Minireview

Why glycine transporters have different stoichiometries

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Abstract In the brain, neurons and glial cells compete for the uptake of the fast neurotransmitters, glutamate, GABA and glycine, through specific transporters. The relative contributions of glia and neurons to the neurotransmitter uptake depend on the kinetic properties, thermodynamic coupling and density of transporters but also on the intracellular metabolization or sequestration of the neurotransmitter. In the case of glycine, which is both an inhibitory transmitter and a neuromodulator of the excitatory glutamatergic transmission as a co-agonist of *N*-methyl D-aspartate receptors, the glial (GlyT1b) and neuronal (GlyT2a) transporters differ at least in three aspects: (i) stoichiometries, (ii) reverse uptake capabilities and (iii) pre-steady-state kinetics. A 3 Na^+/1 Cl^-/gly stoichiometry was established for GlyT2a on the basis of a 2 charges/glycine flux ratio and changes in the reversal potential of the transporter current as a function of the extracellular glycine, Na^+ and Cl^- concentrations. Therefore, the driving force available for glycine uphill transport in neurons is about two orders of magnitude larger than for glial cells. In addition, GlyT2a shows a severe limitation for reverse uptake, which suggests an essential role of GlyT2a in maintaining a high intracellular glycine pool, thus facilitating the refilling of synaptic vesicles by the low affinity, low specificity vesicular transporter VGAT/VIAAT. In contrast, the 2 Na^+/1 Cl^-/gly stoichiometry and bi-directional transport properties of GlyT1b are appropriate for the control of the extracellular glycine concentration in a submicromolar range that can modulate *N*-methyl D-aspartate receptors effectively. Finally, analysis of the pre-steady-state kinetics of GlyT1b and GlyT2a revealed that at the resting potential neuronal transporters are preferentially oriented outward, ready to bind glycine, which suggests a kinetic advantage in the uptake contest. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Transporter; Glycine; Stoichiometry; Driving force; Uptake; Synaptic transmission

1. Transporters of recapture in slow and fast transmission have different constraints

In the central nervous system of Vertebrates, diffusion and uptake of neurotransmitters by specific transporters terminate synaptic transmission at the notable exception of acetylcholine which is hydrolyzed by acetylcholinesterase. Transporters of recapture are located in glial cells and/or neurons and uptake regulates the basal extracellular concentration and spillover of neurotransmitters, thus limiting synaptic cross talk. Though recapture is their principal mode of operation, transporters are bi-directional molecular machines and may also behave as a Ca^{2+}-independent source of neurotransmitters, depending on the direction of the driving force.

Transporters belong to two families of secondary active transporters (see reviews in [1,2]) with a distinct membrane topology and an ionic requirement for Cl^- and K^+ ions [3]. The largest family is formed by Na^+/Cl^- coupled co-transporters that share high sequence homologies in their 12 putative transmembrane segments. This family includes the monoamines transporters (DAT, NET and SERT) and multiple isoforms for GABA (GAT1-4) and glycine (GlyT1-2) transporters. The other family is formed by the five isoforms of glutamate transporters (GLAST, GLT1, EAAC1, EAAT4-5, see review in [4]). These transporters have an unusual membrane topology of 8 transmembrane segments with one [5] or two re-entrant loops [6] and are assembled in pentameric structures [7]. Glutamate uptake is coupled to Na^+ cotransport and obligatory K^+ counter-transport [8,9].

Transporters of recapture play a central role for the volume transmission of monoamines [10] as demonstrated by the psychostimulant and anti-depressant effects of DAT, NET and SERT inhibitors such as cocaine, desipramine and fluoxetine (Prozac®). Accordingly, the rate of clearance of dopamine and norepinephrine from the extracellular space determined by cyclic voltammetry are ~300 times slower in $DAT^{-/-}$ [11] and ~6 times slower in $NET^{-/-}$ knockout mice compared to wild-type animals. In addition to this phasic recapture role, monoamine transporters contribute to the homeostasis of the neurotransmitter as revealed by the profound metabolic changes observed in $DAT^{-/-}$ knockout mice [12].

In contrast, the contribution of transporters to the time-course of fast synaptic transmission and spillover of GABA, glutamate and glycine is intensely debated [13–16]. Mathematical models using elaborated synapse geometries show that diffusion alone can achieve clearance of glutamate at central synapses [17–19] when the diffusion coefficient is not arbitrarily adjusted to slow the process [20]. In addition, the low turnover of amino acid neurotransmitter transporters expressed in *Xenopus* oocytes (GAT: ~5 Hz [21], EAAT2: ~15 Hz [22], GlyT1-2: ~20 Hz [23]) suggests that each transporter may not have a chance to eliminate more than one neurotransmitter molecule during a typical ~10 milliseconds inhibitory or

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excitatory post-synaptic current (IPSC or EPSC). Nevertheless, uptake inhibitors potentiate GABAergic and glutamatergic synaptic transmissions in brain slices [14,16,24–28] and limit the spillover of glutamate [29,30] and the activation of high-affinity metabotropic receptors [31]. Recent experimental evidences explain how glutamate transporters can buffer synaptically released glutamate with sub-millisecond kinetics [24]: (1) transporter densities are higher than previously envisaged [32], with densities of up to 8500 glutamate transporters/μm^2 in the molecular layer of the cerebellum [33]; (2) the synapse size and geometry with the presence or not of surrounding astrocyte processes greatly influence the diffusion and spillover of neurotransmitters ([28]; see review in [30,34]); (3) glial and neuronal glutamate transporters have a 3 Na^+ stoichiometry [35,36] that develops a large driving force for uphill transport; (4) multiple Na^+ binding synchronizes transporters, allowing a rapid binding of glutamate [37]; (5) fast applications of glutamate show that the translocation step of transporters is much faster than the overall cycle and occurs on a sub-millisecond time scale [38,39].

