Glycine receptors: recent insights into their structural organization and functional diversity

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Abstract

Strychnine-sensitive glycine receptors (GlyRs) are known to mediate synaptic inhibition in spinal cord, brainstem and other regions of the CNS. During the past 5 years, considerable progress has been made in delineating structural determinants of ligand binding and channel activation in recombinant GlyRs. Furthermore, immunohistochemical and gene inactivation studies have disclosed distinct distributions and functions of differentially expressed GlyR subtypes in retina, hippocampus and the dorsal horn of the spinal cord. Accordingly, GlyRs regulate not only the excitability of motor and sensory neurones, but are also essential for the processing of photoreceptor signals, neuronal development and inflammatory pain sensitization. Hence, these receptors constitute promising targets for the development of clinically useful compounds.

Keywords: homology modelling, hyperekplexia, ligand-gated ion channel, pain sensitization, synaptic inhibition.


Glycine is the simplest amino acid and has diverse metabolic functions within the mammalian CNS. Similar to GABA, glycine serves as a neurotransmitter at inhibitory synapses, where it activates strychnine-sensitive glycine receptors (GlyRs) which, like GABAA/C receptors, belong to the pentameric nicotinic acetylcholine receptor (nAChR) superfamily. Glycine furthermore acts as a co-agonist at the excitatory NMDA receptor (NMDAR), a member of the tetrameric ionotropic glutamate receptor family. Glycine levels are highest in the medulla oblongata, pons and spinal cord, regions in which GlyRs are prominently expressed. In brainstem and spinal cord, glycinerergic interneurones are involved in the control of motor rhythm generation, the coordination of spinal reflex responses, and the processing of sensory signals. Spinal Ia glycinerergic interneurones mediate reciprocal inhibition in stretch reflex circuits, thus allowing relaxation of antagonist muscles and coordinated contraction of agonist muscles, whereas Renshaw interneurones regulate motoneurone excitability by producing recurrent inhibition via a negative feedback system (overview in Legendre 2001).

The release of glycine into the synaptic cleft is triggered by the Ca2+-dependent fusion of glycine-containing synaptic vesicles with the presynaptic plasma membrane and results in the activation of postsynaptic GlyRs. These receptors respond to agonist binding by increasing the chloride conductance of the postsynaptic cell. Glycinergic synaptic currents have a complex time course, which typically consists of a fast rising phase and a bi-exponential deactivation. The rapid rise of the postsynaptic response during synaptic transmission reflects the highly synchronous opening of multiple GlyR chloride channels by glycine released during neurotransmitter exocytosis, whereas the slower decay phase of the synaptic current is shaped by asynchronous closures of individual GlyR channels and the re-uptake of glycine into presynaptic terminals or surrounding glial processes via Na+-dependent glycine transporters (overview in Laube et al. 2002; Eulenburg et al. 2005). The binding of glycine to the GlyR is antagonized by strychnine, a convulsive alkaloid from the Indian tree Strychnos nux vomica. Strychnine is widely used to distinguish glycinergic from GABAergic inhibition, and constitutes a unique tool for GlyR research.

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Abbreviations used: AchBP, soluble acetylcholine-binding protein; ECD, extracellular domain; GCL, ganglion cell layer GlyR, glycine receptor; ICD, intracellular domain; INL, inner nuclear layer; IPL, inner plexiform layer; mIPSC, miniature inhibitory postsynaptic current; nAChR, nicotinic acetylcholine receptor; NMDAR, NMDA receptor; PGE2, prostaglandin E2; TMD, transmembrane domain; WT, wild type.
Novel insights into GlyR structure and ligand activation

GlyR genes and proteins

The GlyR was the first neurotransmitter receptor protein to be isolated from the mammalian CNS (reviewed in Laube et al. 2002; Lynch 2004). Affinity purification of the detergent-solubilized receptor on aminostrychnine-agarose yielded highly enriched preparations that contained postsynaptic GlyRs composed of α and β subunits together with gephyrin, an anchoring protein required for the postsynaptic clustering of GlyRs and major GABAA receptor subtypes (Kneussel and Betz 2000). Originally, cross-linking experiments with affinity-purified GlyR preparations had indicated a stoichiometry of 3α : 2β (reviewed in Laube et al. 2002). A recent study of recombinant GlyRs using co-expression of wild-type (WT) α or β subunits with an αβ tandem construct revised this stoichiometry to 2α : 3β (Grudzinska et al. 2005) (Fig. 1). GlyR subunits display significant sequence homology to each other and, to a lesser extent, to the subunits of the nAChRs, 5-hydroxytryptamine (5-HT)3 and GABAA/C receptors, which together constitute the group I (or Cys loop) family of ligand-gated ion channels (Barnard 1992; Corringer et al. 2000). All these receptor proteins are comprised of a large N-terminal extracellular domain (ECD), four transmembrane segments (M1–M4), a long intracellular loop connecting M3 and M4, and a short extracellular C-terminus. Sequence homologies are particularly high within the transmembrane segments and a cysteine-bonded motif (Cys loop) located in the ECD. The second transmembrane segment, M2, lines the intrinsic ion channel which, in GlyRs and GABAA/C receptors, displays a strict anion selectivity with a rank order of I− > Br− > Cl− and significant permeability to bicarbonate.

