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Glycine transporters: essential regulators of neurotransmission

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Glycine has important neurotransmitter functions at inhibitory and excitatory synapses in the vertebrate central nervous system. The effective synaptic concentrations of glycine are regulated by glycine transporters (GlyTs), which mediate its reuptake into nerve terminals and adjacent glial cells. GlyTs are members of the Na^+/Cl^--dependent transporter family, whose activities and subcellular distributions are regulated by phosphorylation and interactions with other proteins. The analysis of GlyT knockout mice has revealed distinct functions of individual GlyT subtypes in synaptic transmission and provided animal models for two hereditary human diseases, glycine encephalopathy and hyperekplexia. Selective GlyT inhibitors could be of therapeutic value in cognitive disorders, schizophrenia and pain.

Introduction

Neurotransmission at chemical synapses formed between nerve cells, or by nerve cells on their effector organs like muscle or glands, proceeds with high spatial resolution and extraordinary speed. At individual synapses, the postsynaptic receptor response to transmitter released from the presynaptic nerve terminal lasts for only milliseconds. These rapid kinetics are a prerequisite of high-frequency neuronal firing and require effective clearance of transmitter molecules from the synaptic cleft to prevent both re-binding upon receptor dissociation and spillover to distant synapses.

At the neuromuscular junction, the postsynaptic actions of acetylcholine are terminated by its hydrolysis catalyzed by acetylcholinesterase, the most efficient enzyme known in biology. By contrast, all other classical neurotransmitters used in the nervous system are not removed from the synapse by degradation but through diffusion and re-uptake into the nerve terminal and adjacent glial cells. During the past decade, the plasma membrane transporters that mediate neurotransmitter re-uptake have become accessible to detailed molecular and functional analyses. This review summarizes recent insights into the structures, *in fvo* functions and putative disease aspects of a small subfamily of these membrane proteins, the glycine transporters (GlyTs). Their substrate, glycine, has neurotransmitter functions at both inhibitory and excitatory synapses (Box 1).

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GlyTs: members of the Na^+/Cl^--dependent transporter family

GlyTs mediate the uptake of glycine from the extracellular space into the cytosol. They belong to a large family of Na^+/Cl^--dependent transporter proteins, which includes transporters for monoamines (serotonin, norepinephrine and dopamine) and the inhibitory neurotransmitter γ-aminobutyric acid (GABA) [1]. These polytopic membrane proteins share a common transmembrane topology

Box 1. Neurotransmitter actions of glycine

Glycine is the major inhibitory neurotransmitter in caudal regions of the adult mammalian CNS, with high densities of glycinergic synapses being found in spinal cord and brain stem [55]. Upon release from inhibitory presynaptic terminals, glycine binds to strychnine-sensitive GlyRs that are densely packed in the postsynaptic membrane of the target cell. This causes the opening of a GlyR-intrinsic anion channel and leads to an influx of chloride ions into the postsynaptic cytoplasm. The resulting hyperpolarization raises the threshold for neuronal firing and thereby inhibits the postsynaptic neuron. Notably, during embryonic development, nerve cells contain high intracellular chloride concentrations. Hence, activation of GlyRs at these stages causes chloride efflux and depolarization (i.e. excitation of target neurons). This glycine-evoked depolarization ends around birth, when the neuronal K^+/Cl^- co-transporter KCC2 is expressed and lowers the intracellular levels of chloride ions [56].

In addition to its role as a neurotransmitter at inhibitory glycinergic synapses, glycine is also important for excitatory glutamatergic neurotransmission. Here, glycine serves in addition to D-serine as an essential co-agonist of glutamate at NMDAR subtypes of ionotropic glutamate receptors. NMDARs bind glycine with ~100-fold higher affinity than GlyRs. Therefore, it was postulated that the glycine-binding sites of NMDARs are saturated under physiological conditions because the glycine concentration in the cerebrospinal fluid lies in the low micromolar range. However, recent studies using exogenously supplied glycine have revealed distinct effects of glycine on NMDAR activation and number. First, at 0.5–20.0 μM glycine a potentiation of NMDAR currents was observed. This implies that the glycine-binding site of NMDARs is not saturated at the synapse [57]. Second, higher concentrations of glycine (≧100 μM) 'primed' NMDARs for internalization although endocytosis was ultimately triggered by the activating agonist glutamate [30].

Recently, the range of postsynaptic glycine actions has been further increased by the identification of NMDARs, which require only glycine for channel activation. These receptors are thought to be assembled from the glycine-binding NMDAR subunits NR1 and NR3 (instead of glutamate binding NR2) subunits [58]. The NR3 gene is expressed in selected areas of the spinal cord, therefore, excitatory glycinergic synapses might exist on spinal neurons, which contain postsynaptic NMDARs rather than GlyRs.

with 12 transmembrane domains (TMDs) connected by six extracellular and five intracellular loops (Figure 1a). cDNA cloning has identified two GlyT subtypes, GlyT1 and GlyT2, which share ~50% amino acid sequence identity but differ in pharmacology and tissue distribution (Box 2). Both GlyTs exist in multiple splice variants: three GlyT2 isoforms (a, b, c) differ in the N-terminal region [2], whereas three N-terminal (a, b, c) [3,4] and two C-terminal (d, e) [5] exons have been reported to generate different variants of GlyT1. The N and C termini of these proteins have been shown to be located intracellularly [6]. GlyT2 is a larger protein than GlyT1 due to an N-terminal domain of ~200 amino acids, a unique characteristic of GlyT2 compared with the other transporter family members. Both GlyTs are multiply N-glycosylated within the large second extracellular loop (EL2) connecting TMDs 3 and 4.

