



Review

Atomistic insights into human Cys-loop receptors by solution NMR[☆]

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ARTICLE INFO

Article history:

Received 17 February 2014

Received in revised form 12 March 2014

Accepted 20 March 2014

Available online 28 March 2014

Keywords:

Cys-loop receptors

Channel structures

nAChRs

Glycine receptors

NMR

Anesthetic binding

ABSTRACT

Cys-loop receptors are pentameric ligand-gated ion channels (pLGICs) mediating fast neurotransmission in the central and peripheral nervous systems. They are important targets for many currently used clinical drugs, such as general anesthetics, and for allosteric modulators with potential therapeutic applications. Here, we provide an overview of advances in the use of solution NMR in structural and dynamic characterization of ion channels, particularly human Cys-loop receptors. We present challenges to overcome and realistic solutions for achieving high-resolution structural information for this family of receptors. We discuss how subtle structural differences among homologous channels define unique channel pharmacological properties and advocate the necessity to determine high-resolution structures for individual receptor subtypes. Finally, we describe drug binding to the TMDs of Cys-loop receptors identified by solution NMR and the associated dynamics changes relevant to channel functions. This article is part of a Special Issue entitled: NMR Spectroscopy for Atomistic Views of Biomembranes and Cell Surfaces. Guest Editors: Lynette Cegelski and David P. Weliky.

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Contents

1. Introduction	307
2. Current frontier of solution NMR for channel proteins	308
3. Use of NMR for structures and dynamics of Cys-loop receptors	309
3.1. Challenges and solutions for NMR studies of Cys-loop receptors	309
3.1.1. Spontaneous pentameric assembly	309
3.1.2. Validation of channel functions	309
3.1.3. Optimization of NMR spectra	310
3.2. NMR structures of Cys-loop receptors	310
4. Cys-loop receptor TMDs as drug targets	311
5. Closing remarks	312
Acknowledgements	312
References	312

1. Introduction

Cys-loop receptors, named after the signature 13-residue loop formed between two conserved cysteine residues, are membrane-spanning ion channels that mediate fast neurotransmission in the central and peripheral nervous systems. This family of receptors includes the nicotinic acetylcholine receptors (nAChRs) and serotonin type-3 receptors (5-HT₃Rs) that conduct cations and mediate excitatory neurotransmission. It also includes gamma-aminobutyric acid type-A and type-C receptors (GABA_ARs and GABA_CRs) and glycine receptors (GlyRs) that conduct anions and mediate inhibitory neurotransmission. Malfunction of these receptors is

[☆] This article is part of a Special Issue entitled: NMR Spectroscopy for Atomistic Views of Biomembranes and Cell Surfaces. Guest Editors: Lynette Cegelski and David P. Weliky.

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often associated with various neurological disorders, such as epilepsy, depression, cognitive impairment, nicotine and alcohol addiction, congenital myasthenic syndromes, and startle disease [1–3]. Cys-loop receptors are important targets for many currently used clinical drugs, such as general anesthetics, and for potential therapeutics.

Cys-loop receptors are pentameric ligand-gated ion channels (pLGICs) comprised of five identical or homologous subunits arranged around a central channel axis (Fig. 1). Each subunit consists of a neurotransmitter-binding extracellular domain (ECD), a pore-forming transmembrane domain (TMD) containing four transmembrane helices (TM1–TM4), and a large intracellular domain (ICD) connecting TM3 and TM4 [4]. The ICD has been implicated in receptor assembly, trafficking, and localization [5–8]. It may influence channel conductance and desensitization [9–11], but replacing the Cys-loop receptor ICD with a short TM3–TM4 linker still produced functional channels [12,13]. Neurotransmitter binding to the orthosteric site in the ECD triggers channel opening and allows ions to pass through the cell membrane. Channel activity can also be modulated allosterically by a variety of ligands bound to other regions of these receptors. There is emerging interest in the development of Cys-loop receptor modulators for treating various neurological disorders [14–18].

Given their biological and pharmacological importance, it is highly desirable to gain a better understanding of Cys-loop receptors' structures and functions. Four sources of information have contributed to the overall structural understanding of these receptors. The first source consists of crystallographic studies of ECDs, including acetylcholine binding proteins (AChBPs) [19–24], the mouse $\alpha 1$ -nAChR ECD [25], and an $\alpha 7$ nAChR–AChBP chimera [26]. These structures provide valuable information about ligand binding and the resulting conformational changes in the ECD. The second source includes crystal structures of prokaryotic homologs of Cys-loop receptors from *Erwinia chrysanthemi* (ELIC) in the absence and presence of ligands [27–29], *Gloeobacter violaceus* (GLIC) in an open or closed conformation [30–32], and eukaryotic *Caenorhabditis elegans* glutamate-gated chloride channel α (GluCl) [33]. The prokaryotic homologs do not contain an ICD and have less than 35% sequence homology with Cys-loop receptors, but share a similar structural scaffold to the ECD and TMD of Cys-loop receptors. The third source of structural information for Cys-loop receptors results

