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# Structure, function and regulation of glycine neurotransporters

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**Abstract**

Glycine exerts multiple functions in the central nervous system, as an inhibitory neurotransmitter through activation of specific, $Cl^-$-permeable, ligand-gated ionotropic receptors and as an obligatory co-agonist with glutamate on the activation of *N*-methyl-D-aspartate (NMDA) receptors. In some areas of the central nervous system, glycine seems to be co-released with γ-aminobutyric acid (GABA), the main inhibitory amino acid neurotransmitter. The synaptic action of glycine ends by active recapture through sodium- and chloride-coupled glycine transporters located in glial and neuronal plasma membranes, whose structure–function relationship is being studied. The trafficking and plasma membrane expressions of these proteins are controlled by regulatory mechanisms. Glycine transporter inhibitors may find application in the treatment of muscle tone defects, epilepsy, schizophrenia, pain and neurodegenerative disorders. This review deals on recent progress on localization, transport mechanisms, structure, regulation and pharmacology of the glycine transporters (GLYTs).


*Keywords:* Glycine transport; Neurotransmission; GLYT1 (glycine transporter 1); GLYT2 (glycine transporter 2); NMDA receptor; Central nervous system

## 1. Introduction

Glycine is one of the major inhibitory neurotransmitters in posterior areas of the vertebrate central nervous system (CNS). In the spinal cord and brain stem, glycinergic interneurones provide an inhibitory feedback mechanism that controls the motor rhythm generation during movement and they also play an important role in the coordination of spinal reflex activity (see Grillner et al., 1998; Legendre, 2001 for a review). Glycine is also an important neurotransmitter in the processing of auditive information through cochlear nuclei, the superior oliva complex and the inferior colliculus (Wenthold and Hunter, 1990), and in the processing of visual information in retinal ganglion cells (Han et al., 1997; Protti et al., 1997). Glycinergic synaptic transmission has also been detected in Golgi cells of the cerebellum (Dieudonne, 1995).

The glycine-mediated neurotransmission involves storage of the transmitter in synaptic vesicles (Chaudhry et al., 1998; Dumoulin et al., 1999; Sagné et al., 1997), transmitter release following neuron depolarization (Mulder and Snyder, 1974) and glycine binding to, and activation of, specific, $Cl^-$ permeable, ligand-gated ionotropic receptors on the postsynaptic neuron (Fig. 1). The activation of receptor generates inhibitory postsynaptic potentials as a result of increasing $Cl^-$ conductance that are antagonized by strychnine (Werman et al., 1967). Functional glycinergic transmission appears early in brain development, and activation of glycine and γ-aminobutyric acid (GABA) receptors depolarizes neurons during the last prenatal period and early postnatal days (Cherubini et al., 1991; Flint et al., 1998; Nishimaru et al., 1996; Reichling et al., 1994; Singer et al., 1998; Singer and Berger, 2000). Depolarizing responses induced by inhibitory neurotransmitters are due to a high intracellular $[Cl^-]$ in neonatal neurons. The developmental shift from depolarizing to hyperpolarizing responses occurs when the expression of the neuronal $K^+/Cl^-$ cotransporter KCC2 takes place (about postnatal day 10) producing a decrease of intracellular $[Cl^-]$. (Ehrlich et al., 1999; Hubner et al., 2001; Krupp et al., 1994; Rivera et al., 1999). The depolarization induced by glycine and GABA in the first stages of development provokes $Ca^{2+}$ entry through the voltage-gated $Ca^{2+}$ channels that may be an important signal for maduration of inhibitory synapses (Flint et al., 1998; Kneussel and Betz, 2000; Owens et al., 1996; Reichling et al., 1994; Wang et al., 1994). In addition, glycine released by neurons may serve as a signal between neurons and oligodendrocyte progenitor cells during development (Belachew et al., 2000).

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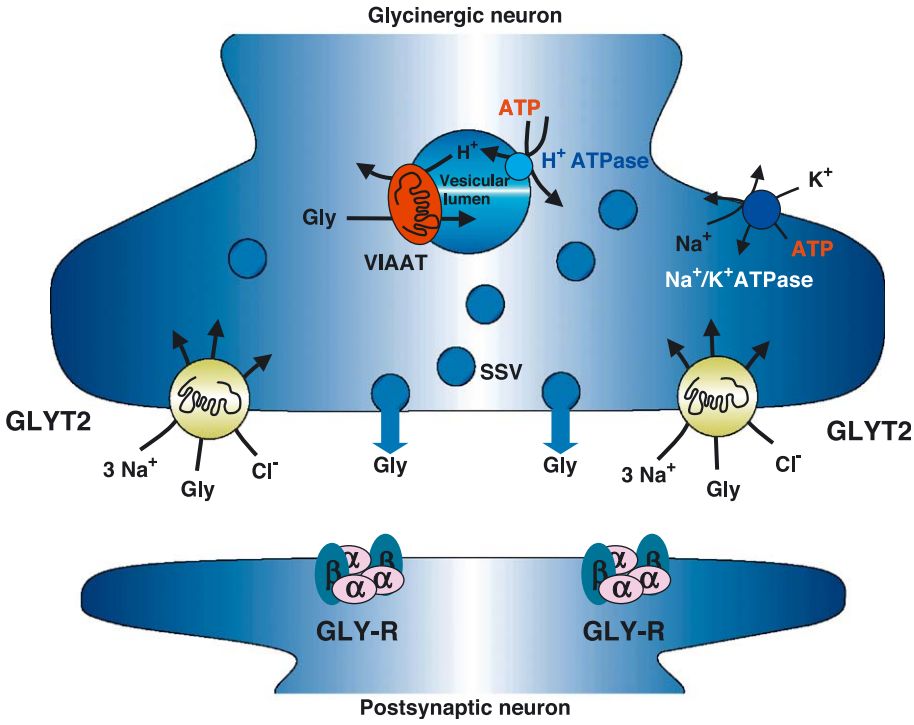


Fig. 1. Schematic representation of a glycinergic inhibitory synapse. VIAAT: vesicular inhibitory amino acid transporter; SSV: small synaptic vesicles; GLYT2: glycine transporter two; GLY-R: strychnine-sensitive glycine receptor.

