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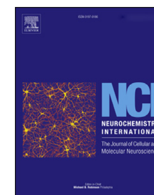

# Advances in understanding the functions of native GlyT1 and GlyT2 neuronal glycine transporters

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ABSTRACT

Glycine can be substrate for two transporters: GlyT1, largely expressed by astrocytes but also by some non-glycinergic neurons, and GlyT2, most frequently present in glycine-storing nerve endings. In morphological studies, GlyT2 expression had been found to be restricted to caudal regions, being almost undetectable in neocortex and hippocampus. Here, we compared the uptake activities of GlyT1 and GlyT2 in synaptosomes purified from mouse spinal cord, cerebellum, neocortex and hippocampus. Although, as expected, $[^3H]$glycine uptake was significantly lower in telencephalic than in caudal regions, selective GlyT2-mediated uptake could be evaluated in all areas. Appropriately, $[^3H]$glycine selectively taken up into hippocampal synaptosomes through GlyT2 could be subsequently released by exocytosis. Native GlyT2, which did not contribute to basal release from cerebellum or spinal cord nerve terminals, could mediate release of $[^3H]$glycine by transporter reversal in synaptosomes exposed to veratridine. Moreover, GlyT2 transporters could perform $Na^+$-dependent homoexchange in response to externally added glycine. In conclusion, transporters of the GlyT2 type exhibited significant uptake also in telencephalic regions, probably because of the elevated driving force related to their stoichiometry. Although glycine release through GlyT2 had been predicted to be a very difficult process, GlyT2 expressed on isolated glycinergic nerve terminals can perform both release by transporter reversal and homoexchange.
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## 1. Introduction

Glycine behaves as an inhibitory neurotransmitter when it activates strychnine-sensitive receptors, especially in spinal cord, brainstem and cerebellum (Betz, 1992; Legendre, 2001). In addition, glycine exerts important excitatory functions throughout the CNS as an obligatory coagonist of glutamate at NMDA receptors (Johnson and Ascher, 1987). Glycine can be substrate for two transporters termed GlyT1 and GlyT2. GlyT1 is largely expressed by astrocytes (Zafra et al., 1995a,b), but significant GlyT1 pools are also present on non-glycinergic nerve endings (Cubelos et al., 2005, 2014; Yee et al., 2006; Raiteri and Raiteri, 2010; Harsing and Matyus, 2013). GlyT2 transporters are mostly localized on glycinergic nerve endings where they are critical for glycine uptake into the presynaptic cytosol for refilling synaptic vesicles with glycine (Zafra et al., 1995a,b; Poyatos et al., 1997; Gomeza et al., 2003; Aragón and López-Corcuera, 2005; Eulenburg et al., 2005).

Glycine transporters are differentially expressed throughout the CNS. In morphological studies GlyT1 was found to be present at the highest concentrations is spinal cord and brain stem, but also, in a lesser degree, in the brain hemispheres. As to GlyT2, its expression was reported to be restricted to spinal cord, brainstem and cerebellum, while it was essentially undetectable in cortex and hippocampus (Luque et al., 1995; Zafra et al., 1995a; Zeilhofer et al., 2005). On the other hand, hippocampal interneurons were shown to be immunopositive, although weakly, for GlyT2 (Danglot et al., 2004; Song et al., 2006), a finding compatible with the existence, yet poorly investigated, of functional glycinergic synapses also in the hippocampus.

Voltage-clamped *Xenopus* oocytes expressing either GlyT1 or GlyT2 were used by Roux and Supplisson (2000) to evaluate the stoichiometry of the two transporters. It was found that GlyT1 has a

*Abbreviations:* BoNT, botulinum toxin; GlyT1, glycine transporter 1; GlyT2, glycine transporter 2; HEK293, human embryonic kidney; NFPS (ALX 5407), *N*-[(3R)-3-([1,1′-biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-*N*-methylglycine hydrochloride; Org25543, *N*-[[1-(dimethylamino) cyclopentyl]methyl]-3,5-dimethoxy-4-(phenylmethoxy)benzamide hydrochloride.

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stoichiometry of $2Na^{+}/Cl^{-}$/glycine, while the stoichiometry of GlyT2 is $3Na^{+}/Cl^{-}$/glycine. Most neurotransmitter transporters are bidirectional devices that, under some conditions, can work in reverse to perform carrier-mediated release (Attwell et al., 1993; Belhage et al., 1993; Levi and Raiteri, 1993; Vizi, 1998; Vizi and Sperlágh, 1999; Jensen et al., 2000; Héja et al., 2009). In their study, Roux and Supplisson (2000) found that GlyT1 and GlyT2 have different reverse transport kinetics. In particular, following a comparable glycine load, the transmitter was easily exported from GlyT1 oocytes but not from GlyT2-bearing cells, indicating that GlyT2 has a kinetic constraint for reverse transport that strongly limits glycine release.

HEK293 cells expressing GlyT1 or GlyT2 were employed by Herdon et al. (2001) in release experiments. Transporter-mediated efflux of preloaded [$^{3}$H]glycine from GlyT1 cells was increased by externally added glycine, indicating homoexchange, which was blocked by the GlyT1 selective inhibitor NFPS. In contrast, external glycine was unable to affect release from glycine-loaded GlyT2 cells. The absence of homoexchange at GlyT2 was explained by taking into account the peculiar kinetic characteristics of GlyT2 described by Roux and Supplisson (2000).

