A tale of ligands big and small: an update on how pentameric ligand-gated ion channels interact with agonists and proteins

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Abstract

Pentameric ligand-gated ion channels (pLGICs, also known as Cys-loop receptors) are a large family of ion channels expressed in all *Bilateria* and in several groups of bacteria and archaea. They are activated by small-molecule neurotransmitters to mediate fast transmission at many central and peripheral nervous system synapses and are the target of several drugs and insecticides. Here we review recent advances in the field, focussing on new insights on the structure of the agonist-binding site and on newly discovered protein-protein interactions involving pLGICs.

Highlights

- Atomic basis for ligand binding and exclusion in $\alpha4\beta2$ acetylcholine receptors
- Identification of new toxins modulators and evolution of toxin resistance
- Proposed chaperones and auxiliary subunits for pentameric ligand-gated ion channels
- Pharmacological targeting of protein-protein interactions

Keywords

Ion channel; ligand binding; membrane protein function; chaperone; auxiliary subunit; toxin

Introduction

In man, pentameric ligand-gated ion channels include both cation-permeable channels, such as nicotinic acetylcholine receptors (nAChRs) and serotonin receptors (5-HT_3Rs), and anion-permeable channels, such as γ-aminobutyric acid type A ($GABA_A$Rs) and glycine receptors (GlyRs).

nAChRs mediate synaptic excitation in the periphery, at the neuromuscular junction between motor neurones and skeletal muscle (muscle nAChRs) and at autonomic ganglion synapses (neuronal nAChRs). In the mammalian central nervous system (CNS), all fast inhibitory transmission is mediated by pLGICs, either by one of the many subtypes of $GABA_A$Rs, or by one of the (relatively few) subtypes of GlyRs. Many subunits are available to form each pLGIC class, especially for $GABA_A$Rs and neuronal nAChRs, and in the CNS many different subunit combinations are expressed in precise spatio-temporal patterns that change during development. Mutations in pLGICs can cause inherited human channelopathies, ranging from congenital myasthenias for muscle nAChRs [1] to various forms of epilepsy (most commonly linked to $GABA_A$R malfunction [2]), to the specific neurological syndrome of startle disease in the case of GlyR mutations. These monogenic inherited channelopathies are relatively rare as a cause of human disease, but their phenotype underscores the physiological importance of pLGICs, and the location of loss-of-function mutations provides useful clues to the functional anatomy of these proteins. Given the physiological importance of pLGICs, it is not surprising that they are the target of many drugs: neuromuscular blockers and nicotine target nAChRs, propofol and benzodiazepines enhance the function of $GABA_A$Rs and the antiemetic ondansetron blocks 5-HT_3Rs. Antiparasitic drugs, such as ivermectin, and insecticides (fipronil, neo-nicotinoids) exploit pharmacological differences between mammalian and invertebrate pLGICs.

The structure of the pLGIC binding site: one heteromer and more homomers…

A pLGIC is formed by five subunits arranged around a central ion-conducting pore. Each subunit has an N-terminal extracellular domain (ECD) consisting of two β sheets sandwiched together. The ECD is connected to the transmembrane domain (TMD), which comprises four membrane-spanning helices, the second of which lines the channel pore. Between the third and fourth transmembrane helices, a linker of variable length forms a cytoplasmic domain, with portals for ion flow and sites for interaction with the cytoskeleton. The binding sites for the neurotransmitter/agonist are at the interface between the ECDs of adjacent subunits, where the anticlockwise subunit contributes the principal (+) side with its three loops A, B and C [3], and the clockwise subunit the complementary (-) side, with loops D, E and F (in reality two β strands and one loop).

