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Review

The glycinergic inhibitory synapse

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Abstract. Glycine is one of the most important inhibitory neurotransmitters in the spinal cord and the brainstem, and glycinergic synapses have a well-established role in the regulation of locomotor behavior. Research over the last 15 years has yielded new insights on glycine neurotransmission. Glycinergic synapses are now known not to be restricted to the spinal cord and the brainstem. Presynaptic machinery for glycine release and uptake, the structure and function of postsynaptic receptors and the factors (both pre- and postsynaptic) which control the strength of glycinergic inhibition have been extensively studied. It is now established that glycinergic synapses can be excitatory in the immature brain and that some inhibitory synapses can corelease γ-aminobutyric acid (GABA) and glycine. Moreover, the presence of glycine transporters on glial cells and the capacity of these cells to release glycine suggest that glycine may also act as a neuromodulator. Extensive molecular studies have revealed the presence of distinct subtypes of postsynaptic glycine receptors with different functional properties. Mechanisms of glycine receptors aggregation at postsynaptic sites during development are better understood and functional implications of variation in receptor number between postsynaptic sites are partly elucidated. Mutations of glycine receptor subunits have been shown to underly some human locomotor disorders, including the startle disease. Clearly, recent work on glycine receptor channels and the synapses at which they mediate inhibitory signalling in both young and adult animals necessitates an update of our vision of glycinergic inhibitory transmission.

Key words. Inhibitory synapse; glycine receptor; synaptogenesis; synaptic current; synaptic vesicular release; receptor-channel kinetics; postsynaptic receptor modulation; glycine uptake; glycine clearance.

Introduction

Glycine has been known to be an inhibitory neurotransmitter for more than 35 years (for review see [1]). Initially, glycine was described to be restricted to the mammalian spinal cord [2] with the first evidence for its possible transmitter status being the inhibition of motoneuron firing [3] when ionophoretically applied [4]. This inhibitory action was shown to be antagonized by strychnine [5], providing the first functional evidence for the antagonist actions of strychnine at inhibitory synapses. The inhibitory actions of glycine result from an increase in chloride conductance similar to GABA [6–9]. Hamill et al. [10] showed that activation of glycine receptors (GlyR) induced opening of chloride channels with multiple subconductance levels distinct from those elicited by GABA receptor activation.

In the spinal cord and brainstem, glycinergic inhibitory interneurons are known to control motor rhythm generation underlying the locomotor behavior (for review, see [11–13]). These inhibitory interneurons play also an important role in the coordination of spinal reflex actions. Ia glycinergic inhibitory interneurons mediate reciprocal inhibition in stretch reflex circuits, allowing relaxation of the antagonist muscle and contribute to the coordination of opposing muscles under the control of higher command centers [14–16]. Renshaw interneurons also release glycine [15, 17], and are known to regulate moto

neuron excitability and firing by producing a recurrent inhibition via a negative feedback system [16].

Understanding the pre- and postsynaptic machinery at glycinergic synapses and its modulation has greatly advanced in recent years. It is now clear that glycine and GlyRs are not confined to the brainstem and spinal cord but are present in several other areas of the central nervous system (CNS) [18, 19]. GlyRs belong to the superfamily of receptor channels, which are generally composed of five subunits. Some subunits possess an agonist binding site, and all of them contribute, at their transmembrane domain, to the pore of the ionic channel (for review, see [20]). Recent molecular and electrophysiological studies have demonstrated that like other neurotransmitters, glycine has several postsynaptic receptors subtypes which have distinct functional properties. GlyR subtypes may be differentially expressed in distinct regions of the CNS, and some of them are developmentally regulated. Electrophysiological and anatomical analysis of glycinergic synapses and receptors has revealed a functional heterogeneity at glycinergic synapses. Moreover, glycine, as well as other endogenous GlyR agonists such as taurine, may sustain functions other than inhibitory neurotransmission. Finally, the discovery that some motor disorders are related to defects in glycine receptors genes has also opened new research opportunities. In the present review, I summarize these new insights on glycine synapses and discuss the possible roles sustained by their functional diversity.

Localization of glycinergic inhibitory neurons, glycine-containing fibers and glycine receptors

Glycinergic synapses were thought for some time to be mainly localized in the spinal cord and brainstem (for review, see [21, 22]). It is now well established that glycine-containing fibers and cell bodies and GlyRs are more widely distributed in the CNS. In the adult rat, mouse, cat and guinea pig, many glycine-immunoreactive (IR) cell bodies and fibers exist in the cochlear nuclei, superior olivary complex and medial nuclei of trapezoid body [23–28], the cerebellar cortex, deep cerebellar nuclei and the area postrema [29–31]. In adult cats, glycine-IR neurons are also described in the vestibular prepositus hypoglossi, in the sensory trigeminal nucleus, in the medullary and pontine reticular nuclei [30, 32–34] and in the brainstem reticular formation [34]. A recent extensive study of glycine-IR in rats reports that glycine-containing cells are also present in the upper brainstem and the forebrain [18]. In the thalamus, glycine-IR cell bodies are mainly localized in the subfornical organ and the lateral habenular nucleus, whereas glycine-IR fibers are present in all thalamic nuclei. Interestingly, an absence of glycine-IR cell bodies is associated with the presence of glycine-IR fibers in the hypothalamus and basal forebrain, suggesting that the projections originate from distant neurons rather than on local glycinergic interneurons.

Glycine receptor expression in the adult and developing rat brain [19] has been examined using in situ hybridization with oligonucleotide probes for specific GlyR subunits [19]. The different α and β subunits (see below) are differently localized in the adult and the developing rat brain (fig. 1). However, expression of these subunits does not always overlap, and some cells expressed only the β subunits [19]. Since only α GlyR subunits possess glycine binding sites (for review, see [35]), the detection of α-subunit transcripts is assumed to indicate the presence of functional GlyRs. In the adult rat brain, GlyR α-subunit messenger RNA (mRNA) was detected in the olfactory bulb, the cerebellar cortex, the hippocampal formation, the thalamus, the hypothalamus the geniculate nucleus, the mesencephalum (in the colliculi and the central grey) in the cerebellum, (granule cells) in the lateral deep nucleus, in the brainstem nuclei and spinal cord neurons [19]. While these morphological studies may suggest that glycinergic inhibition operates more widely in the brain than previously supposed, the existence of glycine IR fibers and transcripts for GlyR α-subunits does not necessarily demonstrate that functional inhibitory synapses exist. At present, glycinergic synaptic transmission has been detected in the mammalian spinal cord [36–44], Golgi cells of the cerebellum [45] and on retinal ganglion cells [46, 47]. Functional glycinergic synapses remain to be demonstrated in other brain areas where glycine fibers and/or GlyR-subunit mRNA transcripts have been detected. Indeed, it now seems likely that some neurons possess functional GlyRs in the absence of apposed glycinergic synaptic terminals: e. g. granule cells in the cerebellum [48], dopaminergic neurons of the juvenile immature substantia nigra pars compacta [49, 50] and developing cortical neurons [51]. The function of these nonsynaptic GlyRs remains unclear, but they may be activated by a nonneuronal release of taurine (which is an agonist at GlyRs) especially during CNS development as observed in the cortex [51].

Presynaptic glycinergic terminals

Inhibitory synapses releasing glycine and/or GABA (type 2 synapses; [52] are characterized morphologically by presynaptic boutons which contain small (10–20 nm) and pleiotropic (ellipsoidal or flattened) vesicles. The width of their synaptic cleft is 10–20 nm (as compared with 30–40 nm at excitatory or type 1 synapses). Inhibitory synapses generally display symmetric pre- and postsynaptic densities, whereas excitatory synapses are characterized by asymmetric densities. In contrast to GABAergic synapses, terminals that contain glycine-loaded

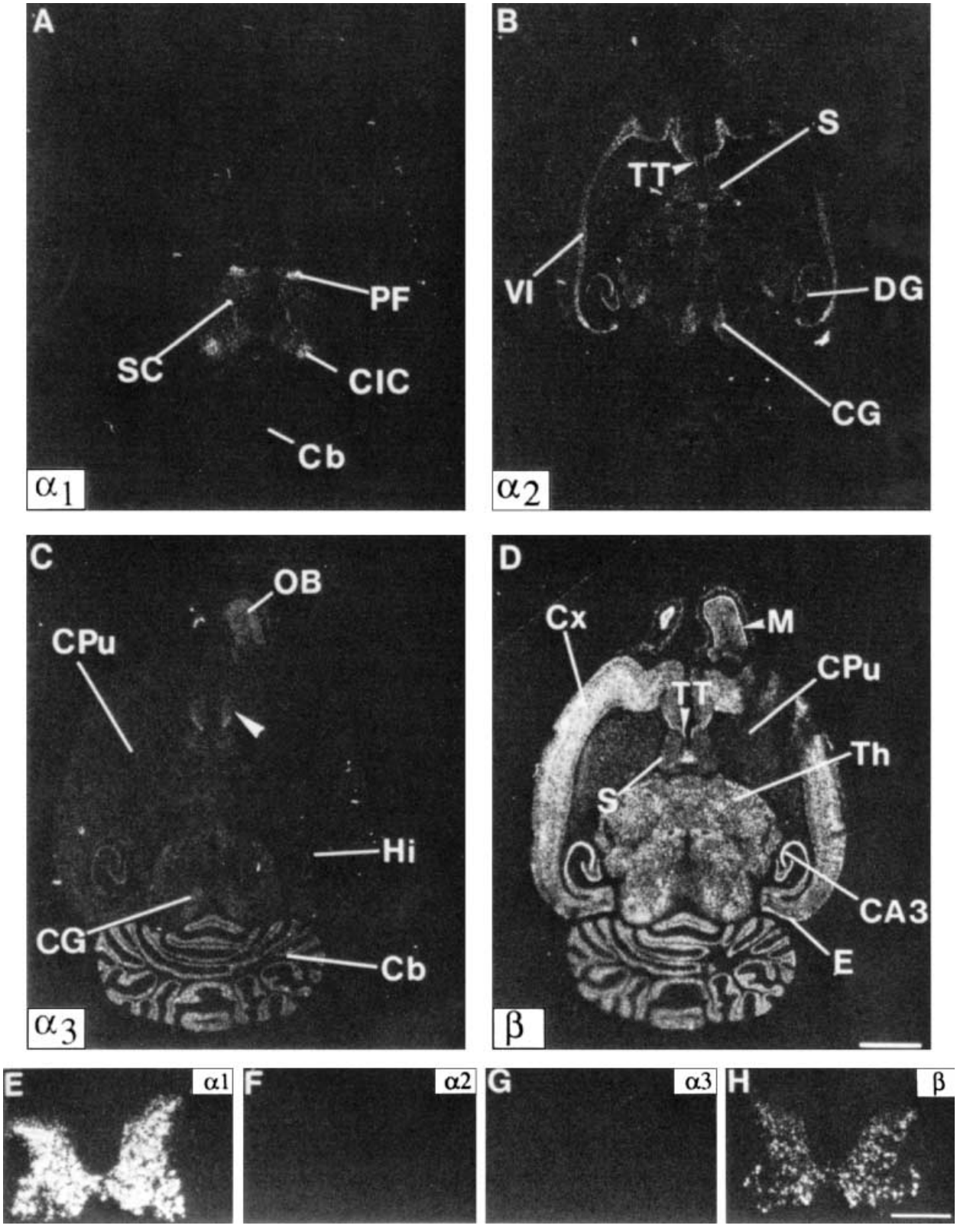


Figure 1. Brain and spinal cord localization of α1, α2, α3 and β GlyR subunit mRNAs by in situ hybridization. (*A–D*) Horizontal brain sections and (*E–H*) coronal spinal cord sections from 40 day old rats. CA3, CA3 fields of the Ammon's horn of the hippocampus; Cb, cerebellum; CG, central gray; CIC, central nucleus of the inferior colliculus; Cpu, caudate putamen; Cx, cortex; DG, dentate gyrus; E, entorhinal cortex; Hi, hippocampus; OB, olfactory bulb; PF, parafascicular nucleus; S, septum; Th thalamus; TT, tenia tacta; VI, sixth layer of cortex. Arrowhead indicates supposed internal layer of the infralimbic cortex. *D*, scale bars: 4 mm; *H*, scale bars, 1 mm. From Malosio et al. [19], with permission.

vesicles present a flat synaptic cleft and are never apposed to dendritic spines. Glycinergic synaptic boutons possess a limited number of presynaptic grids which are usually composed of 8–20 presynaptic dense projections (PDPs) [53, 54]. PDPs are disposed in triangular arrays linked at their bases [55, 65] and referred to as part of a presynaptic grid [57]. They are suggested to guide vesicles to the presynaptic membrane [58]. Synaptic grids can therefore be taken to reflect active zones, suggesting that glycinergic synapses have few release sites (1–5). The number of presynaptic grids may depend on both the synaptic site of the postsynaptic cell and on the cell type. For example, glycinergic synaptic boutons which contact the soma of the goldfish Mauthner cell possess one synaptic grid, whereas dendritically apposed synapses on the same cell possess more grids [59]. It has been proposed, from studies on the goldfish Mauthner cell, that each active zone can release only one vesicle at a time [60].

Presynaptic release machinery and vesicular glycine uptake

The synaptophysin-synaptobrevin complex is present in glycinergic synaptic boutons [61]. The vesicle protein synaptobrevin, which controls vesicle fusion during exocytosis, is certainly involved in release at glycinergic terminals, since tetanus toxin, which selectively cleaves synaptobrevin, blocks glycine release [62]**.** Presynaptic pools of glycine in synaptic vesicles are derived from both metabolic precursors and reuptake. CNS glycine is synthesized by catalysis of serine by the isoenzyme serine-hydroxymethyltransferase [63, 64]. Glycine is largely degraded by the glycine cleavage system, a mitochondrial and cytosolic enzyme complex [65].

Accumulation of glycine in synaptic vesicles implies that a vesicular transporter must exist [66]. While no specific glycine transporter has so far been described, it seems that the vesicular transporter for GABA [67] is also localized to synaptic vesicles in glycinergic neurons [68]. Since this transporter also transport glycine (fig. 2) it may represent a common vesicular transporter for these two inhibitory transmitters [61]. Observations suggesting that inhibitory presynaptic boutons may contain both GABA and glycine favor this hypothesis [68–72]. In the spinal cord, GABA and glycine-containing terminals have been clearly shown to be apposed to postsynaptic sites possessing both glycine and GABA A-type ($GABA_A$) receptors. In consequence, mixed postsynaptic responses have been detected in motoneurons [72] and in lamina I cells [73] as well as in brainstem motoneurons [43].

The ability of this vesicular inhibitory amino acid transporter (VIAAT) to transport GABA or glycine may depend on the relative extravesicular concentration of the two amino acids. Effectively, glycine inhibits GABA uptake and vice versa [70]. This may explain why some terminals contain vesicles loaded with these two inhibitory neurotransmitters. Accordingly, the presence of GABA in synaptic vesicles depends on the concentration of this neurotransmitter in the extravesicular space, which in turn is related to the presence of its synthesis enzyme GAD (glutamic acid decarboxylase) and transporters operating at the plasma membrane. Similarly, vesicular uptake of glycine depends on its extravesicular concentration, which in turn is increased by the presence of specific glycine membrane transporters. Even so, in the lateral olive, a subpopulation of nerve terminals rich in GABA or glycine appear to lack VIAAT. This suggests either that additional vesicular transporters exist or that there are alternative modes of release [68].

Glycine Transporters in the plasma membrane

A high-affinity uptake of glycine from the extracellular space (fig. 2) is mediated by different sodium/chloride transporters systems named GLYT1 and GLYT2 [74, 75]. GLYT1 is blocked by sarcosine only [76], whereas amoxapine inhibits GLYT2a more selectively [77]**.** Doxepin, amitriptyline and nortriptyline block both GLYT1 and GLYT2 [77].