2. Glycine web: connecting excitation and inhibition

To appreciate the role of transporters of recapture in glycine neurotransmission, it is necessary to summarize briefly the complex activity of glycine as a neurotransmitter and a neuromodulator.

2.1. Glycine is an inhibitory transmitter in the adult mammalian CNS

Glycine is one of the major inhibitory neurotransmitters in spinal cord and brainstem interneurons of adult mammalian central nervous systems (CNS; see historical and comprehensive reviews in [40] and [41]). Evidence for glycine inhibition in higher brain structures is elusive, but glycine IPSCs were recorded in Golgi cells, an interneuron of the granular layer of the cerebellum [42], as well as in glycine-evoked inhibitory responses in olfactory bulb neurons [43] and CA3 hippocampal neurons [44,45]. Glycine hyperpolarizes post-synaptic neurons by activating strychnine-sensitive, Cl^--permeable, ligand-gated ionotropic receptors (GlyR) formed by the association of 3 α and 2 β subunits [46,47]. During development and early post-natal days, activation of glycine or GABA receptors depolarizes developing motoneurons (see reviews in [48,49]) until the neuronal transporter KCC2 lowers the intracellular chloride concentration and shifts the Cl^- equilibrium potential toward more hyperpolarized potentials [50,51]. These glycine-evoked depolarizations may bring the post-synaptic neuron to the spiking threshold [51], but an inhibition is often observed because of the large shunting conductance due to GlyR activation [51,52]. Evidence for a pre-synaptic localization of strychnine-sensitive glycine receptors has been reported in giant excitatory synapses (calyx of Held) of the medial nucleus of the trapezoid body [53]. In these brainstem slices from P10-P16 rats, application of glycine evoked a small depolarization of the pre-synaptic terminal, potentiating glutamatergic transmission [53].

A significant number of inhibitory synapses co-released glycine and GABA by the same pre-synaptic inhibitory neuron as first established by Jonas and co-workers in pairs of spinal interneurons [54] and widely confirmed since then [55–58]. This co-release of the two inhibitory transmitters is presumably [59] a direct consequence of the lack of selectivity between GABA and glycine of the vesicular inhibitory amino acid transporter VGAT/VIAAT [60,61], which is localized in pre-synaptic terminals of GABAergic and glycinergic neurons [62,63].

2.2. Glycine is a neuromodulator of excitatory transmission

Glycine is a neuromodulator of excitatory transmission as a high-affinity co-agonist with glutamate of *N*-methyl D-aspartate (NMDA) receptors [64]. NMDA receptors are hetero-oligomers formed by the association of NR1 subunits that contain the glycine binding site, sometimes referred to as the glycine B site (see review in [65]), with at least one of the four NR2 subunits (A–D) that control the apparent affinity of the co-agonist site. In fact, the nature of the endogenous ligand of the NMDA receptors co-agonist site is debated, and cumulative evidence suggests that glycine and D-serine compete for the same site [66].

Since no evidence for a mechanism of co-liberation of glycine and glutamate has been reported, it is assumed that the occupancy of the glycine co-agonist site relies on the resting extracellular glycine ($[Gly]_e$) and D-serine concentrations. At present, specific D-serine transporters have not been reported and it is not clear how D-serine which is synthesized in astrocytes is exported out of the cell, then degraded or recaptured from the extracellular space. Nevertheless, an action of D-serine or glycine requires in the first place that transporters are able to lower $[Gly]_e$ to submicromolar levels in the synaptic cleft. This has been established in a model system of co-expression in *Xenopus* oocytes [67] and in hippocampal slices [68] and brainstem hypoglossal motoneurons [69].

2.3. Glycine activates a cation selective receptor and may be a 'true' excitatory transmitter

Finally a third, truly excitatory, cation-permeable receptor for glycine was identified by the expression of NMDA receptors composed of NR1 and NR3 A or B subunits in *Xenopus* oocytes [70]. These novel NMDA receptor isoforms have unique properties since they are not activated by glutamate, not blocked by Mg^{2+}, are relatively Ca^{2+} impermeable and are inhibited by D-serine [70]. D-Serine-sensitive glycine-evoked action potentials were recorded in cultured cortical neurons but direct confirmation in brain slices is sought.

2.4. Glycine interactions between inhibitory and excitatory transmissions

In spinal cord, brainstem and cerebellum, glycinergic and glutamatergic synapses form contiguous patches on the same post-synaptic neuron. Thus glycine inhibitory receptors and NMDA receptors can be in neighboring micro-environments. An attractive hypothesis would be that spillover of synaptically released glycine from an inhibitory neuron has the capacity to potentiate neighboring NMDA receptors.