Considerable information exists regarding structural domains that are essential for the expression of functional GlyTs at the plasma membrane. In transfection studies, the N- and C-terminal domains have been found to be important for the correct targeting of GlyT1, but not GlyT2, to the cell surface [7]. Di-leucine motifs in the cytoplasmic tail and residues in the N-terminal region are responsible for apical versus basolateral localization of GlyT1 in polarized Madin–Darby canine kidney (MDCK) cells [8]. In addition, extensively glycosylated asparagine residues within EL2 also positively affect transporter insertion into the plasma membrane [7,9]. A C-terminal PDZ-domain-binding motif in GlyT2 might be important for its targeting to the synapse [10].

There is increasing evidence indicating that different Na^+/Cl^--dependent neurotransmitter transporters have an oligomeric structure [11,12]. Co-immunoprecipitation studies, the use of dominant-negative transporter constructs, in addition to cross-linking and fluorescence resonance energy transfer (FRET) assays, all suggest the formation of dimeric transporter complexes. However, oligomerization might not be important for transporter function [13,14]. Hydrodynamic studies on native GlyT2 purified from rat spinal cord [15] and gel-electrophoretic analysis of native and cross-linked recombinant GlyT1 and GlyT2 [13] are consistent with GlyTs in the plasma membrane functioning as monomeric proteins.

Mechanism of GlyT-mediated substrate uptake

The uptake of glycine mediated by GlyTs is energetically coupled to the transmembrane sodium gradient

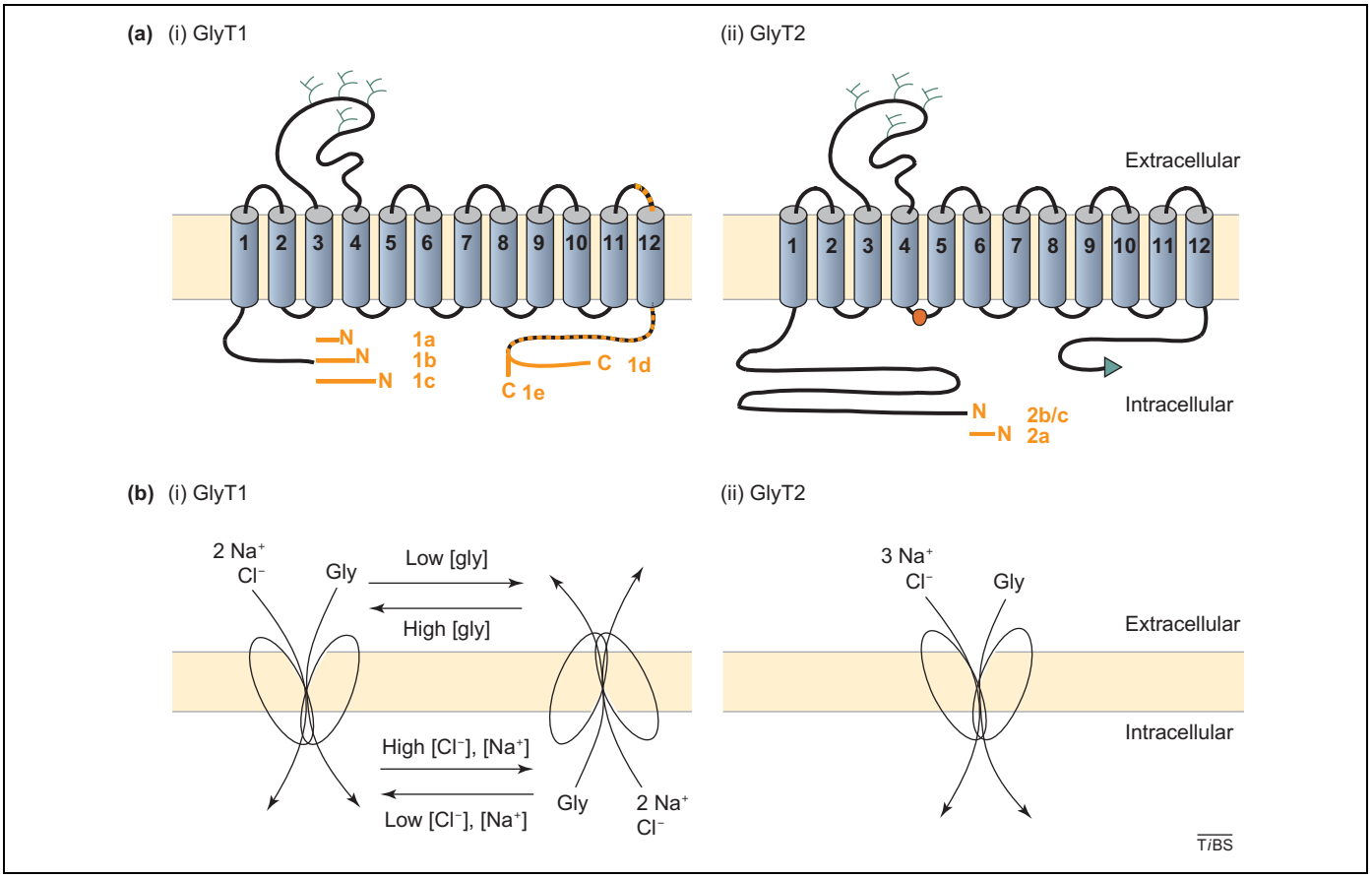

Figure 1. Membrane topology and transport properties of GlyTs. **(a)** GlyTs are characterized by 12 putative TMDs with intracellular N and C termini. Different splice variants are indicated in orange. For GlyT1, three N-terminal splice variants (a–c) and two C-terminal splice variants (d, e) have been identified. The dashed line indicates the shorter GlyT1e variant, identified only for bovine GlyT1, whose TMD12 might cross the membrane not as an α helix. Alternate promoter usage generates three N-terminal GlyT2 isoforms (a–c) with eight additional amino acids for GlyT2a and shorter identical protein sequences for GlyT2b and c. N-linked carbohydrates (green) are attached to the large EL2. Residues implicated in PKC-mediated down-regulation of GlyT2 are located in IL2 (red circle). The C-terminal PDZ-domain-binding motif required for the interaction of GlyT2 with syntenin-1 is drawn as a green triangle. **(b)** Both GlyTs catalyze the symport of glycine in the presence of Na^+ and Cl^- but differ in their ion stoichiometries, with two Na^+ being co-transported for GlyT1 (blue) and three Na^+ for GlyT2 (red). It is therefore suggested that GlyT1 is working close to equilibrium, and that it might either import or export glycine depending on ion gradients and membrane potential. For GlyT2, glycine transport is unlikely to be reversed by changes in the intracellular ionic concentrations due to its higher Na^+ transport stoichiometry.