This picture first emerged from cryo-electron microscopy (cryo-EM) data of *Torpedo* nAChRs [4] refined with high-resolution X-ray data of a soluble homologue of the nAChR ECD, the ACh binding protein [5]. Several crystal structures followed, both of homologous prokaryotic channels (GLIC and ELIC [6-8]) and of eukaryotic pLGICs: *C. elegans* glutamate-gated chloride channel (GluCl) [9], β3 $GABA_A$R [10], 5-HT_3R [11] and α3 GlyR [12]. In 2015, the first application of single particle cryo-EM to a pLGIC, the zebrafish GlyR, broke new ground in the field of pLGIC structural studies [13**]. Cryo-EM will make it easier to obtain structural information for different states of the same channel type. This is important for pLGICs, because sequence homology across different pLGICs is fairly low. The largest range of structurally determined conformations is currently available for GlyR and for GLIC. Structures

of such different conformations provide key start and endpoints for *in silico* molecular dynamics simulations [14**]. The robustness of GLIC as a protein has allowed it to be probed in spin-labelling/EPR spectroscopy [15-18] and with fluorescence quenching [19**]. For recent reviews that cover pLGIC function in greater detail see references [20-25].

2016 has seen the publication of the first X-ray crystal structure of a heteromeric pLGIC, the human $\alpha4\beta2$ neuronal nAChR [26**], the main CNS target of the addictive action of nicotine [27;28]. This channel poses a particular challenge, because it assembles in two stoichiometric forms, with either two or three copies of the α subunit in the pentamer, a peculiarity shared by the peripheral $\alpha3\beta4$ neuronal nAChR [29]. The two forms of the $\alpha4\beta2$ receptor differ in sensitivity to agonists, conductance and calcium permeability [30;31]. Expression can be driven towards either of the two forms by manipulating α:β transfection ratios [32], by employing fully concatenated pentameric constructs [33;34] or by exposing the expression system to nicotine [35].

Morales-Perez and co-workers purified a single stoichiometric form of the $\alpha4\beta2$ receptor, the one that contains two $\alpha4$ subunits (termed 2-α). This was done by monitoring the stoichiometry of expressed receptors with fluorescence tags and optimizing the ratio of α:β carrying baculovirus to be transfected into large scale HEK293 cultures, which were kept in the presence of nicotine [36]. In the resulting structure (Figure 1a), the receptor is likely to be in the desensitised state, as the TMD exhibits a clockwise twist, like the $\beta3$ $GABA_AR$ (also thought to be desensitised [10]). In both structures, the narrowest portion of the pore is at its intracellular end (-1'), a feature attributed to the desensitised state by functional studies [37**].

In the 2-α neuronal nAChR, two of the five possible orthosteric binding sites are at the $\alpha4/\beta2$ interface, where they are formed by the (+) side of $\alpha4$ and the (-) side of $\beta2$ (Figure 1b). In the crystal, these α/β sites are occupied by nicotine, which nestles in a cluster of aromatic side chains, the aromatic box typical of pLGICs [38]. One of the nicotine's positive charges, the protonated pyrrolidine nitrogen, is close to the loop B Trp (TrpB, W156), and is in a good position to form a cation-π interaction with the TrpB aromatic side chain and a hydrogen bond with the TrpB backbone carbonyl. This is an elegant confirmation of the results of 20 years of work by Dougherty, Lester and co-workers, who identified these two features by probing the binding site of pLGICs by unnatural amino acid mutagenesis. This technique makes it possible to weaken cation-π interactions (by decreasing the electronegativity of aromatic rings with fluorine substituents) and to impair hydrogen bonds with the backbone (by decreasing the ability of backbone carbonyls to act as hydrogen bond acceptors; reviewed in [39]). The cation-π interaction with TrpB was seen with all the nicotinic agonists tested in the functional studies, but was particularly important for nicotine. It is the strength of this interaction that makes nicotine much more potent on neuronal nicotinic receptors than on muscle receptors [40]. However, the new crystal structure does not immediately substantiate a third feature identified by functional studies: a proposed network of hydrogen bonds between the pyridine nitrogen of nicotine and the backbone of loop E via a water molecule.