GLYT1 and GLYT2 are derived from different genes, but both proteins belong to the superfamily of sodium/chloride-dependent transporters. They are characterized by 12 putative transmembrane domains [75]. The transmembrane domain III of GLYT2 is suggested to form part of a common permeation pathway for substrate and cotransport ions [78]. In rats and mice, these transporters have two isoforms, GLYT1a and GLYT1b, and GLYT2a and GLYT2b, which are splice variants of the same gene [79–81]. An additional human brain GLYT1 isoform has been cloned (GLYT1c) which contains an additional exon encoding 54 amino acids in the NH_2 terminal [82].

GLYT1 is expressed at highest concentrations in the spinal cord, brainstem, diencephalon and retina, and at lower concentrations in the olfactory bulb and brain hemispheres [83]. GLYT2 is more restricted to spinal cord, brainstem and cerebellum [83], where most functional inhibitory glycinergic synapses have been detected. GLYT2 is generally held to be expressed exclusively by neurons, whereas GLYT1 is localized on glial cells [79, 83, 84]. However, in the cerebellum, GLYT2 is localized both in presynaptic boutons and on glial elements, whereas, in the retina, GLYT1 is expressed by amacrine neurons only [83]. Moreover, a recent study has shown that both types of GLYT are expressed by oligodendrocyte progenitors [85]. During brain development, GLYT2 appears at embryonic days E18–20, shortly after the establishment of functional synapses [86]. GLYT1 is first expressed at late fetal developmental age, and expression reaches a maximum at 2 weeks postnatal [83]. The expression of GLYT1 by glial cell is upregulated by neurons [87] since

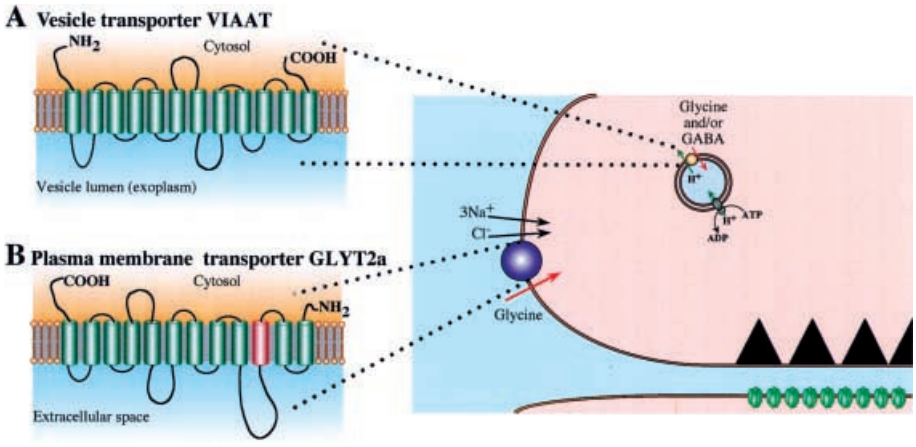


Figure 2. Schematic representation of the transportation of glycine from the extracellular space to the cytosol and from the cytosol into synaptic vesicules. (*A*) Vesicle transporter for GABA and glycine (VIAAT), with its 12 transmembrane spanning regions. (*B*) Plasmic-membrane-specific neuronal glycine transporter, GlyT2. Like VIAAT, GlyTs have 12 transmembrane domains, the third domain being involved in glycine transport.

GLYT1 is not expressed in pure glial cell cultures and increased in mixed neuronal/glial cell cultures, but can be downregulated after selective neuronal elimination of neurons [87]. GLYT2 expression is influenced by neuronal activity since acoustic stimulation, which increases synaptic excitation, evokes a localized increase in GLYT2 mRNA in neurons of the rat dorsal cochlear nucleus [88].

A major function of GLYT2a is to transport glycine from extracellular space into presynaptic boutons [89]. This transporter is unlikely to be reversed by changes in the intracellular ionic concentrations. This is due to its stoichiometry of $3Na^+/Cl^-$/glycine [89, 90]. Although GLYT2 is localized in the plasma membrane of synaptic boutons, it is generally expressed far from the active zones (release sites) localized within the synaptic cleft [91]. This suggests that GLYT2 does not contribute to the clearance of glycine from the synaptic cleft after the release of a vesicle. This is consistent with the lack of effect of glycine transporter blockade on the time course of inhibitory synaptic events in the goldfish Mauthner cell [92].

Although GLYT1 may not participate directly in synaptic transmission, it is likely to control extracellular glycine concentration. In contrast to GLYT2a, the stoichiometry of GLYT1b $2Na^+/Cl^-$/glycine predicts that glycine can be exported or imported depending on the intracellular Na^+ concentration [90]. Surprisingly, this may also be the case for the putative neuronal transporter GLYT2b [80], suggesting that glycine could be released nonsynaptically by glial cells and by some neuronal elements when they are depolarized by, for example, the activation of glutamate receptors [90]. Although the physiological implications of such a nonsynaptic glycine release remain unclear, it is possible that GLYT1b controls the activity of excitatory synapses via the allosteric action of glycine on NMDA receptors [93].

Glycine transport by GLYT1 and GLYT2 can be regulated by phosphorylation mechanisms. Potential phosphorylation sites dependent for protein kinase C (PKC), cAMP-dependent protein kinase A (PKA) and calmodulin-dependent protein kinase have been identified on GLYT2 [75]. In addition to the five consensus phosphorylation sites for PKC determined on GLYT1a, GLYT1b contains two additional potential phosphorylation sites for proline-dependent kinase [75]. However, the functional significance of GLYT phophorylation remains unclear. The activation of PKC by 12-*O*-tetradecanoylphorbol ester (TPA) or phorbol 12-myristate 13-acetate (PMA) inhibits glycine transport by GLYT1b [94, 95], but this inhibition seems unlikely to depend on a direct phosphorylation since removing all five PKC consensus sites did not prevent the inhibitory effect of PMA [95].

Protons also regulate glycine transport by GlYT1. Audrey and colleagues showed that reductions in extracellular pH inhibit the activity of GLYT1 expressed in *Xenopus laevis* oocytes [96]. This effect of protons does not depend on a decrease in the EC_{50} (median effective concentration) for sodium or glycine [96] but rather on a reduction in the rate of maximal transport. The pK_a value for proton inhibition is close to 7 and is related to titration of the histidine 421 residue of the fourth extracellular loop of the transporter [96]. Fluctuations of extracellular pH can result from excitatory neurotransmission [97], and external acidification can lead to accumulation of glycine and could consequently enhance *N*-methyl-D-aspartate (NMDA) receptor activity. However, it is not clear that such mechanisms will influence GlyRs. The increase in glycine concentration, due to extracellular acidification, seems unlikely to exceed 1 µM [96], significantly lower than the k_d for glycine on GlyRs (300–400 µM: [98, 99]).

Syntaxin 1A may modulate the incorporation of GlyTs into the plasma membrane. Syntaxin 1A is a plasma

membrane protein of the SNARE system, which regulates intracellular membrane trafficking [100] and mediates synaptic vesicle exocytosis (for review, see [101]). It has been shown to decrease the number of GLYT1 and GLYT2 proteins in the plasma membrane, but it does not modify glycine transporters expression [101]. GLYTs physically interact with syntaxin1A [102] but SNAP-25 (another SNAREs protein that interacts with syntaxin) has not been shown to participate in this regulation, and glycine does not change interactions between syntaxin 1A and GLYT1 or GLYT2 [102]. This is consistent with the proposal that GLYT2 is not incorporated into presynaptic plasma membrane close to sites of vesicular release [91].

Postsynaptic glycine receptors

Glycine receptors subunits and their encoding genes

The GlyR was the first receptor protein to be isolated from the mammalian brain [103–105]. Affinity-purified GlyR was found to contain two glycosylated integral membrane proteins of 48 kDa and 56 kDa, corresponding to α and β subunits, respectively [106]. Radioligand binding studies showed that glycine and strychnine bind to α subunits only [103–105, 107]. However, complementary DNA (cDNA) sequencing suggests that α and β subunits share similarities in their primary structure [108, 109]. Their transmembrane topology (fig. 3) is similar, and their sequences show considerable homology with nicotinic and $GABA_A$ receptor proteins [110]. Four highly conserved segments have been proposed to correspond to transmembrane-spanning α helices with a specific transmembrane domain TM2 corresponding to the pore wall of an ionic channel [111]. However, this model has been challenged recently. Studies using coupled proteolytic and mass spectrometry analyses [112] suggest instead that transmembrane domains TM1 and TM3 are too short to be membrane-spanning α helices. The TM1 and TM3 segments may instead be composed of both α helices and β sheets, whereas TM2 and TM4 are true α helices**.** Leite and colleagues [112] also provide evidence that the N-terminal domain containing the agonist binding site includes regions that may be associated with the plasma membrane. Although the functional consequences of this GlyR-subunit topology remain unclear, the transmembrane domain TM2 which forms the wall of the anionic channel is confirmed to be a true α helix as for other receptor channels [111].

Molecular approaches have revealed significant diversity of GlyR subunits. Four full-length α subunits, cDNAs have been described so far together with a single β subunit. Chromosomal location and genes have been identified for all α and β subunits. The α2 subunit has been identified as an embryonic form of GlyR. In the embryonic state, α2 mRNA is expressed in various higher brain areas including spinal cord and brainstem [113, 144]. Surprisingly, it is also detected at high densities in embryonic cortex, thalamus and hippocampus [113], areas without functional glycinergic synapses in the mature animal. Perhaps, α2 mRNA expression in these regions is related to a trophic function for GlyRs activated via non-synaptic pathways in early stages of development (see below). Expression of α2 subunits decreases after birth, whereas α1 and α3 expression increases [114–116]. Nonetheless, α2 subunit mRNA is still present at reduced densities in the adult rat CNS [117]. A full-length α4 subunit cDNA that directs the formation of functional GlyRs has recently been described as an embryonic GlyRs subunit isoform [118]. In situ hybridization techniques had shown that this subunit is expressed at high levels only in the spinal cord, sympathetic nervous system and in male genital ridge of the chick embryo [118].

α1 and α3 subunits have different locations in the adult murine brain CNS. α1 subunit mRNA is expressed in the spinal cord, the brainstem, the cerebellar deep nuclei, the hypothalamus and the colliculi [19, 119]. α3 subunit mRNA is restricted to the infralimbic system, hippocampal complex and cerebellar granular layer [19] and is detected at low levels in the spinal cord.

GlyR β subunits cannot form functional receptors alone, but their mRNA is widely expressed both in embryonic and adult brain [19, 116, 120, 121]. The distribution is much wider than that of mRNA for α subunits of GlyRs, and extends to brain areas without [^{3}H] strychnine-binding sites or immunohistochemical evidence for GlyRs. The physiological significance of this widespread β subunit transcript expression is not clear, since there is no evidence that it assembles with α subunits other than those of GlyRs.

Alternative splicing increases GlyR α subunit diversity

Diversity of GlyRs is increased by alternative splicing which generates different isoforms of α1, α2, or α3 GlyR subunits. The two α1 isoforms originate from alternative splice acceptor site selection, at the exon which encodes the intracellular cytoplasmic loop between the M3 and M4 transmembrane domains [119]. The variant of the rat α1 subunit, named $\alpha1^{ins}$, contains eight additional amino acids in the presumed intracellular loop domain close to M4, which represents a potential phophorylation site [119]. Expression of $\alpha1^{ins}$ is restricted to the brainstem and spinal cord of the adult rat. $\alpha1^{ins}$ mRNA represents 30% of the total α1 mRNA, and the ratio of $\alpha1^{ins}$ over α1 mRNA remains constant during postnatal brain development.

Two cDNA variants of the rat α2 subunit have also been described [113]. These two α2 variants, named α2A and

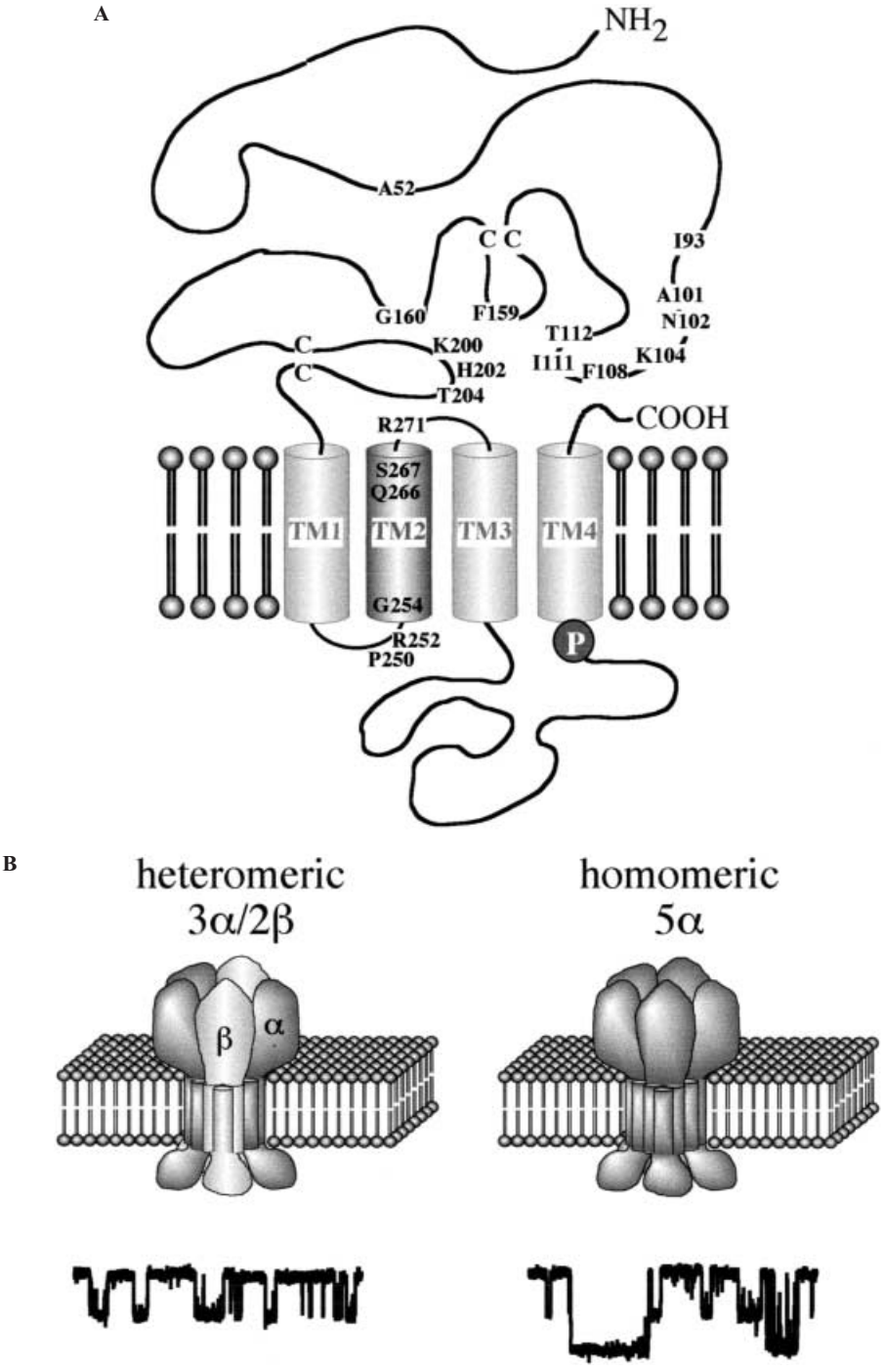


Figure 3. Structure of the glycine receptor. (*A*) Membrane topology of the α subunit with the four transmembrane domains, showing the position of functionally important amino acid residues (see text). The filled circle (black) in the TM3–TM4 intracellular cytoplasmic loop (serine 391) is the phosphorylation site for PKC. The transmembrane domain M2 forming the pore of the chloride channel is in black (*B*) arrangement of α and β GlyR subunits in the heteromeric (left) and homomeric receptors (right). The single-channel recordings (bottom) show the subconductance levels, depending on GlyRs subunit combinations (see text).