from EM data derived from the *Torpedo marmorata* nAChR, which provided a valuable structural model at 4-Å resolution on an intact nAChR, but little structural information for the ICD [4], probably due to an intrinsically unstructured nature of the ICD. To date neither EM nor crystallography has been able to provide high-resolution structures for human Cys-loop receptors, even though encouraging results in protein expression and purification have been reported for some Cys-loop receptors in recent years [34–37]. Finally, NMR has provided high-resolution structures for the TMDs of human $\alpha 1$ -GlyR [38], $\alpha 4\beta 2$ -nAChR [39], and $\alpha 7$ -nAChR [40]. Moreover, NMR has provided insights into drug binding and drug-mediated changes in channel dynamics [16, 17,40–43]. There is no doubt that NMR has established an important position in structure and dynamics determination of Cys-loop receptors and other channel proteins.

In this review, we provide an overview of advances in the use of solution NMR in structural and dynamic characterization of ion channels, particularly human Cys-loop receptors. We discuss challenges and realistic solutions for achieving high-resolution structural information for this family of receptors. We also describe drug binding onto the Cys-loop receptors' TMD, which is a known target for many Cys-loop receptor modulators.

2. Current frontier of solution NMR for channel proteins

In the past decade, NMR has emerged as a powerful tool in investigating structures and dynamics of channel proteins at atomic resolution [44,45]. Although X-ray crystallography has been successfully used to solve numerous channel structures, many systems have not been crystallized or well diffracted even after persistent efforts. Since each channel protein can sample multiple conformations, some discrepancies reflected in NMR and crystal structures from the same protein may be valuable for understanding channel plasticity. Furthermore, NMR can effectively characterize channel motions, in the absence and presence of drug modulation, which are relevant to channel functions. Altogether, NMR is highly valuable and complementary to other tools in providing structural and functional information for channels. This review will focus only on solution NMR's applications to channel proteins (Table 1), particularly to Cys-loop receptors.

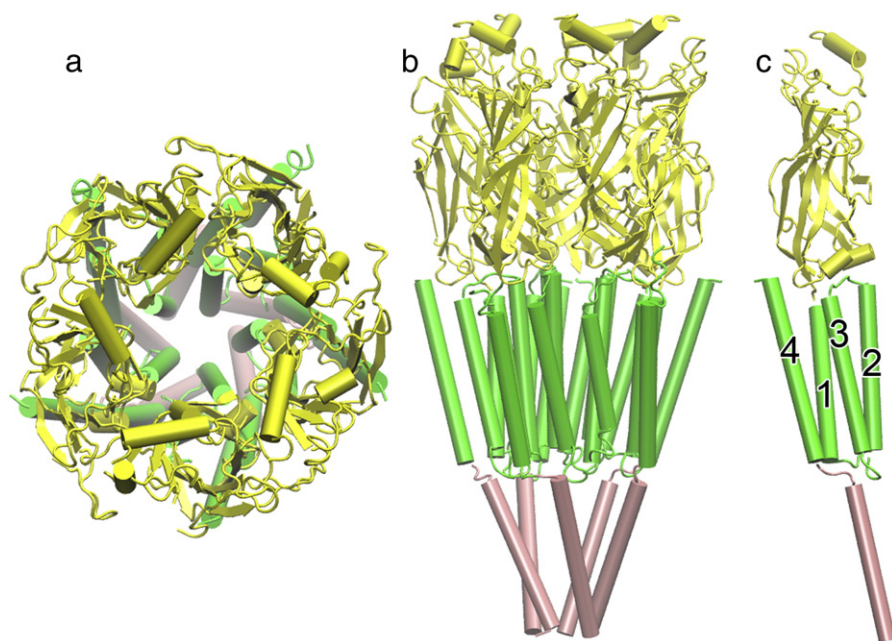


Fig. 1. Topology of the Cys-loop receptor. (a) Top view of a Cys-loop receptor showing the quaternary arrangement of the five subunits around the central channel axis. (b) Side view of the Cys-loop receptor highlighting three domains: ECD (yellow), TMD (green) and ICD (pink). (c) Side view of a single subunit showing the labels for four transmembrane helices, TM1–TM4. The EM structure of the *Torpedo marmorata* nAChR (PDB ID: 2BG9) was used as the model Cys-loop receptor [4].

Table 1

Summary of structures for channels and representative integral membrane proteins determined by solution NMR.