Although glycine has been known to be involved in several important physiological and pathological conditions since decades, some aspects of glycinergic transmission, including glycine release and effects of glycine transporter activation in *native* nervous tissues under physiological and pathological conditions have started to be investigated only in recent years, also thanks to the availability of selective GlyT1 and GlyT2 inhibitors (Saransaari and Oja, 2001, 2009; Raiteri et al., 2002, 2008; Harsing et al., 2003, 2006; Luccini and Raiteri, 2007; Luccini et al., 2008; Oja and Saransaari, 2011, 2013; Lall et al., 2012; Harsing and Matyus, 2013; Milanese et al., 2014; Hanuska et al., 2016), These functional studies have produced substantial information on glycine uptake and on the transporter-mediated release of glycine occurring by reversal of GlyT1 transporters. Other functional aspects of glycine transport, particularly regarding GlyT2, merit to be better understood, considering that glycine transporters, both GlyT1 and GlyT2, have been related to many nervous system disorders (Eulenburg et al., 2006; Carta et al., 2012; Coyle, 2012; Harvey and Yee, 2013; Vandenberg et al., 2015). To improve the knowledge on the function of native glycine transporters, we here used purified nerve ending preparations from adult mouse CNS (i) to analyze the regional distribution of GlyT1-and GlyT2-mediated glycine uptake, with particular attention to neocortex and hippocampus; (ii) to evaluate the ability of native GlyT2 to perform glycine release by transporter reversal and glycine homoexchange.

## 2. Materials and methods

### 2.1. Animals

Adult Swiss mice (weighing 20–25 g; Charles River, Calco, Italy) were used. Animals were housed at constant temperature (22 ± 1 °C) and relative humidity (50%) under a regular light dark schedule (lights 7.00 a.m.–7.00 p.m.). Food and water were freely available. Experimental procedures and animal care complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Italian Ministry of Health in accordance with Decreto Ministeriale 116/1992. All experiments have been performed according to “ARRIVE” guidelines for reporting research. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable results.

### 2.2. Preparation of synaptosomes

Animals were sacrificed by cervical dislocation and spinal cord, cerebellum, cortex and hippocampi were quickly removed. The tissues were homogenized in 10 volumes of 0.32 M sucrose, buffered at pH 7.4 with Tris-HCl, using a glass-teflon tissue grinder (clearance 0.25 mm). Purified synaptosomes were prepared using a discontinuous Percoll® gradient (Dunkley et al., 1988; Nakamura et al., 1993), with some modifications (Luccini and Raiteri, 2007). The homogenate was centrifuged (5 min, 1000×*g* at 4 °C) to remove nuclei and debris and the supernatant was gently stratified on a discontinuous Percoll® gradient (2, 6, 10 and 20%, v/v in Tris-buffered sucrose) and centrifuged at 33,500 × *g* for 5 min. The layer between 10% and 20% Percoll® (synaptosomal fraction) was collected, washed by centrifugation and resuspended in a physiological medium (standard medium) having the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; $NaH_2PO_4$, 1.2; $NaHCO_3$, 5; glucose, 10; HEPES, 10; pH adjusted to 7.4 with NaOH. All the above procedures were performed at 0–4 °C. Protein was determined according to Bradford (1976) using bovine serum albumin as a standard.

### 2.3. Uptake experiments

[$^{3}$H]Glycine uptake was studied according to the following procedure. The synaptosomal pellet was resuspended in standard medium. Aliquots (500 µl) of the synaptosomal suspension (about 25 µg protein) were incubated for 2 min at 37 °C with [$^{3}$H]glycine (0.3 µM) in the absence (control samples) or in the presence of drugs. At the end of the incubation period, samples were rapidly filtered on Whatman glass fibre filters (GF/B; VWR International, Milan, Italy). Each sample was washed three times with 5 ml aliquots of standard medium and filters were counted for radioactivity. Blank values were obtained by maintaining the samples in an ice water bath.

### 2.4. Release experiments

Synaptosomes were incubated at 37 °C for 15 min with [$^{3}$H]glycine (0.3 µM). When appropriate, incubation with the radioactive tracer was performed in the presence of NFPS (0.1 µM). In a set of experiments, synaptosomes were incubated at 37 °C for 90 min in the presence or in the absence of 10 nM Botulinum Toxin $C_1$ (BoNT/$C_1$); the radioactive tracer was present during the last 15 min of incubation. At the end of incubation, identical aliquots of the synaptosomal suspension (each corresponding to about 40 µg protein for spinal cord and cerebellum and 25 µg for hippocampus) were distributed on microporous filters placed at the bottom of a set of parallel superfusion chambers maintained at 37 °C and superfused with standard medium at a rate of 0.5 ml/min (Raiteri and Raiteri, 2000).

In a group of experiments (experiments of depolarization-evoked [$^{3}$H]glycine release), after 36 min of superfusion with standard medium, to equilibrate the system, fractions were collected as follows: two 3-min fractions ($t$ = 36–39 min and $t$ = 45–48 min; basal release) before and after one 6-min sample ($t$ = 39–45 min; evoked release). A 90-s period of depolarization was applied at $t$ = 39 min. Synaptosomes were depolarized with high KCl (substituting for an equimolar concentration of NaCl) or veratridine. Org25543 was added 9 min before depolarization. When appropriate, $Ca^{2+}$ was omitted from the superfusion medium at $t$ = 20 min of superfusion. The $Ca^{2+}$-free medium contained 8.8 mM $MgCl_2$ substituting for an isoosmotic amount of NaCl. Fractions collected and superfused filters were counted for radioactivity by liquid scintillation counting.

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