# Transport activities and expression patterns of glycine transporters 1 and 2 in the developing murine brain stem and spinal cord

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## 1. Introduction

Glycine acts as a fast inhibitory neurotransmitter in caudal regions of the vertebrate central nervous system (CNS). Upon presynaptic release, it activates chloride conducing glycine receptors (GlyRs; [1]). In addition, glycine serves, like D-serine, as an essential co-agonist of the N-methyl-D-aspartate (NMDA) subtype of excitatory glutamate receptors [2]. Extracellular glycine concentrations in the CNS are precisely controlled by two high-affinity glycine transporters (GlyTs), G1yT1 and GlyT2 [3]. GlyT2 is expressed exclusively by glycinergic interneurons [4], whereas GlyT1 is predominantly found in astrocytes and in addition in a subset of presumptive glutamatergic neurons [5,6]. The generation of GlyT-deficient mice has revealed vital roles of these membrane proteins during postnatal development. In mice, GlyT2 deficiency results during the second postnatal week in hyperexcitability and finally death due to reduced presynaptic glycine release [7]. In contrast, GlyT1$^{-/-}$ mice die shortly after birth due to overinhibition resulting from GlyR overactivation [8], and conditional inactivation of GlyT1 expression in glial cells indicates that this phenotype is exclusively caused by the loss of glial GlyT1 [9]. From these results, it was inferred that GlyT1 is primarily responsible for maintaining low extracellular glycine concentrations, whereas GlyT2 is needed for the reuptake of glycine into inhibitory nerve terminals [3]. Notably, some of the mice with glial GlyT1 deficiency survived until adulthood without developing neuromotor symptoms, although GlyT1 expression was efficiently diminished, suggesting that the dependence of CNS function on GlyT1 changes during postnatal development. This view is in line with studies indicating considerable changes in GlyT expression during early postnatal development using Northern blotting [10,11] or immunohistochemistry [11–13]. Quantitative data on the developmental alterations in GlyT protein expression and transport activities, however, are not available. Here we present an analysis of the GlyT1- and GlyT2-mediated glycine uptake activities in membrane preparations of the brain regions with the highest density of glycinergic synapses, i.e. brain stem and spinal cord, during postnatal development and correlate these data with the relative changes in protein expression levels as determined by Western blotting. Our results show that at birth GlyT1 is the prevalent GlyT isoform that is responsible for the major fraction of GlyT-mediated glycine uptake. At later postnatal stages, however, GlyT1 and GlyT2 are both highly expressed and contribute to similar extents to total GlyT activity.

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## 2. Materials and methods

### 2.1. Electrophysiology

*Xenopus laevis* oocytes were isolated as described [14] and injected with 20 ng of cRNA encoding mouse GlyT1 or GlyT2, or left uninjected, and incubated at 18 °C for 3 days in standard ND96 (containing in mM: NaCl 96, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1, HEPES/NaOH 5, and 50 μg/ml gentamycin; pH 7.4). Two-electrode voltage-clamp (TEVC) recordings from oocytes were performed using a Turbo TEC-03X amplifier and CellWorks (V3.7) data acquisition software (all from npi, Göttingen, Germany), with the membrane potential being held at −50 mV. Typically, glycine was applied for 30 s at the indicated concentrations. For determining effective inhibitor concentrations, GlyT expressing oocytes were superfused for 30 s with 20 μM glycine, followed by 5 min superfusion with the respective inhibitor at the concentration indicated. Residual GlyT activity was determined by a second application of glycine (20 μM) in the presence of the inhibitor. Current amplitudes are presented as relative substrate-induced currents, with the respective maximal glycine-induced current being defined as 100%, and are means ± SEM from 6 to 11 recordings using at least three different oocyte preparations.

### 2.2. Preparation of membrane fractions

C57BL/6J mice were obtained from Charles River Laboratories, Germany. To obtain tissue at prenatal developmental stages, pregnant females at the indicated day of pregnancy were killed under isoflurane anesthesia, with the morning of positive plug-check being designated as embryonic day 0.5 (E0.5). Embryos were quickly removed from the uterus and stored on ice. For later developmental stages, the day of birth was defined as postnatal day 0 (P0). Mice were killed by rapid decapitation, brains and spinal cords were quickly removed and kept on ice. After dissection, spinal cord and brain stem membrane fractions were prepared as described previously [8].