Box 2. Tissue expression, subcellular localization and pharmacology of GlyTs

The GlyT1 gene is expressed throughout most regions of the CNS [3,21,59]. Immunohistochemical analysis has revealed intense GlyT1-specific staining of glial cells, in particular astrocytes, and some weak GlyT1 immunoreactivity in selected dendrites and nerve terminals of putative excitatory neurons in spinal cord [21,60]. Similarly, in forebrain regions rich in NMDAR-containing synapses, GlyT1 staining is found on both glia cells and subpopulations of glutamatergic neurons [60]. In the retina, GlyT1 is localized exclusively in selected amacrine and ganglion neurons but is not seen in the glial Müller cells [61]. In conclusion, although astrocytes seem to constitute the major cell type in which the GlyT1 gene is active, selected neuronal subpopulations also express this transporter isoform. Furthermore, GlyT1 is also found in some non-neuronal tissues, for example, liver and pancreas [21].

Analysis of GlyT2 expression indicates an exclusively neuronal expression of this transporter isoform in CNS regions rich in glycinergic synapses, such as the spinal cord, brain stem and cerebellum [20,21,62]. GlyT2 is present only in glycinergic neurons, where it is juxtaposed to GlyR containing postsynaptic specializations [20]. Immunoelectron microscopy demonstrated that GlyT2 is enriched in the plasma membrane of glycinergic nerve terminals but excluded from active zones, such as the neurotransmitter release sites [63]. In addition, GlyT2 has recently also been found in A-cells of the islets of Langerhans in the pancreas [64].

Although the GlyTs share high substrate specificity for glycine, they can be distinguished pharmacologically in ftro and in fvo. GlyT1-driven glycine uptake is inhibited by sarcosine (N-methyl-glycine), which acts as a competitive GlyT1 inhibitor by serving as a substrate. Synthetic sarcosine derivates like NFPS ((R)-N-[3-(4′fluoro-phenyl)-3(4′-phenlyphenoxy]propylsarcosine) and Org-24461 (N-methyl-N-[(4-trifluoromehtyl)phenoxy]-3-phenyl-propyl-glycine) constitute selective high-affinity inhibitors of GlyT1 [65]. GlyT2-mediated uptake is inhibited by the antidepressant amoxapine [66]. In addition, 4-benzyloxy-3,5-dimethoxy[(1-dimethylaminoacylclopentyl) methyl]-benzamide and a series of 5,5-diaryl-2-amino pentenoates have been reported to be potent inhibitors of GlyT2 [67,68].

maintained by the Na^+/K^+-ATPase. Extracellular binding of Na^+ and Cl^- together with glycine is supposed to induce a conformational change in GlyTs that switches the transporters from an 'outward' to an 'inward' facing state (see Box 3). Thus, the glycine-binding site would now be exposed to the cytosol, thereby enabling release of bound glycine and ions. The 'empty' transporter would then return to an outward facing conformation. Accordingly, the substrate-binding site should be accessible alternatively only on either the extracellular or the cytoplasmic side of the plasma membrane. The stoichiometry of substrate/ion co-transport has been determined to be 3 Na^+/Cl^-/glycine for GlyT2, whereas GlyT1 has a stoichiometry of 2 Na^+/Cl^-/glycine [16]. This difference of one Na^+ in ionic coupling implies that, under physiological conditions, the driving force available for uphill glycine transport by GlyT2 is much higher than that by GlyT1 [16]. Consequently, GlyT2 has a higher capacity in maintaining millimolar intracellular versus submicromolar extracellular glycine levels than GlyT1. By contrast, its lower Na^+ transport stoichiometry facilitates GlyT1 to operate in a reverse uptake mode, that is, neurotransmitter release from the cytosol into the extracellular space in case of alterations in substrate or ion concentration gradients or membrane potential [17] (Figure 1b). This is thought to enable Ca^{2+}-independent glycine release in synaptic regions.

Although the identification of transporter domains and residues that participate in substrate binding and translocation is an area of intense research, the precise localization of the substrate permeation pathway has remained elusive. Deletion mutants of GlyTs, in which most of the N- and C-terminal regions had been removed, retained all transport characteristics of the intact transporters upon reconstitution into liposomes, indicating that these intracellular domains are not required for transport function [6]. N-glycosylation is important for stabilizing the active conformation of GlyT2 because treatment with N-glycosidase results in both the loss of its carbohydrate side chains and a substantial decrease in glycine uptake activity [9]. In addition, TMD3 of GlyT2 has been suggested to form part of a common translocation pathway for glycine, Na^+ and Cl^-. Conservative substitutions of TMD3-Tyr289 decreased the apparent Km of glycine uptake and severely altered co-transported ion dependence [18]. Tyr289 is conserved throughout the Na^+/Cl^--dependent transporter family, suggesting a general role of this amino acid in interactions with the amino group found in all substrates of these transporters. The extracellular loops are also thought to contribute to substrate binding and transport function. Cysteine scanning mutagenesis has demonstrated that EL1 of GlyT2 operates as a fluctuating hinge that, upon glycine and ion binding, undergoes sequential conformational changes, which are thought to be essential for substrate translocation [19]. The requirement for extensive conformational rearrangements during the transport cycle is consistent with the comparatively slow substrate turnover of neurotransmitter transporters, including GlyTs. Hence, a high density of GlyTs is required at synaptic sites for the efficient regulation of glycine-dependent neurotransmission.