Perhaps the most striking feature of the $\alpha4\beta2$ receptor structure is what happens in this heteromeric receptor at the remaining three binding sites at the ECD interfaces, i.e. the three interfaces where no ligand is bound. Here, the (+) side is contributed by $\beta2$ subunits and the (-) side by either a $\beta2$ subunit ($\beta2/\beta2$) or by the $\alpha4$ subunit ($\beta2/\alpha4$) (Figure 1c). The structure provides an elegant explanation for why none of these three sites are occupied by nicotine. On the principal side, loop B of the β subunit

contains a bulky Arg (R149), two residues before TrpB (the equivalent position in the α subunit is occupied by a Gly): the positively charged side chain of this Arg juts into the binding pocket, where it is stabilised by cation-π interactions with the aromatic rings of TyrA (Y95) and TyrC2 (Y196). TyrC2 can rotate to take this position because the β subunit does not have a C1 aromatic amino acid. Another difference is that the side chain of TrpB of the β subunit (W151) is rotated out of the binding site (Figure 1c).

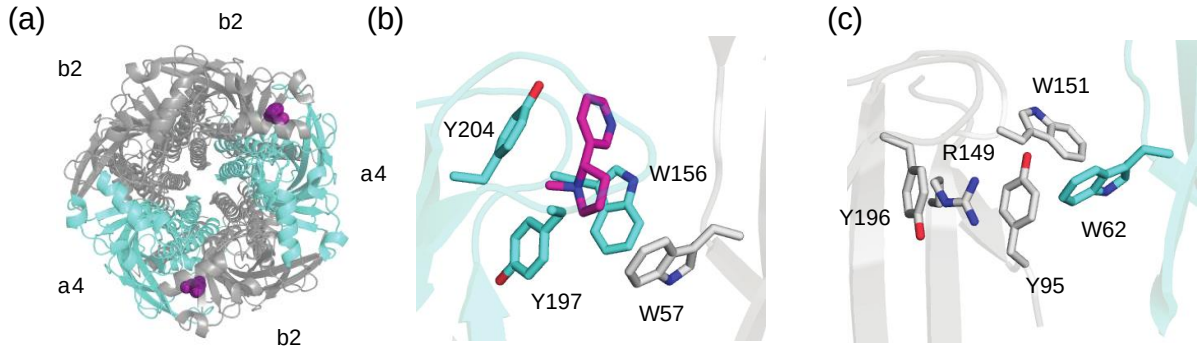

Figure 1: Overview of the α4β2 nAChR structure and two of its orthosteric ligand-binding pockets. (a) Top-down view of the heteromeric α4β2 structure with α4 and β2 subunits shown in cyan and grey, respectively. The bound ligand nicotine is shown in pink. (b)/(c) Close up views of the α4 (+) / β2 (-) (b) and β2 (+) / α4 (-) (c) binding sites with the same colouring scheme as in (a). All panels based on PDB entry 5KXI.

We have known for a while that agonist binding and channel activation are associated with (at least) two motions in the ECD: a quaternary twist of all the ECDs [41] and a closing or capping motion of loop C of the principal subunit [42]. In the β/β and β/α sites, loop C appears relatively open, but the distance between the two sides of the site (loops B and D) looks similar to that seen in the α/β sites that are occupied by a ligand. It may be that this distance is set by the symmetrical quaternary twist of the five ECDs. Both the β/β (and the β/α) sites are occupied by the loop B Arg149 and there is no room for a ligand molecule. It will be interesting to explore whether the cation-π interaction formed by this Arg contributes to the stabilization of the open state, and therefore makes Arg act as an "endogenous agonist", or whether Arg simply occupies the site, much like a competitive antagonist would. Indications that Arg occupancy of the β/β site may not be as efficacious as the binding of an agonist come from comparing the behaviour of the 2-α and the 3-α receptors. In particular, the channel maximum open probability is low for the 2-α form and high (> 80%) for the 3-α form [43-45]. It seems likely that this difference is caused by the binding of a third agonist molecule to the α/α site, and that β/α and β/β sites do not contribute to activation. Differences in how easily the two forms open (i.e. in the allosteric constant) cannot be excluded, as the TMD of the two forms is different. However, work with concatenated subunits [46] shows that switching one TMD from β to α or *vice versa* has no effect on the sensitivity to ACh of the two types of channel. Conversely, the β/β interface has been confirmed to be non-functional, as mutating its aromatic box residues has essentially no effect on the 2-α receptor sensitivity to ACh.