α2B, are highly homologous to the human α2 sequence [122] and are generated by alternative splicing of two homologous exons. Expression of these two isoforms is limited to early CNS development. Structurally, the variant corresponds to two amino acid substitutions in the extracellular NH_2 terminal domains [113]. In both rat and human α2B subunits, a valine is replaced by an isoleucine at position 58, and a threonine is replaced by an alanine at position 59 [113, 122]. Interestingly, α2B shares the same amino acid residues at position 58 and 59 with the rat α1 and α3 subunits [113, 122]. These two α2 subunit isoforms have similar distributions during development

although α2A mRNA is expressed at higher densities. The expression of both these GlyR-subunit isoforms decreases during brain development, but whereas α2A mRNA becomes undetectable in the more mature brain, α2B expression is still present in the adult [113]. No differences in the functional properties of GlyRs corresponding to the isoforms have so far been detected. Homologous screening of a rat brain cDNA library uncovered another α2 cDNA variant, α2* [123], which is not generated by alternative splicing. The deduced protein differs from other α2 subunits in the substitution of a glutamate for a glycine residue at position 167. This substitution results functionally in a 40-fold lower affinity for glycine and a 500-fold lower affinity for the antagonist strychnine [123]. This α2 subunit was first suggested to correspond to the ligand binding subunit of the neonatal GlyR isoform, which also possesses a lower affinity for strychnine binding in the developing spinal cord [124]. However, α2* is unlikely to be the main embryonic GlyRs α subunit since functional GlyRs with a low affinity for glycine or strychnine are absent from developing mouse spinal cord cells in culture [125], from the brainstem of juvenile rats [116] and from the hindbain of the zebrafish embryo [126]. GlyRs with a low affinity for strychnine but not for glycine are expressed by ventral tegmental area neurons during early postnatal development [127], but GlyRs with a low affinity for both glycine and strychnine have only been described in the fetal rat neocortex [51].

Two alternative splice variants of the α3 GlyR subunit have been detected in the human fetal brain [128]. The amino acid sequence of the α3L is mainly identical to the rat α3 subunit [115, 128]. The α3 variant named α3K lacks the coding sequence for 15 amino acid residues located within the postulated intracellular cytoplasmic loop between transmembrane spanning domains M3 and M4 [128]. This results from a splice event where the excision of exon 8A comprising the alternative sequence of 45 bp coincides with the persistence of a large intronic sequence in the 3′-untranslated region [128]. The alternative insert contains a consensus amino acid sequence for casein kinase-II-dependent phosphorylation of serine at positions 370 [129]. These two α3 subunits form functional GlyRs that differ in their desensitization properties, but which are similarly localized in the adult CNS [128].

GlyR quaternary structure and glycine receptor subunit assembly

The different α subunits form GlyRs with different functional properties. In order to understand this functional diversity, the quaternary structure of GlyR has been examined by chemical cross-linking of its subunits [106] (fig. 3). This analysis demonstrated a pentameric quaternary structure with an invariant stochiometry of three α and two β subunits [106]. However, heterologous expression of rat or human GlyR α subunits, in *Xenopus* oocytes or in transfected cells, can create functional receptors consisting of five α subunits and possessing a pentameric quaternary structure [122, 130–135]. Although the α/β heteromeric GlyRs have an invariant stochiometry of 3:2, GlyRs assembled from different α subunits can contain variable α subunit ratios [136]. Assembly boxes in the N-terminal extracellular domain of the GlyR subunits [136] mediate these differences. Assembly boxes are short stretches in which amino acid sequences diverge between α and β subunits [136]. The N-terminal extracellular domain of the β subunit contains the stochiometry assembly signal as well as motifs which prevent homooligomerization [136]. The different lengths of box2 in the α and β subunits may also contribute to their different assembly behavior [137]. Recently eight amino acid residues, located within the assembly boxes of the N-terminal domain of the α1 subunit, were proposed to be important in the formation of homomeric GlyRs [137]. Box 1 must combine with box 3 or boxes 2 and 8 to result in homooligomerization of the α1 subunit [137].

Neurons express both homomeric and heteromeric GlyRs. Homomeric GlyRs are the dominant form in fetal rat spinal cord neurons [138] or in early spinal cord cell cultures [125] and are also expressed by fetal neurons of the rat neocortex [51] and cerebellar granule cells [48, 139], where glycine synapses have not been detected. Homomeric GlyRs seem to be located extrasynaptically, whereas heteromeric α/β GlyRs are predominantly expressed at postsynaptic sites [18, 138, 140]. However, there is evidence that homomeric GlyRs can be synaptically activated in the zebrafish hindbrain [141]. This may also be the case in spinal cord neurons where α1 and α2 mRNA are localised at dendritic synapses, whereas β subunit mRNA is confined to the soma [142].

Overall, these results testify to the existence of numerous subtypes of GlyR and hence to a functional diversity of inhibitory glycinergic synapses. It also seems likely that GlyRs have functions distinct from their role in inhibitory synaptic transmission, at least during brain development (see below).

GlyR agregation, gephyrin and gephyrin-associated proteins

Postsynaptic GlyRs at inhibitory glycinergic synapses are aggregated in clusters whose formation is regulated by a submembrane protein scaffold (fig. 4). The pioneer immunofluorescence experiments of Triller and colleagues [143] revealed that GlyRs are distributed in an exquisite punctuate fashion on neuronal membranes. The size of GlyR clusters varies considerably on a postsynaptic neuron. This variation has been especially well studied for the goldfish Mauthner cell where cluster size increased

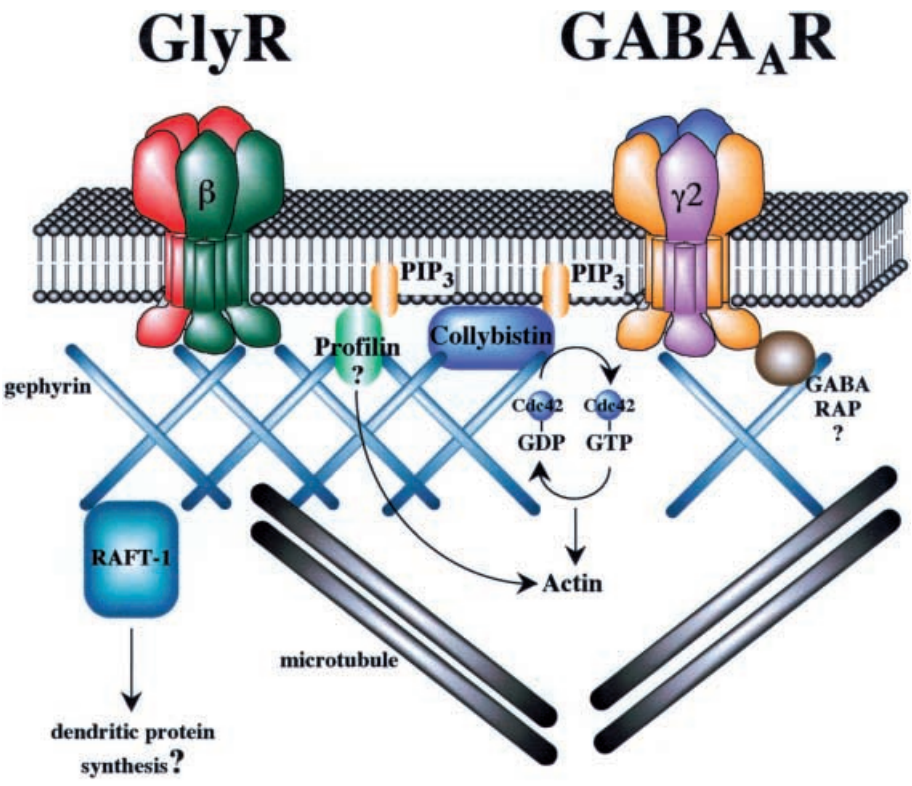


Figure 4. Schematic representation of gephyrin and the corresponding postsynaptic scaffold. Gephyrin directly binds GlyRs and microtubules and is essential for the postsynaptic location of the GlyRs and most of $GABA_A$ receptors. It was proposed that $GABA_A$ receptors might interact with gephyrin via the tubulin-binding protein GABARAP. However, it is not involved in $GABA_A$ receptor anchoring at the synapse [327]. Gephyrin also binds to PIP3 (phosphatidylinositol 3)-binding protein involved in actin dynamics and downstream signalling such as profilin and collybistin. Gephyrin also interacts with RAFT1 (rapamicin and FKBP12 target protein), a candidate regulator of dendritic protein synthesis. Adapted from Kneussel and Betz [140].

systematically with a somatodendritic gradient [144]. Electron microscopy has shown that on this cell, GlyR subunits are restricted to differentiated membrane domains which face inhibitory presynaptic terminals [143, 145, 146].

GlyR segregation depends on the presence of a postsynaptic intracellular scaffold, including a 93 KDa protein named gephyrin, identified as a peripheral membrane protein [147, 148] and first copurified with mammalian GlyRs [103, 104]. Gephyrin binds to the large cytoplasmic loop of the β subunit [149, 150] and anchors receptors to the cytoskeleton [151]. If gephyrin is crucial for GlyR cluster formation, then only α/β heteromeric GlyRs should aggregate at inhibitory synapses [152]. Evidence supporting this proposal has been derived from experiments based on antisense strategies [153] and from gephyrin knockout mice in which synaptic clustering of GlyRs is abolished [154]. However, the situation may be more complex since homomeric GlyRs deprived of β subunit can be synaptically activated, as in the hindbrain of the zebrafish larva [141].

Furthermore recent data show that homomeric GlyRs can cluster at the cell surface independently of gephyrin [155]. Nevertheless, homomeric GlyRs can be partially associated with gephyrin and their accumulation at synapses might depend on hemophilic interactions of newly synthesized GlyRs with endogenous postsynaptic GlyR complexes [155]. Clustering of GlyRs might then depend on different mechanisms from those involved in their accumulation at synapses. Gephyrin probably does act as an anchoring protein at postsynaptic sites but may not be directly involved in the formation of clusters of GlyRs [155]. Seven alternative splicing variants are known, differing in the N-terminal region associated with β subunit binding [156]. Binding depends on short amino acid sequences (cassettes) in the gephyrin N-terminal. Since some splice variants poorly bind to the GlyR β subunit, it seems likely that alternative associations are possible [156].

Gephyrin is also known to colocalise with $GABA_A$ receptors (fig. 4) [157–161]; binding specifically to $\gamma 2$ and $\gamma 3$ $GABA_A$ subunits [159, 162]. It remains unclear whether different gephyrin isoforms bind preferentially to GABA or GlyR subunits. However, multiple gephyrin isoforms may well accumulate at synapses [156], which could explain the coaggregation of $GABA_A$ and glycine receptors observed at synapses terminating on spinal cord motoneurons [72], lamina I neurons [73] as well as brainstem motoneurons [43].

Other proteins such as collybistin [163], profilin [164] and RAFT1 [165] also participate in the postsynaptic scaffold associated with the GlyR aggregate complex (for review, see [140]). Collybistin belongs to the family of the GDP-GTP exchange factors (GEFs) which regulate small G proteins of the Rho/Rac family [163]. Two variants of collybistin (I and II) originate from alternative splicing. Heterologous expression of collybistin II (a covariant lacking the N-terminal SH3 domain and the C-terminal coiled region) modifies the intracellular distribution of gephyrin [163]. Heteromeric GlyRs are recruited to the gephyrin/collybistin coaggregates, and collybistin was proposed to form part of the signalling machinery that controls gephyrin migration to the developing postsynaptic membrane [163].

Profilin is an actin monomer binding protein [166]. It stimulates the ADP-ATP exchange promoting the incorporation of monomeric actin into the filament [167]. Of the two existing profilin isoforms (which are encoded by different genes), profilin II expression is restricted to the brain and skeletal muscle [168]. Profilin-gephyrin interactions have been suggested to regulate the size of GlyR aggregates [140], consistent with evidence that treating spinal cord neurons with the cytoskeleton-disrupting drug cytochalasin D reduces the size of gephyrin clusters [151]. Gephyrin also interacts with rapamycin and the FKBP12 target protein, RAFT1 [165]. The presence of this regulator of translational machinery next to inhibitory synapses suggests that it may control dendritic mRNA translation at this synapse [140]. This can be related to the high levels of GlyR α subunit mRNA located subsynaptically in spinal cord cell dendrites [142].

The anchoring of GlyRs at inhibitory synapses requires gephyrin binding to the cytoskeleton. Gephyrin binds preferentially to polymerized microtubules, a process whose maintenance requires an ATP-dependent autophosphorylation process [169]. Depolymerization of microtubules by demecolcine disperses postsynaptic GlyR clusters and reduces the number of gephyrin clusters per cell, increasing their size but reducing the density of gephyrin molecules in a cluster [151]. These results suggest that the arrangement of submembrane gephyrin clusters depends on gephyrin binding to microtubules. Interestingly, microtubule depolymerization by nocodazole enhances GlyR desensitization, whereas microtubule polymerization by taxol decreases desensitization in spinal cord cell cultures [170]. These effects resemble those described in embryonic chick ventricle cells on L-type calcium currents, which are also linked to microtubules [171]. The effects of nocodazole and taxol on GlyRs desensitization are presumably related to changes in binding of GlyR-gephyrin complex to the cytoskeleton [170], but the exact molecular basis for these effects remains unclear.

Functional properties of GlyR subtypes

The properties of GlyRs presumably depend on their α-subunit combination and on their stoichiometry. However, the functional properties of the distinct GlyR subtypes are far from being fully understood. Different GlyR subtypes have distinct ionic conductance and kinetic properties, affinities for various agonists and/or antagonists as well as different allosteric modulatory binding sites, and they are regulated in distinct ways by phosphorylation processes.

The subconductance levels of the glycine-gated channel depend on GlyR-subunit combination

GlyRs belong to the superfamily of the ligand-gated ion channel receptors, and their activation causes an increase in the chloride conductance of CNS neurons. Studies on mouse cultured spinal cord neurons [172] revealed a relative permeability sequence of $SCN^- > I^- > Br^- > Cl^- > F^-$ for anion permeation through glycine gated channels. In symmetrical anion concentrations, the conductance sequence of GlyR channels is $Cl^- > Br^- > I^- > SCN^- > F^-$. The GlyR channel has a pore diameter of 5.2 Å and contains at least two positive charges to which anions can bind [168]. Each GlyR subunit has four transmembrane domains, with the pore of the ionic channel formed by the TM2 domain [111, 173]. The flanking arginines at the two extremities of the TM2 transmembrane domain have been shown to determine anion selectivity in experiments in which a synthetic protein with the amino acid sequence of the TM2 transmembrane segment was introduced into phospholipid bilayers [111]. Accordingly, the mutations R/L-271 or R/G-271 in the α1 subunit, responsible for the human startle disease, decrease the conductance of GlyR channels [174].

Analysis of GlyRs generated by heterologous expression in HEK-293 cells has shown that different subunit combinations open with different subconductance levels [173]. Homomeric GlyR channels possess many subconductance levels (20, 30, 45, 65, 85, 105–110 pS). α1 homomeric GlyRs have a smaller main conductance state (85 pS) than α2 or α3 homomeric GlyRs (100–110 pS), due to a substitution of a glycine residue at position 221 in the TM2 domain of α1 subunit for alanine in the TM2 domain of the α2, (at position 228), and α3 subunits (at position 221) [173]. However, subconductance levels of heteromeric GlyRs composed from different α subunits alone remain to be determined.