Structural Motif	Protein	Oligomerization state	MW (kDa)	PDB ID	Membrane mimetic	Reference
β -Barrel	OmpX	●	16.4	1Q95	DHPC	[48]
	OmpA	●	19.1	1G90	DPC	[49]
	PagP	●	20.2	1MM4	DPC	[125]
	VDAC-1	●	31.9	2K4T	LDAO	[46]
	OmpG	●	32.8	2JQY	DPC	[47]
α -Helix	KvaP	●●	16.2	2KYH	D7PC	[126]
		●●				
	M2	●●	20.8	2RLF	DHPC	[50]
		●●				
	PLN	●●●●●	30.5	2KYV	DPC	[61] ^a
	p7	●●●	40.6	2M6X	DPC	[63]
		●●●				
	KcsA	●●	71.2	2A9H	DPC	[103]
		●●				
	α 7-nAChR	●●●●●	73.0	2MAW	LDAO	[40]
α -Helix (non-channel)	α 4-nAChR	●●●●●	75.0	2LLY	LDAO	[39]
	β 2-nAChR	●●●●●	75.4	2LM2	LDAO	[39]
	α 1-GlyR	●●●●●	86.5	2M6B	LPPG	[38]
	SRII	●	26.7	2KSY	DHPC	[65]
	PR	●	27.0	2L6X	D7PC	[127]
	DAGK	●●●	39.4	2KDC	DPC	[64]

^a Structure determined by hybrid solution and solid-state NMR methods.

β -Barrels in general have better structural stability and NMR spectral dispersion than α helical proteins. Solution NMR structures for β -barrel channels, including bacterial outer membrane proteins and the voltage dependent anion channel from human mitochondria (VDAC-1), were determined in micelles with protein molecular weights in a range of 16–33 kDa [46–49]. Progress has also been made over the last decade in solution NMR structural determination of α -helical channels. Intensive structural and functional characterization was conducted on the homo-tetrameric proton channels (M2 channels) of influenza A and influenza B in DHPC micelles, yielding NMR structures for AM2 (~21 kDa) and the BM2 transmembrane (~15 kDa) and cytoplasmic (~41 kDa) domains in different functional states [50,51]. However, some structural differences of M2 derived from solid state NMR were reported [52,53]. As a proof of principle, the NMR structure of a “soluble” version of the 46-kDa homo-tetrameric KcsA [54] was determined and proven to agree well with the KcsA crystal structure [55]. The structure of the monomeric subunit of KcsA was also probed in SDS micelles [51]. With the use of solution NMR, several studies revealed K^+ and pH-dependent conformational and dynamics changes of KcsA [56–60]. The structural dynamics and topology of the homopentameric phospholamban (PLN) channel (~31 kDa) were determined using a combination of solution and solid-state NMR methods to reveal the role of PLN in the regulation of calcium transport [61,62]. More recently, an unusual architecture of the 42-kDa homo-hexameric p7 channel, the hepatitis C virus viroporin, was revealed using the latest solution NMR technologies [63].

It is also noteworthy that structures of several relatively large non-channel multi-span transmembrane proteins have been determined in micelles using solution NMR, including the 40-kDa homotrimer DAGK [64] and the seven-helix TM receptor sensory rhodopsin II (~27 kDa) [65]. The reported structures for channels and non-channel transmembrane proteins highlight the advances made in using solution NMR for tackling atomic insights into complex proteins. Ironically, they also mark the current upward limit of protein molecular weights amenable for high-resolution NMR structural studies.

3. Use of NMR for structures and dynamics of Cys-loop receptors

3.1. Challenges and solutions for NMR studies of Cys-loop receptors

A full-length Cys-loop receptor has a typical molecular weight of ~250 kDa, which is too large for structure determination using solution NMR. However, individual domains of Cys-loop receptors can be

studied separately after proper truncation. This is particularly true for the TMD, which spontaneously assembles into pentameric channels in membrane mimetics. Moreover, one can evaluate whether a TMD channel retains functional and pharmacological characteristics of the authentic Cys-loop receptor before investing effort into structure determination.

3.1.1. Spontaneous pentameric assembly

Electron microscopy images [38] and size exclusion chromatography–multi-angle light scattering (SEC–MALS) [39,40] revealed a spontaneous pentameric assembly of the Cys-loop receptor TMD in micelles. The ability to form channel assemblies was observed even for the isolated pore-lining TM2 helix of the GlyR [66], GABA_A receptor [67], and nAChR [68,69]. In contrast, quaternary structural assembly of an isolated ECD is less certain. The isolated ECD of GLIC presents as a hexamer in crystal structure [70] or monomer in solution NMR [71], even though its tertiary structure remains the same as that observed in the pentameric structure of the full-length GLIC [30,31]. Varying oligomerization states were also observed for the ECD α 1-GlyR subunit [72]. The preservation of the native quaternary assembly for an isolated TMD and the destruction observed for the ECD alone suggests that the TMD drives the pentameric assembly of Cys-loop receptors.

