

MINIREVIEW—A LATIN AMERICAN PERSPECTIVE ON ION CHANNELS

Structure and Pharmacologic Modulation of Inhibitory Glycine Receptors

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Received June 21, 2016; accepted July 8, 2016

ABSTRACT

Glycine receptors (GlyR) are inhibitory Cys-loop ion channels that contribute to the control of excitability along the central nervous system (CNS). GlyR are found in the spinal cord and brain stem, and more recently they were reported in higher regions of the CNS such as the hippocampus and nucleus accumbens. GlyR are involved in motor coordination, respiratory rhythms, pain transmission, and sensory processing, and they are targets for relevant physiologic and pharmacologic modulators. Several studies with protein crystallography and cryoelectron microscopy have shed light on the residues and mechanisms associated with the activation, blockade, and regulation of pentameric Cys-loop ion channels at the atomic level. Initial studies conducted on the extracellular

domain of acetylcholine receptors, ion channels from prokaryote homologs—*Erwinia chrysanthemi* ligand-gated ion channel (ELIC), *Gloeobacter violaceus* ligand-gated ion channel (GLIC)—and crystallized eukaryotic receptors made it possible to define the overall structure and topology of the Cys-loop receptors. For example, the determination of pentameric GlyR structures bound to glycine and strychnine have contributed to visualizing the structural changes implicated in the transition between the open and closed states of the Cys-loop receptors. In this review, we summarize how the new information obtained in functional, mutagenesis, and structural studies have contributed to a better understanding of the function and regulation of GlyR.

Introduction

Inhibitory glycine receptors (GlyR) are anion-selective ligand-gated ion channels (LGICs), which together with GABA_A receptors (GABA_AR), the nicotinic acetylcholine receptors (nAChR) and serotonin type 3 receptors (5HT-3) form the eukaryotic Cys-loop family. In mature neurons, the activation of GlyR leads to a fast increase in the passive diffusion of anions, mainly chloride ions, into the neurons, resulting in membrane hyperpolarization and reduction in neuronal excitability (Lester et al., 2004; Miller and Smart, 2010; Zeilhofer et al., 2012). It is well accepted that inhibitory GlyR function is critical for the control of several physiologic processes, namely muscle tone, motor coordination, sensory processing, respiratory rhythms, and pain (Lynch, 2009; Callister and Graham, 2010; Zeilhofer et al., 2012; Alvarez

et al., 2013). In addition, the critical role of GlyR inhibition in normal physiology is further highlighted by genetic studies in humans that have linked mutations in GlyR genes with hyperekplexia (Harvey et al., 2008).

Functional chloride-permeable GlyR ion channels are formed by 5 α subunits (homomeric) or by the mixture of α and β subunits (heteropentameric), which are molecular complexes with either a 2 α –3 β or 3 α –2 β stoichiometry (Grudzinska et al., 2005; Durisic et al., 2012). At present, four α (α 1–4) subunits and one β subunit have been described that have a regionally and temporally controlled expression during development and maturation of the central nervous system (CNS).

The α 1 subunit is widely expressed in the adult CNS, but low levels have been detected in the spinal cord during early development and in newborn animals with an increased expression toward postnatal day 15 (Aguayo et al., 2004; Lynch, 2009). In contrast, α 2 subunits show a high level of expression during embryonic development in the spinal cord, brainstem, and some supraspinal regions such as the cortex, hippocampus, and thalamus (Kuhse et al., 1991; Malosio et al.,

This work was supported by the National Institutes of Health National Institute on Alcohol Abuse and Alcoholism [Grant RO1 AA15150], Fondo Nacional de Desarrollo Científico y Tecnológico [Grant 1140515], and Comisión Nacional de Investigación Científica y Tecnológica [Grant DPI 20140008].
dx.doi.org/10.1124/mol.116.105726.

ABBREVIATIONS: AChBP, acetylcholine-binding protein; CNS, central nervous system; ECD, extracellular domain; ELIC, *Erwinia chrysanthemi* ligand-gated ion channel; 5HT-3, serotonin type 3 receptors; GABA_AR, GABA_A receptor(s); G $\beta\gamma$, G protein $\beta\gamma$ subunits; GLIC, *Gloeobacter violaceus* ligand-gated ion channel; GluCl, glutamate-gated chloride channel; GlyR, glycine receptor(s); IL, intracellular loop domain; LGIC, ligand-gated ion channel; nAChR, nicotinic acetylcholine receptor(s); TM, transmembrane domain.

