Lessons from the Knocked-Out Glycine Transporters

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Abstract Glycine has multiple neurotransmitter functions in the central nervous system (CNS). In the spinal cord and brainstem of vertebrates, it serves as a major inhibitory neurotransmitter. In addition, it participates in excitatory neurotransmission by modulating the activity of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors. The extracellular concentrations of glycine are regulated by Na^+/Cl^--dependent glycine transporters (GlyTs), which are expressed in neurons and adjacent glial cells. Considerable progress has been made recently towards elucidating the in vivo roles of GlyTs in the CNS. The generation and analysis of animals carrying targeted disruptions of GlyT genes (GlyT

knockout mice) have allowed investigators to examine the different contributions of individual GlyT subtypes to synaptic transmission. In addition, they have provided animal models for two hereditary human diseases, glycine encephalopathy and hyperekplexia. Selective GlyT inhibitors have been shown to modulate neurotransmission and might constitute promising therapeutic tools for the treatment of psychiatric and neurological disorders such as schizophrenia and pain. Therefore, pharmacological and genetic studies indicate that GlyTs are key regulators of both glycinergic inhibitory and glutamatergic excitatory neurotransmission. This chapter describes our present understanding of the functions of GlyTs and their involvement in the fine-tuning of neuronal communication.

Keywords Glycine · Glycine uptake · Glycine transporter · Inhibitory glycine receptor · *N*-Methyl-D-aspartate receptor · Transport inhibitors · Knockout mice · Schizophrenia · Pain · Hyperekplexia · Glycine encephalopathy

1
Neurotransmitter Functions of Glycine in the CNS

More than 40 years ago, the amino acid glycine was recognized to have neurotransmitter functions in the mammalian central nervous system (CNS). Initially, glycine was found to be highly enriched in spinal cord and brainstem and to inhibit the firing of spinal cord neurons. This effect was blocked by strychnine, a selective antagonist of inhibitory glycine receptors (GlyRs) (Laube et al. 2002; Lynch 2004). Today it is well established that glycine serves, along with γ-aminobutyric acid (GABA), as a principal inhibitory neurotransmitter in the adult mammalian CNS (Betz et al. 2000). At inhibitory synapses, glycine is released from the presynaptic nerve terminal upon depolarization and Ca^{2+}-influx via the exocytotic fusion of glycine-filled synaptic vesicles with the presynaptic plasma membrane (Legendre 2001). The released glycine binds to postsynaptic GlyRs, thereby causing an increase in the chloride conductance of the plasma membrane (Betz et al. 2000). GlyRs are pentameric membrane proteins composed of α- and β-subunits which, upon glycine binding, undergo a conformational change that opens an intrinsic anion channel. In the adult CNS, GlyR activation results in an influx of chloride ions into the cytoplasm of the postsynaptic cell, thus leading to hyperpolarization and thereby an increase in the threshold for the initiation of action potentials (Lynch 2004). In contrast, embryonic neurons are depolarized, i.e. excited upon GlyR activation. This excitatory action of glycine is due to high intracellular chloride concentrations at early stages of development (Singer et al. 1998), which result in chloride efflux from the cytoplasm upon opening of the GlyR anion channels. Around birth, the intracellular chloride concentration is reduced due to the expression of a very effective chloride export system, the K^+/Cl^--cotransporter KCC2 (Hubner et al. 2001; Rivera et al. 1999). This leads to a conversion of glycine responses from excitatory to inhibitory.

In addition to its role in GlyR-mediated inhibitory neurotransmission, glycine also has essential functions at excitatory glutamatergic synapses. Here, glycine acts, together with glia-derived D-serine, as a co-agonist of glutamate at ionotropic glutamate receptors of the *N*-methyl-D-aspartate receptor (NMDAR) subtype (Johnson and Ascher 1987; Verdoorn et al. 1987). NMDARs are heterotetrameric proteins composed of glycine binding (NR1, NR3) and glutamate binding (NR2) subunits (Hollmann 1999; Laube et al. 1998). The most widely expressed NMDARs are heterotetramers of NR1 and NR2, which display a high binding affinity ($K_d \sim 500$ nM) for glycine. Since the glycine concentration in the cerebrospinal fluid has been estimated to be in the low micromolar range, the high-affinity glycine binding site at the NMDAR was thought to be permanently saturated. However, recent studies have shown effects of exogenously applied glycine on NMDAR-mediated currents, demonstrating that alterations in the ambient glycine concentration have modulatory effects on NMDAR activity. First, the application of up to 20 μM glycine causes a potentiation of the NMDAR component of glutamatergic neurotransmission (Berger et al. 1998), supporting the idea that the glycine binding site of NMDARs is not permanently saturated at synapses. In contrast, application of a high concentration of glycine ($\geq$100 μM) results in a decrease of NMDAR-mediated neurotransmission due to enhanced receptor internalization (Nong et al. 2003).

The neurotransmitter functions of glycine imply that extracellular glycine concentrations have to be tightly controlled. At inhibitory synapses, the released glycine must be removed efficiently after GlyR binding, thereby allowing for high-frequency firing and thus precise temporal control of neurotransmission. Furthermore, cytosolic glycine pools have to be replenished for the reloading of synaptic vesicles after endocytosis. A stringent regulation of extracellular glycine levels is also essential for NMDAR modulation at excitatory synapses. All these tasks are accomplished by highly selective transport systems, the glycine transporters (GlyTs). This chapter describes our present understanding of GlyTs and their involvement in the fine-tuning of neuronal transmission.

2
GlyTs Are Members of the Na^+/Cl^--Dependent Transporter Family

2.1
GlyT Gene Structures

Following the demonstration that glycine acts as an inhibitory neurotransmitter, high-affinity ^{3}[H]glycine uptake into neurons and glial cells was found in rat spinal cord and other regions of the rodent CNS (Lopez-Corcuera and Aragon 1989). This glycine uptake was dependent on Na^+ and Cl^- ion gradients and resembled the glycine transport system previously described for

human erythrocytes, the so-called 'system Gly' (Ellory et al. 1981). A hallmark of 'system Gly' is its inhibition by sarcosine (*N*-methyl-glycine). In the early 1990s, the first purification of a functional GlyT protein from pig brainstem with a molecular weight of about 100 kDa was reported (Lopez-Corcuera et al. 1991). Interestingly, although ^{3}[H]glycine uptake driven by the reconstituted transporter was Na^+- and Cl^--dependent, sarcosine was unable to inhibit it. This was the first convincing evidence that more than one GlyT exists in the mammalian CNS. Subsequently, two GlyT isoforms, GlyT1 and GlyT2, were identified by homology screening with previously cloned Na^+/Cl^--dependent neurotransmitter transporter complementary (c)DNAs (Guastella et al. 1992; Liu et al. 1993; Smith et al. 1992). Both, GlyT1 and GlyT2 exist in different isoforms which result from alternative splicing and/or usage of different promoters (Guastella et al. 1992; Liu et al. 1992).