3.1.2. Validation of channel functions

It is vital to assure that the channels used for structural studies are functional and retain essential pharmacological characteristics of Cys-loop receptors. Several approaches have been used for functional assessments. First, the NMR magnetization-inversion-transfer (MIT) experiment [73,74] is convenient for tracking ion permeation through channels. It has been used to characterize Cl^- transport through the human α 1-GlyR TMD channel reconstituted in vesicles. The MIT experiment demonstrates that the channel conductance could be inhibited by the pore-blocker picrotoxin, a signature for the native pore structure [38]. Second, for Na^+ -conducting channels, such as α 4 β 2- and α 7-nAChRs, the Na^+ flux assay is useful to quickly assess whether the TMD channels are functional [39]. Finally, injection of the α 7-nAChR TMD, purified in LDAO micelles, into *Xenopus laevis* oocytes has yielded functional channels that retain pharmacological features of the native α 7-nAChR [40].

These measurements served not only for the validation of functional structures, but also for the revelation of the interplay between Cys-loop receptor domains. The channels have a much higher opening

probability in the absence of the ECD than in the intact Cys-loop receptors, where the channels are closed until agonist binds to the ECD [75–79]. Based on this observation, we hypothesize that the ECD sets restraints on the intrinsically opened TMD; agonist binding to the ECD relieves the restraints temporarily so that the TMD can rapidly “relax” into its preferred open conformation before transitioning into desensitized conformations.

3.1.3. Optimization of NMR spectra

For a chosen channel protein, the sample preparation and NMR experimental conditions should be optimized in order to obtain adequate quality spectra for structural studies. In particular, one needs to fine-tune protein stability. When ECD or TMD is isolated, the hydrophobic residues at the ECD–TMD interface, protected originally by Van der Waals contacts, become exposed to water and cause protein destabilization. Substituting two or three non-polar residues in the TM2–TM3 linker with hydrophilic residues, as shown in Fig. 2, greatly prevented aggregation of the $\alpha 4\beta 2$ - and $\alpha 7$ -nAChR TMDs [39,40]. Mutations of charged residues at the N- and C-termini were also beneficial for preventing aggregation of the nAChR TMDs at a relatively low pH suitable for NMR data collection [39,40], but such mutations were found unnecessary for the $\alpha 1$ -GlyR TMD [38]. In each case the large ICD was replaced with a glycine linker to limit the molecular size and number of peaks in the spectra. While the ICD plays roles in receptor assembly and trafficking, fully functional receptors could be obtained by replacing the Cys-loop receptor ICD with the short TM3–TM4 linker observed in GLIC [12,13].

Selecting a proper membrane mimetic is another important step for sustaining stable channel folding for the purpose of acquiring good quality NMR spectra. A large number of membrane mimetic options for solution NMR studies of membrane proteins are available [80–82]. Although bicelles have been used in studies of transmembrane proteins [83,84], micelles remain a primary choice for solution NMR. The ability of micelles to sustain stability and functions of membrane proteins has been documented [85]. A specific choice of detergent needs to be tested for each channel protein. For example, LDAO is a good choice for the $\alpha 4\beta 2$ - and $\alpha 7$ -nAChR TMDs [39,40], while LPPG is better for the $\alpha 1$ -GlyR TMD [38]. A molar ratio of detergent to protein concentrations between 150 and 250 is optimal to maintain protein stability and to achieve high quality NMR spectra [38–40].

TROSY-based [86] triple-resonance experiments and TROSY-based NOESY experiments are well suited for the Cys-loop TMD, which typically has ~120 residues. For homo-pentamers, such as the $\alpha 7$ -nAChR

and $\alpha 1$ -GlyR TMD [38,40], the NMR spectra can benefit greatly from magnetic symmetry, in which five identical polypeptides assemble a channel. For hetero-pentamers, such as the $\alpha 4\beta 2$ -nAChR, the isotope labeled $\alpha 4$ subunit was mixed with the unlabeled $\beta 2$ or vice versa to avoid spectral crowding [39]. Selective labeling is often needed to resolve ambiguities of chemical shift assignments [38]. In addition to chemical shift index [87,88] and nuclear Overhauser effects (NOEs) [89–92], long-range spatial restraints from paramagnetic relaxation enhancement (PRE) [93], and orientation restraints from residual dipole couplings (RDCs) [83,94] also aided in determination of the structures of the TMDs [38–40].

3.2. NMR structures of Cys-loop receptors

NMR structures for the full-length TMD of the human $\alpha 1$ -GlyR (PDB ID: 2M6I; 2M6B) [38], human $\alpha 7$ - (PDB ID: 2MAW) [40], $\alpha 4$ - (PDB ID: 2LLY), and $\beta 2$ -nAChRs (PDB ID: 2LM2; 2KSR) [39,40,95] represent the first few atomic resolution experimental TMD structures of mammalian Cys-loop receptors. Although a four- α -helical-bundle scaffold is common to TMDs of all Cys-loop receptors, NMR structures reveal atomic details unique to individual channels that can account for their distinct functional and pharmacological properties.