1991). The predominant $\alpha 2$ expression declines extensively during advanced stages of brain development. However, it has been suggested that $\alpha 2$ expression is maintained in higher brain regions such as the hippocampus and cortex (Kuhse et al., 1991; Chau et al., 2010; Avila et al., 2013; Blednov et al., 2015). The $\alpha 3$ subunits are expressed after the third postnatal week in the hippocampus, retina, and lamina II of the spinal dorsal horn (Malosio et al., 1991; Aguayo et al., 2004; Harvey et al., 2004). Finally, the $\alpha 4$ subunit is found as a pseudogene in humans, but it is expressed in the spinal cord, sympathetic nervous system, kidney, liver, spermatozooids, and retina of other species (Harvey et al., 2000; Simon et al., 2004).

The α subunits, but not the β subunit, can form functional homopentameric ion channels. When present in heteropentameric α/β GlyR, the β subunit displays structural and regulatory functions, including GlyR clustering in synaptic locations through interaction of the intracellular loop domain (IL) of β with the scaffolding protein gephyrin, and control of pharmacologic responses to agonist and several other modulators (van Zundert et al., 2005; Calamai et al., 2009; Dutertre et al., 2012). In the present review, we define the term modulator to refer to a ligand (small molecule or protein) that binds to a site that is distinct to the agonist site, but allosterically affects the function of the protein in question.

The current state of GlyR pharmacology has been reviewed previously elsewhere (Lynch, 2009; Yevenes and Zeilhofer, 2011; Zeilhofer et al., 2012; Burgos et al., 2015b) and is composed of a limited collection of agonists, antagonists, and a growing number of modulators. Until recently, most of our knowledge about individual residues and mechanisms involved

in GlyR function and pharmacologic modulations has been derived from studies using mutated GlyR in combination with electrophysiologic studies and molecular modeling with the nAChR or prokaryotic Cys-loop receptors as templates (Absalom et al., 2003; Bertaccini et al., 2007; Speranskiy et al., 2007; Cheng et al., 2008; Harris et al., 2008; Vijayan et al., 2012; Olsen et al., 2014; Yu et al., 2014). In recent years, however, several reports have shed important new light on the residues and mechanisms at the atomic level associated with the activation, blockade, and modulation of pentameric Cys-loop ion channels. Here, we intend to summarize the most recent advances in GlyR structure with a special focus on the molecular sites underlying its functional regulation by several modulators of physiologic and clinical relevance.

General Structure of Cys-Loop Receptors

Complete atomic structures of Cys-loop receptors, including GlyR, have been very challenging to achieve so far. Technically, there is still a need for optimized expression systems that can produce large concentrations of highly purified proteins with mature posttranslational modifications and high solubility (Carpenter et al., 2008). The principal structures available to date are shown in Table 1. Data from acetylcholine-binding protein (AChBP) from the snail *Lymnaea stagnalis* at 2.7 Å resolution provided the initial experimentally determined data for the agonist binding site on a Cys-loop receptor (Brejci et al., 2001). Later, the nAChR structure obtained by cryoelectron microscopy at 4 Å resolution provided novel information on the atomic structure of one

TABLE 1

Experimentally resolved structures of Cys-loop ion channels

Summary of the principal structures of prokaryote and eukaryote receptors obtained by X-ray diffraction (XRD), electron cryomicroscopy (ECM), or solution nuclear magnetic resonance (NMR). Additionally, the states of the ion channel in each structure are indicated—open (O), closed (C), locally closed (LC), resting (R) states, or not applicable (NA)—with corresponding codes of the Protein DataBank (<http://www.rcsb.org/pdb/home/home.do>).