3. How glycine transporters may interplay with phasic inhibition and tonic neuromodulation of glycine

Biosynthesis of glycine, a non-essential amino acid without stereoisomer, follows the ubiquitous one carbon metabolism in spinal inhibitory neurons [71]. Changes in the relative expression of the mitochondrial bio-synthetic and degradation enzymes, serine hydroxymethyltransferase and glycine cleav-

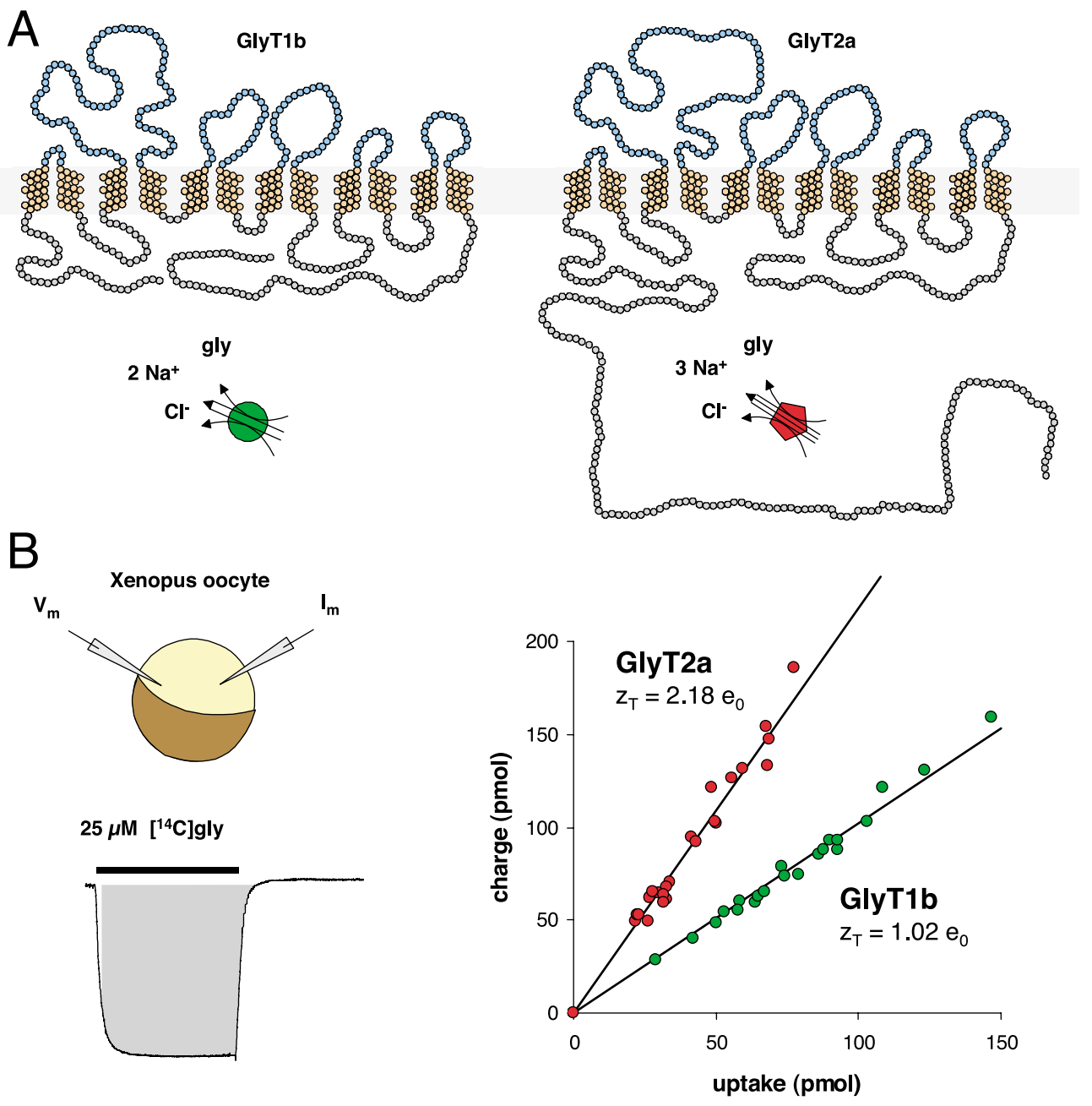

Fig. 1. A: Topology of GlyT1b and GlyT2a. Amino-acids considered extracellular are shown in blue, intracellular in gray, in transmembrane segments in orange. As all members of the Na^+/Cl^- coupled transporters, the two glycine transporters have 12-membrane-spanning segments with intracellular N- and C-termini. The N-terminus of GlyT2a, with over 200 amino-acids, is unusually long. B: Determination of the charge to glycine ratio. *Xenopus* oocytes are large cells that allow simultaneous measurement of the glycine uptake current under voltage-clamp and the radio-labeled glycine uptake (left). Plotting the amount of charge (expressed in pmol of elementary charges) as a function of glycine uptake reveals that the GlyT2a charge to glycine ratio is twice that of GlyT1b (potentials between −120 mV and 0 mV, $n=23$ for GlyT2a, $n=21$ for GlyT1b). Adapted from Ref. [98].

age system, respectively, should increase glycine availability in glycinergic neurons (see review in [72]). However, glycine accumulation in synaptic vesicles by the vesicular transporter requires a high intracellular glycine concentration because of the low affinity ($EC_{50} \sim 20$ mM) and poor selectivity of VGAT/VIAAT (see reviews in [59,73]), and it is not known if the bio-synthetic machinery alone is able to deliver such high glycine concentrations. At the other end of the concentration range, modulation of NMDA receptors via their co-agonist site requires that the synaptic glycine concentration is kept in the submicromolar range, much lower than in the cerebro-spinal fluid (CSF). Therefore, plasma membrane glycine transporters may be important players for glycine homeostasis if they have the capacity to adjust glycine concentrations in the different intracellular and extracellular compartments over a large dynamic scale.