Take the human $\alpha 1$ -GlyR as an example. Several structural and dynamic features of the open-channel $\alpha 1$ -GlyR structures revealed by NMR [38] could not be observed via homology modeling [96,97]. The pore-lining TM2 of $\alpha 1$ -GlyR has a shorter helix (Fig. 3). It is more than two helical turns (9 residues) shorter than TM2 shown in the cryo-EM structure of nAChR [4], and six residues shorter than observed in the NMR structures of $\alpha 4\beta 2$ - and $\alpha 7$ -nAChRs [39,40], and seven residues shorter than in crystal structures of ELIC [27,28], GLIC [30,31,98] and GluCl [33]. Moreover, the C-terminal end of the $\alpha 1$ -GlyR TM2 shows two conformations in NMR spectra and displays conformational flexibility that is likely related to channel functions [99,100]. A kink at A288 changes the $\alpha 1$ -GlyR TM3 helix axis direction by ~33° (Fig. 3). Such a kink has not been observed in structures for nAChRs or other homologous pLGICs [4,27,28,30,31,33,39,40,98]. The shorter TM2 helix with a flexible C-terminal end and the TM3 helical kink are intrinsic to the $\alpha 1$ -GlyR. These structural features were observed in the studies of the $\alpha 1$ -GlyR TMD using different membrane mimetics and varying numbers of TMD helices [38,66,83,101,102].

High-resolution NMR structures for human neuronal nAChRs provide insights into unique characteristics of individual subunits. While the NMR structures for $\alpha 7$ -, $\alpha 4$ -, and $\beta 2$ -nAChRs share a common

	TM1	TM2
$\alpha 4$	SNARRLPFYITINLIIPCLLISCLTVLVFYLPSCEGKITLCISVLLSLTVFLLLIITEIIPSTSLVIPL	
$\alpha 4'$	SNA EE LPFYITINLIIPCLLISCLTVLVFYLPSCEGKITLCISVLLSLTVFLLLIITEIIPSTSS SVSPS	
$\beta 2$	SNARRKLPFYITINLIIPCVLITSLAILVFYLPSCDCGKMTLCISVLLALTVFLLLIISKIVPPTSLDVP	
$\beta 2'$	SNA EE LPFYITINLIIPCVLITSLAILVFYLPSCDCGKMTLCISVLLALTVFLLLIISKIVPPTSS SDSPS	
$\alpha 7$	SNARR-TLYYGLNLLIPCVLISALALLVFLLPADSGEKISLGITVLLSLTVFMLLVAEIMPATSDSVPL	
$\alpha 7'$	SNA EE - E LYYGLNLLIPCVLISALALLVFLLPADSGEKISLGITVLLSLTVFMLLVAEIMP STSDSPS	
	210.....220.....230.....240.....250.....260.....270	
	TM3	TM4
$\alpha 4$	IGEYLLFTMIFVTLISIVITVFVLNVHHRSPRTHT- - - - IDRIFLWMFIIIVCLLGTVGLFLPPWLAG-	
$\alpha 4'$	IGEYLLFTMIFVTLISIVITVFVLNVHHRSP E THTGGGGGIDRIFLWMFIIIVCLLGTVGLFLPPWLAG E	
$\beta 2$	VGKYLMTMVLVTFISIVTSVCVLNVHHRSP TH T- - - - IDRLFLWIFVFCVFGTIGMFLQPLFQ N -	
$\beta 2'$	VG E YLMFTMVLVTFISIVTSVCVLNVHHRSP E THTGGGGGIDRLFLWIFVFCVFGTIGMFLQPLFQ EE	
$\alpha 7$	IAQYFASTMIIVGLSVVVTVIVLQYHHHDPDGGK- - - - VDRLCLMAFSVFTIICTIGILMSAPNFV- -	
$\alpha 7'$	IAQYFASTMIIVGLSVVVTVIVLQYHHHDPDGG E GGGGIDRLCLMAFSVFTIICTIGILMSAPNFV EE	
	280.....290.....300.....450.....460.....470.....	

Fig. 2. Sequence comparison for $\alpha 4$ -, $\beta 2$ -, and $\alpha 7$ -nAChRs and their respective NMR constructs, $\alpha 4'$ -, $\beta 2'$ -, and $\alpha 7'$ -. A few point mutations stabilized the channels under the NMR experimental condition. Glutamate mutations (red) at the N- and C-termini lower the isoelectric point and prevent protein precipitation at pH 4.7. Serine mutations (green) introduced to the TM2–TM3 linker increase sample stability in the absence of the ECD. The sequence numbering corresponds to that of the $\alpha 7$ -nAChR.