Receptor	Structure	State	Method	Reference	Release
ELIC	2VL0: 3.3 Å	C	XRD	Hilf and Dutzler, 2008	2008
	3RQU: 3.09 Å	C	XRD	Pan et al., 2012b	2012
ELIC F246A mutant	2YKS: 3.30 Å	C	XRD	Zimmermann and Dutzler, 2011	2011
ELIC F16'S mutant	4TWH: 3.60 Å	C	XRD	Ulen et al., 2014	2014
ELIC L9' F16' double mutant	3UQ5: 4.2 Å	O	XRD	Gonzalez-Gutierrez et al., 2012	2012
	3UQ7: 3.8 Å				
GLIC	3EAM: 2.90 Å	O	XRD	Bocquet et al., 2009	2008
	3EHZ: 3.10 Å	O	XRD	Hilf and Dutzler, 2009	2008
	4HFI: 2.4 Å	O	XRD	Sauguet et al., 2013b	2013
	4NPQ: 4.35 Å	R	XRD	Sauguet et al., 2014	2013
GLIC H11'F mutant	3TLT: 3.3 Å	LC	XRD	Prevost et al., 2012	2012
GLIC E19'P mutant	3TLS: 3.2 Å	LC	XRD	Prevost et al., 2012	2012
GLIC F14'A mutant	4HFB: 2.75 Å	O	XRD	Sauguet et al., 2013a	2013
GLIC I9'A T25'A double mutant	4LML: 3.8 Å (lig-bounded)	C	XRD	Gonzalez-Gutierrez et al., 2013	2013
GluCl	3RHW: 3.26 Å	O	XRD	Hibbs and Gouaux, 2011	2011
	4TNV: 3.60 Å	C	XRD	Althoff et al., 2014	2014
nAChR 2 α β δ γ	2BG9: 4 Å	C	ECM	Unwin, 2005	2005
	4AQ9: 6.2 Å	O	XRD	Unwin and Fujiyoshi, 2012	2012
	4AQ5: 6.2 Å	C	XRD	Unwin and Fujiyoshi, 2012	2012
nAChR $\alpha 7$ (TMs)	2MAW	NA	NMR	Mowrey et al., 2013b	2013
5-HT3A	4PIR: 3.5 Å	O	XRD	Hassaine et al., 2014	2014
GABA _A $\beta 3$	4COF: 2.97 Å	C	XRD	Miller and Aricescu, 2014	2014
GlyR $\alpha 1$	3JAE: 3.9 Å	O	ECM	Du et al., 2015	2015
	3JAD: 3.9 Å	C			
GlyR $\alpha 3$	5CFB: 3.04 Å	C	XRD	Huang et al., 2015	2015
GlyR $\alpha 1$ (TMs)	2M6B	NA	NMR	Mowrey et al., 2013a	2013
	2M6I	O			
GlyR $\alpha 1$ (TM2–TM3)	1VRY	NA	NMR	Ma et al., 2005	2005
Chimera GlyR-GLIC	4X5T: 3.5 Å	C	XRD	Moraga-Cid et al., 2015	2015
Chimera ELIC-GLIC	4YEU: 4.60 Å	R	XRD	Unpublished	2015

of the most studied LGIC, describing both a detailed structure of the agonist binding-site and proposing some sequential events that might lead to channel opening (Unwin, 2005). More recently, the high-resolution structures of the prokaryotic ligand-gated ion channel (LGIC) activated by GABA from *Erwinia chrysanthemi* (ELIC) (Hilf and Dutzler, 2008; Zimmermann and Dutzler, 2011) and the proton-gated ion channel from *Gloeobacter violaceus* (GLIC) were published (Prevost et al., 2012; Sauguet et al., 2013b). In comparison with GlyR, the analysis showed some differences likely related to the functionality of the proteins. First, they presented a lower sequence identity of 25% ($\alpha 1_{\text{ELIC}}$: 24%; $\alpha 1_{\text{GLIC}}$: 23%). Second, unlike GlyR, they had cationic selectivity and a poor extent of pharmacologic modulations.

A more significant contribution for the understanding of GlyR structural characteristics was recently achieved by studying the crystallographic structure of the glutamate-gated chloride channels (GluCl) from *Caenorhabditis elegans*, a eukaryotic LGIC, which allowed the homology modeling of GlyR because it presented a higher percentage of identity ($\alpha 1_{\text{GluCl}}$: 44%) (Hibbs and Gouaux, 2011). A key advancement in the resolution of GlyR structure was obtained from studies of homopentamers formed by $\alpha 1$ or $\alpha 3$ subunits and the chimeric receptor GLIC/ $\alpha 1$ GlyR called Lily (Du et al., 2015; Huang et al., 2015; Moraga-Cid et al., 2015). All these recent studies have contributed to understand the nature of conformational changes that might occur during open-closed states, desensitization, and pharmacologic modulation.

The resolution of GlyR structures confirmed the classic topology of the Cys-loop family, which includes a large extracellular N-terminal domain (ECD), four transmembrane domains (TM1–4), a large IL between TM3 and TM4, and a small extracellular C-terminal region (Moss and Smart, 2001; Thompson et al., 2010) (Fig. 1A). These protein structures also confirmed the conformation of functional pentameric ion channel complexes, with the TM2 domains shaping the ion channel pore at the central axis and the TM4 domains facing the plasma membrane (Fig. 1B).

In addition, some elements of the secondary structure and most of the residues interacting with extracellular ligands have been identified through the analyses of these structures. The ECD, for example, showed a classic barrel-like structure

formed by 10 β -strands ($\beta 1$ – $\beta 10$) accompanied by two α -helices at the N-terminal between β -strands 3 and 4 (Fig. 1A). The neurotransmitter binding site is located between two neighboring subunits and is formed by three loops (A, B, C) of the principal subunit (+) along with three β -strands (D, E, F) from the complementary subunit (–) (Unwin, 2005; Du et al., 2015; Huang et al., 2015). A more detailed analysis revealed that amino acids R65, T204, Y202, S129, F159, and P207 interact with glycine in the agonist-binding site (the numbers relate to the mature sequence of human $\alpha 1$ GlyR) (Du et al., 2015; Huang et al., 2015) (Fig. 1C). These residues are in agreement with the data obtained from functional and mutagenesis studies regarding agonist binding (Rajendra et al., 1995; Laube et al., 2002; Pless et al., 2011; Yu et al., 2014).