Glycine and GABA seem to share a common vesicular inhibitory amino acid transporter (VIAAT/VGAT) as suggests its localization to synaptic vesicles in glycinergic terminals (Chaudhry et al., 1998; Dumoulin et al., 1999; Legendre, 2001). Mechanistic studies have proven that vesicular GABA/glycine transport depends on both the $\Delta\Psi$ and the $\Delta$pH of the $\Delta\mu_H^+$ (Fig. 1). This protein transports unselectively GABA or glycine towards the vesicle lumen, and shows a lower affinity for the latter (McIntire et al., 1997; Sagné et al., 1997). Although most GABAergic and glycinergic terminals contain VIAAT, subpopulations of nerve endings with high levels of GABA or glycine appear to lack the vesicular carrier, suggesting either that additional transporters may exist or that alternative modes of release can take place (Chaudhry et al., 1998). In addition, a considerable number of synapses in the CNS co-release glycine and GABA by the same presynaptic inhibitory terminal, as demonstrated in spinal cord (Jonas et al., 1998; Keller et al., 2001), brain stem motoneurons (Jonas et al., 1998; Russier et al., 2002), superior olivary complex (Smith et al., 2000) and cerebellar Golgi cells (Dumoulin et al., 2001).

Besides its inhibitory action, glycine exerts a positive modulation on excitatory glutamatergic neurotransmission through *N*-methyl-D-aspartate (NMDA) receptors (Johnson and Ascher, 1987). NMDA receptors are involved in neural development, learning, memory, synaptic plasticity, and neurodegeneration (Cull-Candy et al., 2001; Lipton and Rosenberg, 1994). Glycine behaves as a high-affinity obligatory co-agonist with glutamate on the activation of these heteromeric receptors formed by association of two types of subunits: NR1, where the glycine binding site resides, and any of the four NR2 subunits (A–D) that modulate the affinity of the former binding site (Cull-Candy et al., 2001; Danysz and Parsons, 1998; Nakanishi, 1992). Glycine binding at its NMDA receptor site interacts allosterically with other sites in the receptor increasing the binding of glutamate, and reducing the binding of antagonists to the glutamate-specific site. Conversely, polyamines and glutamate itself increase the glycine site affinity (Leeson and Iversen, 2001). At present, the identity of the endogenous ligand of the co-agonist site on NMDA receptors is controversial and recent evidence suggest that glycine and D-serine compete for the same binding site (Mothet et al., 2000). Nong et al. have reported that the stimulation of the glycine site by glycine or its agonist D-serine, initiates signalling through the NMDA complex, priming the receptor to be endocytosed by a clathrin-dependent mechanism. To provoke endocytosis of the receptor, binding of glycine and glutamate are required. These results demonstrate a transmembrane signal transduction upon activation of the glycine site of NMDA receptors (Nong et al., 2003). But, how can the physiological role of glycine in the regulation of NMDA receptors be achieved if a co-release of glycine and glutamate at glutamatergic synapses has not been proven? One possibility is glycine being released through the reverse operation of the plasma membrane glycine transporters (GLYTs).

The synaptic action of glycine ends by recapture of neurotransmitter by specific high-affinity transporters located in neuronal and glial plasma membranes (Neal and

Pickles, 1969). Glycine reuptake is an active process coupled to the electrochemical gradient of $Na^+$ that controls the level of neurotransmitter in the synaptic cleft (Kuhar and Zarbin, 1978). Furthermore, when the synaptic terminal depolarizes and the intracellular [$Na^+$] increases, the glycine transporter can work in the opposite direction (Aragón et al., 1988), pumping neurotransmitter out of the cell through a $Ca^{2+}$-independent mechanism of release (Adam-Vizi, 1992; Attwell et al., 1993; Chaudhry et al., 1998). Thus, the uptake/release balance could finally modulate the NMDA receptor activity. For this kind of regulation to take place, plasma membrane transporters must be able to keep the glycine concentration in the synaptic cleft below saturating levels for the NMDA receptor. This has been demonstrated in hippocampal slices (Bergeron et al., 1998), brain stem hypoglossal motoneurons (Berger et al., 1998), prefrontal cortex slices (Chen et al., 2003) and in a co-expression system in *Xenopus* oocytes (Supplisson and Bergman, 1997). Recently, an excitatory role of glycine, by itself, through a type of excitatory glycine receptor, has been described. This excitatory receptor contains a third kind of NMDA receptor subunit, named NR3A and NR3B, that has been cloned by Chatterton et al. Oocytes co-injected with cRNAs coding for NR3B and NR1 express novel functional NMDA receptors that show non-conventional (that is, NR1/NR2) unique properties. Glycine fully activates NR1/NR3 receptors without glutamate or NMDA. Moreover, NR1/NR3 receptors form relatively $Ca^{2+}$-impermeable cation channels permeable to small cations such as $Na^+$, which are resistant to $Mg^{2+}$ and inhibited by D-serine. In the same study, action potentials evoked by glycine were recorded in cultured cerebrocortical neurons containing NR3 family members. A burst of firing was observed in the presence of glycine, which was suppressed by D-serine (Chatterton et al., 2002).

As a conclusion of the multiple functions that glycine exerts in the CNS is the possibility to develop compounds able to potentiate the glycine-mediated actions in pathological situations such as altered muscle tone regulation in spasticity or hypofunction of NMDA receptors associated to schizophrenia (Krystal and D'Souza, 1998; Floeter and Hallett, 1993; Simpson et al., 1995; Tsai et al., 1998). Plasma membrane transporters of glycine are now considered targets of future therapeutic drugs.

## 2. Biochemical studies and molecular characterization of glycine transporters

Early investigations identified high-affinity active transport systems for glycine ($K_m$ 20–100 μM) in nerve terminals and glial cells by using brain-derived preparations (Fedele and Foster, 1992; Johnston and Iversen, 1971; Kuhar and Zarbin, 1978; Logan and Snyder, 1972; Mayor et al., 1981; Zafra and Giménez, 1986). These studies provided knowledge about the $Na^+$ and $Cl^-$ dependence, stoichiometry, electrogenicity, and pharmacology of the glycine transport systems (Aragón et al., 1987; Zafra and Giménez, 1988, 1989). A glycine transporter from pig brain stem was characterized in 1991 after solubilization, reconstitution and purification to apparent homogeneity by our group (Alcántara et al., 1991; López-Corcuera and Aragón, 1989; López-Corcuera et al., 1989, 1991, 1993). These studies provided the first direct evidence of the existence of multiple $Na^+$- and $Cl^-$-dependent subtypes differentially inhibited by sarcosine (*N*-methylglycine) (López-Corcuera et al., 1991). The isolated subtype was subsequently proven to be GLYT2. About 30% of their 100-kDa apparent molecular mass consists of sugar chains that are predominantly tri- or tetra-antennary complex N-linked oligosaccharides containing sialic acid residues (Aragón and López-Corcuera, 1998; Núñez and Aragón, 1994).