The human GlyT1 gene (*SLC6A9*) encompasses 14 exons distributed over 44.1 Mb and is found on human chromosome 1 (p31.3–p32) (Jones et al. 1995; Kim et al. 1994). To date, in humans and other mammals three variants, GlyT1 a–c, have been identified which differ only in their extreme N-terminal regions (Adams et al. 1995; Borowsky et al. 1993; Kim et al. 1994). GlyT1a and GlyT1b are generated by differential promoter usage. GlyT1c is an alternative splice product of GlyT1b (Adams et al. 1995; Borowsky and Hoffman 1998). Comparison of the human N-terminal sequences revealed that GlyT1c has an initial 15-residue sequence equivalent to that of GlyT1b, which is followed by a unique 54-residue sequence not found in the other two isoforms (Kim et al. 1994). Additionally, for bovine GlyT1 two C-terminal splice variants (d, e) have been described (Hanley et al. 2000) that have not been found in other species so far.

The GlyT2 gene (*SLC6A5*) is located on human chromosome 11 (p15.1–15.2) (Morrow et al. 1998). Its 16 exons span a region of 20.6 Mb. In mouse, three alternative N-terminal splice variants (GlyT2a–c) have been described and analysed (Ebihara et al. 2004). Mouse GlyT2a contains eight additional amino acids at the extreme N-terminus, whereas GlyT2b and c, which are identical at the amino acid level, have different 5′-untranslated regions (UTRs).

2.2 GlyT Protein Structure

GlyTs share common structural features with other members of the Na^+/Cl^--dependent transporter family (*SLC6)* that utilize neurotransmitters and metabolites as substrates (Nelson 1998). *N*-Glycosylation and cysteine scanning mutagenesis, in addition to in vitro glycosylation reporter fusion assays, have shown that GlyTs are polytopic membrane proteins with 12 transmembrane domains (TMDs), which are connected by six extracellular (EL) and five intracellular (IL) loops (Olivares et al. 1997) (Fig. 1a). Site-directed mutagenesis indicates that GlyT1 and GlyT2 are extensively glycosylated at four asparagine residues within their large EL2 connecting the hydrophobic TMDs 3 and 4

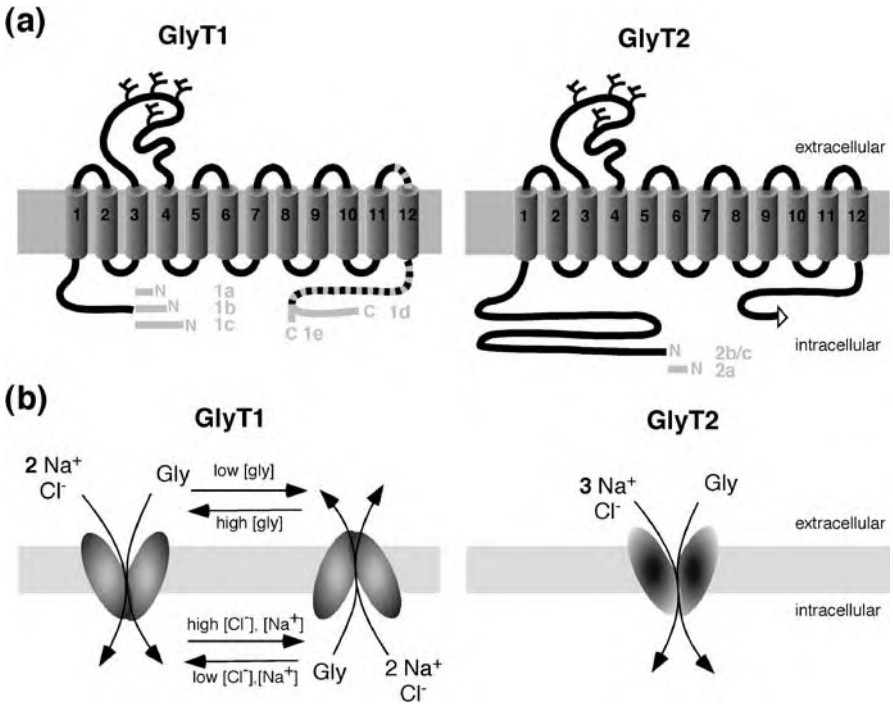

Fig. 1 a,b Membrane topology and transport properties of GlyTs. **a** GlyTs are characterized by 12 putative TMDs with intracellular N- and C-termini. Different splice variants are indicated in grey. For GlyT1, three N-terminal splice variants (a–c) and two C-terminal splice variants (d, e) have been identified. The *dashed line* indicates the shorter GlyT1e variant, identified only for bovine GlyT1, whose TMD12 may 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 are attached to the large EL2. The C-terminal PDZ domain-binding motif of GlyT2 is drawn as *triangle*. **b** Both GlyTs catalyse the symport of glycine in the presence of Na^+ and Cl^- but differ in their transport stoichiometries, with two Na^+ being co-transported by GlyT1 (*left*) and three Na^+ for GlyT2 (*right*). It is therefore suggested that GlyT1 is working close to equilibrium, and that it may 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. (Adapted with permission from Eulenburg et al. 2005)

(Olivares et al. 1995), thereby establishing the extracellular location of this hydrophilic loop region. Immunofluorescence and electron microscopy studies employing sequence-specific antibodies have confirmed that the N- and C-terminal ends of GlyTs are located intracellularly (Olivares et al. 1994). Almost all members of the *SLC6* family show C- and N-termini of comparable length, e.g. approximately 30–90 amino acids for the N-terminus and about 30–45 residues for the C-terminus. However, GlyT2 has a unique N-terminal

region encompassing about 200 amino acids. Moreover, the C-termini of both GlyTs, with 60–75 amino acids, are comparatively long compared with the other family members. Up to now, however, no unique functions of these large intracellular domains have been identified.

2.3 Glycine Uptake: Electrogenic Properties and Transport Mechanism

The uptake of glycine mediated by GlyTs is strictly Na^+-dependent and only partially Cl^--dependent (Lopez-Corcuera et al. 1998). Extracellular binding of both ions together with glycine is supposed to induce a structural change in GlyTs, from an 'outwardly open' to an 'inwardly open' conformation, thus enabling intracellular release of substrates (Rudnick 1998). In transient transfection studies, kinetic analyses of GlyT1 and GlyT2 showed similar K_m values for uptake activity. However, a new combination of electrophysiology and radiotracing techniques demonstrated that GlyTs differ in their ionic stoichiometries (Lopez-Corcuera et al. 1998; Roux and Supplisson 2000). The stoichiometry of substrate/ion co-transport has been determined to be 3 Na^+/Cl^- per glycine for GlyT2, whereas GlyT1 has a transport stoichiometry of 2 Na^+/Cl^- per glycine (Fig. 1b). Therefore, the uptake process is electrogenic, resulting in the intracellular accumulation of two positive charges per transport cycle for GlyT2 and only one positive charge for GlyT1. This difference of one Na^+ in ionic coupling between GlyTs has important consequences for their function. First, it implies that, under physiological conditions, the driving force available for uphill glycine transport by GlyT2 is much higher than for that by GlyT1. Therefore, GlyT2 has a higher capacity in maintaining millimolar intracellular versus submicromolar extracellular glycine levels than GlyT1. Second, it suggests that GlyT1 might function in a reverse uptake mode, i.e. releasing glycine from the cytosol into the extracellular space, in case of alterations in substrate concentration gradients or membrane potential (Sakata et al. 1997). In agreement with this interpretation, GlyT1 expressed in *Xenopus* oocytes generated an efflux of glycine upon intracellular glycine application or depolarization, whereas GlyT2 showed no efflux unless high concentrations of Na^+, Cl^- and glycine were co-injected into the oocyte cytoplasm (Supplisson and Roux 2002). Therefore, it has been proposed that GlyT1 might mediate Ca^{2+}-independent glycine release from cells and thereby contributes to the regulation of the extracellular glycine concentration.