Early investigations identified two Na^+- and Cl^--dependent glycine-specific transporters in rat brain vesicles [74] that dif-

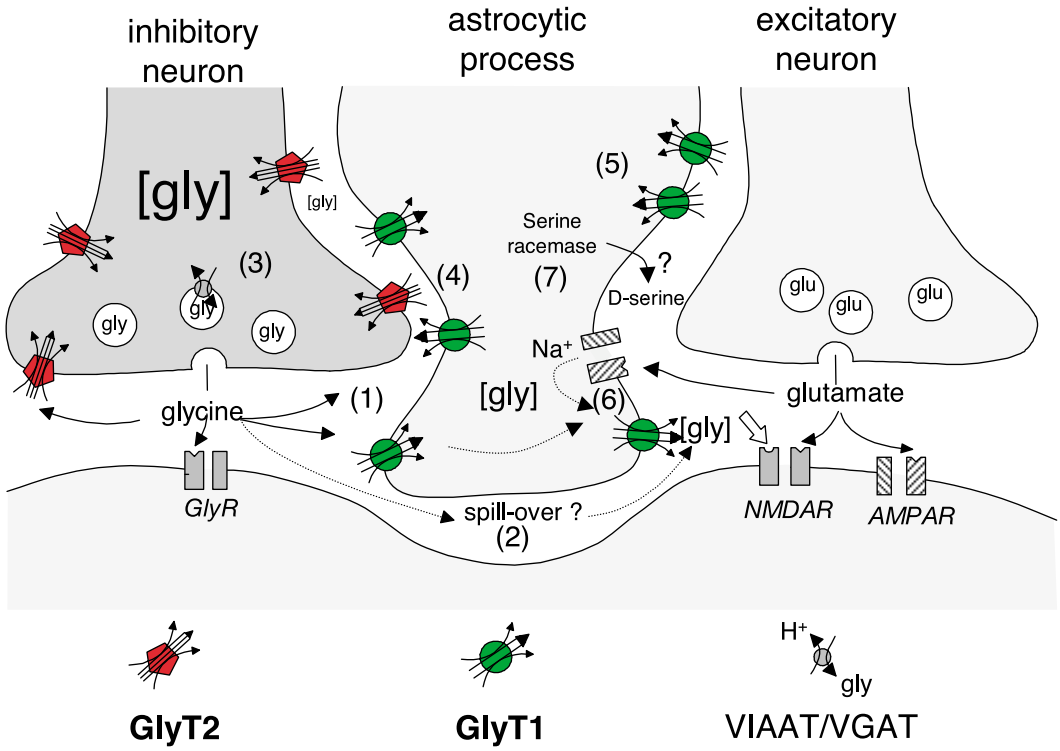

Fig. 2. Roles of glycine transporters. GlyT2a and GlyT1b both contribute to the control of the extracellular [Gly] at the glycinergic synapses (1) and limit spillover to neighboring synapses (2). The more energetic coupling of GlyT2a allows the maintenance of a high [Gly] in pre-synaptic boutons, sufficient to allow VIAAT/VGAT to fill the synaptic vesicles (3). The less energetic coupling of GlyT1b may even allow a glycine shuttle between astrocytes and neurons, maximizing the recycling of released glycine toward neurons (4). At glutamatergic synapses containing NMDA receptors, GlyT1b expressed in surrounding astrocytes are lowering the extracellular [Gly] under the saturating level of the co-agonist site despite the high [Gly] of the CSF (5). However, depolarization of/concentration changes in the astrocytes may be sufficient to reverse GlyT1b function and potentiate NMDA responses (6), alone or in addition to a D-serine release (7), which mechanism has not yet been established. Adapted from Ref. [98].

fered in their sensitivity to the *N*-methyl derivative sarcosine [75]. These observations were confirmed by the successive cloning of two closely related members of the Na^+/Cl^- coupled transporter family (GlyT1 and GlyT2a) [76–78], which show differential sensitivity to sarcosine, a substrate of GlyT1b [67] that does not interact with GlyT2a. Analysis of the gene coding for GlyT1b reveals 3 N- (a,b,c) and 2 C- (d,e) terminus exons [76,77,79,80]. Five GlyT1 variants (a,b,c,e,f) have been described [76,77,79,80] that are generated by different combinations of N- and C- terminal exons (ad, bd, cd, ae, be). Isoforms containing exon e are expressed only in the retina and interact with the GABA receptor ρ1 subunit [80]. A second GlyT2 isoform (GlyT2b), which differs by five amino acids in the N-terminal and does not transport glycine, has also been reported [81]. GlyT2a has a long N-terminal domain of 200 amino-acids which is not found in any other member of the Na^+/Cl^- coupled transporters (Fig. 1A). When this domain was used as a probe in yeast two-hybrid screening, a specific interaction was found with Ulip6, an Unc-33-like phosphoprotein that belongs to a family involved in signaling cascades of axonal guidance [82].