Functions of GlyTs at inhibitory synapses

The localization of GlyT2 parallels that of postsynaptic glycine receptors (GlyRs). Therefore, it was previously thought that GlyT2 is the main isoform mediating the clearance of presynaptically released glycine at inhibitory synapses [20]. However, due to overlapping expression patterns of GlyT1 and GlyT2 in caudal regions of the central nervous system (CNS; see Box 2), GlyT1 might contribute to removing glycine from the synaptic cleft of glycinergic synapses [16,21]. In an attempt to clarify the *in fvo* functional roles of glycine transporters in the CNS, knockout mice deficient in GlyT1 and GlyT2 have been generated recently [22–25]. Both homozygous knockout lines seemed externally normal at birth; no changes in brain anatomy or the expression of other synaptic proteins could be detected. However, electrophysiological recordings from hypoglossal motoneurons in the brain stem revealed abnormal glycinergic inhibitory currents in the mutant animals. GlyT1-deficient mice displayed increased chloride conductances, consistent with a tonic activation of GlyRs by elevated extracellular glycine concentrations [22]. Furthermore, spontaneous inhibitory postsynaptic currents (IPSCs) had longer decay time constants than those in wild-type mice. These observations suggest a

Box 3. GlyT structure: lessons from bacterial transporter proteins

Presently, no structural information is available for any of the neuronal neurotransmitter transporters. Therefore, the precise mechanisms of substrate recognition and translocation are still enigmatic. Recently, however, X-ray structures of two bacterial transporters, lactose permease (LacY) [69] and the glycerol-3-phosphate transporter (GlpT) [70], have been solved. LacY catalyzes the co-transport of lactose and H^+, whereas GlpT exchanges glycerol-3-phosphate for inorganic phosphate. These transporters share a 12-transmembrane topology with the mammalian GlyTs and other Na^+/Cl^--dependent transporters. Notably, in the bacterial proteins, the 12-TMDs form two distinct domains of six N-terminal and six C-terminal helices each, which are separated by a large hydrophilic cavity that extends into the center of the lipid bilayer and harbours the substrate binding residues (Figure Ia). These interact via salt bridges and hydrogen bonds with the bound substrate. Both LacY and GlpT have been crystallized in inward-facing conformations. Consistent with substrate binding to GlpT leading to compaction of the protein, both transporters are thought to adopt several distinct conformational states while catalyzing the transport cycle. For LacY, a model of the conformation, in which the inter-domain substrate-binding site is exposed to the extracellular site of the plasma membrane, has been proposed (Figure Ib). These structural and model data are fully consistent with an 'alternating access' mode of substrate transport. Due to significant topological similarities, the mammalian neurotransmitter transporters are thought to have an overall structure similar to LacY/GlpT and to share analogous conformational transitions.

Rather recently, a high-resolution structure has also become available for a bacterial glutamate transporter homologue [71]. Glutamate transporters constitute a distinct family of neurotransmitter transporter proteins that are structurally unrelated to the Na^+/Cl^--dependent neurotransmitter transporters. The bacterial glutamate transporter has a trimeric structure and differs in its transmembrane organization from the transporters discussed here. However, again the substrate-binding residues are located in a large hydrophilic cavity that reaches down to the middle of the membrane. Thus, ion-driven substrate translocation seems to require a conserved protein architectural design.

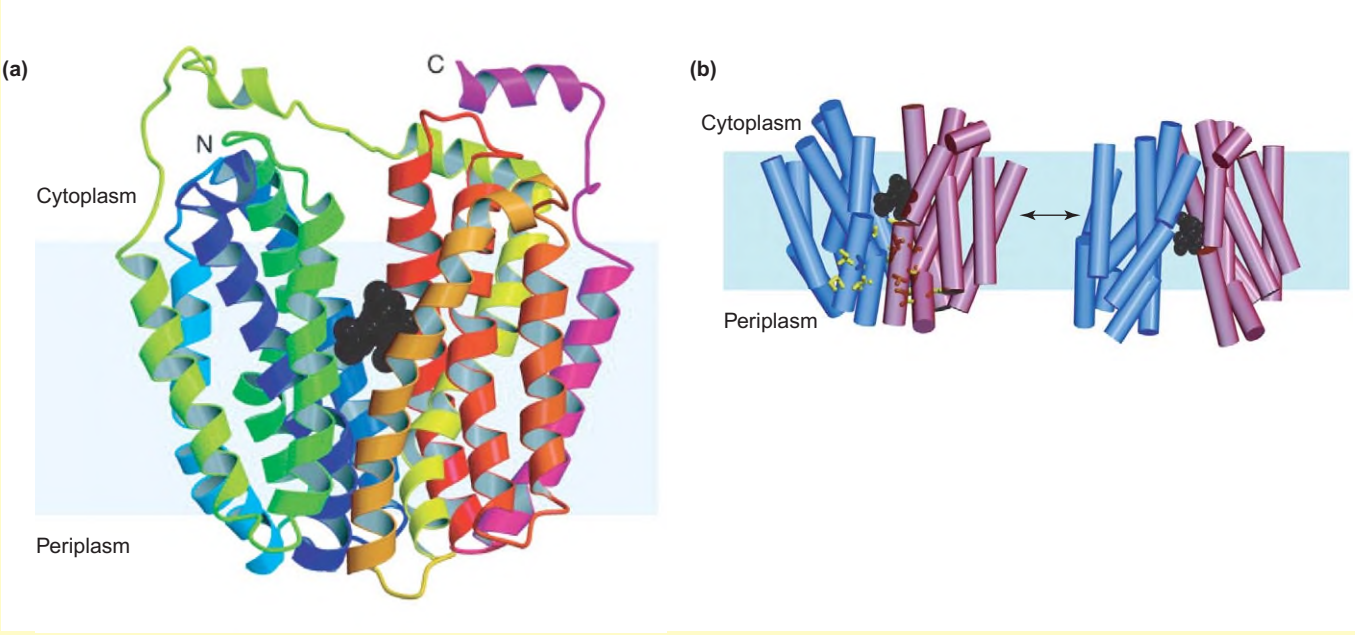

Figure I. Overall structure and proposed conformations of LacY. **(a)** Ribbon representation of the inward-facing substrate-bound structure of the LacY protein. The functional monomer consists of 12 helical TMDs, which form N-terminal (blue to green TMDs) and C-terminal (green to pink TMDs) domains of six helices each. Both domains are connected by a flexible cytoplasmic linker that enables the formation of a large water accessible cavity, in which the substrate (black) and the proton are bound. **(b)** During the transport cycle, LacY must change its conformation to enable access of the substrate-binding site from the periplasm. This requires structural changes, which are thought to involve a rotation of the N- and C-terminal domains around the substrate-binding cavity. Reprinted, with permission, from Ref. [69].