The amino acid side chains and protein domains that participate in the function of GlyTs are not yet well defined. Mutational analysis has identified residues within transmembrane regions which are thought to have key roles in the transport cycle. Conservative substitutions of Tyr289, located in TMD3 of GlyT2, decreased the apparent K_m of glycine and severely altered Na^+ and Cl^- dependence (Ponce et al. 2000). Thus, TMD3 may form part of a common permeation pathway for glycine and co-transported ions. In contrast, deletion of

the N- and C-terminal regions did not affect glycine transport upon reconstitution into liposomes (Olivares et al. 1994), demonstrating that these intracellular domains are not required for transporter function. Likewise, *N*-glycosylation of EL2 of GlyT1 is not important for transport activity because treatment of the purified and liposome-reconstituted transporter with *N*-glycosidase resulted in the loss of its carbohydrate moiety but did not alter transport characteristics (Olivares et al. 1995). Mutation of the four *N*-glycosylation sites of GlyT2, however, induced a substantial decrease in glycine uptake activity, demonstrating a significant role of the carbohydrate moiety in stabilizing the active conformation of this transporter (Martinez-Maza et al. 2001; Nunez and Aragon 1994). The extracellular loop regions are also thought to contribute to substrate binding and uptake activity. Cysteine scanning mutagenesis has demonstrated that the EL1 of GlyT2, although it is not involved in substrate binding, acts as a fluctuating hinge that upon binding of glycine undergoes sequential conformational changes, which are thought to be essential for substrate translocation (Lopez-Corcuera et al. 2001). In summary, all available data indicate that extensive structural rearrangements, involving many different domains of GlyTs, take place during the transport cycle, thereby enabling the transporters to switch in an alternate access mode (Rudnick 1998) from an 'outwardly' to an 'inwardly' facing conformation.

2.4 Plasma Membrane Localization and Modulation of GlyTs

Considerable information exists regarding structural domains that are required for the insertion of GlyTs into the plasma membrane. In transfection studies, the intracellular N- and C-terminal domains have been found to be important for proper targeting of GlyT1 to the cell surface. Deletion of the C-terminal domain of GlyT1, which contains a putative PDZ-domain binding motif, results in impaired trafficking to the plasma membrane (Olivares et al. 1994). In contrast, deletion of the GlyT1 N-terminal region does not affect membrane insertion of the transporter. Interestingly, these N-terminally truncated mutants display an apical distribution in polarized Madin–Darby canine kidney (MDCK) cells as compared to a basolateral localization observed with full-length GlyT1b (Poyatos et al. 2000). Likewise, replacement of two dileucine motifs by alanines in the cytoplasmic tail of GlyT1b induces a relocation of the protein to the apical surface (Poyatos et al. 2000).

Extensively glycosylated asparagine residues within EL2 are also essential for insertion of GlyTs into the plasma membrane. Progressive mutation of the four *N*-glycosylation consensus sequences of GlyT1 results in missorting of the unglycosylated mutants in transfected cells (Olivares et al. 1995). Similar mutations in GlyT2 are responsible for a nonpolarized versus an apical localization in MDCK cells (Martinez-Maza et al. 2001). Furthermore, a C-terminal PDZ-domain binding motif in GlyT2 might be important for its targeting to

the synapse. Biochemical experiments and yeast two-hybrid screening have identified interaction partners of GlyT2, such as the PDZ domain protein syntenin-1, which binds to the GlyT2 cytoplasmic tail (Ohno et al. 2004). In contrast, the presynaptic SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) protein syntaxin 1A (Geerlings et al. 2000) and Ulip6 (Horiuchi et al. 2005), a member of the collapsin response mediator protein family which has been implicated in endocytosis (Nishimura et al. 2003), bind to the N-terminal domain of GlyT2. Co-expression experiments of syntaxin 1A with GlyT1 or GlyT2 in transfected cells modified the number of GlyTs on the cell surface (Geerlings et al. 2000). This might have been due to changes in the rates of exocytosis or internalization of the GlyTs upon their interaction with syntaxin 1A. In neurons, constitutive delivery of GlyT2 to the plasma membrane has been found to be mediated by syntaxin 1A, whereas GlyT2 internalization is independent of this interaction (Geerlings et al. 2001).

Other intracellular regions of GlyTs also contribute to plasma membrane localization. Mutations of charged amino acids in IL2 of GlyT2 have been shown to abolish its internalization upon phorbol ester treatment (Fornes et al. 2004), suggesting that protein kinase C (PKC)-regulated proteins interact with this GlyT2 intracellular domain and modulate its post-plasma membrane trafficking.

In summary, distinct domains of GlyTs are required for proper insertion into and removal from the plasma membrane mainly through interactions with intracellular regulatory binding proteins.

3
Distribution of GlyTs in the CNS

3.1
Tissue and Cellular Distribution

GlyTs share overlapping expression patterns in caudal regions of the CNS (spinal cord, brainstem and cerebellum) (Jursky and Nelson 1996; Luque et al. 1995; Zafra et al. 1995) in which glycine acts as inhibitory neurotransmitter (Fig. 2), thus supporting an important role of both transporters in the regulation of the extracellular glycine levels at inhibitory glycinergic synapses. In addition, GlyT1 transcripts are also detectable in forebrain regions and retina, where GlyT2 is not expressed.

Throughout the CNS, GlyT1 is almost exclusively localized on glial cells, in particular astrocytes (Zafra et al. 1995). However, GlyT1 in mammals is absent from Müller cells, the major glial cell type in the retina. Here, GlyT1 is expressed by a subset of neuronal cells, i.e. amacrine and some ganglion cells (Pow and Hendrickson 1999). Recently, it has been shown that, in forebrain regions, GlyT1 is expressed not only in astrocytes but is found presynaptically

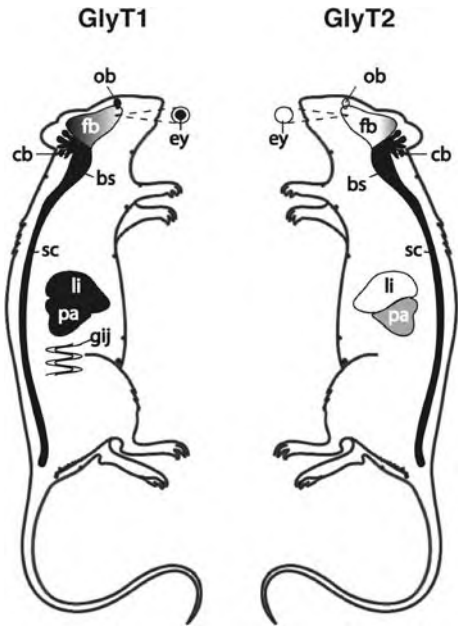

Fig. 2 Schematic representation of the expression patterns of the GlyTs. GlyT1 (*left*) and GlyT2 (*right*) expression patterns overlap in several regions of the CNS, particularly in the spinal cord (*sc*), brainstem (*bs*) and cerebellum (*cb*). In forebrain regions (*fb*), GlyT1 is transcribed, whereas for GlyT2 only very weak immunoreactivity has been reported in the hippocampus. GlyT1 protein can be additionally found in the retina of the eye (*ey*) and predominantly in the olfactory bulb (*ob*). Outside of the CNS, both GlyTs are expressed in the pancreas (*pa*). Furthermore, GlyT1 immunoreactivity is found in the liver (*li*) and the gastrointestinal junctions (*gij*)

in a subset of glutamatergic neurons which synapse onto NMDARs (Cubelos et al. 2005). This is consistent with a role of GlyT1 in the regulation of glycine levels at glutamatergic synapses.