Structure–function studies confirm that GlyT1 and GlyT2 share the landmark membrane topology of the Na^+/Cl^- coupled family (Fig. 1A, see review in [83]). Purification and reconstitution assays of native glycine transporters suggested that the protein was active as a monomer [75], which was confirmed recently by surface labeling of GlyT1b and GlyT2a in *Xenopus* oocytes [84]. Trafficking of GlyT2 at the plasma membrane is regulated by a direct interaction with the SNARE protein syntaxin1a [85,86]. The genetic expression of the different glial isoforms GlyT1a-c is regulated by different promoter usage [87] and neuronal induction is needed [88]. In the brainstem auditory nucleus, GlyT2 expression during development is associated with a shift between GABAergic to glycinergic transmission [89,90].

The cellular and tissular expression of GlyT1 and GlyT2 match the complex glycine activity described above. GlyT1 staining is found in astrocytes while GlyT2 is a reliable marker of glycinergic neurons [91–93] and is localized in axon and terminal boutons [94] (Fig. 2). GlyT2 expression is restricted to the inhibitory system [91,94] while GlyT1b is also found in areas devoid of strychnine-sensitive receptors where it can control the local glycine concentration under the saturating level of the NMDA receptors co-agonist site [77,94]. In the intact retina, neuronal expression of GlyT1 has been described in amacrine cells and some bi-polar neurons [94,95] but not in Müller cells [96]. In the autonomic system, GlyT1b has been reported in pre-synaptic terminals of cholinergic neurons, facing microdomains of GlyRs that they can activate by reverse uptake [97]. Expression of GlyT1 in neurons may thus be correlated with a physiological non-vesicular release of glycine – a task for which GlyT2 is doubly non-adapted, due to its limitation in the reverse uptake mode and its 'higher' stoichiometry [98].

3.1. Pharmacology of glycine transporters

GlyT1 and GlyT2 show differential pharmacological sensitivity for sarcosine, ethanol [99] and the anti-depressant amoxapine [100]. Low affinity glycine uptake inhibitors such as glycyldodecylamide [101] and amoxapine have been reported for GlyT2 [100]. In addition, glycine uptake by the glial transporter is inhibited by arachidonic acid [102] and acidic pH [103].

The lack of specific, reversible inhibitors of GlyT1 and GlyT2 has impaired the analysis of the contribution of glycine transporters to the physiology of inhibitory and excitatory synaptic transmission because the manipulations were limited to the removal of Na^+ or the application of sarcosine or D-serine [15,69,104]. However, high-affinity specific inhibitors have recently been developed for GlyT1b [105,106] and GlyT2 [107,108]. GlyT1 inhibitors are expected to potentiate NMDA receptors via their co-agonist site and may serve to alleviate the negative symptoms of schizophrenia, which presumably involve a reduced activity of NMDA receptors as shown in animal models [109,110]. GlyT2 inhibitors may be helpful to reduce pain if they are able to potentiate glycinergic inhibitory transmission as GABA transporter inhibitors do for GABAergic transmission [16].

GlyT1b inhibitors are irreversible on the timescale of an hour and their mode of action is not fully understood [105,111]. Nevertheless, these molecules are becoming useful tools for blocking recombinant transporters expressed in *Xenopus* oocytes [98,111] or native transporters in synaptosomes [112] and brain slice preparations [68,113,114].

3.2. Electrophysiological characteristics of glycine transporters

Transporters of recapture are electrogenic, carrying at least one net positive charge per transport cycle. Macroscopic recording of these transport-related currents is an attractive method for studying cloned transporters because of the control of the cell membrane potential which greatly influences both the kinetic and energetic of transport. The millisecond time-resolution of this technique is well within the duration of synaptic transmission. However, it requires that transporters are expressed at densities ($>10^6$ for a typical mammalian cell, $>10^9$ for *Xenopus* oocytes) sufficient to overcome the atoscopic current (10^{-18}–10^{-17} A) generated by individual transporters which may be difficult to achieve or may produce over-expression artefacts. The situation is even worse with monoamine transporters, which have a typical turnover 10–20 times lower than amino-acid transporters and generate small macroscopic transporter currents [115,116]. In addition, current and transport were found not be as tightly coupled as first predicted. Electrophysiological characterization of DAT [117], NET [118], SERT [119], GAT [120] and EAAT [121] indicated that tight coupling is more the exception than the rule in both neurotransmitter transporters families [122].

Glycine uptake in vesicles isolated from rat brain or C6 cells was known to be Na^+-, Cl^-- and voltage-dependent with a minimal stoichiometry of 2 Na^+/1 Cl^-/glycine that predicted a charge coupling of +1 charge/glycine [123,124]. The electrogenicity of glycine uptake was confirmed by the recording of glycine-evoked current under voltage-clamp in HEK cells and *Xenopus* oocytes expressing GlyT1 and GlyT2 [67,98,103,125]. Steady-state currents did not reverse up to +50 mV and the current-voltage relationships were linear (GlyT1b) or quasi-linear (GlyT2) [98,125].