Two genes encoding glycine transporters, GLYT1 and GLYT2, have been cloned (Fig. 2) (Guastella et al., 1992; Liu et al., 1992b, 1993a; Smith et al., 1992) taking advantage of their sequence similarity with the GABA transporter GAT1, the first neurotransmitter transporter cloned from the purified neuronal protein (Guastella et al., 1990). GLYT1 and GLYT2 show different sensitivity to sarcosine, a substrate of GLYT1 (Supplisson and Bergman, 1997) that does not interact with GLYT2. Both transporters belong to the family of $Na^+$- and $Cl^-$-dependent neurotransmitter transporter proteins (Amara and Kuhar, 1993; Kanai et al., 1993; Liu et al., 1992a, 1993b,c; López-Corcuera et al., 1992; Malandro and Kilberg, 1996; Pacholczyk et al., 1991; Uhl and Hartig, 1992) that contains transporters for GABA, glycine, proline, monoamines and several "orphan" transporters among others (see Nelson, 1998; Torres et al., 2003b for a review).

GLYT1 has been cloned from different sources. The human GLYT1 gene has been localized to chromosome 1p31.3 and the mouse gene to the murine chromosome 4 (Kim et al., 1994). Five GLYT1 variants (a,b,c,e,f) have been identified (Adams et al., 1995; Borowsky et al., 1993; Borowsky and Hoffman, 1998; Hanley et al., 2000; Kim et al., 1994; Liu et al., 1993a) that are built by combinations of N- and C-terminal exons. Variants containing exon e, coding for a C-tail, are only found in retina and interact with the ρ1 subunit of the GABA receptor c (Hanley et al., 2000). A different gene codes for GLYT2 and has been also isolated from different species (Gallagher et al., 1999; Liu et al., 1993a; Morrow et al., 1998). The human GLYT2 gene has been mapped to chromosome11p15.1–15.2 (Morrow et al., 1998). Two splice variants, GLYT2a and GLYT2b, with five amino acids different N-termini, have been identified for the rat brain DNA (Ponce et al., 1998).

As deduced by hydrophobicity analysis of their amino acid sequence, the family of glycine transporters contains polytopic membrane proteins with 12 transmembrane domains and amino and carboxyl-terminal ends intracellularly oriented (Olivares et al., 1994). Data from different groups have confirmed this topological model (Androutsellis-Theotokis and Rudnick, 2002; Bruss et al., 1995; Chen et

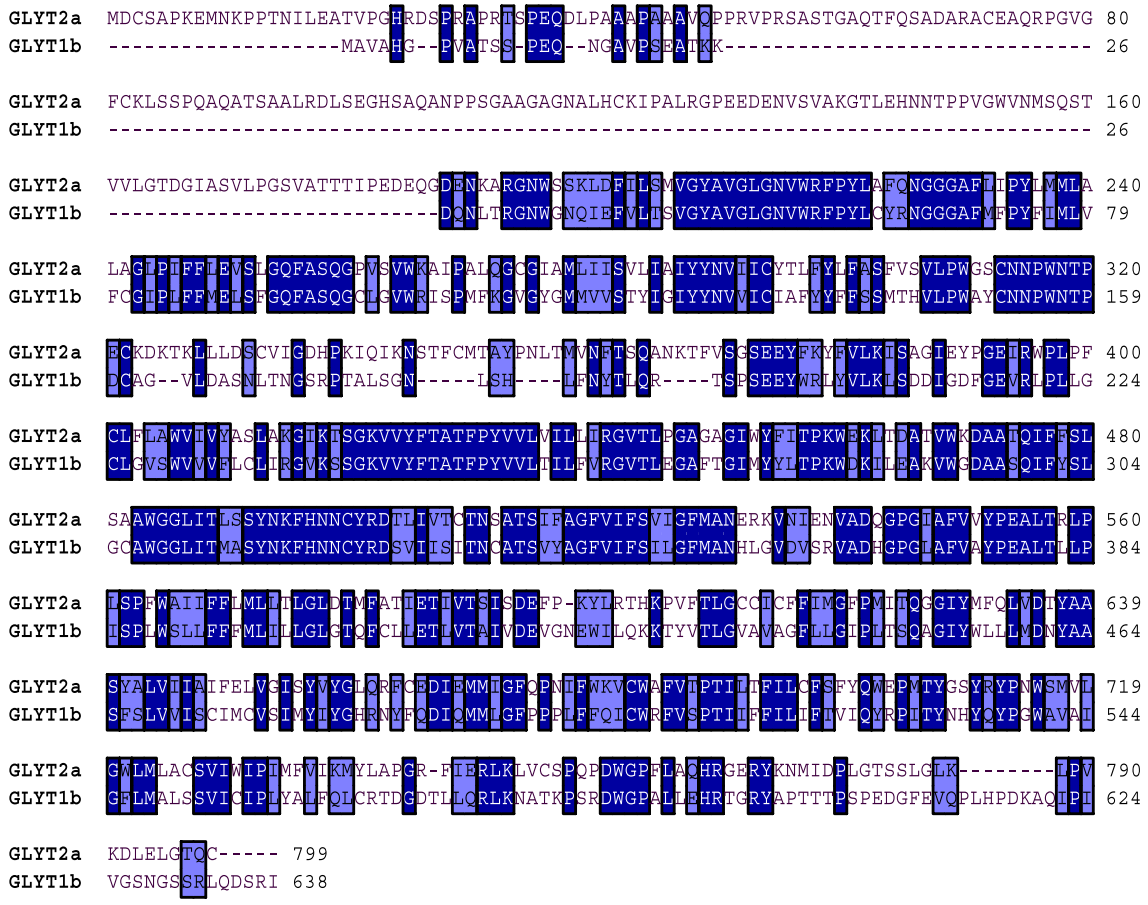


Fig. 2. Alignment of the amino acid sequences of GLYT1b and GLYT2a, the major glycine transporter isoforms in the CNS. Identical residues are shown as white letters on dark blue boxes. Conservative substitutions are indicated as light blue boxes containing black letters.

al., 1998; Hersch et al., 1997). The most conserved amino acid sequences correspond to the transmembrane domains and the lowest homology resides at the amino and carboxyl termini. The large extracellular loop connecting transmembrane domains 3 and 4 has four *N*-glycosylation sites in the glycine transporters. GLYT1 and GLYT2 share 48% and 50% amino acid sequence identity with the proline transporter, respectively. Together with the recently cloned transporters for cationic ($ATB^{0+}$) (Sloan and Mager, 1999) and neutral amino acids (KAAT1) (Castagna et al., 1998), these proteins have been grouped in the subfamily of amino acid transporters within the $Na^+$- and $Cl^-$-coupled transporter family.