α4β2 neuronal nAChRs have been in the limelight recently also because of the discovery that poison frogs evolved resistance to the effects of the nicotinic agonist alkaloids they carry. Poison dart frogs

of tropical Central and South America acquire toxic alkaloids from their diet, and signal the presence of this chemical defence to predators by their distinctive bright colours. The alkaloids include the most potent of all $\alpha4\beta2$ agonists, epibatidine. By sequencing the $\alpha4$ and $\beta2$ genes in several genera, Tarvin and co-workers [47] identified a single Ser-to-Cys point mutation, which is present in all epibatidine-carrying frogs. This residue (Cys108) is located in a β sheet between loop A and loop E of the $\beta2$ subunit, in an area outside the binding site for ACh, which is much smaller than epibatidine. Introducing this mutation into the human $\beta2$ subunit makes the resulting $\alpha4\beta2$ receptor almost 50-fold less sensitive to epibatidine. However, it also reduces the ACh sensitivity of the receptor, but this is thought to be due to the increased presence of the low-sensitivity $3\alpha:2\beta$ stoichiometry (the ACh dose-response curve becomes biphasic). In contrast with these data from human receptors, intriguingly, frog receptors with this Cys residue have a normal sensitivity to ACh, and the authors show that this is due to the evolution of additional, compensatory sequence differences.

2016 yielded another high-resolution view of a pLGIC binding site, that of glycine bound to the homomeric $\alpha3$ GlyR in an X-ray crystal structure at 2.61 Å (the protein is bound also to the positive allosteric modulator AM-3607 and to zinc ions [48**]). This GlyR is also likely to be desensitised, given that the narrowest point of the pore is at its intracellular end [37**], at the Pro residue in -2' (this residue is not present in cationic channels). The modulator binds in a novel binding site, at the top of the ECD interface, sharing loop B with the agonist orthosteric site just below it. The structure shows that glycine is lodged into the pocket with its amino group deep among the aromatic residues. Here, it is stabilised by a hydrogen bond with the backbone carbonyl of the loop B aromatic residue (PheB), by a cation-π interaction with PheC2 and by a network of hydrogen bonds with loop B residues Glu157 and Ser158, via a water molecule. The carboxyl moiety forms hydrogen bonds with the (+) subunit (Thr204 in loop C) and with the (-) subunit (Arg65 in loop D and Ser 129 in loop E). All of these interactions had been hypothesised from functional studies [49;50]. Further, unnatural amino acid mutagenesis [51] suggested PheB as the main aromatic forming a cation-π interaction, but yielded less definitive results for PheC2. Note that particular caution is required in interpreting the effect of mutations in the GlyR binding pocket, because it is relatively small, and results may be confounded by steric clashes. Two other structural features, the presence of a water molecule and the contact of glycine with loop B Ser 129 had been proposed by molecular dynamics work on a GluCl-based homology model (the role of loop B Ser was confirmed by functional mutagenesis [52]).

Small molecule ligands and pLGIC function: outlook

This is just the beginning. The latest high-resolution insights of the interactions between agonist and binding sites lay the foundation for many further questions. How do these interactions differ for agonists with different efficacy? How do these differences then spread to the ECD/TM interface and eventually to the pore? Does transduction need both the ECD quaternary twist and the capping of loop C? What is the basis of the increase in agonist affinity with activation? How exactly does the conformation of the binding site change as the channel moves from the resting state, to the first activation intermediates (identified by single channel biophysics and variously termed flip, primed, catch-and-hold [53-55]) to the open state and from the open to the desensitised state? Is there a progressive increase in agonist affinity? How are these processes influenced by endogenous modulators, such as neurosteroids (i.e. $GABA_AR$-pregnanolone interaction; [56])?