To measure directly the ionic coupling of the two glycine transporters, *Xenopus* oocytes have a unique advantage over all other expression system. Their gigantic size ($\sim$1-mm diameter) allows one to measure simultaneously, under voltage-clamp, radio-labeled uptake and substrate-evoked current *on the same cell* (Fig. 1B). Plot of the charge (time-integral of the glycine-evoked current) as a function of glycine uptake shows a linear relationship for GlyT1b and GlyT2a over a wide range of transporter expression and membrane potentials (Fig. 1C, [98]). However, the slopes were different and the ionic coupling of GlyT2a (+2.1 e/glycine) was twice that of GlyT1b (+1.01 e/glycine) [98]. To test and rule out diverse hypotheses about possible ionic coupling (3 Na^+; Cl^-; Cl^- exchange as proposed for GAT1 [126], glycine-evoked uncoupled current as found for the Cl^- uncoupled conductance of glutamate transporters [121]), the stoichiometries of GlyT1b and GlyT2a were directly determined using a reversal potential slope method developed by Zerangue and Kavanaugh [35] to solve a long lasting controversy about the stoichiometry of glutamate transporters.

If the current is tightly coupled to transport, the reversal potential of the substrate-gated current (E_{inv}) should correspond to the equilibrium potential (E_T) of the transporter (i.e. zero net flux condition). For a glycine transporter that couples the transport of one glycine to n Na^+ ions and m Cl^- ions, the reversal potential is given by the relation:

$$E_{inv} \approx E_T = \frac{2.3RT}{(n-m)F}\log\left(\frac{[Na^+]_e^n[Cl^-]_e^m[Gly]_e}{[Na^+]_i^n[Cl^-]_i^m[Gly]_i}\right) \quad (1)$$

(R, T and F have their usual meaning).

The reversal potential of the transporter current *must* vary accordingly with the change in substrate concentrations. This is a main operational difference with the constant reversal potential observed with ionotropic ligand-gated receptors/voltage-gated channels. If the reversal potential of the substrate-gated current remains constant when the substrate concentration is changed, as reported once with GAT1 [127], the most likely explanation is that the recorded current is not actually coupled to active transport [127,128].

The reversal potential slope method allows a direct determination of the stoichiometric factors n and m by measuring the slope of E_{inv} change as a function of $\log([Na^+]_e)$, $\log([Cl^-]_e$ and $\log([Gly]_e)$. Under these conditions, Eq. 1 can be simplified to a system of linear equations with the slope proportional to the stoichiometric exponents. Experimentally this method has five explicit requirements: (i) a non-transported uptake inhibitor should be available in order to separate by subtraction transporter outward current from all endogenous oocyte conductances; (ii) the approximation $E_{inv} \sim E_T$ must be verified (i.e. the substrate should not activate uncoupled conductances); (iii) the transporter must be able to work in uptake and reverse uptake modes (i.e. can generate inward and outward current); (iv) the reversal potential must be in an experimental accessible voltage range; (v) the charge/substrate coupling should be measured directly with radio-labeled substrate, but this limitation can be overcome in mammalian cells using statistical analysis for the possible stoichiometries [36].

Using specific GlyT1b and GlyT2a inhibitors provided by Organon [106,107], it was shown that glycine does not evoke uncoupled current in GlyT1b- and GlyT2a-expressing oocytes,

indicating that the approximation $E_{inv} \sim E_T$ holds for these transporters [98]. However, the neuronal and glial transporters present a major difference in their ability to function in the reverse uptake mode. GlyT1b generates an outward current after a glycine intracellular load in *Xenopus* oocytes [98] and produces glycine efflux in transfected cells [129] or in synaptosomes [112] while GlyT2 shows a strong limitation for efflux unless all the co-transported substrates glycine, Na^+ and Cl^- were injected at high concentrations into the oocyte cytoplasm with a micropipet [98]. Using the intracellular injection method, it was possible to measure GlyT2a E_{inv} and to analyze the E_{inv} slope as a function of the logarithm of the three co-substrates [98]. The GlyT2a E_{inv} slope as a function of $\log([Gly]_e)$ of GlyT2a was half that of GlyT1b, which confirmed that the extra charge of Gly2a was thermodynamically coupled to transport. The conclusion from these experiments was that glycine uptake is energetically coupled to 2 Na^+ and 1 Cl^- for GlyT1b and to 3 Na^+ and 1 Cl^- for GlyT2a [98], a unique stoichiometry among Na^+/Cl^- coupled transporters (at least, for which it has been determined).

4. Why glycine transporters have different stoichiometries

4.1. Supplying VGAT with glycine

Glial and neuronal transporters compete for fast re-uptake of glycine in a common extracellular environment that undergoes large dynamic variations during synaptic transmission. Their contribution to the phasic uptake depends on their relative capacity to bind and trap rapidly the neurotransmitter, which in turn depends on kinetic parameters that are mostly Na^+- and voltage-dependent, i.e. driving force-dependent.