Up to now, four different genes (called Glrα1–4) encoding GlyR α subunits (α1–α4) have been identified in vertebrates (Matzenbach et al. 1994; Laube et al. 2002). Alternative splicing of exons encoding segments of the extracellular N-terminal and intracellular loop regions has been shown to further contribute to the heterogeneity of GlyR subunits (Kuhse et al. 1995; Lynch 2004). In addition, a recent report has provided evidence for post-transcriptional editing of the GlyR α3 mRNA at a single nucleotide position (Meier et al. 2005). This mRNA editing results in the substitution of a proline residue at position 185 by leucine in the ECD of the α3 subunit and increases the apparent agonist affinity of the GlyR. Therefore, this substitution has been proposed to convey high agonist affinity to extrasynaptically located receptors.

GlyR α subunits display > 80% overall sequence identity and are thought to contain major determinants of agonist and antagonist binding, because their heterologous expression generates functional glycine-gated channels which display properties closely resembling those of native GlyRs. So far, only a single GlyR β subunit gene (Glrβ) has been identified in mammals (reviewed in Laube et al. 2002; Lynch 2004). Its translation product, the β subunit, is required for the postsynaptic clustering of GlyRs, owing to a hydrophobic sequence in its M3–M4 loop region that binds to the postsynaptic scaffolding protein gephyrin with high affinity (Meyer et al. 1995; Kneussel and Betz 2000). Heterologous expression of the β subunit alone does not result in

![Fig. 1 Model of the N-terminal region of the α1 GlyR and organization of the ligand-binding sites in homomeric and heteromeric GlyRs.](image)

(a) View of the ECD of the pentameric α1 GlyR modelled after the structure of the AChBP (Brejc et al. 2001; Laube et al. 2002). Individual α1 subunit backbones are coloured differently. Glycine indicated by capped sticks is bound at the interface of adjacent subunits (Grudzinska et al. 2005). (b) Schematic drawing of the pentameric arrangement of GlyR subunits in homo-oligomeric α1 (left) and hetero-oligomeric α1β (right) GlyRs. Binding sites for glycine are indicated in yellow, and glycine sites also capable of binding strychnine are shown by a red surround. Note the non-equivalence of glycine- and strychnine-binding sites in the hetero-oligomeric receptor. The binding properties of the ββ interface are unknown (indicated by ?).
glycine-activated currents, implying that α subunits are essential for the assembly of functional GlyRs.

The mechanisms that regulate the transmembrane topology and oligomerization of GlyR proteins are poorly understood. N-glycosylation of the ECDs of the α and β subunits appears to be essential for efficient intracellular trafficking of newly synthesized receptors. In addition, sequence differences between the ECDs of the α and β subunits are important for the stoichiometric assembly of GlyR α and β subunits (Griffon et al. 1999). Additional sequence motifs seem to be essential for proper membrane insertion of the individual GlyR polypeptides. Six positively charged residues located in the large cytoplasmic loop following the C-terminal end of M3 are critical for the correct topology of the GlyR α1 subunit (Sadtler et al. 2003).