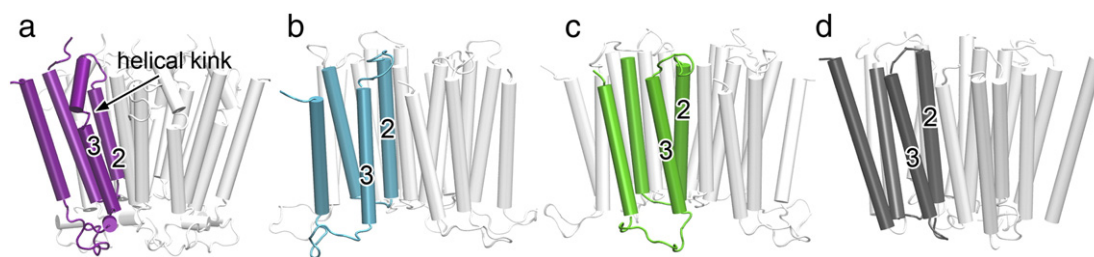


Fig. 3. Structural comparison of Cys-loop receptor TMDs. Representative solution NMR structures for (a) the $\alpha 1$ -GlyR TMD (PDB ID: 2M6I); (b) the $\alpha 4$ - (PDB ID: 2LLY) and (c) $\beta 2$ - (PDB ID: 2LM2) nAChRs; and (d) the cryo-EM structure of the *Torpedo marmorata* nAChR (PDB ID: 2BG9) with the $\alpha 1$ subunit highlighted. In each colored subunit, the TM2 and TM3 helices are labeled 2 and 3, respectively. Note a shorter length of the TM2 helix in $\alpha 1$ -GlyR (a) than those in nAChRs (b–d). Also note the unique helical kink in the $\alpha 1$ -GlyR TM3 that is absent in nAChRs.

scaffold, differences in the lengths and orientations of the TM helices among the nAChR subtypes exist. Such differences can affect intra-subunit cavities, both in size and shape, and ultimately affect drug binding. The $\alpha 7$ - and $\alpha 7\beta 2$ -nAChRs are a good pair of Cys-loop receptors to underscore the importance of structural individuality in shaping the unique pharmacology for each Cys-loop receptor subtype [42]. Even though the $\alpha 7$ and $\beta 2$ TMDs are ~50% identical in sequence, a variation in cavity-lining residues and a less than 10° orientation difference of TM2 helix yield disparity for the cavity at the EC end of the TMD: ~180 Å³ and ~120 Å³ for $\beta 2$ and $\alpha 7$, respectively. Unlike the cavity in $\beta 2$, a smaller cavity in $\alpha 7$ excludes the anesthetic isoflurane from binding. The structural insights offer an explanation as to why $\alpha 7$ is functionally insensitive to isoflurane while $\alpha 7\beta 2$ is hypersensitive [42]. The subtle structural differences, such as those reflected in $\alpha 7$ and $\beta 2$, are often diminished in homology modeling. Thus, comprehensive structure determination for individual subunits is necessary in order to design therapeutics targeting specific subtypes of Cys-loop receptors.

Chemical shift perturbation experiments identified interacting residues between the $\alpha 4$ and $\beta 2$ subunits in the heteromeric $\alpha 4\beta 2$ -nAChR [39]. Such information was used to generate a pentameric assembly for the $\alpha 4\beta 2$ TMD. We notice that leucine–leucine and leucine–isoleucine inter-subunit contacts make up 31% and 39% of the total inter-subunit contacts, respectively. They are the predominant driving force for the pentameric assembly of the $\alpha 4\beta 2$ channel. In addition, other hydrophobic contacts involving valine or methionine make up 22% of the inter-subunit contacts. The role of leucine zippers in folding and/or oligomerization of other membrane-spanning proteins has been well documented [103,104]. The importance of a leucine–isoleucine zipper, interlocking of alternating leucine and isoleucine residues between subunits, has also been demonstrated in structures of PLN [61].

Rich leucine–leucine and leucine–isoleucine contacts between subunits are associated with the abundance of leucine and isoleucine

residues that constitute ~30% of the residues in the $\alpha 4\beta 2$ -nAChR TMD. In contrast, $\alpha 1$ -GlyR has ~10% less leucine and isoleucine in its TMD. Thereby, only ~10% and ~5% inter-subunit leucine–leucine and leucine–isoleucine contacts are observed in the $\alpha 1$ -GlyR structure [38]. Aromatics, however, play a more significant role in assembly of the $\alpha 1$ -GlyR channel [38] than that of the $\alpha 4\beta 2$ -nAChR channel, where only one pair of aromatic contacts between $\alpha 4$ and $\beta 2$ subunits was found [39].

4. Cys-loop receptor TMDs as drug targets

The TMDs of Cys-loop receptors are targets of general anesthetics [41,42,105–112], neurosteroids [113,114], and various positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs) of therapeutic potential [11,18,43,115]. Complementary to photoaffinity labeling [105–112,116] and crystallography [29,48,98,117] used for identifying drug binding sites in Cys-loop receptors and homologous pLGICs, solution NMR is another effective tool for providing atomic details of drug binding sites [16,17,40–42]. Furthermore, solution NMR can identify drug-induced changes in protein dynamics [118–120] and provide insight into potential functional consequences due to drug binding [16,17,42].