Heteromeric α/β GlyRs have a lower principal conductance state than homomeric receptors (fig. 3). $\alpha1/\beta$ and $\alpha3/\beta$ GlyRs have a main conductance state of 44–48 pS (88%), with rare subconductance openings at 30 and 20 pS. $\alpha2/\beta$ heteromeric GlyRs have a similar main conductance state but a distinct subconductance level of 110 pS

Table 1. Binding characteristics of the α1 glycine receptors agonist and antagonist ligands.

Main Agonists	
Glycine	K_d = 2–40 µM EC_{50} = 40–80 µM k_d = 300–400 µM*
β- alanine	K_d = 4–115 µM EC_{50} = 0.6 mM (glycine EC_{50} = 0.2 mM)
Taurine	K_d = 30–180 µM EC_{50} =1.7 mM (glycine EC_{50} = 0.2 mM)
L-alanine	EC_{50} = 3 mM (glycine EC_{50} = 0.2 mM)
D-alanine	EC_{50} = 9 mM (glycine EC_{50} = 0.2 mM)
L-serine	EC_{50} = 5 mM (glycine EC_{50} = 0.2 mM)
D-serine	no agonistic function detectable
β-aminobutyric acid	EC_{50} = 5.6 mM (glycine EC_{50} = 0.2 mM)
β-aminoisobutyric acid (β-AIBA) (partial agonist)	EC_{50} 8.7mM (glycine EC_{50} = 0.2 mM)
Main Antagonists	
Strychnine	K_i = 2–15 nM
Pseudostrychnine	K_i = 8–10 nM
α-colubrine	K_i = 10–20 nM
2-Aminostrychnine	K_i = 8–25 nM
2-Nitrostrychnine	K_i = 50–500 nM
Cacotheline	K_i = 240 nM
N-methylstrychnine	K_i = 30–500 nM
Nipecotic acid	IC_{50} = 0.8 mM (glycine EC_{50} = 0.2 mM)
Isonipecotic acid	IC_{50} = 0.2 mM (glycine EC_{50} = 0.2 mM)
Isobutyric acid	IC_{50} = 2.9 mM (glycine EC_{50} = 0.2 mM)
β-AIBA	IC_{50} = 0.8 mM (glycine EC_{50} = 0.2 mM)
5,7CIQA	IC_{50} =19 µM
Cyanotriphenylborate (CTP). Open channel blocker. Inactive on homomeric α2 GlyR	IC_{50} = 3 µM
3-[2′-phosphonomethyl[1,1′-biphenyl]-3-yl]alanine (PMBA)	IC_{50} = 400–540 nM

Data were obtained from published ligand displacement of ^{3}H-strychnine binding (K_d or K_i) and/or electrophysiological studies (EC_{50}. or IC_{50}) Glycine can have a higher EC_{50} when analyzed on GlyR activity recorded in oocyte (0.2 mM). When necessary, a low EC_{50} value for glycine is mentioned for comparison [99, 186, 189, 324–326].
* Microscopic dissociation constant (k_d) obtained using kinetic analysis of GlyRs [98, 99].

that appears to depend on residues between positions 278 and 296 of the M2 domain of the β subunit [173]. Another amino acid residue, glutamine 297 at position C-terminal to the M2 domain of the β subunit apparently decreases the occurrence of the 85 pS substate in α1/β heteromeric GlyRs [173]. This residue is thought to be located at the extracellular mouth of the GlyR channel [173]. Wild-type homomeric and heteromeric GlyRs show similar conductance states to those of receptors examined in transfected cells, although openings to small subconductance levels are more rare for heteromeric α/β GlyRs [125, 138, 141, 173].

GlyRs ligand binding sites

While most data on the pharmacological profiles of different subtypes of GlyR derive from studies using recombinant techniques in *Xenopus* oocytes or transfected cells, results are very similar to those obtained from native GlyRs.

Glycine, taurine, and β-alanine are the most common agonist ligands at GlyRs, whereas strychnine is a high-affinity antagonist. Glycine and taurine and β-alanine are known to be released in the CNS [4, 174–176], but neither taurine nor β-alanine have yet been shown to activate GlyRs synaptically. Agonists and antagonists of GlyRs are summarized in table 1. EC_{50} values (or IC_{50} for the antagonists) and I_{max} vary between different GlyR-subunit combinations.

Glycine binding sites are localized on the N-terminal of the α subunit [108]. Photoincorporation of [3H] strychnine into membrane fractions from rat spinal cord has shown that binding occurs exclusively to the α subunit [103, 104, 107, 177], and is competitively antagonized by glycine [107]. Most data on amino acid residues involved in agonist or antagonist binding has come from studies on the α1 subunit. In contrast to proposals for the $GABA_A$ and nicotinic acetylcholine receptors, the disulfide loop motif of the extracellular N-terminal of GlyR α subunits does not seem to participate in agonist binding [178]. Several specific amino acids participate in ligand binding by GlyRs, including the alanine residue at position 52 [179, 180], phenylalanine 159 to tyrosine 161 [181, 182] and lysine 200 to lysine 206 [183].

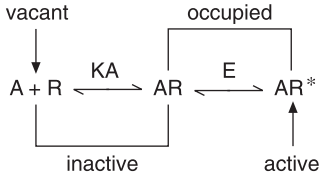
However, glycine and its antagonist strychnine do not seem to bind to exactly the same amino acid residues [184] but rather may bind to distinct but overlapping sites [185] since, for example, glycine responses are much reduced by substitution of threonine 204, whereas strychnine binding is not affected [181]. Two domains of the N-terminal of the GlyR α1 subunit are involved in the strychnine recognition site [181]. The first domain includes glycine 160 and tyrosine 161, and the second domain includes the residues lysine 200 and tyrosine 202 [181]. A β sheet-β turn motif between residues 200 and 212 may be involved in discrimination between agonists and antagonists [131, 181]. Two other domains in the N-terminal of the α1 subunit have recently been implicated in antagonist recognition. The first domain comprises lysine 104, phenylalanine 108 and threonine 112. Substitution of these amino acid residues by alanine reduced the potencies of antagonists, including strychnine, nipecotic acid and isobutyric acid, whereas agonist responses were unaffected or even slightly increased [186]. The second domain comprises isoleucine 93, alanine 101 and asparagine 102. Substitutions of isoleucine 93 and asparagine 102 by alanine and alanine 101 histidine resulted in a 17–44-fold increase in the glycine EC_{50}, whereas Imax values and the antagonist IC_{50} were unaffected [187].

The pharmacology of distinct α subunits differs according to the agonist tested, being largely dependent on the precise sequence of amino acid residues in the N-terminal. Thus, α1 GlyRs are potently activated by glycine, β-alanine and taurine, whereas β-alanine and taurine act only as weak partial agonists on α2 and α3 homomeric GlyRs [123, 131]. These differences were originally proposed to be linked to the substitution of isoleucine 111 and alanine 212 in the α 1 subunit, by a valine residue in the α2 and α3 subunits [123, 131]. This hypothesis now seems much less likely since the pharmacological profile of the recently described α4 subunit [118], which also contains valine at these sites, is similar to that of α1 homomeric GlyRs [118]. Instead, Schmieden and colleagues [186] had proposed that the low efficacy of partial agonist taurine and β-alanine on α1 GlyRs is the result of self-inhibition, implicating that all residues modulating agonistic or antagonistic ligand binding affect the maximal response of partial agonist. This is the case of the amino acid residues lysine 104, phenylalanine 108 and threonine 112 located in the N-terminal, since their replacement by alanine transforms β-alanine and taurine into full agonists [186]. However, the reality may be even more complex; the startle disease-associated mutation Q266H within the TM2 transmembrane domain of the α1 subunit converts taurine from an apparently full agonist into an antagonist [188]. This is also the case for different mutations of residues in the TM1 domain and in the TM2– TM3 extracytoplasmic loop of the α1 subunit [189]. Mutations I244A, K276A or K276E and V277A convert taurine from a relatively full agonist to an antagonist, as does Q226H [189]. These mutations have been suggested to perturb transduction processes between ligand binding and channel activation [189].

α2 subunit variants also have different pharmacological profiles. The α2 subunit variant named α2* [123], which may be expressed specifically during fetal development, has a low affinity for both glycine and strychnine [123]. This subunit possesses a glutamine instead of a glycine residue at position 167. In contrast to the α2 GlyR, responses to glycine of α2* are not inhibited by taurine [123], suggesting that the glutamine residue at position 167 may be involved in agonist and antagonist recognition.

In summary, many electrophysiological, biochemical and molecular cloning studies have permitted localization of α-subunit microdomains involved in agonist or antagonist binding and in the mechanisms that intervene between ligand binding and channel opening. However, there is still no clear picture of binding sites for different agonists and antagonists, due to the difficulty in separating the molecular events that correspond to binding, transduction and channel activation. Most pharmacological studies analyze dose-responses curves for agonists and antagonists and use the parameters EC_{50} (or IC_{50}) and I_{max} (the maximum response to a saturating agonist concentration) to measure the 'apparent' affinity and the efficacy of a given molecule. However, the kinetic meaning of these parameters is complex, and their measurement does not permit the real efficacy and the dissociation constant to be discriminated [190].

The kinetics of interactions involving GlyRs can only be examined when the parameters EC_{50}, IC_{50} and efficacy are clearly defined in terms permitting activity to be linked to molecular structure. Colquhoun [190] recently reviewed this theoretical problem. Briefly, EC_{50} depends on both binding and on conformational changes leading to channel openings. A simple stochastic model can be demonstrated as an example, as follows:



This model possesses a single binding step (AR; where A is an agonist that binds to a receptor R) and one opening step (AR*). In this case, $EC_{50} = K_A/(1+E)$, where K_A is the microsocopic dissociation constant ($K_A = k_{off}/k_{on}$), k_{off} is the dissociation rate constant in s^{-1} and k_{on} is the association rate constant in $M^{-1}s^{-1}$), and E is the equilibrium constant for shut-open isomerization ($E = \beta/\alpha$, where β is the opening rate constant in s^{-1} and α the closing rate constant in s^{-1}). Obviously, the EC_{50} may change indepen-

dent of changes in the affinity or K_A. E refers to a true efficacy and cannot be directly estimated from I_{max}, the maximum current evoked by a saturating agonist concentration (I_{max} = inPo$_{max}$, where i is the unitary current due to a single channel opening, n the number of channels activated and Po$_{max}$ the maximum fraction of receptors in the active state: Po$_{max}$ = E/(1 + E) = $\beta/(\alpha+\beta)$).

However receptor-channel kinetics are generally more complex than this simple example. I_{max} may be influenced by other factors, including fast desensitization processes and variations in subconductance levels. Furthermore K_A can only be determined if E is known, which implies that the kinetic behavior of the receptor channel is well characterized. An analysis of single channel behavior in stationary and nonstationary conditions is needed to calculate values for α and β which can then be used to estimate E (see [99]).

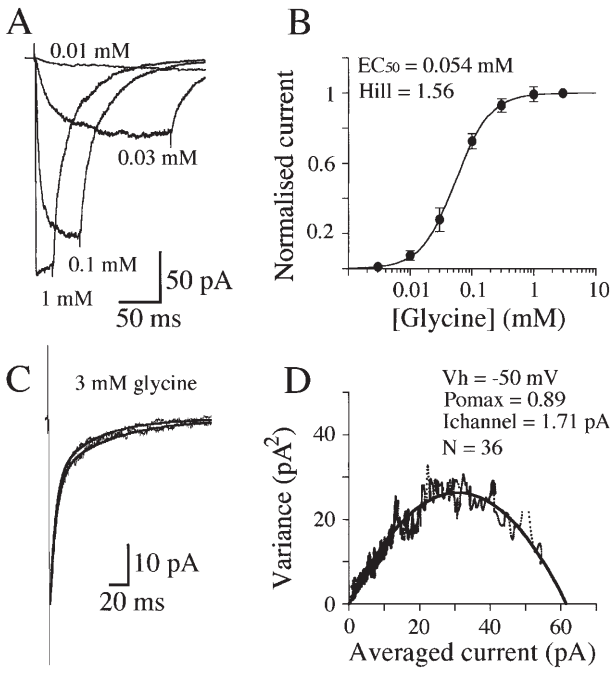


Figure 5. Concentration-response relationship of glycine-evoked current on outside-out patches. (*A*) Example of responses evoked by fastflow application of different glycine concentrations. (*B*) Concentration-response curve from data obtained in 11 outside-out patches. (*C*) Superimposed averaged traces of responses evoked by 1 ms application of a saturating concentration of glycine. (*D*) Variance-amplitude plot computed from 45 outside-out currents, allowing the measurement of the maximum open probability of the glycine-gated channel (P = 0.89). The curve represents the point-per-point relationship between variance (σ^2) and current (I) of the decay phase fitted by the model $\sigma^2 = (iI-(I^2/N))$, where i is the elementary current and N is the total number of available receptors in the patch. From Legendre [99], with permission.

Activation and deactivation kinetic of GlyRs

Experiments to examine the kinetic behavior of GlyRs have relied for some time on outside-out records of single-channel activity in stationary conditions together with whole-cell records of currents induced by moderately rapid agonist applications onto cultured neurons. Evidently fast application techniques, which permit agonist concentrations at an outside-out patch to be changed in less than 0.1 ms, have provided new insights into GlyR kinetics (fig. 5). Most of these recent analyses have been performed on mature wild-type heteromeric GlyRs, so less data exist on other GlyR subtypes. Nevertheless, it is already clear that channel open time duration and desensitization kinetics differ according to the GlyR subunit combinations.

GlyR channels can open with two or more open time durations according to the subconductance level [191]. $\alpha1/\beta$ heteromeric GlyRs open mainly with a single conductance state of 44–48 pS and have two different mean opentimes of 05–0.7 ms and 1.5–1.8 ms, respectively (fig. 6). In constrast to the proposal for nicotinic acetylcholine receptors [192], the fastest mean open time does not arise from a single bound closed state [99]. The existence of two distinct mean open times underlies a complex time course for the deactivation of glycine-induced currents, best described by two exponential components, as are miniature inhibitory postsynaptic responses in both young and mature animals [99, 116, 126, 193]. Embryonic GlyR channels (presumably comprising $\alpha2$ subunits) possess considerably longer mean open times [133], and mIPSCs are correspondingly prolonged [116, 126, 138].

The maximum open probability of the mature form of GlyR is high (0.7–0.9) [99, 193], but at present the efficacy constant, E, is only known for the heteromeric $\alpha1/\beta$ GlyR subtype of the zebrafish brain [99]. In this study, E value ≈12, and the opening and slower closing rate constants were 9000 s^{-1} and 700 s^{-1}, respectively. The closing rate constant is potential dependent, decreasing at depolarized potentials [194] so that the duration of inhibitory synaptic events is increased with depolarization [194–196].