GlyT2 is the main neuronal transporter isoform found in caudal regions of the CNS (Zafra et al. 1995). Ultrastructural analysis of GlyT2 localization using immunogold electron microscopy techniques has shown that GlyT2 is enriched in close proximity to, although excluded from, the presynaptic active zone of glycinergic nerve terminals (Mahendrasingam et al. 2003; Zafra et al. 1995). The overall distribution of GlyT2 parallels that of GlyRs. Therefore, GlyT2 is considered a reliable marker of glycinergic terminals (Jursky and Nelson 1995; Poyatos et al. 1997; Zeilhofer et al. 2005). Interestingly, GlyT2 has recently been detected in the hippocampus, a brain region previously thought to be devoid of glycinergic neurotransmission (Danglot et al. 2004). Although glycine-induced currents have not been observed in adult hippocampal neurons, GlyT2 co-localizes with GlyRs and the vesicular inhibitory amino acid transporter (VIAAT), an additional marker of inhibitory synapses (Danglot

et al. 2004). Also, expression of GlyT2 outside of the CNS has been demonstrated in A-cells of the islets of Langerhans in the pancreas (Gammelsaeter et al. 2004).

During development, both GlyT genes are expressed already prenatally, with GlyT1 transcripts appearing earlier than those of GlyT2. GlyT1 activity has been observed already in the pre-implantation embryo, where glycine is used as an organic osmolyte which regulates the cell volume at the cleavage stage (Steeves and Baltz 2005). After implantation, GlyT1 immunoreactivity (IR) can be identified at embryonic day 10 (E10) in the midbrain floor plate, with its expression spreading to median parts of the spinal cord at E12 (Jursky and Nelson 1996). The expression of GlyT1 extends during E13–14 to radial glial processes in forebrain regions. An exception is the cerebellum, where GlyT1 is detectable only postnatally, correlating with the late onset of synaptogenesis in this brain region (Jursky and Nelson 1996). At E13, GlyT1 expression is already detectable in tissues outside the CNS, i.e. in liver, pancreas and the gastroduodenal junction (Jursky and Nelson 1996).

Compared with GlyT1, GlyT2 immunoreactivity appears at later stages of development. At E12, parallel to the onset of synaptogenesis, GlyT2 can be first detected immunohistochemically in the white matter of the ventral spinal cord (Jursky and Nelson 1996). This might reflect the enrichment of GlyT2 in growth cones of outgrowing axons during this period (Poyatos et al. 1997). The expression of GlyT2 increases and extends upon subsequent development until GlyT2 is also found in the thalamus and the dorsal spinal cord at the time when all circuits are established in the spinal cord (E16–E17). At birth, the overall pattern of GlyT2 expression is basically established, except for the cerebellum, where the onset of synaptogenesis occurs later. During early postnatal development, the expression of GlyT2 increases further, and GlyT2 immunoreactivity changes to punctate structures in grey matter (Jursky and Nelson 1996). Maximal expression of GlyT2 is reached at postnatal day 14 (P14). Thereafter, expression decreases until P21 when the adult expression levels are established (Friauf et al. 1999).

3.2 Transcriptional Control

Although their expression patterns have been extensively studied and their gene structure is established, little is currently known about the transcriptional regulation of GlyT genes. Analyses of their promoter regions have revealed the presence of sequence elements important for transcriptional regulation. However, the precise mechanisms that govern the expression of GlyTs during development and in the mature CNS are currently unknown.

It has been shown that GlyT2 expression is controlled by neuronal activity. In neurons from the rat dorsal cochlear nucleus, a controlled increase in synaptic activity by acoustic stimulation evoked a local increase in GlyT2

messenger (m)RNA levels (Barmack et al. 1999). The expression of GlyT1 is also dependent on the surrounding tissue. In primary cultures, glial GlyT1 expression is only induced in mixed cultures consisting of neurons and glia but not in pure glial cultures (Zafra et al. 1997). Upon cytotoxic killing of the neurons, a down-regulation of GlyT1 mRNA has been observed. This transcriptional down-regulation of the GlyT1 gene could be mediated by members of the HMGN nuclear factor family. The HMGN family comprises small, basic proteins that bind specifically to nucleosomes, thereby promoting chromatin unfolding and modulation of transcription. Two splice variants of one member of this family, HMGN3a and HMGN3b, bind to the GlyT1 gene in vivo, up-regulating its expression (West et al. 2004). Moreover, the HMGN3 genes are highly expressed in glia cells and retina, co-localizing with GlyT1. These results suggest that HMGN3s regulate GlyT1 expression. However, it is currently not known whether HMGN3a and HMGN3b are indiscriminate transcriptional regulators, designed to stimulate overall gene expression during a particularly active period such as differentiation, or whether they specifically target selected genes. Another transcriptional regulator known to increase GlyT1 expression is Trb-1, which is a neuron-specific T-box transcription factor. In cultured hippocampal neurons, overexpression of Trb-1 led to an increase in GlyT1 promotor-dependent luciferase expression (Wang et al. 2004). A Trb-1 binding motif, a non-palindromic so-called T element, is found in the 5′-flanking region of the GlyT1 gene. The in vivo role of this element remains to be elucidated.

4
Functional Roles of GlyTs

4.1
Generation of GlyT-Deficient Mice

GlyTs are supposed to have specialized functions at glycinergic inhibitory synapses. Since the localization of GlyT2 parallels the distribution of GlyRs (Jursky and Nelson 1995; Zafra et al. 1995), it was previously thought that GlyT2 was the main isoform mediating the clearance of presynaptically released glycine at glycinergic synapses. However, GlyT1 also might play a significant role in controlling the extracellular concentration of glycine, as suggested by its overlapping expression patterns with GlyT2 in caudal regions of the CNS (Zafra et al. 1995). Consistent with their locations, glial and neuronal glycine transporters might compete in the rapid re-uptake of synaptically released glycine at glycinergic synapses. Alternatively, the GlyTs could have different but complementary roles in controlling the extracellular levels of glycine. To address this issue, we have generated mouse lines lacking functional GlyT1 and GlyT2 and analysed the effects of GlyT inactivation on glycinergic inhibitory neurotransmission (Gomeza et al. 2003a; Gomeza et al. 2003b).