A novel binding site for cannabinoids was identified in human GlyRs (Fig. 4a) by using solution NMR titration and NOESY experiments, which demonstrated a direct contact between cannabinoids and the residue S296 in $\alpha 1$ -GlyR [16] or in $\alpha 3$ -GlyR [17]. In addition, the NMR experiments revealed two coexisting states of binding on a relatively slow exchange timescale or cannabinoid-induced conformation exchange at S296 [17]. When the binding information is mapped onto the NMR structure of the $\alpha 1$ -GlyR channel [38], it becomes clear that S296 exposes to lipids and the cannabinoid-binding pocket is involved with both TM3 of GlyR and surrounding lipids. The atomic insight revealed by NMR adds a valuable structural basis in pursuing glycinergic cannabinoids for chronic pain treatment [16,17]. The cannabinoid-

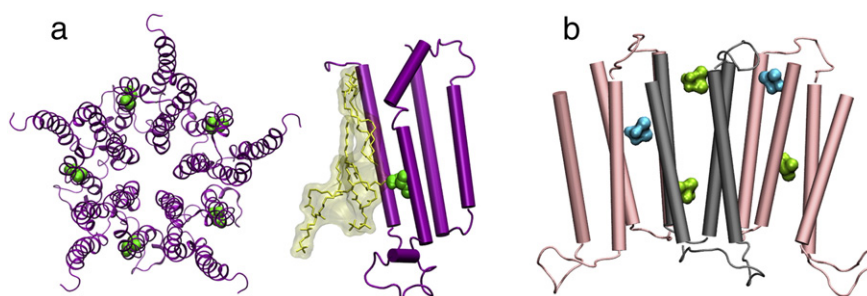


Fig. 4. Drug binding sites in the human $\alpha 1$ -GlyR and $\alpha 4\beta 2$ -nAChR TMDs. (a) Left: top view of the GlyR- $\alpha 1$ TMD (PDB ID: 2M6I) highlighting a novel cannabinoid binding site (green) in each subunit; right: side view of the cannabinoid binding site in an $\alpha 1$ -GlyR subunit, which involves residue S296 (green) in TM3 and the adjacent lipids (yellow) [16]. (b) NMR-identified halothane binding sites in the $\alpha 4\beta 2$ -nAChR TMD. The $\alpha 4$ (PDB ID: 2LLY) and $\beta 2$ (PDB ID: 2LM2) subunits are shown in pink and gray, respectively. Halothane binding was observed at both intra-subunit sites (yellow-green) and inter-subunit sites (cyan) between $\alpha 4$ and $\beta 2$ subunits. Inter-subunit halothane sites were observed exclusively at the EC end of the TMD, while intra-subunit sites were observed at the IC end of the TMD for both $\alpha 4$ and $\beta 2$. Note that the intra-subunit site at the EC end of the TMD was unique to $\beta 2$.