The functional monomeric nature of glycine transporters has been investigated by two different experimental approaches. By a combination of size-exclusion chromatography and $H_2O/D_2O$ sucrose density gradient sedimentation analysis, the hydrodynamic properties of the purified native GLYT2 were compatible with a functional 85–100 kDa protein suggesting that, in its native state, the transporter is active as a monomeric protein (López-Corcuera et al., 1993). These results are in good agreement with a recent work performed with heterologously expressed GLYT1 and GLYT2 in *Xenopus* oocytes. Upon blue native gel electrophoresis and crosslinker glutaraldehyde treatment, the surface-localized transporters seem to exist only in complex-glycosylated monomeric form (Horiuchi et al., 2001). However, since an oligomeric structure has been proven for related transporters, this issue perhaps deserves further attention (Hastrup et al., 2001; Kilic and Rudnick, 2000; Schmid et al., 2001; Torres et al., 2003a).

## 3. Localization of glycine transporters

The cellular and tissular expression of glycine transporters is parallel to the complex glycine activity described before. Several studies using in situ hybridization and immunocytochemistry techniques have identified the populations of cells that express GLYT mRNA and protein, respectively (Adams et al., 1995; Borowsky et al., 1993; Guastella et al., 1992; Jursky and Nelson, 1995; Luque et al., 1995; Smith et al., 1992; Zafra et al., 1995a,b). Quantitative analysis reveals that both GLYT1 and GLYT2 (mRNA and protein) are expressed in caudal areas of the brain, indicating a clear association of both proteins with the inhibitory glycinergic neurotransmission. GLYT1 is also found in areas devoid of strychnine-sensitive receptors such as diencepha-

lon, retina, olfactory bulb and brain hemispheres (Zafra et al., 1995a,b), which matches its role in the control of the local glycine concentration near NMDA receptors. GLYT2 distribution is more restricted to spinal cord, brain-stem and cerebellum, finding the highest transporter levels in the dorsal and ventral horn, in the auditory system, and in the nuclei of the cranial nerves (Zafra et al., 1995a,b). More precise immunocytochemical analysis has shown that GLYT1 is mostly found in astrocyte-like perikarya and processes, both in gray and white matter, whereas GLYT2 is expressed almost exclusively in axons and presynaptic terminals. However, an exception is found in the cerebellum where GLYT2 is localized both in presynaptic terminals and glial elements as well as in the retina, where GLYT1 is found only in amacrine and some bi-polar neurons (Menger et al., 1998; Zafra et al., 1995a). In spinal dorsal horn, GLYT2 has been visualized by electron microscopy in the plasma membrane of the axonal boutons where it appears as discrete patches outside the active zone (Spike et al., 1997). Importantly, double-immunofluorescence experiments have shown a perfect co-localization of GLYT2 and glycine immunoreactivities, indicating that GLYT2 is a reliable marker of glycinergic neurons (Poyatos et al., 1997).

The existing antibodies for GLYT1, in the reported experimental conditions, are not able to detect a clear expression of GLYT1 in the plasma membrane of neurons except in the amacrine cells of the retina. However, on the basis of in situ hybridization observations, the existence of a neuronal form of the protein cannot be ruled out. Although the distribution of the recently identified GLYT1e and GLYT1f isoforms have not been reported yet, it is possible that they represent the atypical neuronal forms present in amacrine neurons of the retina and other CNS locations (Borowsky et al., 1993; Smith et al., 1992; Zafra et al., 1995a). A moderate to high expression of GLYT1 mRNA is found in neurons not only in spinal cord, brainstem or cerebellum, but also in forebrain regions such as the cortex, the hippocampus, the thalamus, the hypothalamus or the olfactory bulb (Borowsky et al., 1993; Smith et al., 1992; Zafra et al., 1995b), areas where no inhibitory glycinergic neurons have been found. The apparent discrepancy between GLYT1 protein detection and GLYT1 mRNA distribution suggests the existence of a neuronal form of the transporter that cannot be identified by the available antibodies. As long as this matter is not clarified, this issue constitutes an open question.

In the autonomic system, the presence of GLYT1 has been reported in presynaptic terminals of cholinergic neurons, facing microdomains of glycine receptors that can be activated by reverse uptake (Tsen et al., 2000). Expression of GLYT1 in neurons can be correlated with a physiological non-vesicular release of glycine (Roux and Supplisson, 2000; Supplisson and Roux, 2002). In rodent brain development, GLYT2 appears at embryonic days 18–20, immediately after the establishment of functional synapses (Friauf et al., 1999). GLYT1 is expressed at the end of foetal period and reaches a maximum at 2-week postnatal age (Jursky and Nelson, 1996; Zafra et al., 1995b). The expression of GLYTs seems to depend on neuronal factors. In mixed neuronal/glial cell cultures, GLYT1 expression is upregulated by neurons and can be down regulated by selective neuron elimination (Zafra et al., 1997). In neurons of the rat dorsal cochlear nucleus, GLYT2 expression is influenced by neuronal activity as acoustic stimulation evokes an increase in GLYT2 mRNA (Barmack et al., 1999).

## 4. Transport mechanism

The mechanism by which transporter proteins mediate uptake of neurotransmitters such as glycine, GABA or amines involves binding and co-transport of $Na^+$ and $Cl^-$ ions. The $Na^+$ concentration gradient is generated and maintained by the plasma membrane $Na^+/K^+$-ATPase. The alternating access mechanism appears as a suitable model for neurotransmitter transporters (Rudnick, 1998). The binding of substrate and ions to the transporter induces a conformational change that permits the interconversion between external and internal access to the binding site with the consequent translocation of the substrate and ions from the one side to the opposite side of the membrane. This mechanism ensures the coupling between neurotransmitter, $Na^+$, and $Cl^-$ fluxes and permits the accumulation of substrate against its concentration gradient which is driven by the different ion concentrations in both sides of the membrane. Transporters can also function in the reverse direction mediating the release of neurotransmitter. Efflux and exchange of glycine through the transporters has previously been studied (Aragón and Giménez, 1986; López-Corcuera et al., 1989; Sakata et al., 1997).