Structure of the GlyR and its ligand-binding sites
Based on significant sequence homology within the group I ligand-gated ion channel family, all members are thought to share a common phylogenetic origin and structural organization (Barnard 1992; Corringer et al. 2000). Composite ligand-binding sites, conserved throughout this receptor family, are located at the (+) and (−) interfaces between subunits and involve contact residues that belong to distinct loops of two adjacent subunits (Fig. 1a). Homology modeling of GlyR proteins based on the crystal structure of the soluble acetylcholine-binding protein (AChBP) from the snail *Lymnea stagnalis* (Brookhaven Protein Data Bank entry 119B) (Brejc et al. 2001) and high-resolution three-dimensional micrographs of the nAChR (Brookhaven Protein Data Bank entry 1OED) (Unwin 2005) have provided intriguing insights into the architecture and ligand-binding pockets of the GlyR (Fig. 1a). Previously, extensive mutational analysis of the recombinant homo-oligomeric α1 GlyR has allowed identification of several key residues important for agonist and/or antagonist interaction (Laube et al. 2002; Lynch 2004). A recent study now also revealed a major role of the β subunit in ligand binding to heteromeric synaptic GlyRs (Grudzinska et al. 2005). Here, highly conserved residues of both the α and β subunits are involved in ligand binding. Specifically, homologous arginines located at the (−) interface of the α and β subunits (α1Arg65, βArg86) are found to form strong ionic interactions with the α-carboxyl group of glycine, whereas acidic residues at their (+) interfaces (α1Glu157, βGlu180) interact with its α-amino group. Interestingly, similar side-chain interactions have been shown to mediate co-agonist binding in the glycine-binding pocket of the NMDAR (Laube et al. 1997; Furukawa and Gouaux 2003), although the subunit structures and ligand-binding sites of ionotropic glutamate receptors are phylogenetically unrelated to those of the group I ligand-gated ion channels. As shown in Fig. 1b, the work of Grudzinska et al. (2005) also highlighted differences in the number of agonist- and antagonist-binding sites between homo- and hetero-oligomeric receptors. First, homomeric GlyRs have five identical ligand binding sites for agonists and antagonists at all subunit interfaces (Fig. 1b). In contrast, the interfaces of hetero-oligomeric GlyRs are heterogeneous (αβ, βα and ββ), and evidence that all interfaces contribute to agonist binding is lacking. Second, although glycine and strychnine share overlapping but non-identical binding determinants, in hetero-oligomeric GlyRs strychnine preferentially interacts with the ββ interface (Fig. 1b). The pharmacology of the unique ββ interface is presently unknown but might be exploited as a target for synaptic GlyR modulation, in analogy to the benzodiazepine-binding site formed by the single γ subunit of the related GABA<sub>A</sub> receptors (Cromet et al. 2002).

Mechanisms coupling ligand binding to channel opening
Structural studies of the group I ligand-gated ion channel family indicate that the extracellular agonist-binding site and the channel gate located in transmembrane segment M2 are separated by a distance of approximately 30 Å (Unwin 2005). Therefore local conformational changes induced by ligand binding must be propagated to distant domains of the receptor protein. Although much is known about the structure of the ECDs homologous to AChBP, the conformational transitions coupling neurotransmitter binding to channel gating are not understood. High-resolution electron microscopy by Unwin and collaborators (2003, 2005) suggests that agonist binding to the nAChR induces a clockwise 10° rotation of the inner strands of the extracellular ligand-binding domain and a

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**Fig. 2** Structural model of the ECDs and TMDs of two adjacent subunits in the α1 GlyR homo-oligomer. GlyR α1 subunits were modelled based on the structures of AChBP for the ECD (Brejc et al. 2001) and the nAChR for the TMD (Miyazawa et al. 2003; Unwin 2005). The polypeptide backbones are displayed as tubes, and regions of the putative gating interface are colour coded as follows: the extracellular loop 2 is highlighted in red, loop 7 in blue and loop 9 in white. The remainder of the ECD is shown in green. The pre-M1 and post-M4 regions are highlighted in orange, the M2–M3 linker region in yellow, and the intracellular domains (ICDs) in orange–red. The pore-forming transmembrane segment M2 is coloured magenta, the TMDs 1 and 3 in blue–green, and M4 in cyan. Residues implicated in the coupling of the ECD with the TMDs (ECD–TMD coupling) are indicated (Glu53, Phe145, Asp148, Lys276). Dashed lines indicate the approximate borders of the lipid bilayer. The agonist-binding site is labelled by a yellow circle; residues crucial for glycine recognition are indicated (Glu157 at the (+) side, Arg65 at the (−) side). Cysteine residues forming the disulphide-bonded Cys loop (Cys138, Cys152) are indicated; in addition, a disulphide bridge unique to GlyRs (Cys198, Cys209) lies in the immediate vicinity of the agonist-binding pocket. The intramembrane binding site for anaesthetics is indicated by a red circle including residues Ile229, Ser267, Arg271 and Ala288. Channel blockers bind close to the inner mouth of the ion channel (green circle); residue Gly254 in M2 is required for efficient cyanotriphenylborate and ginkgoide B inhibition.
dislocation of its Cys loop. These conformational changes are transduced to the channel-lining M2 helix via an interaction of loop 2 of the ECD by the linker region connecting segments M2 and M3 (Miyazawa et al. 2003; Unwin 2005). Mutational data obtained for the α1 GlyR are consistent with a role of charge interactions between the N-terminal ligand-binding domain and the M2–M3 loop in coupling ligand binding to channel gating, i.e. Asp148 and Lys281 (Absalom et al. 2003; Kash et al. 2004; Schofield et al. 2003, 2004). Furthermore, additional interactions between uncharged residues located in loop 7 and the M2–M3 linker have recently been shown to determine the activation rate of channel opening (Grutter et al. 2005). As summarized in a model displaying two adjacent subunits of the GlyR (Fig. 2), loops...
2, 7 and 9 of the ECD are orientated towards the four \( \alpha \)-helical transmembrane segments M1–M4. Helix M1 and post-M4 that appear to be capable of interacting with the loops 2, 7 and 9 of the ECD (Fig. 2). In conclusion, the present experimental data suggest that coupling of agonist binding to channel opening in the GlyR is triggered by a conformational change initiated at the agonist-binding site that is transduced to transmembrane segment M2 via interactions between specific residues located at the interface between the ECDs and the loops connecting the transmembrane segments.