To generate mice lacking functional GlyT1 and GlyT2, we targeted both GlyT genes in mouse E14 (129/OLA) embryonic stem (ES) cells via homologous recombination (Gomeza et al. 2003a, b). The GlyT1 gene was inactivated by replacing its exon 3, which encodes the second transmembrane region of the GlyT1 protein (Adams et al. 1995), with a neomycin-resistance cassette. Likewise, the GlyT2 gene was inactivated by removing the exon encoding the fourth transmembrane domain of the transporter (Ebihara et al. 2004). Through standard transgenic and mouse breeding techniques, chimeric mice were obtained and used to generate F1 offspring heterozygous for both GlyTs. These animals appeared phenotypically normal and showed undisturbed development and fertility. Intercrossing of the heterozygous mice generated wild-type (+/+) as well as heterozygous (+/−) and homozygous (−/−) knockout (KO) mutant mice at the expected Mendelian ratios, excluding gross defects during embryonic development. The absence of the wild-type GlyT transcripts and proteins in the homozygous KO animals was confirmed by RT-PCR and Western blot analysis, respectively. Membrane fractions prepared from both forebrain and brainstem regions of GlyT1$^{-/-}$ mice exhibited significantly reduced [^{3}H]glycine uptake (Gomeza et al. 2003a). Similarly, GlyT2$^{-/-}$ mice displayed low glycine transport activity in brainstem and spinal cord, whereas no change was observed with membrane fractions prepared from frontal cortex (Gomeza et al. 2003b), where GlyT1 is the major transporter isoform expressed (Jursky and Nelson 1996). Hence, as expected from the normal distribution of both GlyT isoforms, glycine uptake was selectively impaired, consistent with a complete loss of the respective GlyT isoform in the different homozygous mutant mice.

4.2 GlyTs Are Essential for Vital Postnatal Functions

Both homozygous GlyT1$^{-/-}$ and GlyT2$^{-/-}$ mice appear grossly normal at birth. However, loss of either transporter severely shortens the lifespan of the animals. GlyT1$^{-/-}$ mice die on the day of birth, demonstrating that GlyT1 is dispensable for embryonic development but essential for postnatal survival. They fail to suckle, as is obvious from a lack of milk in their stomachs, and show an abnormal body posture with dropping forelimbs (Gomeza et al. 2003a). In addition, they display weak spontaneous motor activity in response to mild tactile stimuli and react poorly to intense pain stimuli. Plethysmographic recordings (Chatonnet et al. 2002) demonstrated that dysfunction of motor activity extends to the respiratory system. Breathing patterns and respiratory frequencies are severely depressed in GlyT1$^{-/-}$ newborn animals, resulting in long periods of apnoea interrupted by gasp-like inspirations instead of the regular breathing characteristic of wild-type animals (Gomeza et al. 2003a). In summary, GlyT1$^{-/-}$ newborn mice display motosensory deficits characterized by lethargy, hypotonia, hypo-responsivity and a severe respiratory deficiency.

GlyT2-deficient mice also show a lethal phenotype, although death occurs only during the second postnatal week and after developing an acute neuromotor disorder (Gomeza et al. 2003b). GlyT2 KO mice display a readily observable tremor in their limbs. Also, when suspended by the tail, they show an abnormal behaviour, clasping their hind feet and holding the front paws together. These are typical symptoms of muscular rigidity (Becker et al. 2000, Hartenstein et al. 1996). Finally, GlyT2$^{-/-}$ mice are unable to right themselves onto all four legs after being turned on their backs, indicating reduced motor co-ordination (Hartenstein et al. 1996). In conclusion, GlyT2-deficient mice display a lethal neuromotor disorder whose symptoms are entirely different from those seen upon GlyT1 deletion, and include tremor, muscular spasticity and impaired motor co-ordination.

The severe phenotypes observed in GlyT1 and GlyT2 KO mice cannot be attributed to anatomical malformation or insufficient synaptic differentiation of the CNS. GlyT KO mice show no histological defects in skeletal muscle or visceral organs. Similarly, no differences were found when examining specific regions of the CNS where GlyTs are expressed, such as the brainstem, spinal cord and forebrain. Western blot and immunohistochemistry analyses revealed no major changes in the expression level, distribution and density of proteins localized in glycinergic and glutamatergic synapses. Further analysis showed that absence of GlyT2 caused no change in GlyT1 expression in GlyT2 KO animals. Likewise, GlyT2 remained unaffected in homozygous GlyT1$^{-/-}$ animals. This demonstrates that ablation of GlyT genes does not cause major adaptive alterations in synapse biochemistry and excludes the possibility that loss of one specific transporter might lead to compensatory changes in the expression of the other GlyT isoform.

4.3
Essential Functions of GlyTs at Glycinergic Synapses

The neuromotor disorders observed in the homozygous KO mice suggested wide-ranging deficits of glycinergic neurotransmission in those animals. To examine the effects of GlyT gene deletion on glycinergic neurotransmission, electrophysiological recordings (Hulsmann et al. 2000) were performed from hypoglossal motoneurons in brainstem slices of GlyT mutant mice. Loss of either transporter induced abnormal glycinergic inhibitory currents in the KO animals. Neurons from GlyT1-deficient mice generated glycinergic spontaneous inhibitory postsynaptic currents (IPSCs) with increased frequencies and longer decay time constants than those in wild-type mice (Gomeza et al. 2003a). In addition, augmented tonic chloride conductances mediated by GlyRs were also recorded. These findings demonstrate hyperactive glycinergic signalling in GlyT1 KO mice and indicate that deletion of GlyT1 causes elevated extracellular glycine concentrations which result in a sustained activation of postsynaptic GlyRs. Furthermore, they suggest a fundamental role of GlyT1 in

lowering extracellular glycine levels at inhibitory glycinergic synapses. In contrast, GlyT2-deficient hypoglossal neurons displayed glycinergic IPSCs with markedly reduced amplitudes compared to those from wild-type cells, a finding that revealed reduced glycinergic activity in the KO animals (Gomeza et al. 2003b). As mentioned above, no postsynaptic changes in the distribution and localization of GlyRs were found in the mutant animals. This excludes the possibility that postsynaptic receptor currents are reduced due to malfunction or down-regulation of GlyRs and indicates that glycine release from glycinergic terminals is impaired in the GlyT2 KO mice. Apparently, loss of GlyT2 decreases the cytosolic glycine concentration in the presynaptic terminal, leading to inefficient synaptic vesicle refilling with glycine, and hence reduced transmitter