Glycine uptake in plasma membrane vesicles from rat brain and C6 cells showed a $Na^+$, $Cl^-$ and voltage dependence with a stoichiometry of at least 2 $Na^+$/1 $Cl^-$/glycine. (Aragón et al., 1987). In a stable expression system on human embryonic kidney (HEK-293) cells, the functional properties of GLYT1 and GLYT2 were compared through biochemical and electrophysiological methods (López-Corcuera et al., 1998). The two glycine transporters require 1 $Cl^-$ ion per transport cycle, but their apparent chloride affinities are different, showing that GLYT1a has three times higher apparent affinity than GLYT2. The $Na^+$ dependence of glycine transport shows a sigmoidal pattern for the two transporters but the data indicate a higher $Na^+$ coupling for GLYT2 than GLYT1. The voltage dependence of GLYT2 was higher in either, the glycine evoked and the capacitative currents recorded in the absence of substrate. These data suggested a stoichiometry of 2 $Na^+$ and 3 $Na^+$ for GLYT1 and GLYT2, respectively (Fig. 3). By combined electrophysiological and radio-tracing techniques on *Xenopus* oocytes expressing GLYT1 or GLYT2, the different $Na^+$ stoichiometry has been proven (Roux and Supplisson, 2000). By plotting the charge movement as a function of glycine

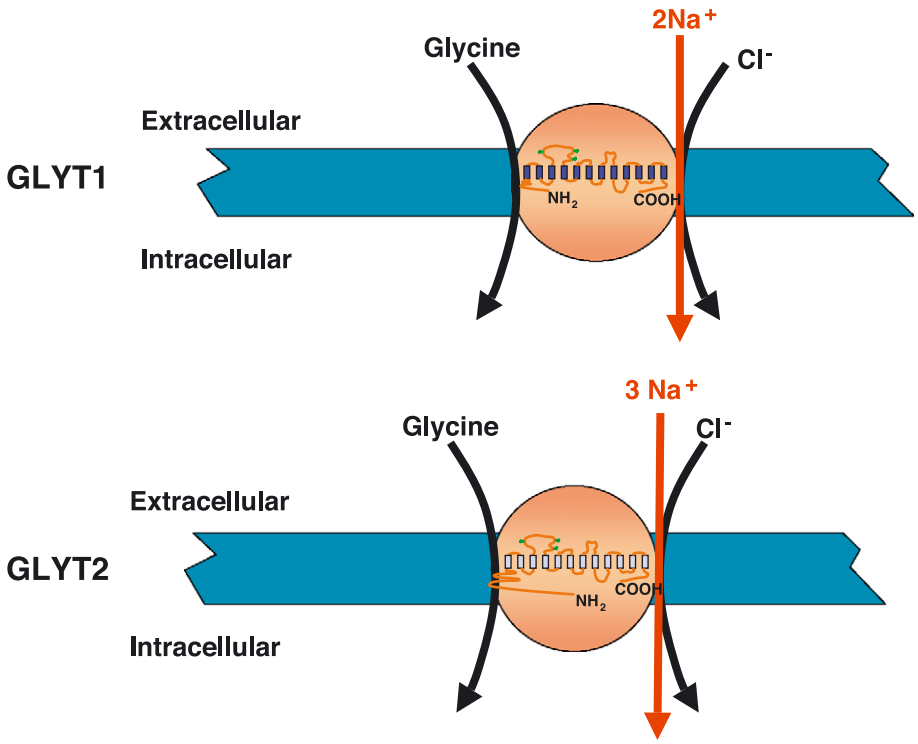


Fig. 3. Stoichiometry of the glycine transporters. The transport of one glycine molecule involves the transport of one chloride ion per transport cycle for the two glycine transporters but different number of sodium ions: two for GLYT1 and three for GLYT2.

uptake, a linear relationship for GLYT1 and GLYT2 was observed. However, plot slopes were different, indicating a GLYT2 ionic coupling twice than that of GLYT1. Therefore, GLYT2 has a stoichiometry of 3 $Na^+$/1 $Cl^-$/1 glycine which allows GLYT2 to maintain a high glycine concentration inside the synaptic terminal (20–40 mM). This condition is essential to ensure the filling-up of synaptic vesicles by the low-affinity VIAAT/VGAT. A coupling ratio of 2 $Na^+$/1 $Cl^-$/1 glycine predicts that GLYT1 is working close to equilibrium and that glycine can be exported or imported depending on the ionic or electrical environment. These observations support the role of GLYT1 in the modulation of glycine concentrations around receptors in glutamatergic NMDA-mediated synapses (López-Corcuera et al., 2001b; Roux and Supplisson, 2000).

Some neurotransmitter transporters, in addition to the classic substrate transport mechanism, also catalyze a substrate-dependent uncoupled ion flux showing channel-like activity. These conductances, which cannot be accounted for the fixed stoichiometry of substrate translocation, have been observed for serotonin transporter (SERT) (Mager et al., 1994), glutamate transporter EAAT-4 (Fairman et al., 1995), norepinephrine transporter (NET) (Galli et al., 1996), dopamine transporter (DAT) (Sonders et al., 1997) and the GABA transporter GAT1 (Cammack and Schwartz, 1996). However, GLYT1 and GLYT2 do not exhibit substrate-dependent uncoupled currents, as demonstrated by using specific inhibitors for either transporter when expressed in *Xenopus* oocytes. These transporters present a reversal potential of the substrate-gated current which is similar to the transporter equilibrium potential (i.e. zero net flux condition), indicating that the substrate-induced current is tightly coupled to transport (Roux and Supplisson, 2000; Supplisson and Roux, 2002).

## 5. Structure/function analysis

The predicted structural model derived from hydropathy analysis of the $Na^+$ and $Cl^-$-coupled neurotransmitter transporter sequences suggests 12 transmembrane domains, hydrophilic amino- and carboxyl-termini inside the cytoplasm, and a large second extracellular loop that is glycosylated. The glycine transporters are paradigms of this structural model, although GLYT2 presents an unusually long N-terminus (Liu et al., 1993a). The structure and the topology of the glycine transporters have been experimentally examined. Using antibodies against the terminal ends of GLYT1, it has been shown that either amino- or carboxyl-termini of the protein are intracellularly located as predicted. A large part of the carboxyl-terminus of GLYT1 is needed to avoid retention in the endoplasmic reticulum, and to allow progression to the Golgi system, and complete glycosylation (Olivares et al., 1994; Zafra et al., 1995a). In addition, the topology has been studied on GLYT1 by glycosylation scanning mutagenesis and in vitro transcription/translation techniques. Although the topological results confirmed a 12-transmembrane domain model, an important rearrangement

in the amino-terminal third of the transporter was proposed (Olivares et al., 1997). Since similar results were obtained for GAT1 transporter (Bennett and Kanner, 1997), this question remained for some time controversial until the originally predicted topology was confirmed by chemical labelling of extracellular residues on the serotonin transporter (Chen et al., 1998). The reported topological discrepancies might be due to the dynamic nature of the transporter proteins or to a dissimilar conformational state of the different transporters (see below).