Beyond the agonist-binding pocket: discovery of new interaction partners, chaperones and accessory subunits

It is becoming increasingly recognised that under physiological conditions, ion channels do not function in isolation, but interact with one or more partner proteins to form multi-component complexes [57]. Recent high-throughput screening, protein labelling and mass spectrometry (MS) data show that pLGICs are no exception. For pLGICs, several novel interacting proteins have been discovered in the last few years, not only offering a first glimpse into the fascinating functional consequences of these interactions, but also opening up new pharmacological avenues, distinct from those directly targeting the ion channel protein (Figure 2).

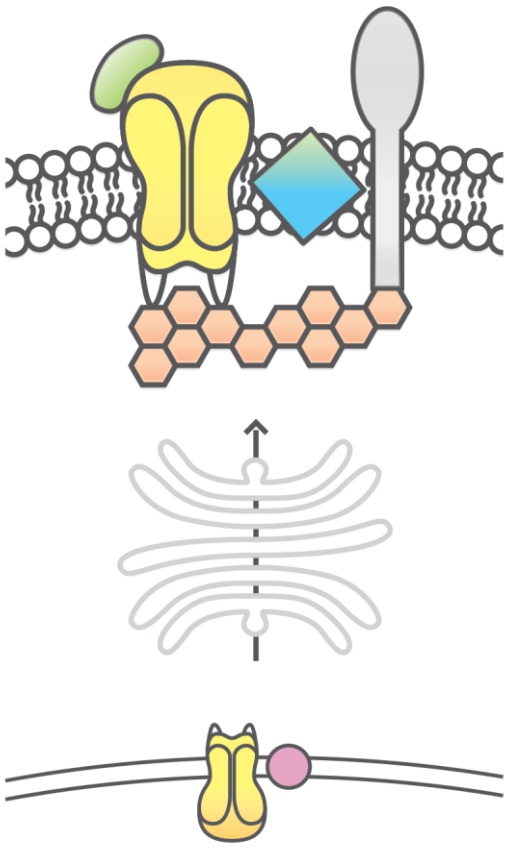

Figure 2: Examples of recently identified protein-protein interactions involving pLGICs. An exemplary pLGIC is depicted in yellow in both the ER (lower panel) and the plasma membrane (upper panel). New proposed interactions involving GABAR-specific toxins (green) targeting the ECD, nAChr α7 and the ER resident protein NACHO (pink), putative GABAR auxiliary subunits from the GARLH family (blue) that form a tripartite complex with GABARs and neuroligin-2 (grey). Interactions of the scaffolding protein gephyrin (orange) and GlyRs/GABARs have been deciphered in great detail and recently targeted using synthetic peptides.

The majority of pLGICs express readily in various cell lines to form functional receptors. The most striking exception is perhaps the homomeric α7 nAChR, which does not properly oligomerise and functionally express in most expression systems. The transmembrane protein RIC-3 (Resistance to Inhibitors of Cholinesterase-3) interacts with nAChRs and 5-HT_3R subunits and promotes receptor maturation [58] (for a review see [59]), but even when co-expressed with RIC-3, α7 nAChRs express

poorly. Given their physiological importance [60], a recent study employed high throughput screening to pinpoint what proteins determine α7 nAChR expression, and screened nearly all human transmembrane and secreted proteins (a total of ca. 4000 clones [61**]). This identified a previously uncharacterised four-pass transmembrane protein, termed NACHO (novel nAChR regulator), which resides in the endoplasmic reticulum and promotes folding and surface expression of α7 nAChR and other nAChRs [62]. Using NACHO knockout mice, this neuronal protein was shown to be critical to α7 nAChR assembly and function in the brain. NACHO is the first essential chaperone specific to a mammalian neurotransmitter receptor.