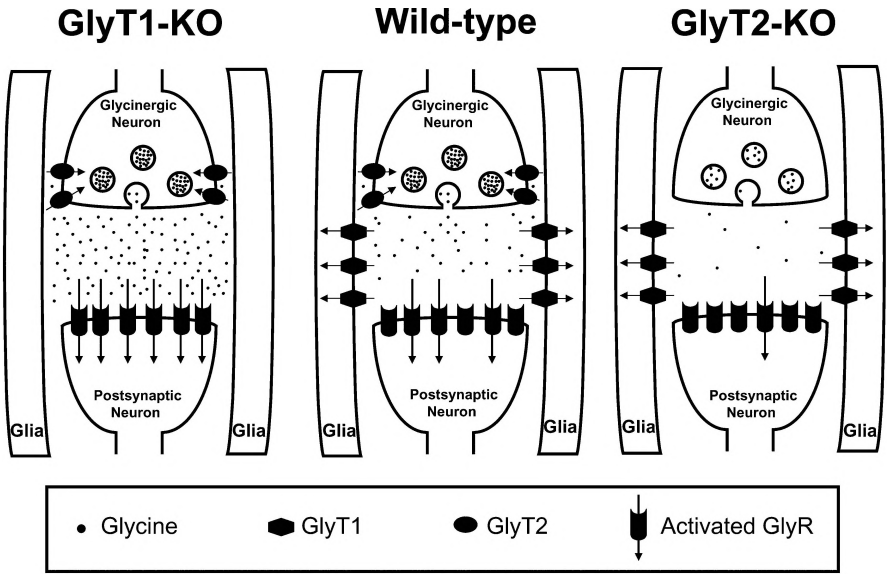

Fig. 3 Models of glycinergic synapse structure in GlyT knockout mice. The scheme depicts glycinergic synapses from GlyT1 knockout (*left*), wild-type (*centre*) and GlyT2 knockout (*right*) mice, respectively. The neuronal and glial location of GlyT2 and GlyT1 are indicated. In the wild-type (*centre*), GlyT2 is essential for glycine uptake into the nerve terminal cytosol, thereby replenishing the supply of presynaptic transmitter. Glycine released from the presynaptic terminal is removed from the synaptic cleft by GlyT1, which terminates glycinergic postsynaptic currents and maintains a low extracellular glycine concentration. In the GlyT1 KO mouse (*left*), the absence of GlyT1 leads to an increased glycine concentration in the synaptic cleft, which results in a sustained activation of postsynaptic GlyRs, and thus a potentiation of glycinergic inhibitory neurotransmission. In the GlyT2-KO mouse (*right*), deletion of GlyT2 results in an impairment of presynaptic vesicle loading with glycine. This leads to a low content of the neurotransmitter within synaptic vesicles. Hence, the amount of glycine released into the synaptic cleft upon stimulation is reduced compared with the wild-type synapse, which in turn results in diminished activation of GlyRs and reduced glycine-mediated neurotransmission

release upon presynaptic stimulation, which results in smaller postsynaptic currents. Together, these data show that GlyT2 does not play an important role in clearing glycine from the synaptic cleft of glycinergic synapses but is essential for glycine uptake into the presynaptic cytosol, and hence glycine recycling.

The perturbation of glycinergic neurotransmission found in GlyT-deficient mice explains the strong phenotype displayed by these animals. Slice recordings of neuronal activity in the GlyT1 KO brainstem circuitry responsible for generating the respiratory rhythm revealed a slowed and irregular pattern that was normalized upon application of the GlyR inhibitor strychnine. This indicates that the motosensory deficits caused by the deletion of GlyT1 are a result of glycinergic over-inhibition induced by the sustained activation of GlyRs in the presence of high levels of extracellular glycine. In contrast, loss of GlyT2 causes severe glycinergic under-inhibition that produces the 'spastic' phenotype observed in GlyT2 KO mice.

In summary, GlyT1 and GlyT2 have different roles at glycinergic synapses (Fig. 3): GlyT1 eliminates glycine from the synaptic cleft, thus terminating glycine neurotransmission and maintaining a low extracellular glycine concentration throughout the caudal regions of the CNS. This prevents excessive tonic activation of GlyRs. On the other hand, GlyT2 is required for replenishing the pool of cytosolic glycine from which vesicles are filled for release. Thus, both GlyTs have complementary functions at glycinergic synapses, which fine-tune the efficacy of glycinergic inhibition in the mammalian CNS.

4.4
Pharmacology of GlyTs

Important functions of different neurotransmitter transporters have been disclosed by pharmacological approaches, and the identification of potent transport inhibitors has contributed essentially to our recent understanding of their in vivo functions. Furthermore, transporters are known to constitute important drug targets. Widely used drugs, which act on neurotransmitter transporters, include the tricyclic antidepressants, which block transporters specific for serotonin and norepinephrine (Blakely et al. 1994), and anticonvulsants such as tiagabine, which selectively inhibits the neuronal GABA transporter GAT1 (Ashton and Young 2003).

Also in case of the GlyTs, the initial hypothesis that two different transport systems exist emerged from differences in susceptibility to *N*-methyl-glycine (sarcosine). Sarcosine competitively inhibits GlyT1, but not GlyT2 (Lopez-Corcuera et al. 1998). Electrophysiological and radioactive tracer experiments revealed that this glycine analogue acts as a substrate, thereby competing with glycine for GlyT1 binding. Further substitutions at the amino group of sarcosine with lipophilic heterocycles has led to several novel high-affinity blockers of GlyT1, like ALX-5407, its stereoisomer (*R*)-*N*-[3-(4′-fluorophenyl)-3-(4′-

phenylphenoxy)propyl]sarcosine ((*R*)-NFPS) (Aubrey and Vandenberg 2001), (3-(4-chloro-phenyl)-3[4-(thiazole-2-carbonyl)-phenoxy]-propyl-methyl-amino)-acetic acid) (CP 802,079) (Martina et al. 2004) and (*R*,*S*)-(±)-*N*-methyl-*N*-[(4-trifluoromethyl)phenoxy]-3-phenyl-propyl-glycine (Org-24461) (Harsing et al. 2003; Fig. 4). Although the mode of action of these compounds is not fully understood, these inhibitors have been shown to act as noncompetitive inhibitors, which do not change the accessibility of the Na^+- and Cl^--binding sites (Mallorga et al. 2003). Moreover, these substances appear to be very potent, as glycine uptake by GlyT1 expressed in heterologous systems or in synaptosomal preparations was irreversibly inhibited for up to 1 h.

Initially reported inhibitors of GlyT2 comprise the antidepressant amoxapine and several alkanols, including ethanol (Nunez et al. 2000a; Nunez et al. 2000b). Due to their low affinity and selectivity for GlyT2, these compound are not suitable for pharmacological studies. The first inhibitors of high-affinity and specificity for GlyT2 were generated recently. To date, several organic compounds, including 4-benzyloxy-3,5-dimethoxy-*N*-[(1-di-methylaminoacyclopentyl)methyl]-benzamide (Ho et al. 2004), a series of 5,5-diaryl-2-amino-4-pentenoates (Isaac et al. 2001) and 4-benzyloxy-3,5-dimethoxy-*N*[(1-dimethyl-aminocyclopentyl)methyl]benzamide (Org-25543) (Caulfield et al. 2001), have been found to potently inhibit GlyT2 without affecting the activity of GlyT1, GlyR or NMDAR (Fig. 4). All these inhibitors constitute new tools for the more detailed in vivo and in vitro analysis of GlyT functions.