Glycine transporters are proteins with N-linked carbohydrates. The implication of N-linked glycosylation of GLYT1 and GLYT2 in transporter expression and function has been investigated. The four potential glycosylation sites (Asn-X-Ser/Thr consensus sequences) present on the second extracellular loop of the glycine transporters are actually used. Since the totally unglycosylated proteins remain in the intracellular compartment, sugar chains seem to be necessary for the protein trafficking to the plasma membrane (Olivares et al., 1995). Additionally, a new role not previously reported for the sugar moiety of a transporter as a determinant of the GLYT2 apical sorting in polarized cells has recently been revealed (Martínez-Maza et al., 2001).

The direct involvement of transmembrane domain 3 in the mechanism of transport has been proven for several members of the glycine transporter family such as GAT1, SERT, DAT and creatine transporter (CREAT) (Bismuth et al., 1997; Chen et al., 1997; Chen and Rudnick, 2000; Dodd and Christie, 2001; Lee et al., 1998; Lin et al., 1999). In a study on GLYT2, the tyrosine residue at position 289 in this very transmembrane domain has been shown to be crucial for ion coupling, glycine affinity, and sodium selectivity. Even its conservative substitution to aromatic residues rendered transporters unable to catalyze glycine uptake. However, glycine-evoked steady-state currents could be measured in mutant-transfected HEK293 cells by whole cell patch clamp, allowing the functional study of T289 substitutions. The apparent affinity for glycine and sodium were severely altered showing, in addition, a minor cooperative behaviour for this ion. Accordingly, sodium selectivity was lost and chloride dependence was decreased in the T289 mutants. These results support that the T289 is likely to lie in or near the permeation pathway (Ponce et al., 2000).

Recently, the functional involvement of the first extracellular loop, a short protein stretch connecting transmembrane domains 1 and 2, has been studied by substituted cysteine accessibility method (SCAM) (López-Corcuera et al., 2001c; Roux et al., 2001). The sensitivity of GLYT1 and GLYT2 to small, charged sulfhydryl-specific methanethiosulfonate derivatives was tested under voltage clamp and uptake conditions. In *Xenopus* oocytes expressing GLYT1, application of non-permeant methanethiosulfonate compounds re-

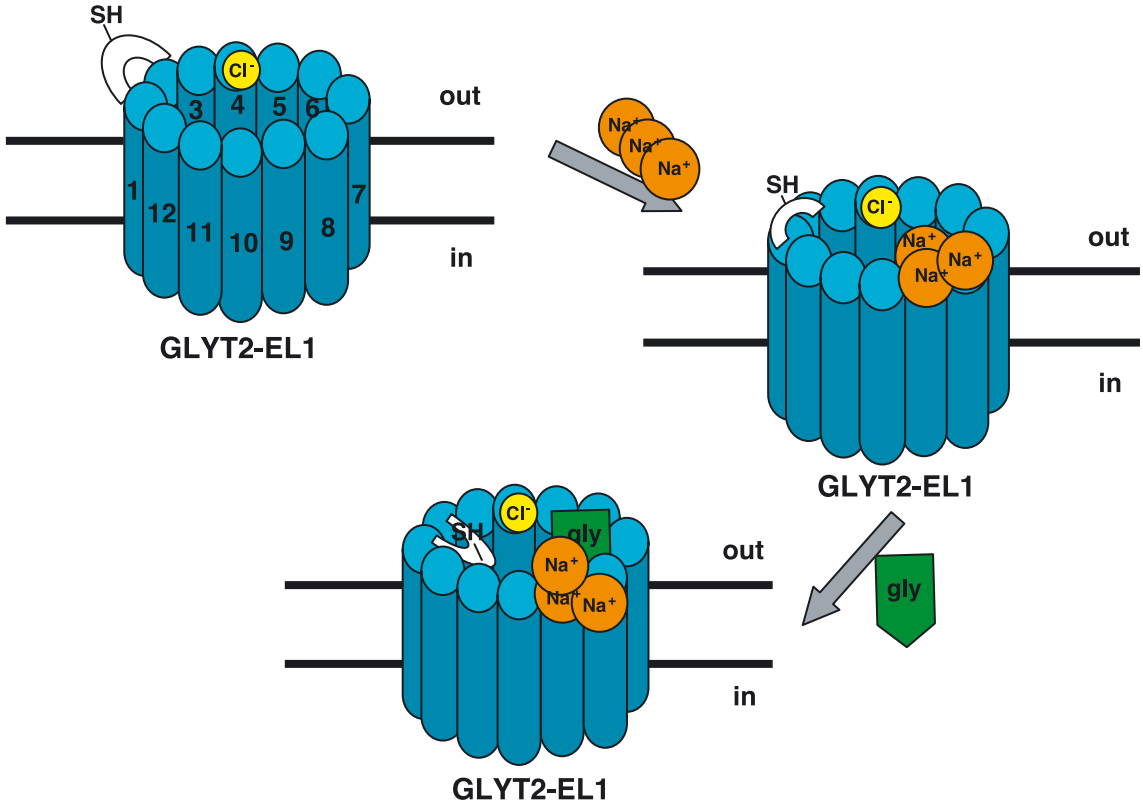


Fig. 4. Scheme of the substrate-induced conformational changes of the first external loop in the glycine transporters. The external loop one of the glycine transporters is externally exposed in the outward-facing conformation but is occluded upon ion binding and further upon glycine binding, becoming inaccessible from the outside in the inward-facing conformation.

duced the $Na^+$-dependent charge movement, the glycine-evoked currents and the glycine uptake, indicating an inactivation of the transporter following cysteine modification. In contrast, GLYT2 was resistant to the methanethiosulfonate compound. The methanethiosulfonate derivative sensitivities of the two transporters can be exchanged by constructing a GLYT2-EL1 chimera where the first extracellular loop (EL1) of GLYT1 (containing the active cysteine 62) was introduced within the corresponding sequence of GLYT2. By studying the protection requirements of the methanethiosulfonate compound inhibition, it has been shown that EL1 region of GLYT2 is less accessible in the presence of co-transported ions and substrates, this protection being clearly dependent on temperature. These results indicate that EL1 of glycine transporters acts as a fluctuating hinge undergoing sequential conformational changes during the transport cycle (Fig. 4) (López-Corcuera et al., 2001c; Roux et al., 2001). Moreover, the differential protection requirements between EL1 of SERT and GLYTs indicated that EL1 seems to be a source of structural heterogeneity among the $Na^+$ and $Cl^-$ neurotransmitter transporters (Ni et al., 2001; López-Corcuera et al., 2001c).

## 6. Regulation of glycine transporters

Analysis of the amino acid sequence of glycine transporters reveals several consensus sites for some protein kinase phosphorylation as protein kinase C (PKC), protein kinase A, and $Ca^{2+}$/calmodulin-dependent protein kinase. This suggests that GLYT1 and GLYT2 may be regulated by diverse signal transduction systems. The PKC activator phorbol ester 12-myristate 13-acetate (PMA) causes a dose- and time-dependent inhibition of GLYT1 activity in several experimental systems (Gomeza et al., 1995). Although the mode of GLYT1 regulation by PKC remains to be elucidated, it cannot be ruled out that is due to the redistribution of the transporter from the cell surface to intracellular compartments as described for GABA and monoamine-related transporters (see Zahniser and Doolen, 2001 for a review).