Nonstationary analysis of the kinetic behavior of heteromeric GlyRs, with rapid application techniques, reveals that two binding sites exist [99], consistent with previous electrophysiological studies [98, 197, 198]. This result contrasts, however, with the stoichiometry proposed for heteromeric GlyRs consisting of three α and two β subunits [106], suggesting that subunit stoichiometry determined from cross-linking studies may not be easily related to estimates of the number of functional binding sites based on the analysis of currents elicited by glycine. The microscopic dissociation constant (K_A) of the site controlling channel opening has been estimated from studies on heteromeric zebrafish GlyRs and mouse spinal cord GlyRs [98, 99]. The value of KA was 0.3–0.4 mM, and the dissociation and association rate constants were ~1500 s^{-1} and ~5 $\mu M^{-1} s^{-1}$, respectively [94]. I recently

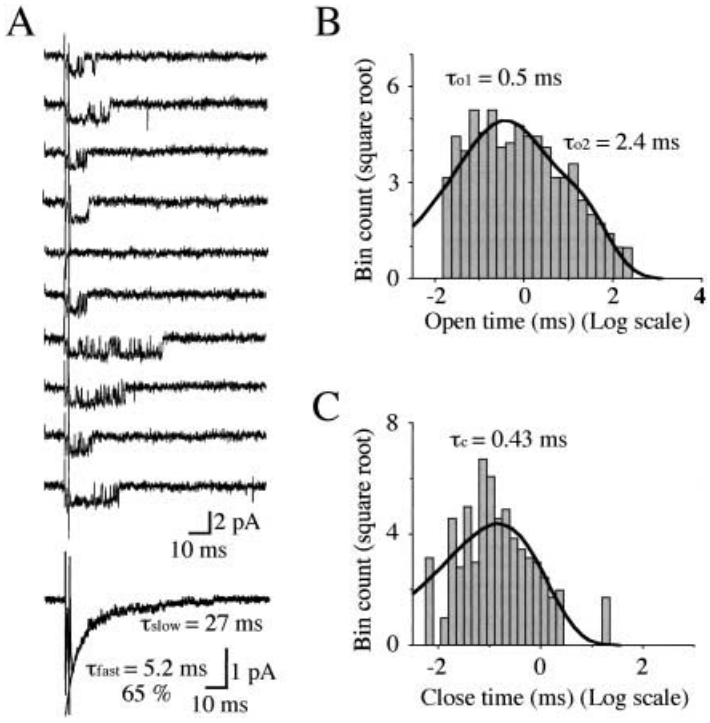


Figure 6. Openings of a single GlyR evoked by a short step application of 3 mM glycine. (*A*) Example of responses evoked by successive 2 ms glycine application. Bursts of short and long openings occurred after the end of the glycine application. Bottom trace is the average of 26 responses, showing that GlyR reopenings control the mIPSCs time course. (*B*) Open time histogram (ms log intervals/square root scale) with the two open time constants for short and long openings. (*C*) Close time distribution of short closures within a burst of openings. From Legendre [99] with permission.

proposed a relatively complete kinetic model of the activation and deactivation behavior of heteromeric GlyRs [99, 194]. It includes two binding sites, a doubly liganded open state and a reluctant gating mode leading to another open state (fig. 7). The model does not consider slow desensitization, which seems unlikely to affect the amplitude or duration of single postsynaptic events [99, 193].

Desensitization of GlyRs

Wild-type GlyRs desensitize slowly (0.5–5 s) [99, 193, 198, 199] in contrast to some other ionotropic receptors , including some $GABA_A$ (50–100 ms) [200] and AMPA receptors (5–10 ms) [201]. The time course of GlyR desensitization is complex and follows two or more exponential components [99, 198, 199]. During continuous application of agonist, glycine-evoked responses decline with time to 90% of the peak amplitude in the last exponential component (fig. 8), and desensitization also appears to be voltage-dependent [195,198]. The physiological role of this slow desensitization remains to be elucidated. It seems much too slow to influence the time course or amplitude of single postsynaptic events [99, 193], but might be recruited during repetitive or epileptic discharges of glycinergic presynaptic cells [195].

There is little data relating GlyR desensitization to variation in subunit composition. The homomeric α2 GlyR appears to desensitize more completely than the α1 receptor in the zebrafish [202]. Human homomeric GlyRs composed of the α3 subunit splice variant, α3K, have been reported to desensitize more rapidly than those composed of α3L subunits [128]. This difference presumably depends on the deletion of 15 amino acid residues from the postulated intracellular cytoplasmic loop linking membrane-spanning domains TM3 and TM4 of the α3K subunit. There is evidence that amino acid residues located within the TM1–TM2 intracytoplasmic loop of the α1 GlyR subunit also control desensitization. Mutations I244A [189] and P250T [203] accelerate desensitization. Overall, these results suggest that GlyR desensitization depends on interactions between the two intracellular loops of α subunits.

Phosphorylation of GlyRs

GlyRs are modulated by activation of PKC, PKA and calcium-dependent calmodulin kinase II (CaMKII).

There is a clear consensus sequence for PKC phosphorylation consisting of a serine residue localized at position 391 in the M3–M4 intracellular cytoplasmic loop close to the fourth transmembrane domain of the GlyR α1 subunit [204]. While some results are contradictory, the evidence suggest that PKC activation, which modulates GlyRs, is calcium independent [205, 206].

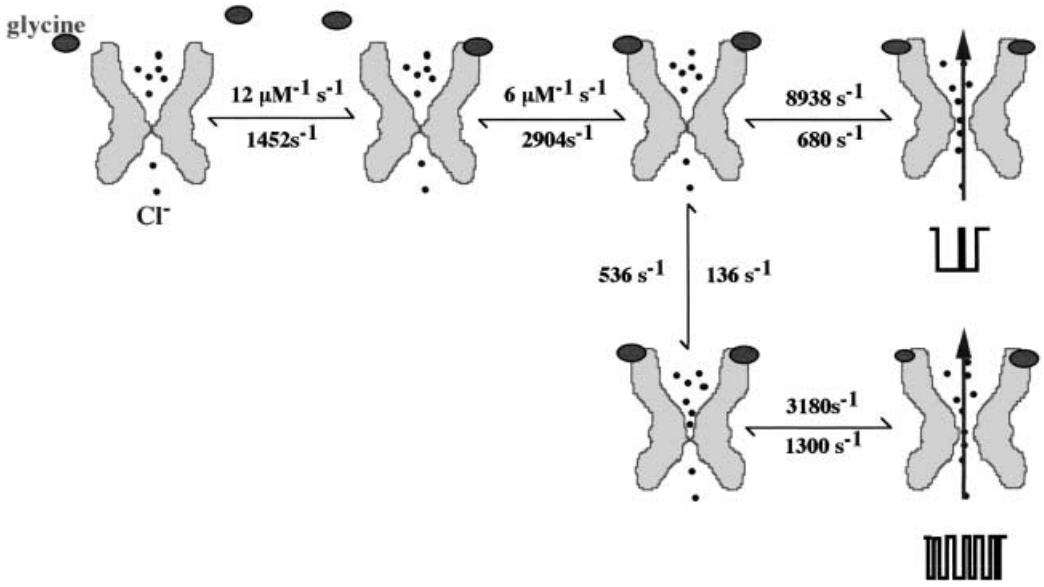


Figure 7. Kinetic model with rate constants reproducing the gating properties of the zebrafish heteromeric $\alpha 1/\beta$ GlyR. This model possesses two sequential equivalent binding steps, the doubly liganded closed state providing a direct access to a doubly liganded open state and to a reluctant closed state. The reluctant closed state also provides access to an independent doubly liganded closed state that gives a long burst of short openings (see [99]).

The activation of PKC by phorbol esters such as TPA, decreases GlyRs activity in *Xenopus* oocytes injected with poly(A)$^+$ mRNA isolated from nervous tissue [207, 208]. Similar results were derived from primary spinal cord cell cultures when PKC was activated by PMA [206], from murine hypothalamic neurons when phorbol 12,13-dibutyrate (PDBu) was used [209] and on activation of PKC by TPA in spinothalamic tract neurons [210]. In contrast, glycine-evoked responses of rat sacral dorsal commisural neurons were enhanced on activation of PKC by phorbol ester or diacylglycerol (DAG) [205], and similar increases were recorded from acutely dissociated rat periaqueducal gray neurons [211], and hippocampal neurons [212]. PKC activation also enhances GlyR responses to taurine in substantia nigra neurons [213] and to glycine in rat sacral dorsal commissural neurons [214]. More recently, Gu and Huang [215] showed that glycinergic responses were directly enhanced by intracellular perfusion of PKA or PKC. They also demonstrated a cross-dependance of PKA and PKC effects. The PKC action on GlyRs was conditional on the modulation of glycinergic responses by PKA and vice versa. One possible explanation of these contradictory results on PKC is that phorbol esters might act directly on GlyRs in some conditions, as suggested by Nishizaki and Ikeuchi [216] from studies on homomeric $\alpha 1$ GlyRs and $\alpha 2$ GlyRs expressed in oocytes. Activation of endogenous oocyte PKC by pretreatment with serum enhanced the activity of both $\alpha 1$ and $\alpha 2$ GlyRs, an effect which was blocked by the specific PKC inhibitor GF109203X. In contrast, GF109203X did not suppress decreases in GlyR responses elicited by the PKC activator, TPA. It seems possible that TPA may act on GlyRs directly and that this direct action may mask effects due to activation of PKC [216]. Although PKC has similar effects on homomeric $\alpha 1$ GlyRs and $\alpha 2$ GlyRs [216], differences in the GlyR subunit combinations might partly underly these contradictory results. In mature neurons most GlyRs are heteromeric, consisting of three α and two β subunits [136]. Although the GlyR β subunit contains a putative consensus cytoplasmic site for PKC phosphorylation at position 388 [120], the effects of this phosphorylation have not been studied.

Song and Huang [217] were first to show that PKA activation modulates GlyR function. However, as for PKC, subsequent results on the functional effects of modulation by PKA have been contradictory. PKA activation increased glycine-evoked responses in *Xenopus* oocytes expressing GlyRs [207], in spinal trigeminal neurons [217], in spinal cord cell culture [206], in acutely dissociated trigeminal neurons [215] and in acutely dissociated rat ventral tegmental area neurons [218]. PKA had the opposite effect in ventromedial hypothalamic neurons [219], in rat substantia nigra neurons [220, 221], in rat sacral dorsal commissural neurons [214] and in acutely dissociated rat periaqueductal neurons [211]. Modulation of GlyR by PKA is at first sight surprising, since most of α subunits possess no consensus sequences for PKA phosphorylation. Nevertheless $\alpha 1_{ins}$, an alternatively spliced variant of the $\alpha 1$ subunit described by Malosio and colleagues [119] does possess a potential PKA phosphorylation site [119]. $\alpha 1_{ins}$ represents 30% of the total α subunit in the adult spinal cord [19], and it seems likely that PKA phos-

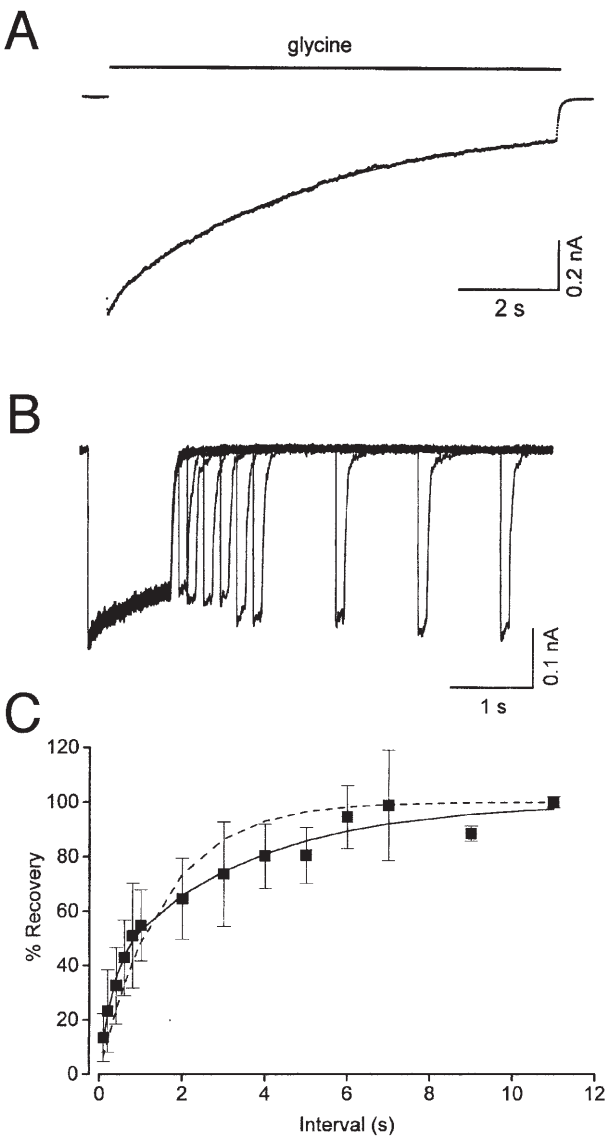


Figure 8. Desensitization of outside-out currents evoked by a long application of 1 mM glycine (9 s) in the ventral cochlear nucleus of the guinea pig. (*A*) Long applications of glycine reveal an additional slow component of desensitization with time constant of 4.22 ms. (*B*) Recovery from desensitization. 1 s application of glycine was followed, at various intervals, by 100 ms step of glycine to analyse the recovery from desensitization. (*C*) Recovery function was analyzed by plotting the relative amplitude of the second response as a function of the intervals between the two applications. Data points were best fitted with the sum of two exponential curves with time constants of 3.45 ms and 305 ms, respectively. From Harty and Manis [199] with permission.

phorylates this specific subset of GlyRs. Furthermore, the GlyR β subunit also contains a putative consensus cytoplasmic site for PKA phosphorylation at position 363 [120]. It is tempting to suggest that, as for PKC, the apparently contradictory results on the effects of PKA phosphorylation derive from distinct subtypes of GlyR with different subunit combinations.

A third kinase, CaMKII, has also been shown to modulate GlyRs. Intracellular application of the α subunit of CaMKII increases glycine-evoked responses in acutely isolated rat spinal neurons [222]. This effect is mimicked by calcium entry, via activation of either AMPA or NMDA receptors [223, 224]. The increase in intracellular calcium concentration is suggested to activate CaMKII, which in turn enhances the Imax of glycine-evoked responses without changing the EC_{50} [223, 224]. The phosphatase calcineurin inhibits the calmodulin-stimulated phosphodiesterase activity by competing with the enzyme for calmodulin, and appears to be coactivated with CaMKII providing a pathway to reverse the effects of CaMKII [223, 224]. While these results are functionally consistent, no known α or β subunit possesses a consensus sequence for CaMKII, phosphorylation. Xu et al. [223] suggested that CaMKII may act directly by phosphorylating an undescribed GlyR splice variant. Alternatively CAMKII might act indirectly to catalyze the phosphorylation of other proteins such as adenylyl cyclase [225], cyclic nucleotide phosphodiesterase or calcineurin [226].

Allosteric and nonallosteric GlyR modulation

GlyR activity is allosterically modulated by multiple compounds, some which have been shown to possess specific binding sites on GlyRs subunits. This is the case for zinc, some alcohols and anaesthetics, picrotoxin, some 5HT3 antagonits, cocaine and some anticonvulsants.

Zinc modulation of GlyRs

Zinc is probably an endogenous modulator of GlyRs. It is released together with neurotransmitters at some synapses, and zinc ions are known to modulate several voltage-gated and ligand-gated channels (for review, see [227]). The effects of zinc on GlyRs are concentration dependent (fig. 9); at low concentrations, zinc enhances, whereas at concentrations higher than 10 µM it depresses glycinergic currents [125, 228–235]. Two allosteric zinc binding sites are suggested to be located on the α subunits [230]. The zinc binding site on GlyRs is also recognized by Pb^{2+} and La^{3+} with the potency sequence $Zn^{2+} > Pb^{2+} > La^{3+}$ [235]. The low-affinity zinc binding site on the human $\alpha 1$ subunit was proposed to be a histidine-hydrophobic residue motif at positions 107 and 109 near the apex of a β sheet in the N-terminal [234]. While the high-affinity binding site is not yet definitively localized, it also seems likely to involve histidine residues on the NH2 terminal of α subunits [234].