**Intracellular GlyR domains: roles in receptor modulation and anchoring**

Activity-dependent changes in synaptic efficacy are thought to be crucial for higher brain functions. Although different forms of plasticity at glycineergic synapses have been reported, the underlying mechanisms are unclear. For example, increases in postsynaptic Ca\(^{2+}\) concentration after synaptic activity are thought to affect the rates of insertion and retrieval of GlyRs into and from the plasma membrane. This allows modulation of the strength of glycineergic inhibition (overview in Legende 2001). The number of GlyRs at synaptic sites is regulated by factors that control exocytotic receptor insertion into the postsynaptic membrane, anchoring of the receptors at synaptic sites by binding to the scaffolding protein gephyrin, and removal of the GlyR by endocytosis and subsequent proteolysis (overview in Moss and Smart 2001). The kinetics of GlyR trafficking at the level of individual receptor molecules have been studied by single-particle tracking, which allowed single recombinant \( \alpha \)1 receptors in the membrane of transfected neurones to be followed (Meier et al. 2001). To assess the effect of gephyrin binding on the motility of the GlyR, an 18-amino acid intracellular motif of the GlyR \( \beta \) subunit mediating high-affinity gephyrin binding was inserted into the intracellular loop of the GlyR \( \alpha \)1 subunit. Comparison of the motility of \( \alpha \)1 WT and \( \alpha \)1 engineered GlyRs containing this gephyrin-binding site in neurones revealed that GlyR mobility of the engineered but not the WT GlyRs is slowed at postsynaptic sites. However, heteromeric GlyRs are not irreversibly trapped at synaptic sites but can escape from the subsynaptic gephyrin scaffold, as elegantly demonstrated by labelling endogeneous GlyRs in hippocampal neurones with antibodies coupled to quantum dots (Dahan et al. 2003). Thus, GlyRs immobilized at synaptic regions of the neuronal plasma membrane are in a highly dynamic equilibrium with a diffusive extrasynaptic state that allows receptor endocytosis and subsequent proteolytic cleavage. The latter processes are triggered upon ubiquitination of lysine residues located within the cytoplasmic loop of the GlyR subunits, as demonstrated for recombinant \( \alpha \)1 GlyRs generated in *Xenopus* oocytes (Büttrup et al. 2001).

**Emerging functions of GlyR subtypes**

**Developmental and regional expression**

The expression of the Gira1–4 genes is developmentally and regionally regulated. Biochemical and in situ hybridization studies have shown that GlyR \( \alpha \)1 mRNA and protein are prominently expressed in spinal cord, brainstem and colliculi of adult rodents, whereas \( \alpha \)2 transcripts are abundant at birth and found only at low levels in adult hippocampus, cerebral cortex and thalamus (Malosio et al. 1991; Sato et al. 1992). Moderate levels of \( \alpha \)3 transcripts are detected in spinal cord, cerebellum and olfactory bulb (Malosio et al. 1991). Presumably because of its low abundance, \( \alpha \)4 mRNA has so far escaped localization in the mammalian CNS (Harvey et al. 2000). The Gibr gene is widely expressed throughout the embryonic and postnatal CNS. The Gira genes show distinct developmental regulation: \( \alpha \)1 GlyRs accumulate, and \( \alpha \)2 GlyRs decrease after birth. Expression of \( \alpha \)3 GlyRs also occurs postnatally, whereas \( \alpha \)4, like \( \alpha \)2, transcripts appear to be mainly expressed at embryonic stages. The neonatal form of the GlyR is thought to be a homopentamer of \( \alpha \)2 subunits (Hoch et al. 1989; Takahashi 2005) that are mainly found extrasynaptically in vivo, whereas adult synaptic GlyRs are composed of \( \alpha \)1 and \( \beta \) subunits (Meyer et al. 1995).