By using *Xenopus laevis* oocytes and electrophysiological techniques, a pH regulation of GLYT1 activity has been shown (Aubrey et al., 2000). Protons inhibited glycine transport by a noncompetitive mechanism, with half-maximal inhibition exerted by concentrations found in physiological and pathological situations. The decrease of the registered glycine-evoked current corresponds to a lower glycine influx showing no alteration in the coupling ratio of transport. The inhibition effect was membrane potential independent. Mutation of histidine 421 in the fourth extracellular loop of GLYT1 rendered the transporter insensitive to the proton inhibition with no alteration of kinetic parameters. Since the $pK_a$ value for proton inhibition is close to 7, this effect is physiologically relevant for the fluctuations of extracellular pH that may occur during the neurotransmission; therefore, proton modulation of GLYT1 can be important in determining the dynamics of glycine neurotransmission (Aubrey et al., 2000).

A characteristic of the $Na^+/Cl^-$-dependent family of plasma membrane transporters is the existence of an asymmetrical distribution on the cell surface of neurons. This uneven distribution is thought to be important for the optimal removal of neurotransmitters from the extracellular compartment and suggests potential mechanisms for the regulation of neurotransmitter transport. The subcellular distribution of GLYT1 and GLYT2 has been studied upon expression of the proteins in polarized cells such as primary hippocampal neurons and Madin-Darby canine kidney (MDCK) epithelial cells (Poyatos et al., 2000; see Zafra and Giménez, 2001 for a review). By site-directed mutagenesis and immunofluorescence methods, it has been demonstrated that the subcellular distribution of glycine transporters is dependent on either the protein isoform or the cell type. Additionally, signals for basolateral/somatodendritic localization were found in the amino terminal tail of GLYT1 and in two di-leucine motifs located on its carboxyl terminal tail. In MDCK cells, GLYT2 was exclusively detected in the apical part of the membrane, and the *N*-glycosylation sites located on the second extracellular loop of the transporter were involved in the polarized distribution of the protein (Martínez-Maza et al., 2001).

The 200 amino acids long N-tail of GLYT2 was used as a bait in yeast two-hybrid screening, allowing to detect a specific interaction of the transporter with Ulip6 a novel unc-33 protein that is involved in neuronal differentiation and axon growth (Horiuchi et al., 2000).

Recent studies have demonstrated that syntaxin1 is somehow involved in the neuronal trafficking of GLYT2 (Geerlings et al., 2000, 2001; see López-Corcuera et al., 2001a for a review). GLYT1 and GLYT2 interact functionally and physically with syntaxin1A as demonstrated by co-expression of syntaxin1A and GLYT1 or GLYT2 followed by co-immunoprecipitation either from transfected cells or brain tissue. The interaction disappears in the presence of the syntaxin1A-binding protein Munc 18, showing that the observed effect is specific. Since syntaxin is involved in the release of neurotransmitter, the physiological role of the syntaxin1–GLYT2 interaction has been studied in brain-derived synaptosomes under conditions of exocytosis stimulation. When vesicular glycine release was experimentally triggered, GLYT2 is rapidly trafficked first toward the plasma membrane and then internalized. When the synaptosomes were inactivated for syntaxin1 by the neurotoxin Bont/C, GLYT2 was unable to reach the plasma membrane. From these results, the existence of a soluble *N*-ethyl-maleimide-sensitive attachment factor receptor (SNARE)-mediated and $Ca^{2+}$-dependent regulatory mechanism that controls the surface expression of GLYT2 becomes evident Syntaxin1 is involved in the transporter arrival to the plasma membrane but not in its retrieval (Fig. 5). In the same work, GLYT2 was detected in small synaptic-like vesicles by immunogold labeling of purified synaptic vesicle preparations derived from synaptosomes. These vesicles may represent glycine

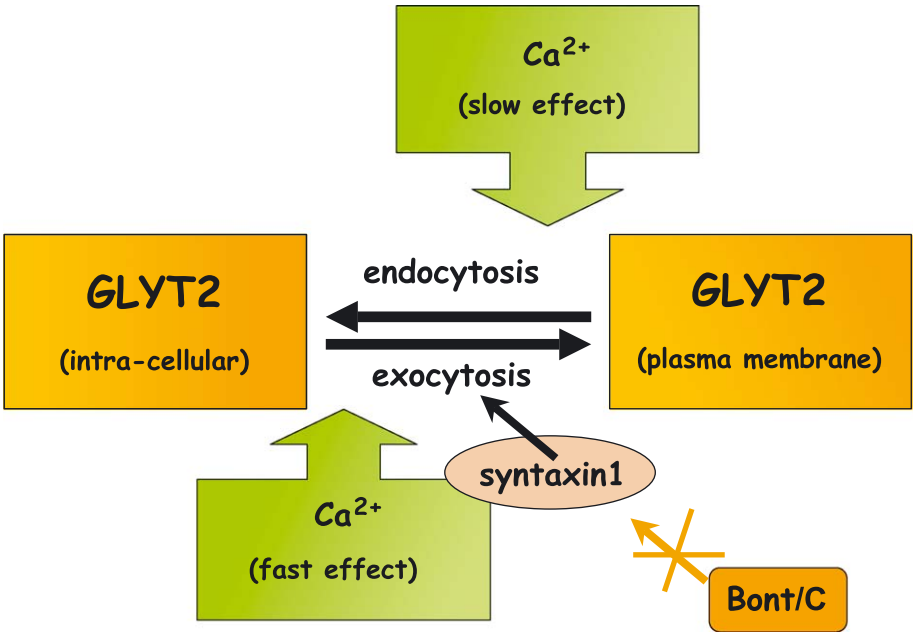


Fig. 5. Summary of the $Ca^{2+}$ and syntaxin-dependent regulation of GLYT2. In conditions that promote the vesicular release of glycine, $Ca^{2+}$ first stimulates the arrival of GLYT2 to the plasma membrane, immediately followed by a retrieval. Arrival of GLYT2 to the plasma membrane is mediated by syntaxin1, the retrieval is not. GLYT2 is being trafficked in small synaptic-like vesicles.

transporter that is being trafficked, suggesting a parallelism between exocytosis of neurotransmitter and its reuptake from the synaptic space (Geerlings et al., 2000, 2001).