fundamental role of GlyT1 in lowering extracellular glycine levels at glycinergic synapses (Figure 2).

By contrast, neurons from GlyT2-deficient mice generated glycinergic IPSCs with markedly reduced amplitudes, compared with those from wild-type mice [23]. No postsynaptic changes were found in the mutant, which indicates that glycine release from glycinergic nerve terminals is impaired in GlyT2 knockout mice. Apparently, the loss of GlyT2 decreases the cytosolic glycine concentration in the presynaptic terminal, leading to inefficient synaptic vesicle refilling with glycine, and thus smaller postsynaptic currents. Therefore, GlyT2 does not have an important role in clearing glycine at glycinergic synapses but is essential for glycine uptake into the presynaptic cytosol, and hence the recycling of glycine. The presence of GlyT2 in glycinergic boutons adjacent to the active zones (see Box 2) and the high Na^+-dependence of GlyT2-mediated substrate transport [16,17] further corroborate this conclusion. In summary, GlyT1 and GlyT2 have complementary functions at glycinergic synapses: GlyT1 eliminates glycine from the synaptic cleft and thereby terminates glycine neurotransmission, whereas GlyT2 enhances its efficacy by providing cytosolic glycine for vesicular release (Figure 2).

Modulation of excitatory neurotransmission by GlyT1

In addition to regulating glycine concentrations at inhibitory synapses, GlyTs have been implicated in the control of glycine levels at glutamatergic synapses [26]. Alterations in the ambient glycine concentration are known to have modulatory effects on N-methyl-D-aspartate receptors (NMDARs), where glycine acts besides

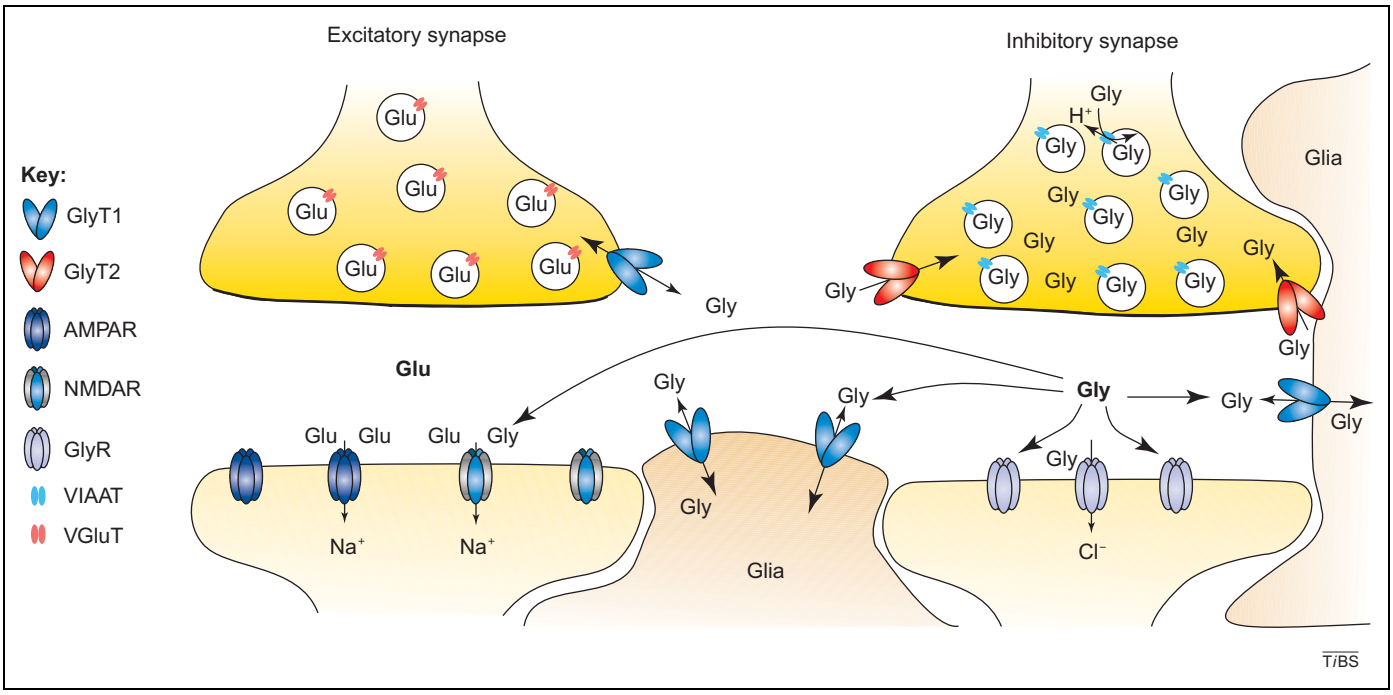

Figure 2. Localization and proposed functions of GlyTs at excitatory and inhibitory synapses. At inhibitory synapses, glycine release from the presynaptic terminal activates postsynaptic GlyRs and thereby induces Cl^- influx – hyperpolarization – of the postsynaptic cell. At excitatory glutamatergic synapses, glycine acts as an essential co-agonist of postsynaptic NMDARs, whereas neighbouring glutamate receptors of the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic-acid receptor (AMPAR) subtype require only glutamate for channel activation. Here, glycine might be derived from neighbouring glycinergic terminals or even be released from astrocytes via non-vesicular mechanisms (e.g. reverse transport by GlyT1). GlyT2 is localized in the presynaptic plasma membrane of glycinergic neurons and transports glycine into the terminal, thereby enabling the refilling of synaptic vesicles with glycine by the H^+-dependent vesicular inhibitory amino acid transporter (VIAAT). GlyT1 is mainly expressed by glia cells surrounding both inhibitory and excitatory synapses. In addition, GlyT1 has been found on terminals of some excitatory neurons. Thus, GlyT1 mediates the clearance of glycine from the synaptic cleft of inhibitory synapses and, in addition, participates in the regulation of the glycine concentrations at excitatory synapses.