Allosteric processes involved in the potentiation of GlyR responses by zinc are complex. They are impaired by mutations both in the first transmembrane domain and in the

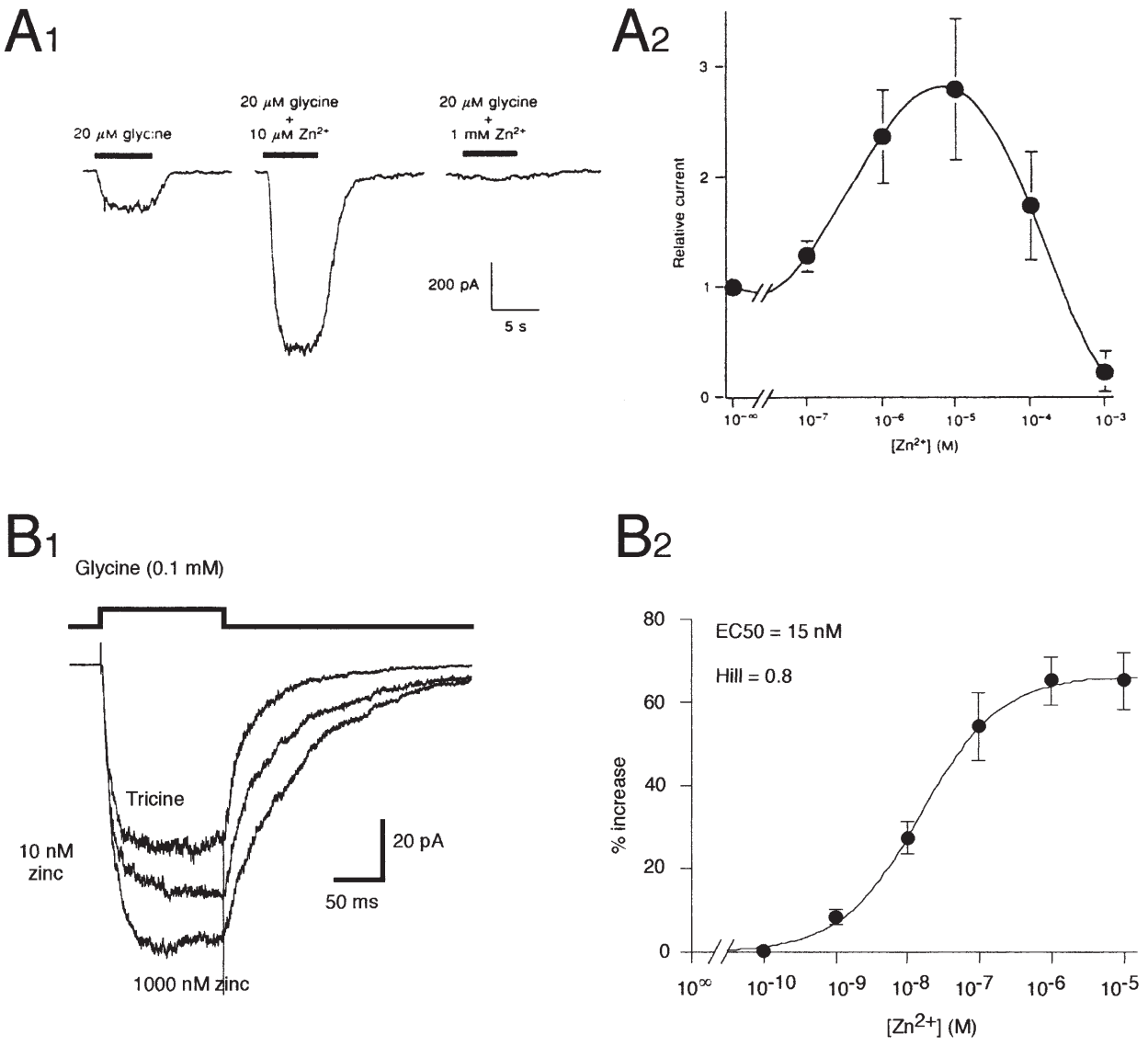


Figure 9. Effect of zinc on chloride current evoked by glycine. (*A*) Dose-response curve of zinc effects on the amplitude of glycine evoked current recorded in spinal cord cell cultures. Note that zinc decreased glycine-evoked response amplitude at concentration higher than 10 μM, whereas lower concentrations of zinc increased GlyRs activity. From Laube et al. [230] with permission. (*B*) Dose-response curve of zinc effect on outside-out currents in the presence of a heavy metal chelator in the zebrafish hindbrain. In these conditions, zinc evoked a high-affinity potentiation of glycine-evoked response. From Suwa et al. [237], with permission.

M2–M3 extracellular loop [231]. Mutations of aspartate 80 and threonine 112 also modify zinc mediated potentiation of glycine responses, but do not affect currents evoked by taurine [236]. Despite this complexity, zinc potentiation of GlyR activity clearly involves interactions with agonist gating [225, 230]. Recent studies show that zinc potentiates glycinergic currents by decreasing the microscopic dissociation rate constant for glycine [236, 237].

Although zinc enhances GlyR activity at concentrations ≤1 μM (the extracellular concentration that zinc is estimated to reach after synaptic release) [227], glycinergic terminals have not yet been shown to release zinc. Zinc release from terminals containing other transmitters would need to diffuse to adjacent glycinergic synapses to affect GlyR function. The apparent affinity initially described for zinc binding with GlyRs, 0.5–1 μM, seems to be too low for such mechanisms to operate [125, 228–234, 236]. However, we recently documented a lower EC_{50} (15 nM) of the high affinity zinc binding site on GlyRs than previously thought [237], as is also the case for NMDA NR1–NR2A receptors [238]. Using heavy metal chelators such as tricine or TPEN, this work shows that heavy metal contamination, at 10–15 nM, in recording solutions may significantly influence GlyR activity [237]. With a high-affinity zinc binding site on GlyRs, cross-talk may occur between glutamatergic and glycinergic systems. An increase in activity at glutamatergic

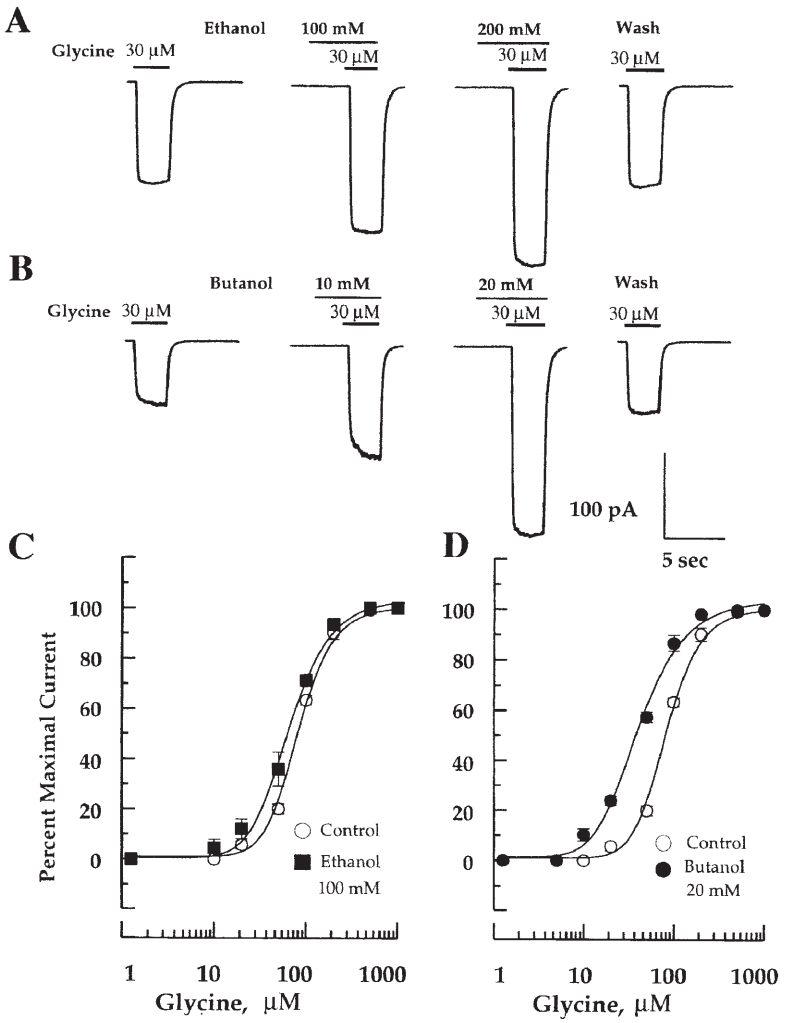


Figure 10. Alcohol pharmacology of homomeric α1 GlyRs expressed in HEK 293 cell. (*A*) Enhancement of submaximal glycine-induced currents evoked by ethanol. (*B*) Enhancement of submaximal glycine-induced responses evoked by butanol. (*C* and *D*) concentration-response curves of glycine-evoked currents in the absence or in the presence of ethanol (*C*) or in the presence of butanol (*D*). Note that ethanol and butanol shifted the dose-response curve for glycine to the left and that butanol was more efficient than ethanol. From Ye et al. [255], with permission.

synapses, which release zinc [227], would be functionally balanced by an increase in the duration of glycinergic postsynaptic currents [237] due to lateral zinc diffusion, as well as the direct effects of zinc on glutamate receptors.

Allosteric modulation of GlyRs by Alcohols and Anaesthetics

Ethanol and anaesthetics modify the function of $GABA_A$ and glycine receptors in a similar way (for review, see [239]). Alcohols [240–244] and volatile anesthetics [245–249] increase GlyR activity in a variety of preparations (fig. 10), and these effects may partly account for their depressing central nervous system (CNS) actions [250]. Increasing size of homologous n-alcohols is correlated with an increase in their potency to enhance GlyRs activity [239], although beyond a certain size, potency does not increase further (the phenomenom of alcohol cutoff). Dodecanol is the largest n-alcohol to have an anaesthetic potency [251]. Studies on the effects of alcohols on glycine-evoked actions on homomeric α1 GlyR and α2 receptors show that dodecanol is no more potent that decanol, whereas tridecanol has little or no effect [244]. However, low alcohol concentrations have more potent actions on homomeric α1 receptors than on α2 GlyRs [244].

The hypothesis that the effects of alcohol on the CNS are mediated via membrane lipids rather than by interactions with neurotransmitter receptors has recently been contradicted by several lines of evidence. Ethanol enhances GlyR activity in a concentration-dependent manner [240, 241]. It decreases the EC_{50} for glycine without changing I_{max} [252] and at 10–200 mM ethanol concentrations that

increase responses to glycine [253] does not modify membrane lipid order**.** Mihic and colleages [254] were the first to suggest that $GABA_A$ and GlyRs possess a similar binding site for alcohol and volatile anaesthetics, including enflurane and isoflurane. Using mutagenesis and chimeric receptors, they identified two amino acid residues in transmembrane domains TM2 and TM3 (serine 267 and alanine 288 for the α1 GlyR subunit) critical for the allosteric modulation of these two receptors [254]. Interestingly, the homomeric αl GlyR with mutation A288W was tonically active. Substitution of serine 267 by tyrosine results in GlyR insensitivity to ethanol and enflurane, whereas substitution by the smaller amino acid isoleucine does not modify potentiation by enflurane. In contrast, the M287L mutation enhances potentiation by enflurane but does not change potentiation by ethanol. More recent evidence suggests that serine 287 forms part of a common allosteric binding site for alcohol and volatile anaesthetics in α1 GlyR subunits. For ethanol to potentiate glycine response, position 267 must be occupied by a small amino acid residue [255], whereas insertion of larger amino acids at this site decreases the alcohol cutoff [256]. Modified subunits with a larger amino acid at position 267 appear to possess a smaller alcohol binding site [239]. A different approach using covalent labelling with alkanethiol, after mutating serine 267 and alanine 288 of the α1 subunit to cysteine, has confirmed that GlyRs possess a specific anaesthetic binding site [257], which is suggested to form a cavity between the M2 and M3 transmembrane domains at a site inside the membrane.

In summary, volatile anaesthetics and alcohol clearly affect GlyRs by allosteric mechanisms, but the kinetics of the interactions are still poorly understood, and there may be multiple allosteric binding sites. The potentiation of GlyR responses by ethanol depends also in part on phosphorylation of the α1 subunits by PKC, but there is no evidence that PKA-mediated modulation occurs even in homomeric $\alpha 1_{ins}$ GlyRs [252, 258]. However, partial inhibition of the effect of ethanol by the PKC-inhibitors staurosporine or GF 109203X, does not occlude enhancement of glycine-evoked responses by halotane or propofol [258], suggesting that GlyRs may possess another binding site for anaesthetics. In fact, GlyR activity is enhanced by several different classes of anaesthetics, including intravenous anaesthetics such as propofol and etomidate, barbiturates such as pentobarbital and thiopental, and the gaseous anesthetics nitrous oxide and xenon [248, 259].

Multiple effects of picrotoxin on GlyR activation

Picrotoxin, commonly used as a $GABA_A$ receptor antagonist, also suppresses GlyR responses to glycine [134, 260]. Interestingly, the TM2 transmembrane domain of the β subunit considerably reduced the IC_{50} for picrotoxin actions on heteromeric GlyRs [173]. Picrotoxin binding sites on GlyRs seem likely to differ from those present on $GABA_A$ receptors. Picrotin and picrotoxinin, the two molecular components of picrotoxin [261], block GlyR α1 homomers with similar potencies [134], whereas they have differing effects on $GABA_A$ receptors. Several approaches suggest that picrotoxin does not act as a classical competitive antagonist on homomeric GlyRs. It decreases both the mean open time and the relative frequency of the 80–88 pS main conductance state of homomeric GlyRs [141, 161], whereas these two parameters are not concentration dependent. Picrotoxin does not displace bound [^{3}H] strychnine and/or modify the displacement of bound [^{3}H] strychnine on GlyR by glycine [134]. Furthermore, picrotoxin has other concentration dependent actions on GlyRs. At concentration $\geq$100 µM it both induces a flickering block of smaller subconductance states of heteromeric GlyRs and reduces the mean open time of the main conductance state without significantly changing the relative open probability, NPo [141, 262]. Apparently, the absence of a change in the NPo of GlyRs with a main conductance state of 40–46 pS reflects a balance between two opposing effects on channel kinetics. One of these effects of high picrotoxin concentrations is an increase in the relative frequency of longer openings [141, 262], presumably due to an increase in the affinity of glycine for its binding site [134]. The other and opposing effect of picrotoxin is to decrease the mean open time, resulting in flickering and increase of the duration of bursts of openings [141, 262]. The weak antagonist effect of picrotoxin on α/β heteromeric GlyRs seems to result from the very small probability that this channel opens to conductance states higher than 44 pS [141, 262]. These data are consistent with the proposal that the multiple effects of picrotoxin all result from modifications in transduction processes between agonist binding and channel activation [134], and suggest that picrotoxin should only be effective in certain GlyR conformations. Transduction between agonist binding and channel activation depends critically on amino acids of the extracellular loop between the M2 and M3 transmembrane domains [134]. This extracellular domain is important for conformational changes which correspond to distinct subconductance levels [173]. The mutations of α1 subunits associated with the human startle disease, R271L or R271G [263, 264], both decrease the average conductance of the glycine-gated channel [183, 264] and disrupt transduction between agonist binding and channel activation [265, 266]. They also convert picrotoxin into an allosteric potentiator without changing its antagonist-like properties [134], effects which are very similar to those on 40–46 pS GlyR subconductance levels [141, 262]. However, these residues appear not to contribute to the picrotoxin binding site, since neither the R271G nor the R271L mutations change the IC_{50} value significantly [134].

Other GlyRs modulators

GlyRs are modulated by several other molecules, including antagonists of 5HT3 receptors and NMDA receptors, neurosteroids, cocaine and certain anticonvulsants. Some of these molecules have been shown to possess specific allosteric binding sites. Chesnoy-Marchais [267, 268] first described effects of 5HT3 antagonists on GlyR activity, showing that MDL-72222, ICS-205,930 and LY-278,584 all potentiate glycinergic responses. The potentiation seems to result from an increased affinity of GlyRs for glycine. However, at higher concentrations (>10 μM) both MDL-72222 and ICS-205,930 suppress glycinergic currents. The identity of the binding sites involved in this potentiation is not known, but it seems to be different from other sites involved in potentiating GlyRs [268].