The subcellular localization of glycine transporters was further studied in PC12 cells that were stably transfected with the fusions of GLYT1 and GLYT2 with green fluorescent protein (GFP). As demonstrated by confocal microscopy and surface biotinylation experiments, either protein was targeted to large dense-core vesicles and endosomes, but the two transporter isoforms differ in subcellular distribution. Whereas GFP-GLYT2 was mainly present on the intracellular compartment (75–80%), GFP-GLYT1 was mostly localized on the plasma membrane (75–80%). Although little is known about the cellular mechanism that target proteins to different classes of neurosecretory vesicles, glycine transporters should have targeting signals which direct them to the large dense-core vesicles in PC12 cells, and signal sequences responsible for the different localization should be present on GLYT1 and GLYT2 (Geerlings et al., 2002). Future work will probably reveal those determinants.

## 7. Pharmacology of glycine transporters

Small molecule inhibitors of the neurotransmitter transporters have been thought to find application in the treatment of several CNS pathologies such as epilepsy, depression, psychosis, anxiety, pain and neurodegenerative disorders. The need for the precise regulation of glycine concentrations around the multiple glycine-modulated receptor systems implicates glycine transporters as potential therapeutic targets. Specific GLYT1 inhibitors would find therapeutic applications in the treatment of schizophrenia and dementia through modulation of NMDA receptor function. In addition, GLYT2 inhibitors would be useful in the treatment of epilepsy and pain by interfering with strychnine-sensitive glycine receptor systems (see Armer, 2000 for a review).

GLYT1 and GLYT2 activities can be pharmacologically distinguished by the sensitivity of the former to sarcosine (López-Corcuera et al., 1989). The lipophilic sarcosine derivative *N*[3-(4′-fluorophenyl)-3-(4′phenylphenylphenoxy)propyl]sarcosine (NFPS) has reported to be a potent and selective GLYT1 inhibitor and it has been used to study the contribution of GLYT1 to the modulation of the NMDA receptor function in hippocampal pyramidal neurons (Bergeron et al., 1998). The mechanism of action of NFPS on glycine transport has been studied in *Xenopus laevis* oocytes (Aubrey and Vandenberg, 2001) and in a stable expression system by using a quail fibroblast cell line (Atkinson et al., 2001). NFPS inhibits the activity of GLYT1a, b and c subtypes with an $IC_{50}$ value of 3 nM (Atkinson et al., 2001) but has no effect on the GLYT2a. The inhibition of transport is time and concentration dependent and NFPS acts as a non-competitive and long lasting inhibitor. Recently, an analogue of NFPS, *N*[3-phenyl)-3-(4′-(4-toluoyl)phenoxy)-propyl]sarcosine, has been synthesized proving to be a suitable radioligand for GLYT1 binding assays (Lowe et al., 2003). These sarcosine derivatives will be useful tools to study the role that glycine and NMDA receptors play in CNS neurotransmission.

A selective GLYT2 inhibition could be used for developing novel muscle relaxants that act by decreasing muscle

hyperactivity, compounds that would be useful in treating diseases associated with increased muscle contraction such as spasticity and epilepsy. Also, the selective inhibition of GLYT2 can offer an approach to develop novel analgesic compounds by diminishing neuropathic pain transmission in the spinal cord. As either Gly receptor or GLYT2 presents a discrete localization within the spinal cord and brain stem, an inhibitor of this transporter is expected to act without the serious CNS side effects characteristic of the used μ-opioid analgesics. Two classes of glycine reuptake inhibitors selective for GLYT2 have been developed, a series of 5,5-diaryl-2-amino-4-pentenoates with $IC_{50}$ values of 0.3–0.4 μM (Isaac et al., 2001) and a series of amide compounds of which the 4-benzyloxy-3,5-dimethoxy-*N*-[(1-dimethylaminocyclopentyl)methyl]benzamide is the most potent inhibitor ($IC_{50}$ = 16 nM) (Caulfield et al., 2001). However, the complete characterization of the inhibitory effect of these compounds awaits to be elucidated.

Several tricyclic antidepressant drugs have been tested on the glycine uptake by GLYT1 and GLYT2 in stably transfected HEK293 cells (Núñez et al., 2000a). Amoxapine displayed a selective inhibition of GLYT2 behaving as a 10-fold more efficient inhibitor of this transporter than of GLYT1. Amoxapine displayed a competitive inhibition of either glycine or chloride transport and a mixed-type inhibition with respect to sodium. By using the former stable expression system, the effect of several alkanols has been investigated as well (Núñez et al., 2000b). Ethanol, at clinically relevant concentrations, modulated allosterically GLYT2 but showed no effect on GLYT1. A probable direct interaction of alcohols with a discrete site on GLYT2 that could accommodate alkyl chains of at least four methylene groups was deduced from "cut-off" experiments. Ethanol chronic treatment caused differential adaptive responses on glycine transporters. A decrease in activity and plasma membrane expression was detected for GLYT2, whereas GLYT1 increased in function and cell surface density. Together with the changes described for other neurotransmitter systems, these changes may account of some of the modifications observed in glycinergic and/or glutamatergic neurotransmission under ethanol intoxication (Núñez et al., 2000b).

## 8. Conclusions and perspectives

Glycinergic synapses are now seen to be as complex and variable as those releasing other neurotransmitters. The fact that glycinergic synapses are excitatory in early stages of life suggests new functions of these synapses in the brain development. The importance of glycine transporters in the control of extracellular neurotransmitter concentration, and its contribution to the modulation of NMDA receptor function is now well established. The need for the precise regulation of glycine levels around the multiple glycine-modulated receptors implicates glycine transporters as potential therapeutic targets in several CNS disorders.

As we continue to unravel the glycine transporter biology, several areas remain unknown. The apparent discrepancy between GLYT1 protein detection and GLYT1 mRNA distribution in neurons must be clarified. The enigmatic nature of the vesicular transporters for glycine in some subpopulations of glycinergic terminals lacking VIAAT/VGAT is an interesting area for future research. Other challenges are the role of glycine transporters in the structural organisation of the presynapse or the knowledge of primary and secondary structures of glycine transporters at the atomic level as well as the dissection of the substrate and modulator binding sites. Moreover, the final structure of these proteins as monomers or oligomers needs to be readdressed by using novel experimental approaches. A clear understanding of all the factors that contribute to glycine transporter regulation is also needed. The physiological meaning of the reverse function of the glycine transporters or the role of intracellular transporters present in small synaptic-like vesicles must also be clarified. Further studies of the plasma membrane glycine transporters, perhaps through the development of transgenic animals, will help to understand the neuronal role of glycine and hence develop new therapeutic approaches for glycine-mediated neurotransmission pathologies.

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