glutamate as an essential co-agonist (Box 1). Recently, Ahmadi *et al.* demonstrated that stimulation of inhibitory synapses in brain stem slices could potentiate neighboring glutamatergic synapses due to 'spillover' of glycine released from glycinergic nerve terminals [27]. Evidence that GlyT-dependent processes contribute to the regulation of effective glycine levels at NMDAR containing excitatory synapses mainly comes from uptake inhibition studies. Bradaia *et al.* found that blockade of both GlyT1 and GlyT2 with the specific inhibitors Org-24598 and Org-25543, respectively, resulted in a larger NMDAR component of glutamatergic neurotransmission in spinal cord [28]. Similarly, in hippocampal slice preparations partial inhibition of GlyT1 caused an increase in the extracellular glycine concentration and thereby a facilitation of NMDAR currents, resulting in enhanced long-term potentiation [29]. These findings are consistent with GlyT1 activity preventing saturation of the glycine-binding site of synaptic NMDARs (Figure 2). However, both complete inhibition of GlyT1 with the uptake inhibitor CP-802079 and addition of high concentrations of glycine (100μM) led only to a transient facilitation of NMDAR responses, which was reversed at later time-points [29]. This reduction of NMDAR-mediated currents was attributed to internalization of NMDARs via clathrin-mediated endocytosis [30]. Thus, GlyT1 might be important for both enabling facilitation and preventing downregulation of NMDARs. Moreover, under particular physiological conditions it might even positively affect NMDAR responses by enabling glycine release from astrocytes through reverse transport.

Recently, evidence for a role of GlyT1 in the regulation of NMDAR efficacy has also been provided by genetic studies. Heterozygous mice carrying only one functional GlyT1 allele are phenotypically normal [22,24,25] but [^{3}H]glycine uptake into brain membrane fractions isolated from these animals is reduced to ~50% of that obtained with wild-type samples [22,25]. Notably, in brain slices prepared from the heterozygous animals NMDAR-evoked postsynaptic currents were significantly larger than in wildtype, and glycine failed to potentiate current amplitudes [24,25]. Moreover, behavioural studies revealed changes in the pharmacology of prepulse inhibition, the reduction of the sound-induced startle response by preexposition to a milder acoustic stimulus [25]. Because this test paradigm has been associated with NMDAR function, these findings further underline the crucial regulatory role of GlyT1 in glutamatergic neurotransmission.

Regulation of GlyT activities and numbers

The previous data indicate that GlyT1 and GlyT2 have essential functions at both inhibitory and excitatory synapses. Therefore, changes in the transport activity and/or number of GlyTs in the plasma membrane might profoundly affect the efficacy of neurotransmission. For instance, in spinal cord, the downregulation of GlyT1 would potentiate glycinergic inhibition, whereas reducing GlyT2 expression would depress it. For this reason, GlyTs are believed to be tightly regulated through different mechanisms.

GlyT1 is directly inhibited by arachidonic acid, a second messenger released following phospholipase A2 activation