Another notable advance has been in the field of accessory proteins. It has long been known that trafficking of several subtypes of nAChRs from the endoplasmic reticulum is enhanced by the human membrane-anchored proteins Lynx1 and 2 (lymphocyte antigen-6 protoxin), which also modulate nAChR gating [63-65]. However, no *bona fide* auxiliary subunits were known for pLGICs until the recent discovery of the GARLH (GABA$_A$ Regulatory Lhfpl)-type transmembrane proteins. GARLHs control GABA$_A$R synaptic localisation and GABA-mediated synaptic transmission by anchoring γ2-containing GABA$_A$Rs to synaptic neuroligin-2 (NL-2), another synaptic transmembrane protein [66]. Specifically, the authors propose that GARLH4 forms a trimeric complex with (γ2-containing) GABA$_A$Rs and NL-2. Evidence of a direct GARLH4/GABA$_A$R interaction is still missing and we do not know if the formation of this complex has functional consequences (the proposed auxiliary subunit does not appear to affect the action of agonists or antagonists). Another recent report suggests that GARLHs may determine some of the (notoriously complex) assembly rules for GABA$_A$Rs [67]. A key question for the future is exactly how and when the proposed trimeric LN-2/GARLH4/GABA$_A$R complex interacts with the intracellular scaffold protein gephyrin. While gephyrin is already the best characterised of all intracellular partners of pLGICs, such as GABA$_A$Rs and GlyRs [68], new studies have recently offered insight into the nature of the gephyrin/receptor interactions at atomic and cellular levels [69;70]. These advances were enabled by the development of peptides that disrupt the gephyrin/receptor interaction with high affinity and specificity [71**]. These tools allowed Maric and co-workers [69] to show that GlyR-mediated inhibitory synaptic transmission is impaired by the loss of the interaction between the receptor and the intracellular scaffold (and by the resulting loss of receptor accumulation at the synapse). Another recent report showed that the small molecule antimalarial drug artemisinin can stabilize gephyrin to enhance GABA$_A$R signalling and thereby promote conversion of pancreatic α cells into functional β cells [72]. Together, this opens up a novel route for pharmacological targeting of pLGICs and other ion channels by using peptides to disrupt physiologically relevant protein-protein interactions (see also [73] for a recent review). This route has considerable potential for enabling greater receptor specificity, given that they target cytoplasmic loops, which are much less conserved across different pLGIC subunits and subtypes than orthosteric ECD binding sites.

In general, peptide-derived drugs are able to recognise and bind biological targets with greater specificity than small molecule drugs/ligands. In many cases, nature provides useful blueprints for such drug candidates in the form of peptide toxins that are already highly evolved to develop high affinity and specificity to their protein target. However, examples among pLGICs have remained scarce, with the exception of the reasonably well-characterised interactions of the orthosteric binding site of nAChRs with α-bungarotoxin and conotoxins [74]. A recent breakthrough came from the discovery of the first GABAR-specific toxins from coral snake venom [75], which potently modulate GABA$_A$R activity. Although these particular peptides will likely serve mostly as tool compounds to

decipher GABAergic synaptic transmission, they could serve as a starting point to target the dazzling variety of $GABA_A$Rs with more specific therapeutics in the future. The development of new computational approaches for toxin docking and virtual screening using pLGIC structures or models as templates is therefore a highly timely development [76].

Protein-protein interactions in pLGICs: outlook

These examples highlight the discoveries of numerous new interaction partners of pLGICs in recent years. With evermore-elaborate proteomic and MS tools to discover novel synaptic interactions [77**;78], there is no doubt that the pace of discovery in this field will continue to accelerate. Such efforts are highly timely, as the identification of pLGIC protein-protein interactions is still in its infancy, and most examples of interactions with chaperones, auxiliary subunits and scaffold proteins are at present limited to a single receptor subtype or subunit (Figure 2). The key question is whether all pLGIC subtypes will turn out to have a similar complement of interacting proteins, especially in the light of the low sequence conservation of their intracellular loops. Research into protein-protein interactions will be complemented by the recent surge in cryo-EM structures of ion channel complexes, which (in contrast to most X-ray structures) will hopefully provide much-needed structural information on intracellular loops. This will help clarify the structural basis of protein-protein interactions, further facilitating and complementing their functional elucidation. This is particularly important, as our knowledge of these interactions lags far behind the level of detail known for the interactions occurring in and around the agonist-binding pocket.

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