### 2.3. [$^3$H]Glycine uptake

Aliquots of the brain stem or spinal cord membrane fractions (20 μl, equivalent to 30–50 μg of protein) were mixed with 80 μl of Krebs–Henseleit Medium (KHM; containing in mM: NaCl 125; KCl 5; $CaCl_2$ 2.7, $MgSO_4$ 1.3, Glucose 10, HEPES/Tris 25, pH 7.4) and preincubated with or without 1 μM ALX 5407 (Tocris, Minneapolis, MN) and/or 300 nM ORG 25543 (Organon Laboratories Ltd., Cambridgeshire, UK) for 4 min at 37 °C. Uptake was initiated by the addition of 100 μl prewarmed KHM containing a mixture of 3.96 μM glycine and 0.04 μM [$^3$H]glycine (MovasekInstru Biochemicals, Brea, CA) and the respective inhibitors. After 1 min incubation with gentle agitation, uptake was terminated by diluting the incubation mixture with 3 ml of ice-cold KHM followed by rapid filtration through moistened cellulose acetate filters (Sartorius, Gottingen, Germany). Filters were rinsed twice with 5 ml of KHM and filter-bound radioactivity measured by scintillation counting (Beckmann Coulter, Krefeld, Germany). In all experiments, total [$^3$H]glycine uptake, GlyT1- and GlyT2-mediated uptake as well as total GlyT-mediated uptake were determined from respective triplicate assays and are presented as means ± SEM of at least four independent experiments.

### 2.4. Western blot analysis

P2 membrane fractions (35 μg protein/lane) were analyzed by SDS–PAGE on 8% gels followed by Western blotting using antibodies against GlyT2 (rabbit, 1:2000, [7]), GlyT1 (rabbit, 1:1000, [9]), vesicular inhibitory amino acid transporter (VGAT, rabbit, 1:1000, Synaptic Systems, Göttingen, Germany), the alpha subunits of the GlyR (GlyRα, mouse, mAb4, 1:200, Synaptic Systems) and anti-glucose-regulated-protein 75 (GRP75), (mouse, JG1 2799, 1:1000, Abcam, Cambridge, U.K). Bound antibodies were visualized by the ECL system (Pierce ECL Western-blotting detection reagent, Rockford, IL, USA). For quantification, films were digitalized by scanning, and integrated band intensities were determined by densitometry using the ImageJ software (http://rsbweb.nih.gov/ij/). Results are presented as ratios of GlyTs, GlyR or VGAT immunreactivity to GRP75 immunoreactivities after normalization to parallel values obtained with samples from adult animal and are means ± SEM from at least four independent experiments.

## 3. Results and discussion

### 3.1. Selective inhibition of GlyT1- and GlyT2-mediated glycine uptake

In this study, we analyzed developmental changes in the transport activities and protein expression profiles of GlyT1 and GlyT2 in membrane fractions from mouse brain stem and spinal cord, i.e. regions that are known to be rich in glycinergic synapses [1]. In order to pharmacologically discriminate GlyT1- and GlyT2-mediated uptake activities, TEVC recordings were performed on *X. laevis* oocytes injected with mouse GlyT1 or GlyT2 cRNA in the absence and/or presence of GlyT-specific inhibitors, ALX 5407 [15] and ORG 25543 [16]. Glycine-induced currents were readily detected in GlyT expressing oocytes with $EC_{50}$ values of 18 ± 2 μM and 13 ± 1 μM for GlyT1 and GlyT2, respectively (Fig. 1A). Non-injected oocytes did not produce any glycine-induced currents (data not shown). Subsequently the efficacies and specificities of the GlyT-specific inhibitors were determined at a glycine concentration of 20 μM. ALX 5407 inhibited glycine-induced currents with an $IC_{50}$ of 90 ± 6 nM, whereas GlyT2-mediated currents were unaffected even at concentrations up to 10 μM (Fig. 1B). The affinity of ALX 5407 for mouse GlyT1 determined here is lower as reported for the human GlyT1 [15], possibly reflecting species differences and/or a lower biological activity of the batch used here. Inversely, ORG 25543 efficiently inhibited GlyT2-mediated uptake with an $IC_{50}$ of 13 ± 4 nM, whilst GlyT1-mediated uptake was not inhibited up to concentrations of 1 μM (Fig. 1C). Taken together, these data confirm for mouse that GlyT1- and GlyT2-mediated uptake activities are reliably distinguished by the GlyT inhibitors ALX 5407 and ORG 25543, which at 1 μM and 300 nM, respectively, inhibit exclusively either GlyT1 or GlyT2.