The driving force for glycine uptake can be rewritten from the thermodynamic equilibrium equation of transport using the electrochemical ion gradients as follows:

$$\frac{\Delta\tilde{\mu}_T}{F} = \underbrace{(n-m)\,V_m - n\,E_{Na} + m\,E_{Cl}}_{\text{Driving Force}} - 59\log\left(\frac{[Gly]_e}{[Gly]_i}\right) \quad (2)$$

(E_{Na}, E_{Cl} are the Nernst potentials for Na^+ and Cl^-, respectively, V_m is the membrane potential and F the faraday's constant).

A difference of one Na^+ in ionic coupling implies that the available driving force for glycine uptake for GlyT2a is two orders of magnitude larger than for GlyT1b under physiological conditions. Because GlyT1 and GlyT2 share a 'common' extracellular space in spinal cord and brainstem, it can be stated that the thermodynamic equilibrium for both transporters corresponds to the condition:

$$\frac{[Gly]_{i,Neuron}}{[Gly]_{i,Astrocyte}} = 10^{\left(\frac{E_{Na}-V_m}{59}\right)} \quad (3)$$

where $[Gly]_{i,Neuron}$ and $[Gly]_{i,Astrocyte}$ are the intracellular glycine concentrations in glycinergic neurons and astrocytes, respectively. For simplification, E_{Na}, E_{Cl} and V_m were assumed to be equal in astrocytes and glycinergic neurons. The prediction of Eq. (3) is in good agreement with the fact that glycinergic neurons have a higher glycine content than astrocytes or other types of neurons.

Considering a resting glycine concentration in the both glycinergic and glutamatergic synaptic clefts of 0.1–0.2 μM that

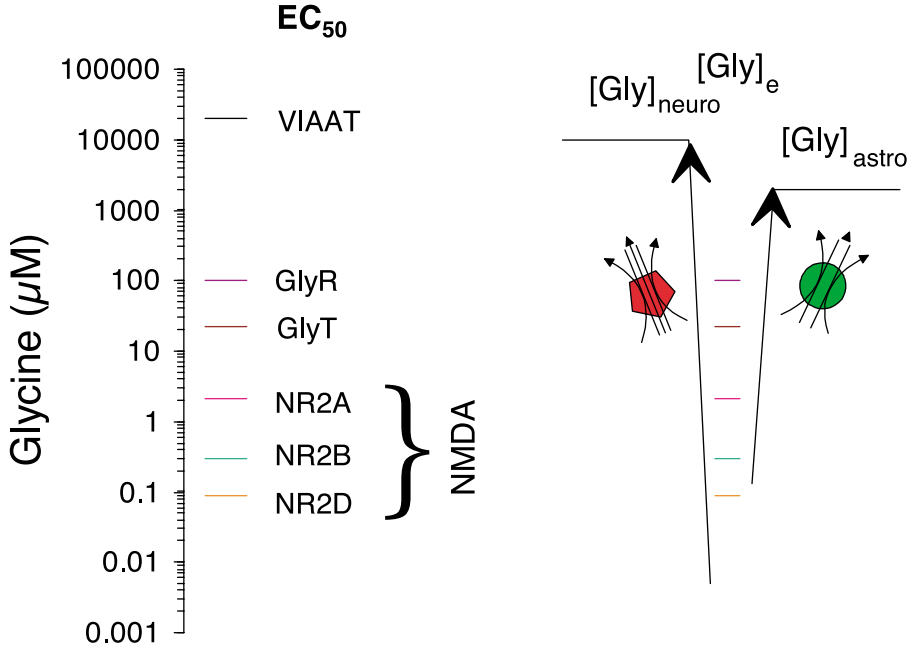

Fig. 3. Affinities of glycine 'receptors' and transporters (left) and the comparison between these affinities and the gradients that can be maintained by GlyT2a and GlyT1b if equilibrium is reached.