Glycine is a major inhibitory neurotransmitter in the adult CNS, whereas it is excitatory during embryonic development and around birth. This is due to a positive chloride equilibrium potential of the postsynaptic neurones, which results in chloride efflux upon GlyR activation, causing depolarization (Reichling et al. 1994). This excitatory GlyR function is important for synaptogenesis, because GlyR-triggered rises in intracellular Ca\(^{2+}\) have been found to be crucial for the correct formation of postsynaptic glycineergic membrane specializations (Kirsch and Betz 1998). At early postnatal stages, the chloride equilibrium potential shifts to negative values owing to active chloride extrusion by the K\(^{+}\)/Cl\(^{-}\) co-transporter KCC2. Thus, GlyR activation becomes hyperpolarizing, and therefore inhibitory (Rivera et al. 1999). This developmental switch from excitatory to inhibitory glycine action is paralleled by changes in the biophysical characteristics of the postsynaptic glycine response, such as a faster rising phase and faster decay kinetics. These changes arise from a concomitant switch in GlyR subunit composition. Recombinant homo-oligomeric \( \alpha \)2 GlyRs show slower response kinetics and larger subconductance state distributions than \( \alpha \)1\( \beta \) heteromeric receptors, consistent with the *in vivo* properties of neonatal compared with adult GlyRs (overview in Takahashi 2005). The transition from (extrasynaptic) neonatal homo-oligomeric \( \alpha \)2 GlyRs to (synaptic)
adult hetero-oligomeric αβ receptors thus correlates with the shortening of the mean open time of adult GlyR channels and the faster decay times of inhibitory glycineric postsynaptic currents seen at early postnatal stages.

Early developmental roles

In addition to glycine, the endogenous amino acid taurine displays inhibitory activity when applied to neurones. Taurine is released non-synaptically from immature cortical neurones, and has been found to influence cortical development by activating extrasynaptic GlyRs (Flint et al. 1998). Recently, Young and Cepko (2004) reported that taurine regulates the number of rod photoreceptors in the developing retina by activating α2 subunit-containing GlyRs expressed by retinal progenitor cells. These data implicate extrasynaptic GlyRs in the regulation of neuronal development. Although neurotransmission between nerve cells occurs predominantly at synapses, extrasynaptic receptors are thought to mediate non-focal tonic transmission caused by non-vesicular release and/or transmitter spillover from adjacent synapses. Consistent with this, some persistent expression of the α2 subunit in the adult auditory brainstem, hippocampus and retina (Danglot et al. 2004; Haverkamp et al. 2004) may be required for tonic taurine inhibition mediated by extrasynaptically localized α2 GlyRs. In conclusion, GlyRs may produce both ‘phasic’ inhibition, triggered by high concentrations of glycine released from synaptic sites that activate synaptic GlyRs, and ‘tonic’ inhibition, as a result of persistent activation of extrasynaptic GlyRs by comparatively low concentrations of glycine or taurine in the extrasynaptic space.

GlyR diversity in a model circuitry: the mammalian retina

Although heterologous expression of different GlyR subunits revealed only comparatively subtle pharmacological differences between the receptor isoforms, evidence for differential roles of specific GlyRs in neuronal circuits has recently emerged from studies on the retina. Glycinergic transmission has a pronounced role in retinal processing of light signals (overview in Wässle 2004). In situ hybridization and single-cell polymerase chain reaction analyses showed that GlyR α subunit mRNAs are differentially distributed in the mammalian retina: α1 transcripts are predominantly localized in bipolar cells and some ganglion cells, α2 mRNA in amacrine cells of the inner nuclear layer (INL) and in the ganglion cell layer (GCL) and α3 transcripts in the entire INL and, to a lesser extent, in the GCL. As in the CNS, the β mRNA shows a widespread distribution. More recently, the localization of GlyR α subunit proteins in the retina has been investigated immunocytochemically using specific antibodies (Haverkamp et al. 2004; Jusuf et al. 2005). All antibodies produced a punctate immunofluorescence, corresponding to GlyRs clustered at synaptic sites in bipolar, amacrine and ganglion cells. Although different GlyR α subunits may exist throughout the synaptic layers of the retina, these immunocytochemical studies revealed that < 10% of the glycineric synapses show a co-localization of different α subunits within the same synapse (Fig. 3a). Hence, most retinal glycineric synapses contain only one type of GlyR channel (Haverkamp et al. 2003, 2004), consistent with a selective targeting of different α subunit isoforms to distinct synaptic sites. A recent publication (Ivanova et al. 2006) indeed indicates that different GlyR subtypes are linked to distinct neuronal circuits and consequently serve distinct functions in the retina. These authors demonstrated that the α1 subunit is present in all synaptic GlyRs in bipolar cells. In contrast, amacrine cells express mainly α2 subunits, whereas ganglion cells express both fast (α1 and α3) and slow (α2) subunits at different loci, indicating that differential expression and processing of the α subunits to discrete synaptic regions is crucial for proper functioning of the retinal network.