There are five identical agonist-binding sites on homopentameric $\alpha 1$ GlyR. In heteropentameric GlyR, the presence of α/β and β/β binding interfaces play a role in modifying the agonist affinity and pharmacologic properties (Dutertre et al., 2012; Shan et al., 2012). The TM, on the other hand, consist of four amphipathic α -helices (TM1–4), where TM2 helices form the channel pore while TM4s are located on the external face corresponding to the region that interacts with lipid components of the neuronal membrane (Ma et al., 2005; Mowrey et al., 2013a) (Fig. 1, A and B). Unfortunately, to facilitate crystallization of these proteins, the large intracellular domain that connects TM3 and TM4 was truncated or replaced by short peptide linkers, so it is currently not experimentally resolved (Thompson et al., 2010; Langlhofer et al., 2015).

Despite the lack of detailed structural information, data from 5-HT₃ receptors and nAChR strongly suggest the presence of α helices in intracellular regions (Unwin and Fujiyoshi, 2012; Hassaine et al., 2014). In addition, a recent study using in silico and circular dichroism methodologies has proposed the existence of a helical conformation at both the N- and C-terminal regions of the IL in $\alpha 1$ GlyR near the TMs (Burgos et al., 2015a).

Transitions between Open and Closed States

The available structures of eukaryotic Cys-loop receptors in expected open/close conformations have allowed depicting some events that could well represent key steps leading to

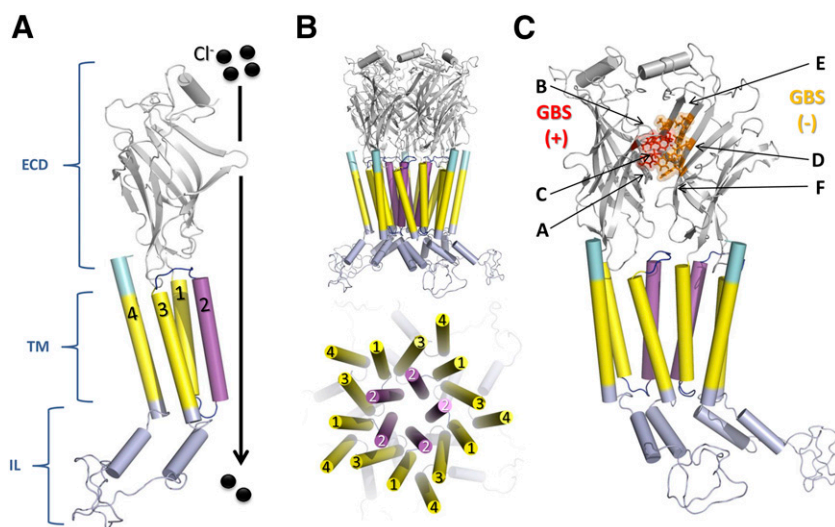


Fig. 1. Topology and schematic structure of GlyR. (A) Representation of a monomer of $\alpha 1$ GlyR where the different regions of the receptor are presented: extracellular domain (ECD, gray), transmembrane domains (TM, yellow) highlighting the TM2 which is part of the channel pore (magenta), the intracellular loop domain (IL, light blue), and C-terminal region (cyan). (B) Pentameric arrangement of subunits to form functional GlyR (C) Representation of dimer $\alpha 1$ – $\alpha 1$ GlyR where the glycine binding site (GBS) is located. The loops (A–C) and β -strands (D–F) that comprise the GBS are also labeled. The amino acids from the principal (F44, F63, R65, L117, L127, S129) and complementary subunit (F158, Y202, T204, F207) are colored red and orange, respectively. The IL is based on the $\alpha 1$ GlyR full model described previously by Burgos et al. (2015a). All images were created using PyMOL.

ion channel opening. For instance, structures for nAChR (Unwin and Fujiyoshi, 2012), GluCl (Hibbs and Gouaux, 2011; Althoff et al., 2014), and $\alpha 1$ GlyR (Du et al., 2015) have been described in the agonist-bound open state and in the antagonist-bound closed state. Structural alignments of each receptor between its open and closed conformations showed that the major conformational changes in the Cys-loop receptors are well conserved.