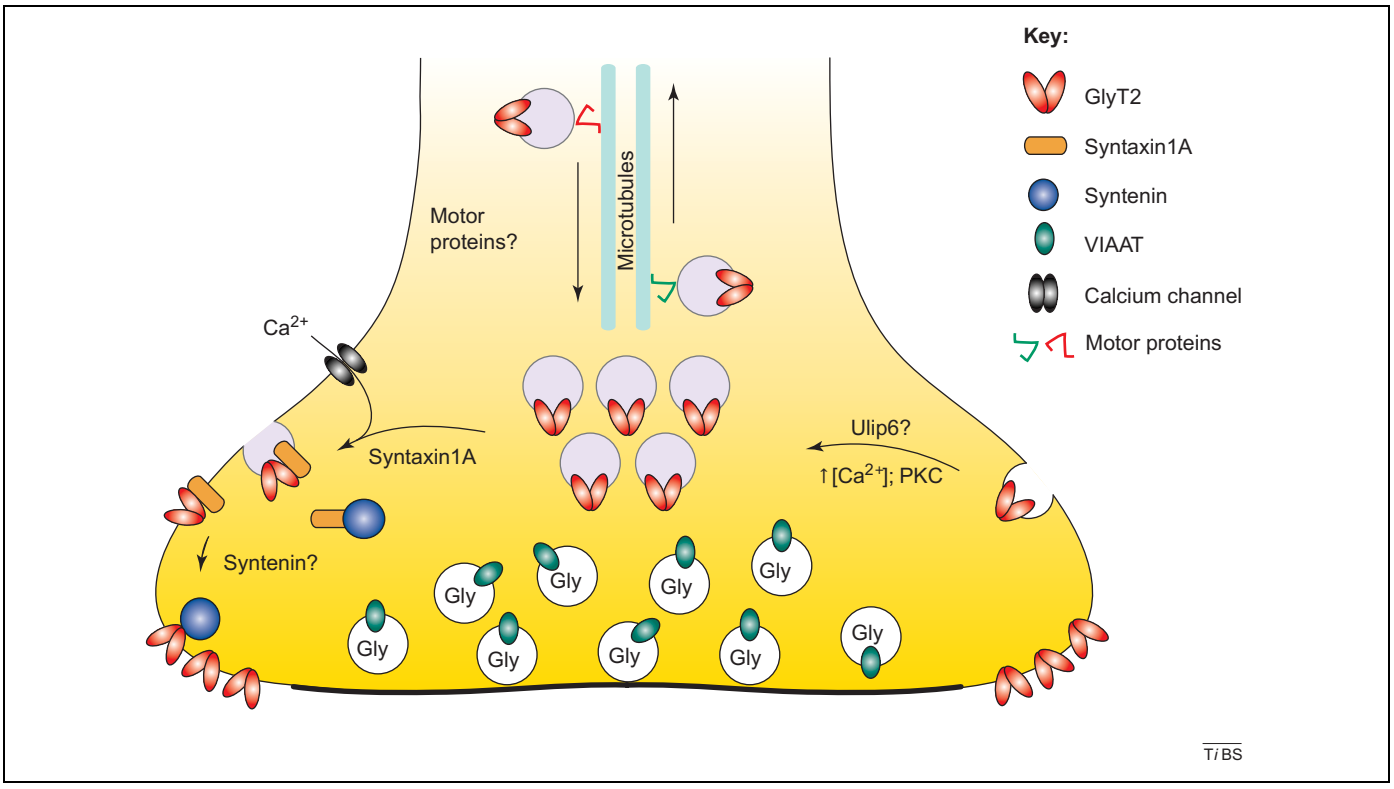

Figure 3. Model of GlyT2 trafficking in the presynaptic terminal. GlyT2-containing transport vesicles travel from the nerve cell body by motor protein driven anterograde transport along microtubules to the presynaptic terminal, where they form a reserve pool of intracellular GlyT2-containing vesicles. Depolarization-induced Ca^{2+}-influx promotes the syntaxin 1A-dependent insertion of GlyT2 into the plasma membrane surrounding the active zone (thick black line). This process might be facilitated or terminated by binding of syntenin-1 to the C-terminal tail of GlyT2. Upon sustained Ca^{2+}-influx or PKC activation, GlyT2 is endocytosed rapidly. Ulip6, another interacting protein, might support GlyT2 internalization. Endocytosed GlyT2-containing vesicles are removed from the nerve terminal via retrograde motor protein-dependent transport along microtubules. Note that, according to this model, GlyT2-containing transport vesicles are distinct from synaptic vesicles, which are filled with glycine by VIAAT.

[31,32]. In addition, changes in pH modulate GlyT1 activity, and this modulation involves residue His421 [33]. Low doses of Zn^{2+}, a metal ion that is co-released with glutamate by different types of excitatory neurons, also inhibit GlyT1 but have no effect on GlyT2 [34]. Two histidine residues on the extracellular face of GlyT1, one within EL2 and the other within EL4, coordinate Zn^{2+} binding, thus inhibiting conformational changes that are crucial for glycine translocation [35].

Intracellular Ca^{2+} levels modulate GlyT2 numbers on the cell surface [36]. Rapid membrane depolarization in the presence of extracellular Ca^{2+} stimulates the delivery of GlyT2 into the plasma membrane of spinal cord synaptosomes, subsequently followed by its constitutive internalization (Figure 3). In addition, GlyT2 removal from the neuronal cell surface is enhanced under conditions favoring sustained Ca^{2+} influx [36]. This could be due to activation of protein kinase C (PKC) because stimulation of this enzyme by phorbol esters decreases the density of GlyT1 and GlyT2 on the cell surface [37,38]. Mutation of all consensus PKC phosphorylation sites in GlyT1 did not abolish phorbol ester-induced downregulation [39], suggesting that PKC does not function by phosphorylating GlyT1 directly but that intermediate substrate proteins are involved in this mechanism. This is supported by the observation that mutations of charged amino acids in the second intracellular loop (IL2) of GlyT2 abolish its internalization upon phorbol ester treatment [38].

Biochemical and transfection experiments have disclosed a physical and functional interaction between GlyTs and the presynaptic SNARE protein syntaxin 1A. Co-expression of GlyT1 or GlyT2 with syntaxin 1A results in a reduction in the number of GlyTs on the cell membrane [40]. In addition, syntaxin 1A has been found to be essential for constitutive and Ca^{2+}-triggered insertion of GlyT2 into, but not its retrieval from, the plasma membrane of neurons [36]. Yeast two-hybrid screening has identified additional binding partners of GlyTs, such as Ulip6, a member of the collapsin response mediator protein family [41] that has been implicated in endocytosis [42], and the PDZ domain protein syntenin-1 [10]. Interestingly, syntenin-1 also binds syntaxin 1A, revealing a close network of interactions between GlyT2 and these proteins, which might have an important role in the intracellular trafficking and/or presynaptic localization of GlyT2. A model depicting the potential functions of the known interactors and regulatory enzymes of GlyT2 in glycinergic terminals is shown in Figure 3.

Implications for human neurological disease

The perturbations of glycinergic neurotransmission observed in GlyT-deficient mice have fatal consequences for the survival of these animals. Loss of either transporter shortens their life spans. Newborn GlyT1 knockout mice die on the day of birth [22]. During the short time of postnatal survival, the newborn mutant mice display severe motosensory deficits characterized by lethargy,

hypotonia and hyporesponsivity to tactile stimuli. Dysfunction of motor activity extends to the respiratory system, in which rhythmic breathing is severely depressed. Recordings of neuronal activity in the brain stem circuitry responsible for generating the respiratory rhythm disclosed a slowed and irregular pattern that was normalized upon application of strychnine [22]. These findings demonstrated that these motosensory deficits caused by the loss of GlyT1 are a result of glycinergic over-inhibition induced by sustained activation of GlyRs in the presence of high levels of extracellular glycine. GlyT2-deficient mice also show a lethal phenotype, although death occurs during the second postnatal week and after developing an acute neuromotor disorder, whose symptoms are entirely different from those seen upon GlyT1 deletion [23]. Here, diminished glycinergic neurotransmission caused by the loss of GlyT2 induces a severe under-inhibition phenotype characterized by muscular spasticity, impaired motor coordination and tremor.