α3 GlyRs: molecular substrates of inflammatory pain sensitization

Physiological symptoms of strychnine poisoning include disinhibition of motor neurones and sensory afferents as well as enhanced pain perception. In the dorsal horn of the spinal cord, nociceptive afferents coming from the periphery make synaptic connections with neurones located in the superficial laminae I and II, the first site of synaptic integration in the pain pathway. Here, a local network of glycine inhibitory interneurones regulates propagation of nociceptive signals to higher brain regions. Hence, relief from glycineric inhibition can elicit and exaggerate nociceptive responses. Therefore, compounds that enhance glycine responses should have therapeutic promise for peripheral analgesia. So far, however, this concept has received little attention because of an apparent lack of GlyR isoform specificity in spinal cord. Recently, the central component of sensitization to inflammatory pain has been shown to originate from disinhibition of dorsal horn neurones, which are relieved from glycineric inhibition by the inflammatory mediator prostaglandin E2 (PGE2) (Ahmadi et al. 2002). PGE2 activates prostaglandin E receptors of the EP2 subtype and leads to a protein kinase A-dependent phosphorylation and inhibition of synaptic GlyRs containing the α3 subunit (Harvey et al. 2004). Originally, the α3 subunit was considered a minor GlyR protein with an expression pattern similar to that of the α1 subunit. However, immunocytochemistry with specific antibodies revealed that in spinal cord α3 GlyRs are distinctly expressed in the superficial layers of the dorsal horn, where nociceptive afferents terminate (Fig. 3b). Again, GlyR α3 immunoreactivity showed little overlap with GlyR α1, indicating that the two adult GlyR α subunit isoforms are targeted to distinct synaptic sites, as found in retina (Fig. 3c). Notably, inactivation of the murine Gria3 gene abolished both the inhibition of glycine currents in dorsal horn neurones seen upon PGE2 application in spinal cord slice preparations as well as PGE2-mediated pain.
sensitization in the intact animal (Harvey et al. 2004). Therefore, therapies aimed at specifically potentiating α3 GlyR currents, for example by enhancing the post-translational RNA editing process leading to high-affinity α3-containing GlyRs (Meier et al. 2005) or by administering α3-specific drugs, may prove effective in providing analgesia.

**GlyR pharmacology: unravelling antagonist and modulator actions**

Although the different GlyR α subunits exhibit differential synaptic distribution patterns and functions, particularly evident in the retina and the superficial dorsal horn of the spinal cord, the physiological consequences of different GlyR isoform distribution patterns are difficult to establish pharmacologically because of a lack of specific antagonists. The neurosteroid pregnenolone sulphate, although not specific for the GlyR, is more potent on α1 than α2 receptors and can be used to distinguish between these isoforms. Tropis-teron and colchicines are more potent blockers at α2 than α1 GlyRs. The organic anion cyanotriphenylborate acts in a use-dependent manner, with better affinities for α1 than α2 GlyRs. However, although these differences may be helpful in discriminating receptors containing different α subunits, they are far from being subtype-selective probes (see overview in Laube et al. 2002). Picrotoxin, which is widely

![Fig. 3](image_url) Differential expression of GlyR α subunits in mouse retina and spinal cord. (a) Confocal fluorescence photomicrograph showing punctate GlyR α1, α2 and α3 distributions in the inner plexiform layer (IPL) of the mouse retina. Retinal layers are indicated. Note segregation of the different fluorescence signals to distinct sublayers of the IPL. (b) Fluorescence micrograph taken from a single section through the dorsal horn of the thoracic spinal cord double-immunostained for GlyR α3 and GlyR α1. Punctate labelling shows that the GlyR α3 subunit (green) is restricted to laminae I and II of the dorsal horn, whereas the α1 subunit (red) is expressed throughout the grey matter. (c) High-resolution images and superposition show that only a few synaptic GlyR α3 clusters co-localize with GlyR α1 subunit puncta (indicated by yellow colour and arrows). Courtesy of Liane Heinze and Heinz Wässle.
used as a non-competitive GABA_\text{A} receptor antagonist, also inhibits GlyR currents. Both the glycine responses of neurones and of recombinantly expressed hetero-oligomeric αβ GlyR are relatively resistant to picrotoxin, whereas homo-oligomeric α-containing GlyRs are sensitive to low micromolar concentrations of this alkaloid. Although picrotoxin distinguishes between homomeric and heteromeric GlyRs, its lack of specificity for GlyRs restricts its usefulness in detecting the presence of heteromeric GlyRs. Recently, ginkgolide B, a component of the extract from leaves of the Ginkgo biloba tree, has been shown specifically to inhibit glycine-induced currents of hetero-oligomeric GlyRs in a non-competitive fashion with IC_{50} values in the nanomolar range, with an up to 20-fold higher affinity for hetero-oligomeric than homo-oligomeric GlyRs (Kondratskaya et al. 2005). Both picrotoxin and ginkgolide B bind to the channel region of the GlyR, as deduced from the analysis of substitutions within transmembrane segment M2. Because ginkgolide B is inactive at GABA_\text{A} receptors, it may become a useful tool with which to (i) discriminate homo- from hetero-oligomeric GlyRs, (ii) separate extrasynaptic from synaptic glycine currents, and (iii) block selectively the GlyR but not the GABA_\text{A} receptor chloride channel. The latter property is of particular interest, because GlyRs and GABA_\text{A} receptors are often co-localized at the same postsynaptic sites in spinal cord and brainstem (Bohnhalter et al. 1994). It has been demonstrated that miniature inhibitory postsynaptic currents (mIPSCs) resulting from the simultaneous co-activation of both types of receptor decay with a ‘mixed’ time course; this raises new opportunities for affecting inhibitory synaptic integration by specific modulators (overview in Laube et al. 2002). In addition, the relative strength of glycineergic and GABAergic synapses is known to change during postnatal development, such that GABA_\text{A} receptor-mediated inhibition is prevalent embryonically and GlyR-mediated transmission is dominant postnatally.

