determines the length of all its sides. Surprisingly, the five lower-pentagon vertices are located between pre-M1 and the apex of the Cys-loop, a key position in the coupling of the ECD to the TMD, while the lower apical vertex overlaps with the 9′ ring of residues that hosts the channel gate and is the binding site of several open-pore channel blockers (Fig. 9B). Aligning the five vertices of the upper-pentagon onto the intrasubunit benzodiazepine binding-site (by adjusting both its radius and its rotation angle around the five-fold axis) generates a second icosahedron, named Nicosahedron-ECD-intra. Interestingly, the five lower-pentagon vertices of this new icosahedron match with the ELIC Ca^{++}-binding site (Fig. 9B).

In the TMD, two additional icosahedrons named Nicosahedron-TMD-inter and Nicosahedron-TMD-intra can be generated by respectively superimposing the lower pentagon vertices onto the inter- and intrasubunit GAs modulation sites. The upper pentagon vertices of the Nicosahedron-TMD-intra are found again to match the ELIC Ca^{++}-binding site and are thus somehow connected to the Nicosahedron-ECD-intra, while its lower apical vertex overlaps with the selectivity filter and the picrotoxin-binding site. On the other hand, the upper pentagon vertices of the Nicosahedron-TMD-inter match with the C-terminal part of M2 and the beginning of the M2–M3 loop a key actor on the signal transduction pathway.

In total, just four Nicosahedrons (two of which are clearly linked) successfully permitted the clustering of all the modulatory sites discussed in this review as well as the most critical regions known to be involved in gating. Furthermore, they are readily transferable to all pLGICs. The growing numbers of novel ligand-bound pLGIC crystal structures from various species urge the need for classifying the newly discovered binding sites. In this purpose, Nicosahedrons might become a helpful nomenclature in the pLGIC field for unifying the many topographically distinct pharmacological sites displayed by these receptors.

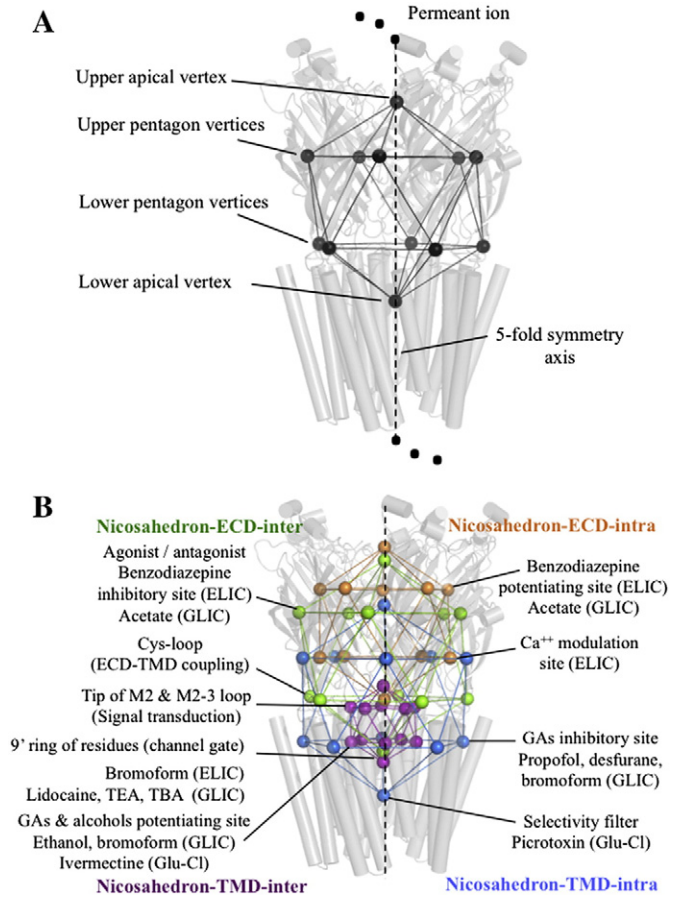

Fig. 9. Nicosahedrons: a geometric tool for classifying pharmacological sites in pLGICs. (A) The archetypical structure of an icosahedron. (B) Superimposition of the four (N)icosahedrons that allow clustering of all the pharmacological sites observed by X-ray crystallography in pLGICs. The upper-pentagon-vertices of the Nicosahedron-ECD-inter (in green) and the Nicosahedron-ECD-intra (in orange) were respectively aligned and size adjusted on the orthosteric agonist binding and on the benzodiazepine potentiating site observed in ELIC. The Nicosahedron-TMD-inter (in purple) and Nicosahedron-TMD-intra (in blue) were generated by superimposing the lower pentagon vertices onto the inter- and intrasubunit GAs modulation sites in the TMD, respectively.

7. Conclusion and perspectives

Decades of pharmacological research have revealed that pLGICs are the targets of structurally diverse allosteric modulators that bind to topographically distinct sites. Modulation by GAs, alcohols, benzodiazepines and metal ions is thus the net effect of several competitive allosteric-binding sites. The expanding field of structural molecular biology of pLGICs not only rationalizes the mechanism of existing-drugs but also identifies novel drug binding sites, such as, for instance, the intrasubunit benzodiazepine binding-site identified in ELIC and the acetate binding sites found in GLIC.

In concluding this review we wish to stress that the structures of all allosteric states of these receptors must be considered in order to understand the mechanisms of allosteric modulators that selectively favor one conformation of the receptor versus the other. For example, ethanol and GAs were shown to potentiate GLIC currents upon binding to an intersubunit cavity embedded within the TMD. Having at our disposal several GLIC structures solved in distinct conformations (open, resting and locally closed) helped rationalize the potentiation mechanism by GAs and alcohols. However, the quest for the structural determination of novel conformations continues. In addition to a basal closed-state and an active open-state, pLGICs exist in several desensitized states, as well as several intermediate states whose structure is still unknown. Obviously, it would be most interesting to determine the crystal structure of a desensitized form of GLIC.

One might ask to what extent conclusions derived from prokaryotic receptors are readily extendable to human pLGICs, which are the true pharmacological targets. Despite a low sequence identity, prokaryotic and eukaryotic pLGICs display a remarkably conserved architecture. The activation mechanism that was deduced from the GLIC open and resting state structures might very well be extendable to human pLGICs given that the most critical regions involved in gating (Cys-loop, M2–3 loop, pre-M1) are well conserved and include several canonical residues. Consistent with this hypothesis, GA, alcohol, benzodiazepine and channel blocker binding sites that were identified from prokaryotic pLGIC ligand-bound X-ray structures are in very good agreement with former biochemical and functional data collected on human pLGICs. Nevertheless, the structure determination of hetero-pentameric full-length mammalian pLGICs at atomic resolution is a goal of considerable importance that remains very challenging. Indeed, X-ray structures of integral pLGIC are limited, so far, to prokaryotic and lower-eukaryotic homo-pentameric receptors. The atomic structure determination of the additional cytoplasmic domain, which is absent in prokaryotic pLGICs but present in all eukaryotic pLGIC, will probably open the way to a largely unexplored territory for drug design. Expanding the detailed structural knowledge of pLGICs allosteric modulation paves the way towards a structure-based approach for drug-design that should impact positively the treatment of neurological disorders in the near future.

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