Interestingly, the symptoms observed in each line of mice are similar to those associated with a group of human hereditary diseases, which develop in early postnatal life or during adolescence. Hyperglycinergic GlyT1 knockout mice present symptoms similar to glycine encephalopathy, a disease often associated with mutations in the mitochondrial glycine cleavage system (GCS), which degrades excess intracellular glycine [43,44]. Hypoglycinergic GlyT2 knockout animals resemble patients suffering from hyperekplexia. This neuromotor disorder results in exaggerated startle responses and, in severe cases, a 'stiff baby syndrome' and has been shown to involve mutations of the α1 and β subunit genes of the GlyR [45]. Notably, in several patients diagnosed with those illnesses no defects in GCS or GlyR genes have been found [43,46]. This indicates that glycine encephalopathy- and hyperekplexia-like syndromes can be caused through different genetic mechanisms and suggests that some yet unclassified forms of these diseases might involve mutations in the human GlyT genes.

GlyT1 inhibitors – potential therapeutics?

Recently, several studies have been published that describe the *in fvo* application of GlyT1 inhibitors and their consequences on NMDAR function. Here, the acute application of such inhibitors led to significant increases in the glycine concentration of the cerebrospinal fluid in forebrain and caudal regions of the CNS, whereas the concentrations of other amino acids were not changed [29,47]. Electrophysiological recording revealed enhanced NMDAR currents after inhibition of GlyT1 [29,48]. Together these findings demonstrate that GlyT1 has an important role in the regulation of extracellular glycine in the CNS. Furthermore, Whitehead *et al.* reported that as a consequence of GlyT1 inhibition the level of citrulline was remarkably increased [47]. This was attributed to an increased activity of nitric oxide synthase, which generates citrulline as a stoichiometric by-product of NO synthesis and is associated with postsynaptic NMDARs.

A severe and widespread psychiatric disorder that is thought to involve NMDAR hypofunction is schizophrenia. Schizophrenic patients show enhanced motor activity, cognitive deficits and an increase in stereotyped behaviours. Rather similar symptoms can be induced in healthy humans and rodents by NMDAR blockers, such as ketamine and phenylcyclidine (PCP) [49]. Notably, the effects of NMDAR inhibition by PCP or the glycine-binding-site antagonist HA-966 were reversed upon inhibition of GlyT1 [50,51]. Therefore, it was proposed that GlyT1 inhibitors might be beneficial in the therapy of schizophrenic patients [52]. The behavioural analysis of animals treated with agents that induce schizophrenia in humans indeed disclosed positive effects of GlyT1 inhibitors on schizophrenia-associated symptoms, like hyperactivity or an impaired pre-pulse inhibition [50,53]. Hence, these studies have been extended to clinical trials, and a high glycine diet and the competitive GlyT1 substrate sarcosine (Box 2) have been recommended as co-medication of schizophrenia. Whether GlyT1 inhibitors could be of wider use as general cognitive enhancers is under investigation.

GlyT1 inhibitors might also have the potential to increase the efficacy of inhibitory neurotransmission in different pathological situations by transiently potentiating postsynaptic GlyR currents. For example, raising glycine levels at spinal synapses should reduce spontaneous motor activity [22,45] and decrease pain perception [54]. This might be exploited for muscle relaxation during narcosis or acute spastic syndromes, and for analgesia. Whether subsaturating doses of GlyT2-selective inhibitors might also be beneficial is not clear at present.

Concluding remarks and perspectives

The studies summarized here document that major progress has been made in unravelling the physiological roles of GlyTs in the mammalian CNS. Although our understanding of the overall structure and conformational transitions of these membrane proteins is still largely based on comparisons to bacterial transporters (Box 3), inhibitor studies and the generation of GlyT knockout mice have disclosed highly specialized synaptic functions of GlyT subtypes. GlyT1 is essential for regulating glycine concentrations at synaptic receptors. At glycinergic synapses, it shortens the duration of the postsynaptic response by lowering glycine concentrations at inhibitory GlyRs, and its inactivation or blockade results in over-inhibition. In addition, GlyT1 prevents saturation of the glycine-binding site of NMDARs, thereby enabling glycine potentiation of excitatory glutamatergic neurotransmission. GlyT2 by contrast is uniquely designed for neurotransmitter recycling at inhibitory glycinergic synapses, and loss of GlyT2 function generates a severely hyper-excited state.

The clear dichotomy of GlyT subtype functions at central synapses provides the basis for future therapeutic approaches, which selectively target these membrane proteins. It should, however, be noted that both GlyT1 and GlyT2 are also expressed in non-neural tissues and, hence, possible side effects of GlyT1 inhibitors will have to be examined carefully. Furthermore, studies are required to clarify whether changes in the metabolic state of

astrocytes could result in increases of synaptic glycine concentrations due to GlyT1-driven reverse transport.

The most pertinent questions to be solved in GlyT research, however, concern the roles of the individual GlyT subtypes and splice variants in adult brain function and behaviour, in addition to the mechanisms that regulate transporter number and activity. So far, GlyT gene inactivation has not been variant-specific, and the early postnatal lethality of constitutive GlyT knockout mice has precluded studies at later developmental stages. Different lines of evidence suggest that changes in the activities, densities and localizations of GlyTs in glial and nerve terminal plasma membranes could importantly contribute to the regulation of synaptic efficacy, and hence neuronal plasticity, within the CNS. Also, GlyT2 is the only gene product to date known to be expressed exclusively in glycinergic neurons, and activation of the GlyT2 gene could, therefore, constitute a key event in the development of inhibitory circuits. Deciphering the transcriptional and post-translational mechanisms involved in GlyT regulation should provide further insight into how GlyTs regulate neurotransmission in the developing and adult CNS.

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