**Cannabinoids – new modulatory compounds acting at the GlyR**

It has been proposed recently that significant sequence similarities exist between partial GlyR subunit sequences and putative ligand-binding regions of cannabinoid receptors (Lozovaya et al. 2005). Indeed, the cannabinoids anandamide and 2-arachidonoylglycerol inhibit glycine currents elicited in isolated hippocampal neurones at high glycine concentrations (100 µM; Lozovaya et al. 2005). Interestingly, at low glycine concentrations (3 µM), nanomolar concentrations of anandamide and 2-arachidonoylglycerol were found to potentiate glycine-mediated currents of both recombinant and native GlyRs (Hejazi et al. 2006). Furthermore, the main psychoactive constituent of Cannabis sativa, Δ9-tetrahydrocannabinol, has recently been reported to mimic the activity of endogenous cannabinoids at the GlyR (Hejazi et al. 2006), supporting the idea of a dual regulation of these receptors by cannabinoid compounds. Δ9-tetrahydrocannabinol and synthetic cannabinoid receptor agonists have analgesic activity in acute pain models, suggesting that cannabinoids may modulate GlyRs within pain pathways.

**Positive modulators acting via the transmembrane domains (TMDs)**

Potentiation of GlyR function in the CNS is a considerable therapeutic target (overview in Laube et al. 2002). Anaesthetic concentrations of propofol, an intravenous anaesthetic, volatile halogenated hydrocarbons, such as halothane, enflurane, isoflurane, methoxyflurane and sevoflurane, and ethanol enhance GlyR currents elicited by low concentrations of agonist. Although the mechanism of action of these drugs is still unclear, different lines of evidence suggest that the TMDs of the GlyR are involved in its modulation by anaesthetics (overview in Lynch 2004). Homology modelling suggests the existence of a water-filled cavity between transmembrane segments M2 and M3 that acts as an anaesthetic-binding site (Bertaccini et al. 2005). Using site-directed mutagenesis, residues within segments M2 and M3 of the α1 subunit (Ser267 and Arg271 in M2, and Ala288 near the extracellular end of M3; Fig. 2), have been shown to be important for the potentiating effect of both volatile and intravenous anaesthetics and alcohol. A defined anaesthetic-binding site capable of modulating receptor function thus appears to exist with the membrane-embedded region of the GlyR.

**Modulation of GlyR function in vivo**

Recent progress in understanding the physiological roles of distinct GlyR subtypes has fostered efforts to terminate the ‘therapeutic orphan status’ of the GlyR (Laube et al. 2002). Transgenic mice carrying the hyperekplexia mutation Arg271Gln show spontaneous or handling-induced tremor and an exaggerated startle response (Becker et al. 2002), which can both be alleviated by treatment with subanaesthetic doses of the positive modulator propofol (O’Shea et al. 2004). Indeed, the agonist response of recombinant GlyRs harbouring the hyperekplexia 271 mutation is strongly potentiated by subanaesthetic concentrations of propofol (O’Shea et al. 2004), showing that propofol has the potency to rescue patients with hyperekplexia from acute syndromes. The potential of drugs enhancing GlyR responses is further emphasized by studies with the divergent cation Zn^{2+}. Zn^{2+} is a physiological allosteric modulator of ionotropic receptors (Smart et al. 2004) that exhibits biphasic effects on both native and recombinantly expressed GlyRs (Laube et al. 1995). Low concentrations of Zn^{2+} (1–10 µM) potentiate submaximal glycine-induced currents, whereas higher concentrations cause competitive inhibition. These differential effects have been attributed to distinct Zn^{2+}-binding sites in the ECD of GlyR α subunits (Lynch et al. 1998; Harvey et al. 2006).
et al. 1999; Laube et al. 2000; Nevin et al. 2003; Miller et al. 2005). Because Zn\(^{2+}\) is present within different subpopulations of synaptic vesicles and co-released with neurotransmitters upon stimulation, Zn\(^{2+}\) modulation is thought to be of physiological significance (overview in Smart et al. 2004). Indeed, GlyR-mediated mIPSCs have been shown to be affected by Zn\(^{2+}\), indicating that Zn\(^{2+}\) regulation is effective at synaptic sites (Laube 2002). However, whether Zn\(^{2+}\) modulation is important for neurotransmission in vivo has remained elusive. A recent study provides the first in vivo evidence for Zn\(^{2+}\) modulation of a ligand-gated ion channel in the intact mammalian CNS (Hirzel K., Muller U., Betz H. and Laube B., unpublished observation). By using gene targeting, a point mutation (Asp80Ala) was introduced into the murine Glr1 locus, which selectively suppresses Zn\(^{2+}\) potentiation. Knock-in mice homozygous for Glr1 (D80A) show a phenotype that mimics that of patients with hyperekplexia (hereditary startle disease) and of spasmodic or spastic GlyR mutant mice, and thus is indicative of decreased glycine inhibitory. These findings point to an essential role of Zn\(^{2+}\) in regulating glycine transmission in the mammalian CNS and highlight the therapeutic potential of GlyR subunit-specific modulators.