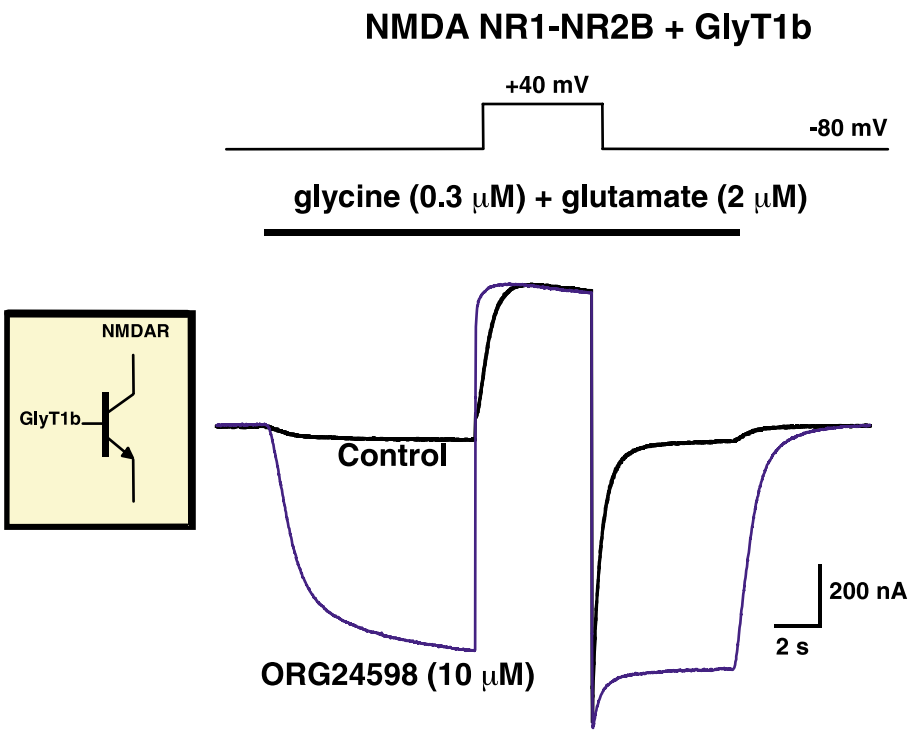

Fig. 4. Control of the NMDA response by GlyT1b activity in a co-expression system. Oocytes expressing GlyT1b were subsequently injected with mRNA of the NMDA receptors NR1 and NR2B subunits. Application of 0.3 μM glycine and 2 μM glutamate evoked only a small response in control conditions (black trace) at negative potentials, but a full response during and shortly after a depolarization to +40 mV, potential for which glycine uptake is strongly reduced. When GlyT1b is blocked by the specific inhibitor ORG24598, NMDA response occurs at all potentials (blue trace).

is non-saturating for NMDA receptors, a coupling to 3 Na^+ allows GlyT2a to maintain a concentration of 20–40 mM in the synaptic boutons, while a coupling to only 2 Na^+ would allow an intracellular concentration of only ~1 mM, probably too low to ensure an efficient filling-up of synaptic vesicles considering the low affinity of VIAAT/VGAT (Fig. 3). On the other hand, this internal glycine concentration is close to what has been reported for astrocytes, meaning that GlyT1b is working close to equilibrium.

A more speculative hypothesis is that GlyT2a may deplete glycine from the extracellular space down to a concentration that forces GlyT1b to operate in reverse uptake mode, thus producing a transcellular glycine flux from astrocytes to neurons, similar to the glutamate/glutamine shuttle. This would allow a co-operative action of glial and neuronal transporters to quickly lower extracellular glycine after synaptic release while maximizing the amount of glycine finally recaptured by the neurons. The limitation in efflux observed with GlyT2a also contributes to glycine accumulation, preventing a temporary reversal of transport during neuronal activity.

The additional Na^+ gives another advantage to GlyT2a over GlyT1b in the uptake contest. The higher voltage-dependence of the protein rearrangements ($z\delta = 1.48$ vs. $z\delta = 0.55$ e_0 for GlyT2a and GlyT1b, respectively [130]) favors an extracellular orientation of the glycine binding site at negative potentials, giving a kinetic advantage to the neuronal transporter. This may be particularly important in spaces with limited diffusion as in the vicinity of synapses as long as equilibrium is not reached: a concentration of 1 μM in a synaptic cleft of surface of 0.5×0.5 μm and a 20 nm width corresponds to only three molecules.

4.2. *Gating NMDA receptors activation by GlyT1b activity: a biological transistor*

Indeed, the high compactness of brain structures, with an estimated extra/intracellular volume ratio of 0.2, facilitates a rapid extracellular depletion over a slow intracellular accumulation by transporter, as the effect of uptake in terms of concentration change depends on the volume ratio of the two compartments. This depletion opposes the accumulation of substrate observed in structures with high external/internal volume ratio, such as synaptic vesicles, epithelia or in vitro uptake preparations.

Glycine depletion produced by glycine transporters can be demonstrated in a *Xenopus* oocyte model system, in which GlyT1b and NMDA receptors are co-expressed [67]. NMDA receptors are used as a sensor of the local, juxtamembrane glycine concentration when the transporter is active or blocked by specific inhibitors. Application of submicromolar glycine concentration (0.3 μM with 2 μM glutamate), a hundred times below the transporter EC_{50}, evokes only a small response that increases when transporters are inactivated by depolarization or 10 μM ORG24598 (Fig. 4). In a provocative image, NMDA-receptor activation is here under the control of glycine transporter activity.

In the CNS, considering an intraglial glycine concentration of 2 mM, GlyT1b is working close to equilibrium. Thus, small concentration/potential changes in its vicinity may reverse the net glycine flux. Astrocytes are usually considered to be clamped at the equilibrium potential of K^+. However, it has been suggested that activation of AMPARs in glial cells by glutamate may produce a depolarization and a rise in $[Na^+]_i$ that could be sufficient to reverse GlyT1b [131]. AMPARs and

glutamate transporter currents activated by synaptic release of glutamate have been recorded in situ in glial cells in the cerebellum [132,133] and the hippocampus [134,135]. The amplitude of the AMPA currents recorded in the Bergmann cells are around 100 pA, which predicts a depolarization of ~7 mV assuming a membrane resistance of 70 MΩ [132]. The extent of depolarization that may occur in unclamped cells is not known and may be larger. Moreover, a rise in intracellular Na^+ following AMPAR activation by kainate is able to block two of the glial K^+ conductances [136,137]. The depolarization and the change in intracellular Na^+ concentration (which could reach the 20–40 mM range because of the low intracellular volume of fine astroglial processes) may effectively be sufficient to reverse GlyT1b locally.

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