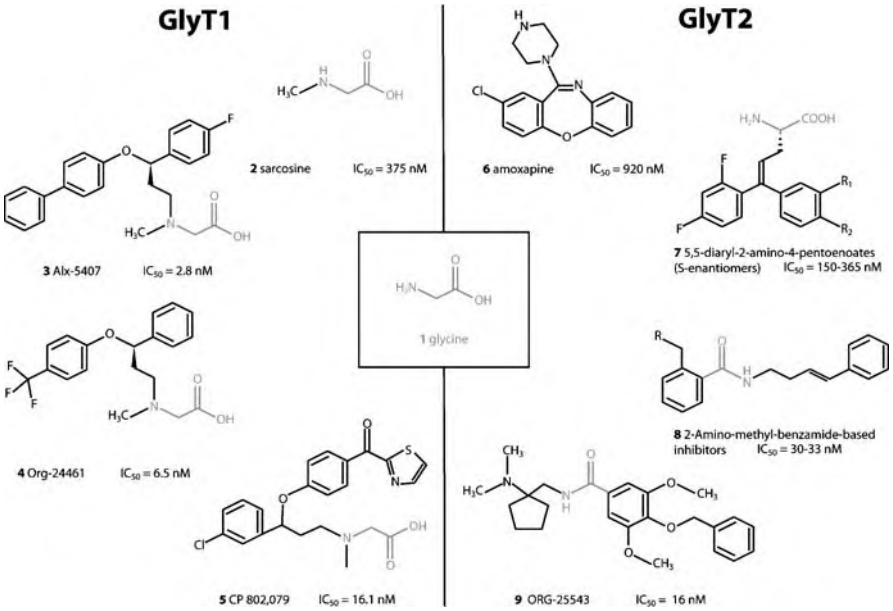

Fig. 4 Inhibitors of GlyT1 and GlyT2. For details, see text

4.5
Function of GlyTs at Glutamatergic Synapses

4.5.1
Pharmacological Studies In Vitro

As described above, glycine not only constitutes a major inhibitory neurotransmitter but is also essential for glutamatergic neurotransmission by serving as a co-agonist of the NMDAR. It is now widely accepted that the glycine concentration at glutamatergic synapses is close to the K_m of the glycine binding site of the NMDARs, thereby allowing for modulation of NMDAR activity by small changes in the ambient glycine concentration. Since glycine is not co-released with glutamate from synaptic vesicles, the source of glycine at excitatory synapses is at present unclear. In brain areas which are rich in glycinergic synapses, i.e. brainstem and spinal cord, glycine might reach NMDARs through spill-over from highly active neighbouring glycinergic synapses (Ahmadi et al. 2003). In these locations, insufficient clearance of glycine from the synaptic cleft (or small distances between excitatory and inhibitory synapses) might allow for diffusion of the neurotransmitter to adjacent glutamatergic synapses. However, such mechanisms do not seem to play a role in higher brain regions lacking strong glycinergic neurotransmission, where GlyTs might then have a pivotal role as regulators of glutamatergic neurotransmission by modulating synaptic glycine concentration.

Supporting evidence for this hypothesis came from studies using the GlyT-specific inhibitors Org-24958 and Org-25543, which specifically block GlyT1 and GlyT2, respectively (Bradaia et al. 2004). Application of these compounds led to a facilitation of NMDAR activity in spinal cord slice preparations. Comparable results were obtained in recordings performed on forebrain neurons (i.e. cortex or hippocampus), where the application of low doses of the GlyT1 inhibitor CP 802,079 caused a strong facilitation of the NMDAR component of glutamatergic neurotransmission (Martina et al. 2004). This included some forms of synaptic plasticity such as long-term potentiation (LTP), which has been shown to be NMDAR-dependent. Strikingly, the application of high concentrations of GlyT1 inhibitors or glycine only produces a transient facilitation of the NMDAR currents. At later time points, however, this effect is inversed (Martina et al. 2004). This has been attributed to priming of NMDARs for internalization and their subsequent removal from the cell membrane by clathrin-mediated endocytosis (Nong et al. 2003). In summary, these findings support the assumption that GlyT1 controls extracellular glycine levels at glutamatergic synapses, resulting in modulatory effects on NMDAR function.

4.5.2 Pharmacology and Genetics In Vivo

Acute in vivo application by reverse dialysis or systemic application of GlyT1 inhibitors like sarcosine, NFPS or ALX5407 has been shown to cause a significant increase of the glycine concentration in the cerebrospinal fluid, whereas the concentration of other amino acids was not changed (Martina et al. 2004; Whitehead et al. 2004). Electrophysiological recordings showed, comparable to the in vitro experiments, increased NMDAR-mediated glutamatergic currents (Lim et al. 2004). Interestingly, the concentration of citrulline was also remarkably increased upon the application of GlyT1 inhibitors (Whitehead et al. 2004). At synapses, citrulline can be generated as a stoichiometric by-product of NO synthesis by neuronal NO synthase. The activity of this enzyme has been shown to be dependent on NMDAR-mediated Ca^{2+}-influx (Brenman and Bredt 1997); the elevated levels of citrulline thus provide further evidence for an increased NMDAR activity, resulting from GlyT1 inhibition.

Subsequent studies of heterozygous GlyT1-deficient animals also indicate a role of GlyT1 in NMDAR regulation. Membrane preparations from $GlyT1^{+/-}$ animals display approximately 50% reduced [^{3}H]glycine uptake activity compared to wild-type samples (Gabernet et al. 2005; Gomeza et al. 2003a; Tsai et al. 2004). Thus, the loss of one functional GlyT1 allele is not compensated by up-regulation of the second allele. In contrast to what is seen in wild-type animals, the glycine binding site of NMDARs seems to be saturated in $GlyT1^{+/-}$ mice, because application of exogenous glycine or D-serine did not lead to a facilitation of NMDAR-mediated currents (Gabernet et al. 2005; Tsai et al. 2004). The constitutive saturation of the NMDAR glycine binding site results in an increase in the ratio of NMDAR/AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor)-mediated currents. However, whether this effect is due to a stronger NMDAR response or a reduction of the AMPAR component of glutamatergic neurotransmission is an unsolved issue.

Kinetic analysis of spontaneous and evoked synaptic events in $GlyT1^{+/-}$ animals revealed faster deactivation time constants of the NMDAR currents compared with their wild-type litters (Martina et al. 2005), suggesting a different subunit composition of NMDARs. This hypothesis received support from an altered susceptibility to the NMDAR modulators ifenprodil and zinc, which inhibit NMDARs containing NR2B or NR2A subunits, respectively. Together, these results demonstrate that constitutive saturation of the glycine binding site of the NMDAR leads to compensatory changes at glutamatergic synapses.

Although heterozygous GlyT1-deficient animals do not display any obvious phenotype (Gabernet et al. 2005; Gomeza et al. 2003a; Tsai et al. 2004), the behavioural analysis of $GlyT1^{+/-}$ animals revealed additional differences when compared with wild-type litters. Assessment of spatial orientation in the Morris water maze revealed a better performance of the mutant mice (Tsai et al. 2004). Additionally prepulse inhibition (PPI), the reduction of the sound-

induced startle response by pre-exposition to a milder acoustic stimulus, was tested. Although PPI was not changed in GlyT$^{+/-}$ animals, Tsai et al. demonstrated changes in the pharmacology of this behavioural paradigm (Tsai et al. 2004). In GlyT1$^{+/-}$ animals, PPI was partially resistant against disruption with amphetamines, but displayed an increased susceptibility for the NMDAR open channel blocker MK801. In addition, a recent study has reported that in mice the application of the GlyT1 inhibitor ALX 5407 caused different effects depending on dosage (Lipina et al. 2005). A low concentration of the inhibitor was able to restore the disruption of PPI induced by subsaturating concentrations of MK801, thus mimicking the results obtained with mice displaying reduced GlyT1 activity. The application of high concentrations, however, resulted in a reduction of PPI. Comparable results were obtained in electrophysiological records in which high concentrations of the GlyT1 inhibitors reduced the NMDAR current amplitude (Martina et al. 2004). This is consistent with a removal of NMDARs from synaptic sites. Taken together, these data further underline the importance of GlyT1 in glutamatergic neurotransmission. Here, lowering of the extracellular glycine concentration by GlyT1 does not only allow a glycine-mediated potentiation of NMDAR activity, but additionally prevents NMDARs from internalization.