Another 5-HT3 antagonist, odansetron, inhibits responses to glycine [269] in competitive fashion. Dextromethorphan, the NMDA receptor antagonist, also appears to exert a competitve inhibition on GlyR activity. At concentrations less than 3 μM, it increases the glycine EC_{50} for GlyR without changing the I_{max} [270]. Of the neurosteroids, pregnenolone sulfate inhibits glycinergic responses competitively [271], whereas inhibition by progesterone is noncompetitive [271]. A noncompetitive inhibition of GlyR activity by cocaine was also reported [272], but as for progesterone, the existence of a specific allosteric binding site for cocaine has not yet been proved.

An interesting recent report suggests that actions of the anticonvulsant thiobutyrolactone on glycinergic responses depend on the GlyR subunit combination [273]. Responses to glycine mediated by homomeric α1 and heteromeric α1/β GlyRs or α3/β GlyRs are potentiated, but those of homomeric α3 GlyRs are suppressed. The actions of thiobutyrolactone on the α3 subunit probably result from allosteric interactions with an overlapping set of amino acid residues in the TM2 transmembrane domain [273]. However, it is not clear whether a specific allosteric binding site is implicated in the potentiation of glycine responses by thiobutyrolactone.

Glycine receptors and genetic deseases

Alterations of glycinergic synaptic function are involved in the pathogenesis of several neurological disorders. Specific mutations of postsynaptic GlyR subunits underly hereditary motor disorders. In humans, [203, 263, 274, 275], horses [276], cattle [276], and mice [180, 277–279], defects in GlyR subunit genes result in complex motor disorders characterized by hypertonia and hyperexcitability.

GlyRs mutations in mice

Three autosomal recessive mutations in murine GlyRs α or β subunit genes lead to disease phenotypes (fig. 11). While all three mutant animals, spastic (spa), spasmodic (spd) and oscillator (spd^{ot}) are born without detectable symptoms, their specific phenotype becomes apparent two weeks after birth. Homozygous spa [280], spd [281], and spd^{ot} [279] mutants present muscle rigidity, tremor, myoclonic jerks and exaggerated startle reactions.

The spa mutation, localized on chromosome 3, results in dramatically reduced GlyR expression in the spinal cord and brainstem of homozygous mice. The spa gene is a mutant allele of the β subunit gene with a transposable element (LINE-1) inserted into intron 5 [277, 279]. This mutation results in a truncated β subunit mRNA, since the LINE-1 element interferes with splicing of β subunit pre-mRNA, leading to a severe reduction but not to a structural alteration of the residual full-length transcript. The amplitude of glycinergic inhibitory synaptic events impinging on motoneurons is considerably reduced, suggesting a diminution in the mean number of GlyRs at inhibitory synapses.

The spd mutant mice bear a missense mutation in the α1 subunit gene located in chromosome 11 [281]. The mutation causes a substitution of the alanine residue at position 52 of the N-terminal domain [179, 180], which increases glycine EC_{50}.

The spd^{ot} mutation, allelic to the spd gene, corresponds to a microdeletion of seven nucleotides from exon 8 of the α1 subunit gene [279]. The microdeletion starts at amino acid residue 308 in transmembrane domain M3 and initiates a translational frame shift. GlyR receptor expression is completely suppressed in these animals [282] which do not survive beyond 3 weeks after birth [279].

GlyRs mutations in human hyperekplexia

Hyperekplexia, also known as startle syndrome, or stiff baby syndrome is a congenital human motor disorder with a phenotype similar to that seen in the mouse [280, 283, 284]. Dominant and recessive forms of this rare disease result from missense mutations of the α1 GlyR subunit gene. The first mutation was identified from extensive genomic mapping studies as a point mutation in the human α1 subunit gene. It results in the substitution of an arginine 271 located in the M2 transmembrane domain [263, 264]. This arginine 271 residue is crucial in the control of agonist signal transduction [174], and its substitution by leucine or glycine, increases glycine EC_{50} and reduces single-channel conductance [134, 174, 265, 266, 285].

Hyperekplexia syndromes also result from several other missense mutations of the α1 subunit gene. They include the recessive I244A mutation [274] and the dominant P250T mutation [197] located at the intracellular border

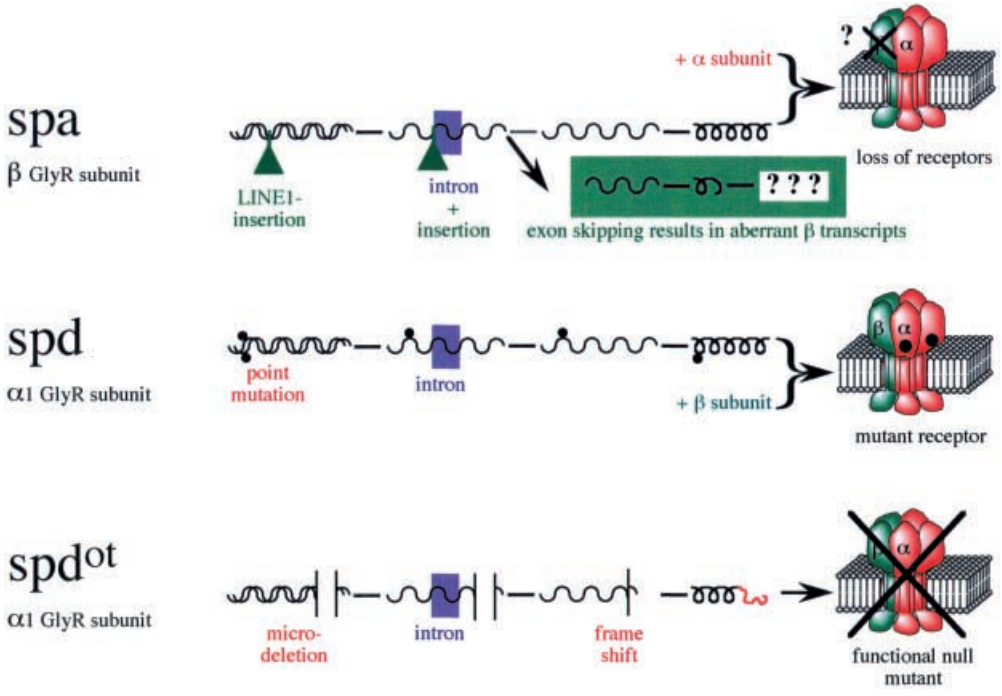


Figure 11. Murin mutations affecting GlyRs expression. Position of the different mutations leading to abnormal GlyRs activity or lost of postsynaptic receptors in spastic (spa), spasmodic (spd) and oscillator (spd^{ot}) mutants. Adapted from Vannier and Triller [319].

of the M2 transmembrane domain, the Q266H [286] located within the pore-forming TM2 domain, and mutations K276E and Y279C, which lie within the TM2–TM3 extracellular loop [287, 288]. Analysis of single-channel and whole-cell responses to glycine have shown that these mutations affect GlyR activity in several ways. The main effect of the α1 mutation K276E is to reduce the duration of channel openings and has been proposed to result from an impairment of channel gating rather than of glycine binding [289]. The Q266H mutation also decreases mean GlyR channel open time without affecting subconductance levels [188]. In contrast, the missense mutation which substitutes proline 250 by threonine (P250T) results in GlyRs with a much-reduced single-channel conductance and fast desensitization (fig. 12), but with normal glycine binding [203].

These data show that recombinant analysis of mutated α1 GlyR subunit alleles has advanced our understanding of how the hyperekplexia syndrome is linked to GlyR dysfunction. However, differences exist in mouse and human phenotypes such that while the α1 GlyR subunit null mutation is lethal in spd^{ot} mice, an α1 GlyR subunit null mutation is effectively compensated in a human hyperekplexia syndrome [290]. Further analysis requires both a closer correlation of defects in GlyR function with the properties of inhibitory synaptic networks, and the development of animals that model human pathologies more closely.

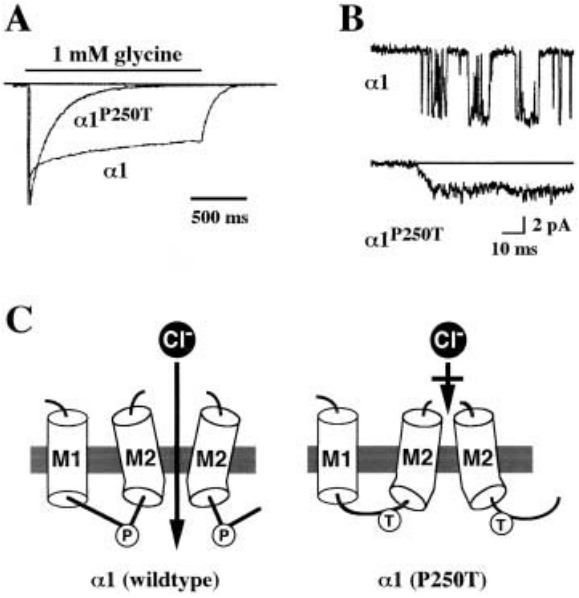


Figure 12. Changes in the functional properties of human GlyRs evoked by α1 subunit mutation P205T. (*A*) P205T mutation is localized in the TM1–TM2 cytoplamic loop. This mutation increased the desensitization rate of GlyRs. (*B*) This mutation also impaired channel gating and decreased the conductance level of the glycine-gated channel. (*C*) Topological predictions for the cytoplamic M1–M2 loop of recombinant α1 and α1 (P205T) GlyRs. From Saul et al. [203], with permission.

Functional heterogeneity of glycinergic synaptic activity

At single central synapses, the duration, amplitude and variance of postsynaptic events depend on multiple pre- and postsynaptic factors, such as the time course of neurotransmitter concentration in the synaptic cleft, the number of molecules released, the number and proportion of postsynaptic receptor subtypes and their gating properties [291]. Detailed analysis of miniature postsynaptic events, evoked by the release of a single vesicle, is essential to understand how these factors combine to control synaptic strength. A further step towards linking receptor and synaptic activity is to ask how miniature events reflect the kinetic behaviour of the ensemble of channels opened by transmitter released from a single site. The recent association of such electrophysiological data with confocal images of GlyR clusters has provided new insights into the morphofunctional properties of glycinergic synapses.

Functional properties of mature glycinergic synapses

Miniature inhibitory synaptic currents (mIPSCs) at glycinergic synapses have a complex time course, which typically consists of a fast rising phase and a bi-exponential deactivation [99, 116, 193, 292]. mIPSCs of the zebrafish hindbrain have a 20–80% rise time close to 0.3 ms, and the two time constants associated with their decay phase are 3–4 ms and 20–30 ms, respectively [99]. mIPSCs of motoneurons and superior olivary cells of the rat brainstem have similar kinetics [116, 193, 292], which resemble those of the deactivation of glycinergic currents evoked by the application of short pulses of saturating glycine concentrations to outside-out patches [99, 193]. This similarity suggests that rebinding of glycine to its receptor does not contribute significantly to the duration of glycinergic mIPSCs [99]. This is consistent with the proposal that free neurotransmitter is rapidly cleared from the cleft at central synapses [293]. If rebinding is not involved, then the complex kinetics of the decay seems likely to depend on reopening of GlyR channels occuring before glycine dissociates from its binding site [99].

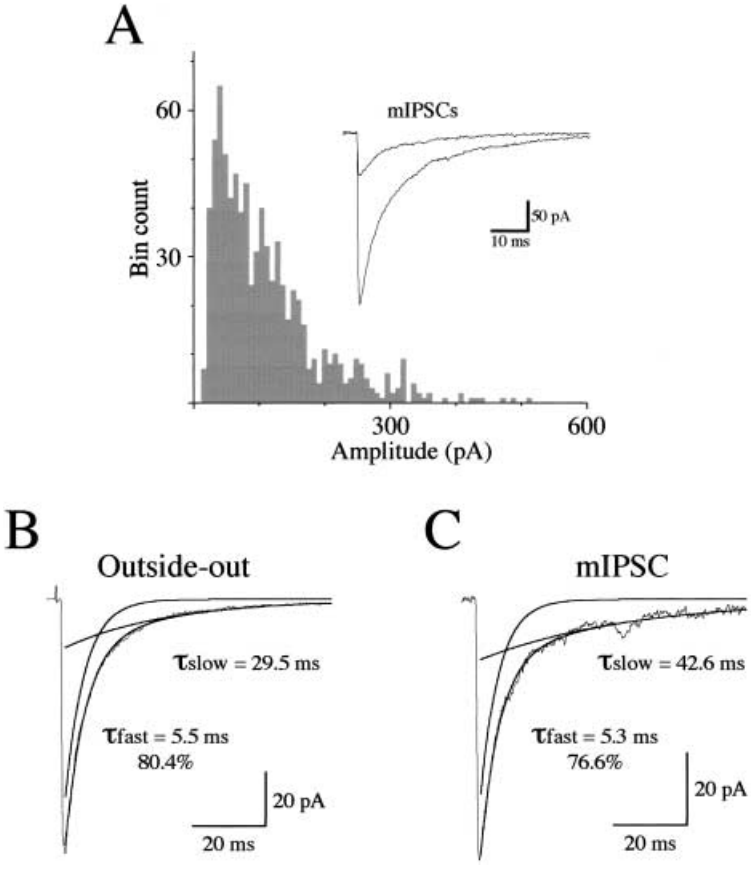


Figure 13. Amplitude fluctuation and time course of miniature inhibitory synaptic events recorded from the zebrafish Mauthner cell. (*A*) Skewed amplitude distribution of mIPSCs. (*B*) Time course of mIPSCs is identical to the time course of outside-out responses evoked by a short step (2 ms) of 3 mM glycine, which suggests that mIPSC duration is not due to glycine rebinding after the release of one vesicle. From Legendre [99], with permission.

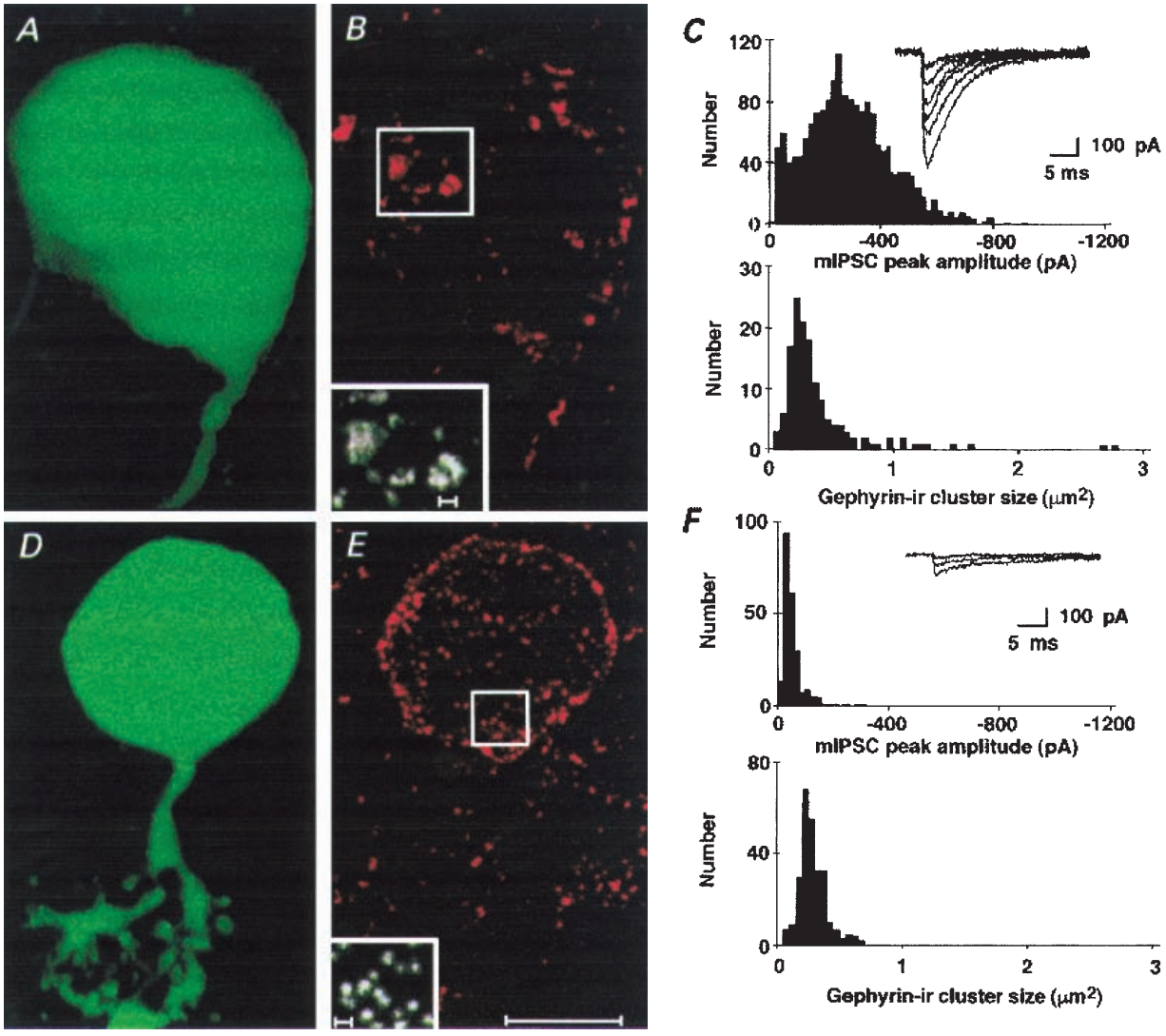


Figure 14. Comparisons between the GlyR cluster size and the mIPSC amplitude fluctuation. (*A* and *D*) Recorded cells visualized with neurobiotin fluorescence labelling. (*B* and *E*) Immunolabelling of gephyrin clusters on cells shown in *A* and *D*, respectively. Scale bars in *A*, *B*, *C* and *D* are 10 µm. (*C* and *F*) Comparison of the mIPSC amplitude histogram and the gephyrin-ir cluster size distribution obtained on cells shown in *A*, *B* and *D*, *E*, respectively. mIPSCs amplitude fluctuation appears to be reduced when gephyrin-ir cluster size distribution is less variable as shown in *F*. Inserts in *C* and *F* are examples of superimposed mIPSCs. From Lim et al. [303], with permission.