Briefly, upon activation by agonist binding to the ECD, the upper section of ECD twists around the pore axis while the lower ECD section tilts toward the center of the pore, accompanied by a significant displacement of loop C (Althoff et al., 2014; Du et al., 2015). To transfer the conformational changes in the ECD to the TM domains and open the ion channel, the upper section of TM2 seems to rotate outward because of the displacement of the Cys-loop through $\beta 10$, producing the coupling of the TM2–TM3 extracellular loop to the $\beta 1$ – $\beta 2$ loop.

It was also shown that the lower section of TM2, connected to TM1 and coupled to the $\beta 8$ – $\beta 9$ loop, moves toward the pore axis as a consequence of displacement of the $\beta 8$ – $\beta 9$ loop and subsequent rotation of TM1 (Du et al., 2015). Additionally, TM3 and TM4 rotate clockwise around the center of the helix bundle, producing the apparent complete rotation of the TM domains (Althoff et al., 2014).

It is currently assumed that all these conformational changes gated by the binding of the agonist to ECD might actually lead to the opening of the ion channel. Nevertheless, a finer picture, where intermediate and desensitized states of the receptor are also incorporated, is pending further studies.

Molecular Sites for the Functional Regulation of GlyR

The availability of Cys-loop family receptor structures provides information about the location and possible mechanisms of action of several physiologically and other clinically relevant modulators (see Table 2). For instance, these studies have provided structural information for GLIC and ELIC bound to key pharmacologic modulators such as general anesthetics (desflurane, isoflurane, propofol, ketamine) (Nury et al., 2011; Pan et al., 2012a; Kinde et al., 2015), benzodiazepines (Spurny et al., 2012), ions (Hilf et al., 2010; Hibbs and Gouaux, 2011; Zimmermann and Dutzler, 2011), and ethanol (Sauguet et al., 2013a). Interestingly, some of these modulators have opposite pharmacologic effects to those reported in GlyR (Table 2). Unfortunately, only a single study has provided the structure of GlyR bound to one modulator, ivermectin, showing the molecular interactions of this antiparasitic drug with the TM domains of GlyR $\alpha 1$ subunits (Du et al., 2015). Despite these limitations, the current evidence makes it possible to describe the molecular sites for the regulation of GlyR by important modulators.

One chemically simple ligand able to modulate the activity of GlyR is Zn^{2+} , which is stored in synaptic vesicles in glycinergic, GABAergic, and glutamatergic neurons. When this ion is released during neurotransmission, it can reach concentrations greater than 100 μM , which allows for the modulation of different receptors, including GlyR (Birinyi et al., 2001; Frederickson and Bush, 2001; Trombley et al., 2011). Zn^{2+} modulates $\alpha 1$ GlyR in a biphasic concentration-dependent manner. Potentiation of glycine-evoked currents

TABLE 2

Experimentally obtained structures of prokaryotic LGIC with pharmacologic and physiologically relevant compounds
All modulators described here were cocrystallized with their respective receptors allowing the analysis and description of each binding site.