5
GlyTs and Human Diseases

5.1
GlyT Genes: Candidate Disease Loci?

The symptoms observed in GlyT1- and GlyT2-deficient mice are similar to those associated with human hereditary diseases which develop in early postnatal life or during adolescence. Hyperglycinergic GlyT1 KO mice present symptoms similar to glycine encephalopathy or non-ketotic hyperglycinaemia (NKH) (Applegarth and Toone 2001). This disorder is characterized by muscular hypotonia, lethargy and poor feeding with hiccups (Boneh et al. 1996; Tada et al. 1992). NKH can rapidly progress to a lethal symptomatology associated with respiratory insufficiency, apnoea, coma and death. Often patients with NKH have a primary defect in the glycine cleavage system (GCS) (Applegarth and Toone 2001). The GCS consists of four protein components (named as P-, H-, T- and L-proteins) (Sakata et al. 2001), which are located in the inner mitochondrial membrane and catalyse the degradation of intracellular glycine. As a consequence, the affected individuals accumulate elevated levels of glycine in the blood and, particularly, in cerebrospinal fluid. The high glycine concentration is supposed to cause potent activation of GlyRs, thus leading to symptoms of glycinergic over-inhibition. On the other hand, hypoglycinergic GlyT2 KO animals resemble patients suffering from hyperekplexia (Becker et al. 2000;

Zhou et al. 2002). This neuromotor disorder is characterized by exaggerated startle responses and, in severe cases, a 'stiff baby syndrome'. Some dominant and recessive forms of hyperekplexia are known to be associated with mutations in the GlyR α1- and β-subunit genes that impair postsynaptic GlyR function (Laube et al. 2002; Lynch 2004) and cause a phenotype characterized by spasticity, muscular rigidity and tremor.

Although neither of the human GlyT genes has been linked to a disease phenotype, it is noteworthy that about 50% of the patients diagnosed with NKH or hyperekplexia carry no mutations in GCS or GlyR genes (Applegarth and Toone 2001; Vergouwe et al. 1997). This indicates that glycine encephalopathy- and hyperekplexia-like syndromes may be caused through other genetic mechanisms, and it is consistent with the idea that mutations in the human GlyT gene might cause such hereditary neurological disorders. If so, GlyT-deficient mice should constitute valuable animal models to analyse the underlying pathomechanisms.

5.2 GlyTs as Potential Drug Targets

The facilitation of the NMDAR component of glutamatergic neurotransmission upon acute application of GlyT1 inhibitors identified this transporter as a prime pharmacological target for diseases thought to be associated with NMDAR hypofunction, such as schizophrenia (Jentsch and Roth 1999). This widespread psychiatric disorder is characterized by hyperactivity, cognitive deficits and stereotyped behaviour. Partial NMDAR inhibitors like ketamine or phenylcyclidine (PCP) are known to induce some of the symptomatology characteristic for schizophrenia in healthy humans (Jentsch and Roth 1999). In rodents, these compounds cause hyperactivity and stereotyped behaviour, as well as impaired spatial orientation. Therefore, ketamine- and PCP-treated animals are thought to constitute useful pharmacological models of schizophrenia. Notably, in these animals both the application of glycine and the (partial) inhibition of GlyT1 by its antagonists have positive effects on most symptoms (Javitt 2002; Javitt et al. 2003; Kinney et al. 2003; Sur and Kinney 2004). These animal studies have recently been extended to clinical trials by using a diet containing a high glycine content or the GlyT1 substrate analogue sarcosine (Javitt 2002). Both strategies, in combination with classical medication, seem to have a beneficial effect on the symptoms in human schizophrenic patients.

The use of high-affinity GlyT1 inhibitors like NFPS (*N*-[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl]sarcosine) as therapeutic compounds is controversial. Up to now, possible side-effects have prevented clinical trials. First, a strong increase in the extracellular glycine concentration might lead to a facilitation of glycinergic neurotransmission, which could result in severe side-effects such as respiratory suppression. Second, increased glycine concentrations at glutamatergic synapses might result in a persistent saturation

of the glycine binding site of NMDAR. In heterozygous GlyT1-deficient mice, which seem to be a good genetic model for the long-term application of GlyT1 inhibitors, the chronic saturation of the glycine binding site of the NMDAR apparently led to adaptive changes in the electrophysiological properties of glutamatergic synapses (Martina et al. 2005). Although GlyT1$^{+/-}$ animals do not display any obvious phenotypic changes (Gabernet et al. 2005; Gomeza et al. 2003a; Tsai et al. 2004), this does not exclude negative effects in humans. Furthermore, in rodents the application of high concentrations of GlyT1 inhibitors induces hyperactivity and strong stereotyped behaviour in vivo, symptoms that are also seen after the administration of partial NMDAR inhibitors (Lipina et al. 2005). Together with studies showing a reduction in NMDAR amplitude upon GlyT1 inhibition (Martina et al. 2004), these data suggest that a strong inhibition of GlyT1 could result in a down-regulation of NMDARs. Thus, overmedication with GlyT1 inhibitors might boost the disease phenotype.

Data obtained in GlyT1-deficient mice, as well as pharmacological studies, indicate that GlyT1 inhibitors also have the potential to increase the efficacy of inhibitory neurotransmission (Bradaia et al. 2004; Gomeza et al. 2003a). This increased inhibitory tone in spinal circuitries should reduce motoneuron activity and decrease pain perception. Therefore, the local application of GlyT1 inhibitors in spinal cord might be useful for muscle relaxation and analgesia during narcosis. Whether a partial inhibition of GlyT2, which transiently may also increase the activity of postsynaptic GlyRs (Bradaia et al. 2004), could also be beneficial is not yet clear.

6
Conclusions and Perspectives

In summary, use of newly developed antagonists and the generation of mouse models have greatly improved our understanding of the precise functions of GlyTs in the CNS. At inhibitory glycinergic synapses, GlyT1 is essential for the rapid removal of the neurotransmitter from the synaptic cleft, thereby terminating neurotransmission via GlyRs. Inhibition or the loss of GlyT1 leads to an accumulation of glycine in the synaptic cleft, resulting in overinhibition. Furthermore, GlyT1 prevents the saturation of the glycine binding site of NMDARs, thereby enabling glycine potentiation of glutamatergic neurotransmission. Inactivation of a single GlyT1 allele leads to full saturation of the NMDAR glycine binding site, which then results in major changes in the physiology of glutamatergic synapses. This modulatory function of GlyT1 makes it a highly promising drug target in psychiatric diseases such as schizophrenia, for which no effective medication is currently available. However, the possible side-effects, which may arise from the dual functions of GlyT1 at inhibitory and excitatory synapses, as well as its functions in non-neuronal tissues, will have to be examined carefully. In contrast, the major function of GlyT2 seems

the reuptake of neurotransmitter into the inhibitory presynaptic terminals to allow efficient refilling of synaptic vesicles with glycine. The loss of GlyT2 activity results in a strong reduction of glycinergic inhibition and causes a severe hyperekplexia phenotype.

An area of research that will have to be extended in future studies concerns the potential role of GlyT1-mediated glycine release from astrocytes or even neurons. Such studies are essential for further understanding of the functional role of these transporters. Furthermore, although the analysis of GlyT-deficient mice has already provided important details on GlyT functions, more sophisticated genetic models will be required to dissect the variant- and region-specific tasks of GlyTs. These approaches need to overcome the early lethality of GlyT deficiency, a prerequisite for studying GlyT functions in the adult nervous system.

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