Analysis of the rising phase of mIPSCs permits an estimate for the peak glycine concentration in the synaptic cleft after the release of one vesicle. This value is ≥1 mM at synapses in the zebrafish hindbrain [99], comparable with values determined for other central synapses [293].

Amplitude distributions for glycinergic mIPSCs are not well fitted by a single gaussian [116, 193, 294, 295] but are rather skewed or even multimodal (fig. 13) as observed at other central synapses [296–300]. One exception is the gaussian distribution described for mIPSCs recorded from the hindbrain of the adult goldfish [301]. Broad amplitude distributions for mIPSCs recorded from zebrafish hindbrain [141] may depend on an intersynaptic variability in contributions from more than one GlyR subtype [141].

Fluctuations in miniature synaptic event amplitude at several other synapses have been attributed to variations in the number of postsynaptic receptors [302], including glycinergic synapses (fig. 14). Using imaging and whole-cell recordings from the same neuron, the variability in mIPSC amplitudes was compared with that of the surface area of gephyrin or GlyR clusters [44, 237, 303]. Results, from spinal motoneurons and brainstem cells of the rat and zebrafish, suggest that GlyR cluster size is the major determinant of amplitude variability. But other factors might also contribute. The covariation of mIPSC amplitude and GlyR cluster size does not necessarily imply that

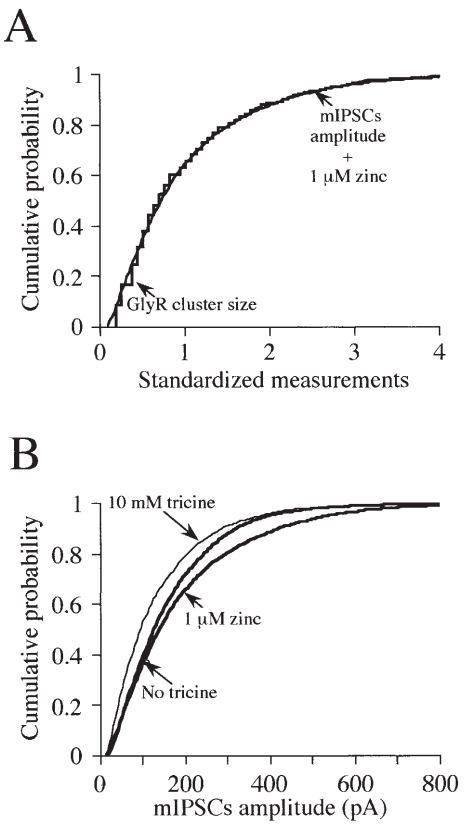


Figure 15. Low zinc concentrations can influence the occupancy level of the postsynaptic GlyRs on the zebrafish Mauthner cell. (*A*) Standardized cumulative averaged amplitude histogram of mIPSCs in the presence of zinc (1 μM) compared with standardized cumulative histogram of all GlyR-ir cluster sizes. Note that mIPSC amplitudes and GlyR-ir cluster sizes covaried in the presence of zinc. (*B*) Averaged cumulative mIPSC amplitude histograms obtained in the presence or in the absence of a heavy metal chelator (tricine) and in the presence of 1 μM zinc. Note that zinc strongly enhances the amplitude of mIPSCs and that addition of tricine to the bath revealed the presence of traces of heavy metals in the extracellular medium. From Suwa et al. [237], with permission.

the release of a single vesicle saturates postsynaptic glycine receptors. The question of receptor saturation has been examined in the zebrafish hindbrain using zinc (fig. 15), which allosterically increases the GlyR affinity for glycine [237]. Zinc application caused an increase in mIPSC amplitude, arguing against saturation, yet the estimated peak concentration of glycine in the cleft (1 mM) should suffice to saturate synaptic receptors [99, 237]. A Markov model of glycine interactions with GlyRs in the absence and presence of zinc was used in an attempt to resolve these contradictions. Simulations of synaptic currents with reasonable estimates for kinetics of glycine in the synaptic cleft and for its rapid clearance (time constant ≈ 0.3 ms; [293]) suggested that the lack of saturation may depend on fast clearance and the kinetics of GlyR currents. Specifically, simulations predicted a lower open probability for postsynaptic GlyRs following the release of a single vesicle than that observed in fast, saturating applications of glycine to GlyRs of outside-out patches [237]. Effectively, the activation kinetics of GlyRs is slower that the clearance of the glycine from the synaptic cleft [237]. These simulations predicted that small variations in the number of released glycine molecules might also contribute to fluctuations in mIPSC amplitude. Thus, whereas intersynaptic differences in the number of GlyRs probably accounts for most of the variation in efficacy of glycinergic synapses, other factors such as allosteric modulators of GlyR function may tune glycinergic synaptic efficacy.

The efficacy of glycinergic synapses is also modifiable by high-frequency presynaptic activity. IPSPs evoked disynaptically in the teleost Mauthner neuron [304] show a long-term potentiation apparently triggered by an increase in postsynaptic calcium concentration. This inhibitory LTP seems to result from activation of inhibitory synapses which were largely silent before tetanic stimulation of the afferent eighth nerve [305]. Such weakly functional junctions may represent a 'reserve' pool whose modulation permits variation in the threshold for the escape reaction reflex which the Mauthner cell initiates.

Glycinergic inhibitory synapses are also diverse in the different cotransmitters that they contain. GABA was first shown to be coreleased with glycine in paired recordings from synaptically connected neurons in slices of the rat spinal cord [72]. This work showed that both GABA and glycine released by single lamina I interneurons activate functionally distinct receptors of postsynaptic target cells, as has subsequently been demonstrated at inhibitory synapses on brainstem motoneurons [43] and superior olive cells [292]. Although it has been suggested that GABA-glycine cotransmission may be specific to early developmental stages [292], the same phenomenon also occurs in lamina I neurons of the adult rat spinal cord [73]. Interestingly, $GABA_A$ receptors seem to be extrasynaptic, whereas GlyRs face synaptic sites [73], a situation which may confer different functional properties on the inhibition that these two transmitters mediate.

Glycinergic synapses during brain development

Glycinergic synapses become functional early in brain development, and glycine or GABA depolarize neurons in embryonic and immature animals [51, 116, 127, 306–310]. This paradoxical 'excitation' by inhibitory transmitters is also observed for GABA and results from a depolarized chloride equilibrium potential in postsynaptic cells [116, 127, 308, 310, 311]. The chloride equilibrium potential shifts in a hyperpolarizing direction during early postnatal development [311–313] due to the replacement of immature homeostasic mechanisms [314, 315] by either a passive chloride distribution or by an active chloride extrusion [316]. The latter hypothesis was

recently verified in hypocampal neurons [317]. The K^+/Cl^- cotransporter KCC2 expression occurs at 10 days postnatal and produces a marked negative shift in the reversal potential of $GABA_A$ responses [318].

Glycine-mediated depolarization of immature neurons induces calcium entry via voltage-gated calcium channels [51, 307, 311, 313], which may represent an important signalling mechanism for both neuronal development and maturation of inhibitory synapses [51, 140].

Whereas analysis of single-channel records has described how GlyR activity changes during development, relatively little is known of functional changes at glycinergic synapses during embryogenesis. In early embryos of the zebrafish, glycinergic mIPSCs have a slow rising phase [126], which is probably independent of immature GlyR kinetics or low vesicular glycine concentrations. Slowly rising mIPSCs may depend instead on an enlarged synaptic cleft at immature synapses and/or interactions with extrasynaptic GlyRs [126]. The slow decay kinetics of glycinergic mIPSCs in embryonic or juvenile neurons [116, 126, 138, 318] results from the presence at immature synapses of $\alpha 2$ subunits [19], which exhibit long mean open times [138]. Initially, immature GlyRs were proposed to consist of homomeric receptors [19, 125]. Although this is certainly true for extrasynaptic receptors [125], homomeric GlyRs with multiple subconductance levels have not been detected at glycinergic synapses terminating on the zebrafish Mauthner cell or brainstem motoneurons [116, 126]. Instead, immature glycinergic synapses have been suggested to express $\alpha 2/\beta$ heteromeric receptors [116, 126], consistent with the proposal that the anchoring protein, gephyrin, is expressed at synapses before GlyRs [319].

As glycinergic synapses develop, mIPSC duration is progressively reduced [116, 126, 138, 318]. The reduction in decay time may reflect a progressive transition between the expression of GlyRs with slow and fast kinetics (fig. 16) [116, 126, 138, 193]. During intermediate developmental stages, glycinergic synapses probably express a variable proportion of slow ($\alpha 2$) and fast ($\alpha 1$) GlyRs, which results in mIPSCs of intermediate duration [126]. Alternatively, individual GlyRs composed of several types of α subunit might be responsible for similar changes in mIPSC duration, since $\alpha 2$ and $\alpha 1$ subunits are known to form heteromeric GlyRs [136]. Data on the kinetic properties of heteromeric GlyRs composed of different α subunits are needed to test this hypothesis.

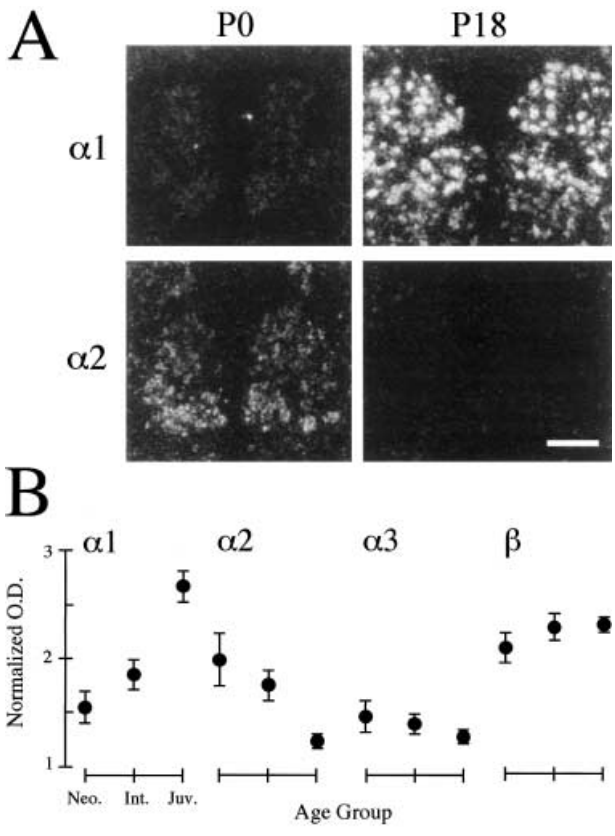


Figure 16. Postnatal change in GlyR subunit mRNA expression in the developing brainstem motoneurons, revealed by in situ hybridization. (*A*) In situ hybridization of $\alpha 1$ and $\alpha 2$ GlyR subunit mRNA expression was examined using two oligonucleotide probes for each GlyR subunit. At birth (P0) $\alpha 2$ GlyR mRNA is dominant, whereas its expression decreases to background level at 18 days postnatal (P18). Conversely, $\alpha 1$ mRNA expression is not detectable at P0, whereas it increases dramatically at P18. (*B*) Summary of the postnatal changes in $\alpha 1$, $\alpha 2$, $\alpha 3$ and β GlyR subunit mRNAs plotted as optical density (O.D.). Note that the $\alpha 3$ subunit is not significantly expressed by hypoglossal motoneurons, whereas the β subunit is expressed at a high level throughout the developmental stages examined. From Singer et al. [116], with permission.

Conclusion

We now have an impressively detailed knowledge of glycinergic synaptic transmission. Glycinergic synapses are as functionally heterogeneous as GABAergic or glutamatergic synapses. The diversity of GlyR subtypes is now clearly revealed as a major determinant of the variability of glycinergic synaptic strength during development and in different brain areas by extensive work showing how the kinetic properties of different GlyRs subtypes shape inhibitory postsynaptic events. It also becomes more evident that the morphology of the synaptic cleft, the size of the postsynaptic GlyRs aggregates, their location on postsynaptic cell as well as the glycine clearance after the release of a vesicle can play important roles in controlling glycinergic inhibitory synapse activity. However, further investigations are needed to uncover how these factors combine to control glycinergic synaptic efficacy in a physiological context.

Although extensively described, the physiological role of the diversity of GlyR subtypes is not yet fully understood. There has been important progress in analysis of relations between subunit combinations and the functional

properties of GlyRs subtypes, but correlations between the structure of the different GlyR subunits and their functional properties is far from being well understood.

The discovery that glycinergic synapses can be excitatory in early development revealed new functions of glycinergic synapses during brain development. Although the molecular mechanism for the switch between the immature and the mature form of synaptic GlyRs remains unclear, we are starting to understand how the complex postsynaptic scaffold controls the postsynaptic aggregation of GlyR clusters during development. These discoveries emphasize the plastic nature of glycinergic synapses, and preliminary data suggest that a continuous modification of their structural and functional properties may exert a dynamic control on neuronal integration. The awakening of silent glycinergic synapses by tetanic afferent stimulation provides an excellent example, perhaps related to the recently demonstrated mobility within the membrane of receptors, ionic channels and other transmembrane proteins [320–323, 328]. GlyR subtypes may also move laterally within the membrane if they can escape anchoring to the postsynaptic intracellular scaffold [321, 328]. Reversible changes in the number and position of GlyRs via lateral receptor diffusion may therefore provide a novel mechanism to dynamically modulate glycinergic synaptic efficacy.

In summary, glycinergic synapses are now seen to be as complex and variable as synapses releasing other neurotransmitters. Acceptance and exploitation of this complexity and variability will be crucial in understanding how glycinergic synapses contribute to signal integration in the CNS.

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