Receptor	Modulator	PDB Code	Molecular or Pharmacologic Effects	GlyRa	Reference
GLIC	Desflurane	3P4W	Inhibition ($IC_{50} = 27 \pm 13 \mu M$)	+	Nury et al., 2011
		3P50	Inhibition ($IC_{50} = 24 \pm 6.3 \mu M$)	+	Nury et al., 2011
	Propofol	4F8H	Inhibition ($IC_{50} = 58 \mu M$)	+	Pan et al., 2012a
		4HFD	Potentialiation by 1 mM (F14'A ethanol-sensitive mutant)	+	Sauguet et al., 2013a
	Ketamine	4HFD	Potentialiation by 1 mM (F14'A ethanol-sensitive mutant)	+	Sauguet et al., 2013a
		5HCM	Locally closed conformation (2–21' cross-linked mutant)		Laurent et al., 2016
	Bromoforn	2XQ8	Inhibition by pore blocker	+/-	Hilf et al., 2010
		4ZZB	Inhibition ($IC_{50} = 1.05 \text{ atm}$)	+	Weng et al., 2010;
	Zinc	4ZZC			Sauguet et al., 2016
		2XQ6	Inhibition by pore blocker	WE	Hilf et al., 2010
	Xenon	4ILA	Inhibition by pore blocker (GLIC A237F mutant)		Sauguet et al., 2013b
		4HFE	Potentialiation by low ethanol $\geq 20 \mu M$ (F14'A ethanol-sensitive mutant)	+	Sauguet et al., 2013a
ELIC	Isoflurane	4Z90	GLIC wild type insensitive to ethanol	+	Chen et al., 2015
		4Z91	Resting state, in absence of agonists	+	Chen et al., 2015
	Bromo-flurazepam	4A98	Desensitized state – in presence of agonists		
		2YOE	Inhibition on ELIC ($IC_{50} = 21.9 \pm 1.5 \mu M$)	+	Spurny et al., 2012
	GABA and flurazepam	4A98	Potentialiation similar to flurazepam	+	Spurny et al., 2012
		2YOE	Potentialiation flurazepam $\leq 50 \mu M$ and inhibition at higher concentrations (biphasic effects)	+	Spurny et al., 2012
	Zopiclone	4A97	Inhibition ~90% by 10 μM	NA	Spurny et al., 2012
		3ZKR	Inhibition ($IC_{50} = 125 \pm 10 \mu M$)	+	Spurny et al., 2013
	Bromoforn	2YN6	Inhibition by decreasing agonist efficacy and blocking channel opening	WE	Zimmermann et al., 2012
		4TWD	Inhibition by decreasing agonist efficacy and blocking channel opening	WE	Ulens et al., 2014
	Barium	4TWD	Inhibition by decreasing agonist efficacy and blocking channel opening	WE	Ulens et al., 2014
		4TWF	Inhibition by decreasing agonist efficacy and blocking channel opening	WE	Ulens et al., 2014
Memantine	4TWD	Inhibition by decreasing agonist efficacy and blocking channel opening	WE	Ulens et al., 2014	
	4TWF	Inhibition by decreasing agonist efficacy and blocking channel opening	WE	Ulens et al., 2014	
Bromo-memantine	4TWD	Inhibition by decreasing agonist efficacy and blocking channel opening	WE	Ulens et al., 2014	
	4TWF	Inhibition by decreasing agonist efficacy and blocking channel opening	WE	Ulens et al., 2014	

+, Potentialiation, +/-, biphasic effect; NA, not available; WE, without effect.

^aRefers to the most reported effect.

occurs at low concentrations ($<10 \mu\text{M}$) whereas higher concentrations ($>10 \mu\text{M}$) produce inhibition (Bloomenthal et al., 1994; Laube et al., 1995).

This biphasic modulation appears to be associated with different binding sites within the GlyR structure. The binding sites for Zn^{2+} associated with potentiation of glycinergic currents are located on the outer face of the N-terminal domain of $\alpha 1$ GlyR subunits and are composed of amino acids forming β -strands in the ECD (Fig. 2, A and B). These include the amino acids D80, E192, and D194 complemented by H215 and T151 (Laube et al., 1995; Laube et al., 2000; McCracken et al., 2013).

The binding of Zn^{2+} to GlyR produces an increase in channel open probability and burst duration, indicating an open state stabilization after agonist binding, even turning taurine, a partial agonist of GlyR, into a full agonist (Laube et al., 2000; Miller et al., 2008; Trombley et al., 2011; Farley and Mihic, 2015). On the other hand, the amino acids associated with the inhibitory effect of Zn^{2+} in $\alpha 1$ GlyR are located on the inner side of the ECD oriented toward the vestibule, and they include residues H107, H109, T112, and T133 (Fig. 2, A and C) (Miller et al., 2005b).

The inhibition of the glycine-evoked current is associated with reduced channel opening (gating) and a decrease in agonist efficacy (Miller et al., 2005a; Eto et al., 2007). Interestingly, in recent years it has been shown that modulation by Zn^{2+} is related to the effects of ethanol on GlyR, where Zn^{2+} increases the potentiation of glycinergic currents in presence of ethanol, which occurs in brain regions associated with the development of alcohol addiction (McCracken et al., 2010b, 2013; Morud et al., 2015).

Another important group of modulators are general anesthetics, which produce a marked depression in several CNS functions resulting in muscle relaxation, amnesia, and loss of

consciousness (Rudolph and Antkowiak, 2004). Volatile anesthetics such as isoflurane, halothane, and enflurane potentiate glycine-evoked currents in $\alpha 1$ GlyR (Harrison et al., 1993; Downie et al., 1996; Mascia et al., 1996). The potentiation elicited by this group of pharmacologic agents is associated with a positive modulation on glycinergic synapses, which display increased decay time constant kinetics and frequency in glycinergic inhibitory postsynaptic potentials (Yamashita et al., 2001; Cheng and Kendig, 2002).

From mutagenesis and physiologic studies, it is believed that the binding site for volatile anesthetics on GlyR consists of amino acids located in TM2 and TM3 that form an intrasubunit cavity (Mihic et al., 1997; Roberts et al., 2006; Duret et al., 2011) and include residues S267 (TM2) and A288 (TM3) (Horani et al., 2015) (Fig. 2A,D). A similar cavity was described in GABA_AR where amino acids S270 (TM2) and A291 (TM3) in the $\alpha 1$ subunit appeared to be important for the potentiation (Mascia et al., 2000; Jung et al., 2005; Jung and Harris, 2006). This binding site is shared with general anesthetics and alcohol and has been discussed previously elsewhere (Lobo and Harris, 2005; Howard et al., 2014; Olsen et al., 2014; Trudell et al., 2014).