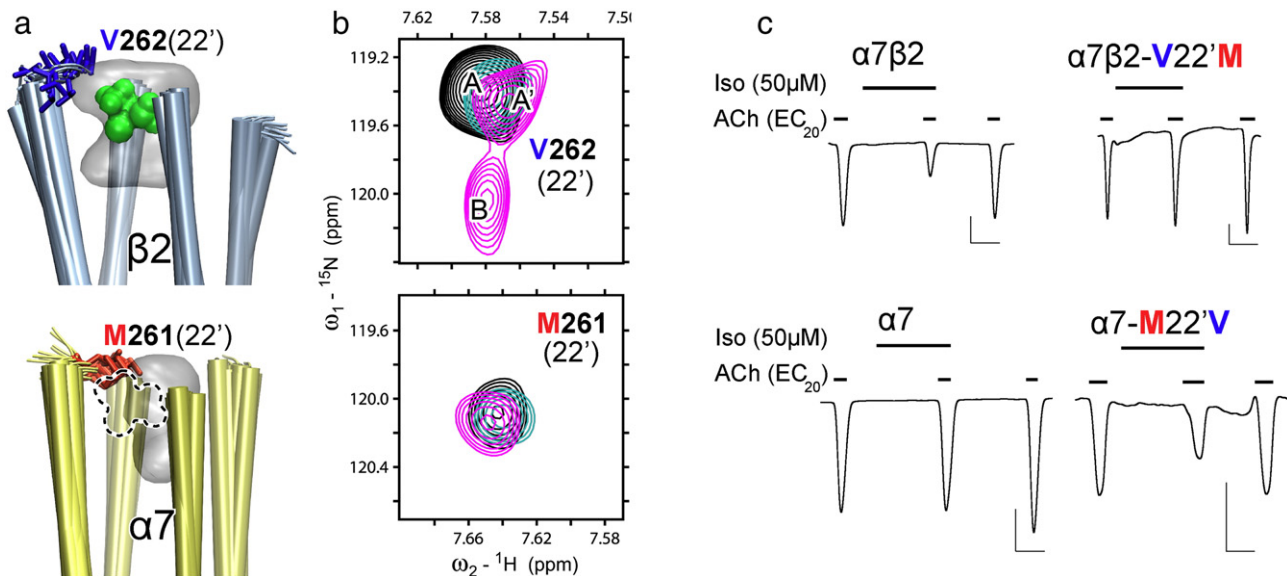


Fig. 5. Dynamics as a critical link between drug binding and changes in channel function. (a) NMR structures of $\beta 2$ - (top) and $\alpha 7$ - (bottom) nAChRs show different sizes of cavities (transparent surface) at the EC end of their respective TMDs. The anesthetic isoflurane (green surface) binds to this cavity in $\beta 2$, but to a different site in $\alpha 7$. The cavity-lining residue 22' is shown as blue and red sticks for $\beta 2$ and $\alpha 7$, respectively. (b) NMR spectra for residue 22' of $\beta 2$ (top) and $\alpha 7$ (bottom) in the presence of 0 mM (black), ~1.5 mM (cyan), and ~3.0 mM (magenta) isoflurane. Note that only in $\beta 2$ was isoflurane-induced peak splitting observed, indicating a dynamics change for the 22' residue on the μ s time-scale. In contrast, the isoflurane-induced peak splitting was not observed in $\alpha 7$. (c) Isoflurane (Iso) can inhibit the current elicited by acetylcholine (ACh) in *Xenopus laevis* oocytes expressing $\alpha 7\beta 2$ (top), but cannot affect currents in the case of $\alpha 7$ (bottom). The $\alpha 7\beta 2$ -V22'M (top) and $\alpha 7$ -M22'V (bottom) mutants have reversed functional responses to isoflurane in comparison to their parent channels. The functional sensitivity to isoflurane (c) is correlated to the dynamics modulation by isoflurane (b) on these channels. The currents were recorded by two-electrode voltage clamp held at -60 mV. The vertical and horizontal scales represent 25 nA and 1 min, respectively.

binding pocket found in human $\alpha 1$ - or $\alpha 3$ -GlyRs also highlights the appropriateness of shallow and lipid-facing pockets for drug binding to Cys-loop receptors.

Most drug-binding pockets found in the TMDs of Cys-loop receptors and the homologous pLGICs were found more deeply embedded into the proteins. For low affinity drugs, such as volatile general anesthetics, multiple binding sites often exist in one channel. For example, the anesthetic halothane binds to both intra- and inter-subunit sites in the $\alpha 4\beta 2$ -nAChR TMD (Fig. 4b). Similar binding sites were also found separately in the muscle type nAChR with the photo-labeled halothane [112], the fluorescence quenching of GLIC by halothane [121], crystal structures of GLIC bound with the anesthetics desflurane or propofol [117], crystal structures of the GLIC F14'A mutant bound with alcohols [48], and the crystal structure of ELIC bound with a derivative of chloroform [29]. The binding at both intra- and inter-subunit sites inhibited these cation-conducting channels except the GLIC F14'A mutant, in which ethanol binding to an inter-subunit site caused potentiation of the channel current [48]. Thus, the relation between binding sites and functional impact varies among individual channels. The binding to an inter-subunit site can potentiate function in one channel, but inhibit function of another.

Channel function is closely associated with channel motion. The drug binding relevant to a channel's functional impact is often associated with the drug-induced changes in channel dynamics that can be observed in NMR spectra. Take the recent study on the $\alpha 7$ - and $\alpha 7\beta 2$ -nAChRs as an example (Fig. 5). The $\alpha 7$ -nAChR is functionally insensitive to volatile anesthetics [122,123], such as isoflurane. The presence of $\beta 2$ makes the $\alpha 7\beta 2$ -nAChR hypersensitive to isoflurane [42]. We identified different isoflurane binding sites in $\alpha 7$ and $\beta 2$ by using NMR experiments [42]. More importantly, we observed that isoflurane induced a transition of several $\beta 2$ residues from a single peak to double peaks in NMR spectra, which suggested a shift in the motional timescale or conformational equilibrium [124]. Such transitions were observed for both the V22' residue lining the isoflurane-binding-site and the channel gate residue L9' in $\beta 2$. In contrast, similar dynamics changes were not detected in $\alpha 7$. Only isoflurane binding to the $\beta 2$ -specific site could affect the channel dynamics and produce a functional consequence.

5. Closing remarks

There are many subtypes of Cys-loop receptors covering a wide range of functions and pharmacology. It remains a great challenge to determine atomic-resolution structures of these receptors. Recent technological advances in NMR, crystallography, and cryo-EM have rapidly improved our ability to study complex channel proteins. Integrated information generated by these tools will further accelerate our understanding of Cys-loop receptors. Solution NMR will continue to provide atomistic insights into channel structures and dynamics, drug binding (especially at low affinities), and allosteric modulation.

Acknowledgements

The authors thank Ms. Sandy Hirsch for her editorial assistance. The research on human nAChRs and GlyRs was supported by grants from the National Institutes of Health (R01GM056257 and R01GM066358 to P.T. and R37GM049202 to Y.X.). M.K. was supported by an NIH Ruth L. Kirschstein National Service Award (T32GM075770 to Y.X.).

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