It is noteworthy that GlyRs are the only members of the group I ligand-gated ion channel family that do not have a counterpart in the metabotropic receptor families. Thus, glycine transmission apparently involves only ionotropic receptors. Curiously, however, given the lack of any direct link to G protein-coupled signalling pathways, GlyRs can be modulated by G protein \(\beta\gamma\) subunits, which increase the apparent affinity for glycine and the duration of glycineergic synaptic currents via a direct interaction with the GlyR \(\alpha1\) subunit (Yevenes et al. 2003). This mechanism may also be exploited in further attempts to potentiate GlyR currents.

Role of GlyRs in pathology

As outlined above, sublethal strychnine poisoning leads to motor disturbances, increased muscle tone, and hyperacuity of sensory, visual and acoustic perceptions. Similarly, mutations in the human Glr1 and Glrb genes cause an impairment of glycineergic transmission, which results in hyperekplexia, a hereditary neuromotor disorder (Schofield 2002). Hyperekplexia is characterized by an exaggerated startle reaction in response to unexpected auditory or visual stimuli, often followed by a short period of generalized stiffness. Consciousness, however, remains intact. Genetic studies have identified different mutations in the Glr1 gene of patients with hyperekplexia. Substitutions of the highly conserved charged residue Arg271 by uncharged amino acids (leucine or glutamine) underlie autosomal dominant hyperekplexia and result in reduced glycine affinities and chloride conductances of the respective recombinant receptors (Langosch et al. 1994). Additional autosomal recessive inheritance patterns or compound heterozygosity have also been described for other hyperekplexia mutations in Glr1 (Gilbert et al. 2004; Tsai et al. 2004; overview in Breitinger and Becker 2002; Lynch 2004). Consistent with an important role of the \(\beta\) subunit in postsynaptic GlyR function, a hyperekplexia mutation in the Glrb gene has also been identified, which significantly reduces the apparent glycine affinity of the GlyR (Rees et al. 2002). In keeping with the distinct functions of GlyR \(\alpha2\) and \(\alpha3\) subunits, no pathogenetic mutations have been detected in the corresponding Glra genes. Besides the well documented roles of GlyRs in motor control and sensory processing that are impaired in hyperekplexia, recent studies implicate GlyRs in pain perception (Ahmadi et al. 2002), the pathology of autism (Ramanathan et al. 2004), human immunodeficiency virus-associated dementia (Gelman et al. 2004) and generalized epilepsy (Sobetzko et al. 2001). Together, these results indicate a significant clinical need for novel ligands that display high selectivity for and potency at GlyRs. Two recently developed fluorescence-based assays, which allow efficient monitoring of GlyR-mediated anion influx into cultured cells, offer considerable promise for high-throughput screening (Jensen and Kristiansen 2004; Kruger et al. 2005).

Conclusion and perspectives

Remarkable progress has been made over recent years in understanding of the structural organization and functional properties of different types of GlyRs. Although GlyRs do not display the high molecular diversity characteristic of ionotropic GABA\(_{A}\) or glutamate receptors, the cell type-specific expression and differential assembly of the different GlyR subunits into distinct receptor isoforms allows for considerable specialization of glycineergic synapses. Based on the advances made in understanding of the structural basis of ligand binding in particular to synaptically localized hetero-oligomeric GlyRs, subtype-selective compounds are expected to emerge that will allow dissection of specific GlyR isoform functions in various regions of the mammalian CNS.

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