Additionally, recent studies have reported that the endovenous anesthetic propofol binds to this cavity in a more superficial position compared with desflurane. However, this site is not the only one that is important for general anesthetics. For example, another study reported the presence of at least one residue (F380) outside this cavity located in the MA-stretch of the IL that is essential for propofol actions on $\alpha 1$ GlyR (Moraga-Cid et al., 2011). However, it has been reported that this putative binding site is exclusive for propofol because substitution of residue F380 by alanine attenuates the potentiation of glycinergic currents to propofol without altering the basal activity of the receptor or the modulation by

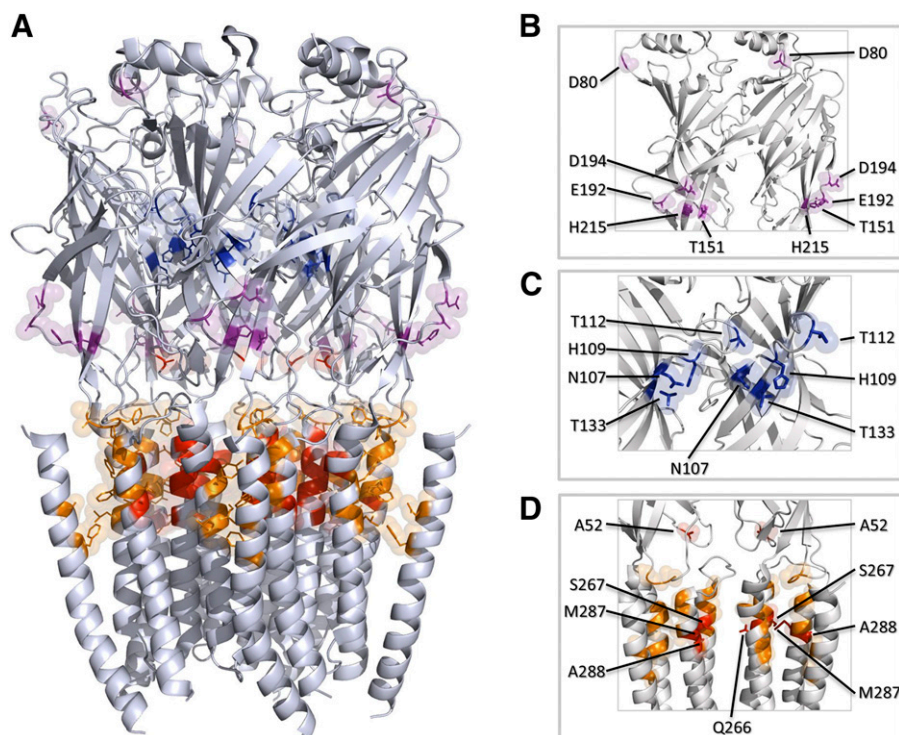


Fig. 2. Putative binding sites for selected important modulators in the $\alpha 1$ GlyR structure. (A) Representation of a homopentameric $\alpha 1$ GlyR (PDB:3JAE) where the binding sites for low (magenta) and high (blue) Zn^{2+} concentrations are shown. Amino acids forming the shared cavity for ethanol (red) and general anesthetics (orange) are also shown. (B) Detailed view of amino acids involved in Zn^{2+} potentiation by low concentrations ($<10 \mu\text{M}$), and (C) an internal pore view that show amino acids related to the inhibitory effects of Zn^{2+} . (D) Ethanol and general anesthetics binding site; principal amino acids are colored red while the residues that complement the cavity are colored orange. All images were generated using PyMOL.

other anesthetics such as isoflurane or etomidate (Moraga-Cid et al., 2011). More interestingly, this mutation does not affect ethanol actions on GlyR, supporting the notion that the pharmacologic actions of propofol and ethanol are possibly mediated by different sites in the protein.

The other pharmacologic compound that affects the regulation of GlyR is ethanol, one of the most popular drugs of abuse worldwide. The acute effects of alcohol in the CNS can vary from a decrease in sensorial reflexes, disinhibition in social behaviors, and euphoria, to depression, mental incoherence, coma, and death (Spanagel, 2009; Perkins et al., 2010). Despite the significant impact of ethanol on health and society, its mechanism of action has been difficult to elucidate, and several theories have been proposed.

One of the most accepted molecular targets to explain the complex effects of ethanol in mammals are the LGIC (Harris et al., 2008; Howard et al., 2014). Studies in cultured mammalian neurons and recombinant receptors have determined that several LGIC are modulated by clinically relevant ethanol concentrations (Lovinger et al., 1989; Aguayo, 1990; Lovinger, 1991; Aguayo and Pancetti, 1994; Cardoso et al., 1999; Moykkynen et al., 2003). Additional analysis found that low ethanol concentrations (≤ 50 mM) potentiate glycinergic currents by increasing the apparent affinity of GlyR with no change in efficacy (Aguayo et al., 1996; Crawford et al., 2007; Perkins et al., 2008), and showed that alcohol effects on glycinergic currents were developmentally regulated in cultured spinal neurons (Tapia et al., 1997; Tapia and Aguayo, 1998). Other studies have demonstrated that ethanol affects GlyR expressed in hypoglossal motoneurons (Eggers and Berger, 2004; Aguayo et al., 2014), the spinal cord (Celentano et al., 1988; Mariqueo et al., 2014), and ventral tegmental area neurons (Ye et al., 2001).

Two main hypotheses have been offered to explain the actions of ethanol on GlyR. The first and most studied and recognized mechanism proposes that the functional modulation of glycine-activated currents occurs by a direct interaction of alcohol with a group of amino acids that form a binding pocket, which is shared with other long-chain alcohols and general anesthetics (Lobo and Harris, 2005; Harris et al., 2008). This binding site for ethanol (Fig. 2D) comprises the S267 (TM2) and A288 residues (TM3) complemented by Q266 and M287. Besides this site, additional binding pockets for ethanol have been proposed in the TM4 domain (i.e., I409, Y410, and K411) (Lobo et al., 2006; McCracken et al., 2010a) and in the ECD (i.e., A52) (Crawford et al., 2007; Perkins et al., 2008). Thus, it is likely that several binding pockets for ethanol exist on GlyR, and they should become occupied as the concentration of the ligand increases.

Another hypothesis was proposed to explain the effects of ethanol at pharmacologic concentrations (≤ 100 mM) involving an intracellular mechanism and G protein modulation. These studies in recombinant and native GlyR determined that the potentiation of the receptor by ethanol is associated to an intracellular modulation by $G\beta\gamma$ (Yevenes et al., 2008, 2010). The binding of $G\beta\gamma$ to the IL of $\alpha 1$ subunits facilitates channel opening of GlyR, showing an increase in amplitude of glycine-evoked and GlyR open probability (nPo) (Yevenes et al., 2003). More detailed analysis determined that the physical contacts between two groups of basic amino acids in the IL of the $\alpha 1$ subunit ($^{316}\text{RFRRK}^{320}$ and $^{385}\text{KK}^{386}$) are pivotal for the interaction between GlyR and $G\beta\gamma$ (Yevenes

et al., 2006, 2008). Substitutions of these basic residues by alanines generated GlyR with low ethanol sensitivity in the pharmacologic range (≤ 100 mM) and did not display changes in ion channel function or the sensitivity to other agents such as isoflurane, propofol, zinc, alphaxolone, or longer n-alcohols, indicating a great degree of selectivity (Yevenes et al., 2008; Yevenes and Zeilhofer, 2011).

Future Directions for the Study of Regulatory GlyR Mechanisms

Although a considerable amount of information has been generated in the field of GlyR in the recent years, there are important issues that need to be addressed: 1) the composition of GlyR must be mapped throughout the CNS in terms of subunit composition and neuronal localization. 2) Selective agonists, antagonists, and modulators must be developed for specific GlyR subunits. Such compounds will shed light on the physiologic and pathologic roles of different GlyR subtypes in animals and humans. 3) Structural studies must be performed with pharmacologic concentrations of the ligands. It is likely that future technique refinements will show the existence of several binding sites for most of the modulators within GlyR.

Conclusion

In summary, GlyR offer a largely unexplored new dimension for the understanding of how inhibitory receptors play distinct roles in the CNS from the spinal cord to the cortex. Additional structural and functional data on different GlyR subtypes and key accessory proteins, together with the development of subunit selective ligands acting on synaptic and nonsynaptic receptors and genetically engineered animals, should increase our knowledge of these dimensions. One can expect that glycinergic-based pharmacotherapy will be developed for muscle tone, motor control, pain transmission, sedation, cognition, and reward.

Acknowledgments

The authors thank Lauren Aguayo for revising the manuscript and for expert technical assistance during the years. ASPET thanks Dr. Katie Strong for copyediting of this article.

Authorship Contributions

Contributed to the writing of the manuscript: Burgos, Yévenes, Aguayo.

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