### 3.2. GlyT1 and GlyT2 uptake activities during postnatal development

In order to determine how GlyT1 and GlyT2 contribute to glycine uptake at different stages of mammalian postnatal development, we performed [$^3$H]glycine uptake assays with membrane fractions prepared from mouse spinal cord or brain stem in the absence or presence of 1 μM ALX 5407 or/and 300 nM ORG 25543. The contributions of the individual GlyTs to total [$^3$H]glycine uptake were defined as fractions of total uptake sensitive to ALX 5407 for GlyT1, to ORG 25543 for GlyT2, and to both inhibitors for total GlyT-mediated transport, respectively. In all experiments performed, the GlyT-mediated uptake, as defined by the fraction of total uptake inhibited by the combination of ALX 5407 and ORG 25543, was comparable to the sum of the GlyT1- and GlyT2-specific uptake activities determined in the presence of the individual inhibitors alone (Table 1). The residual glycine uptake activity observed in the presence of both inhibitors was

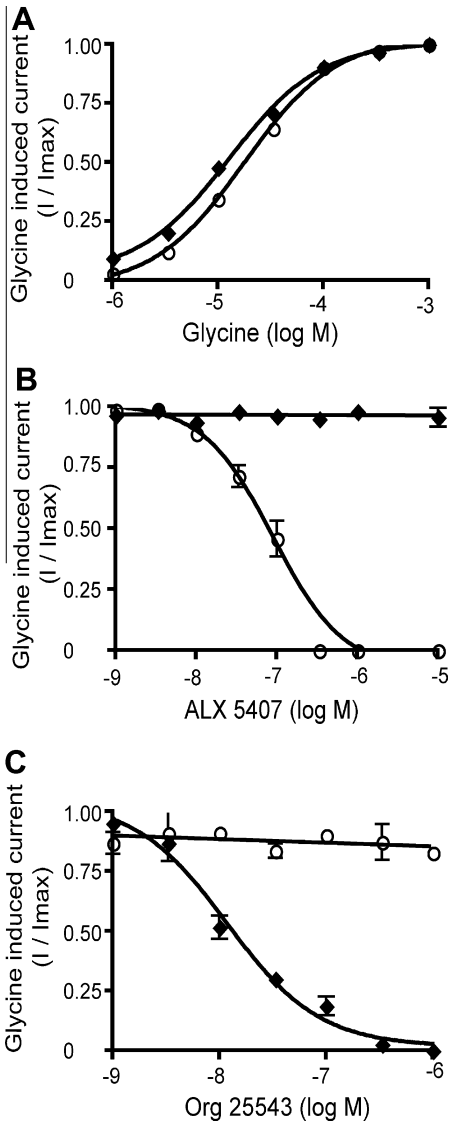


**Fig. 1.** Inhibition of recombinant GlyT1 and GlyT2 by the GlyT inhibitors ALX 5407 and ORG 25543. (A) Glycine dose-response curves recorded from *Xenopus laevis* oocytes expressing mouse GlyT1 (open circles) or GlyT2 (black squares), respectively. Currents were normalized to the maximal glycine-induced current. (B and C), ALX 5407 and ORG 25543 inhibition curves determined on GlyT1 or GlyT2 expressing oocytes. Current amplitudes, evoked by application of 20 μM glycine after pre-incubation with the respective inhibitor, were normalized to the current elicited by 20 μM glycine prior to inhibitor treatment. Bars indicate SEM when larger than symbol ($n$ = 6–11).

defined as "unspecific" and likely reflects the presence of other uptake systems, like system A or system N [17,18].

In membrane fractions from spinal cord, high GlyT1-specific transport activity was observed already at P0 which increased further in P5 membrane fractions, consistent with a postnatal upregulation of GlyT1 expression (Table 1). In membranes from older animals (P10-adult), however, the apparent specific uptake activity of GlyT1 decreased again, with the lowest GlyT1-specific uptake value being found in membranes derived from adult spinal cord (Table 1). GlyT2-specific uptake also was readily detectable in P0 spinal cord membrane fractions, although its activity was lower as compared to that of GlyT1 (Table 1). At the subsequent stages analyzed (P5–P15), GlyT2-specific uptake increased with age. In membrane preparations isolated from adult spinal cord, however, the lowest GlyT2-specific uptake activity was found. Comparison of the relative contributions of GlyT1 and GlyT2 to the total GlyT-mediated glycine uptake into spinal cord membranes revealed that at P0 GlyT1 accounted for about 60% and at P5 > 65% of the total GlyT-mediated uptake (Suppl. Fig. S1A). However, from P10 onwards the relative contribution of GlyT1 to total transport activity declined, while the fraction of GlyT2-specific glycine uptake increased with both transporters contributing about equally to GlyT-mediated transport in membrane fractions from adult spinal cord (Suppl. Fig. S1A).

Parallel [$^3$H]glycine uptake experiments with membrane fractions from brain stem similarly disclosed significant GlyT1-mediated transport already at P0 although the specific transport activity was much lower than that observed in the age-matched spinal cord fractions (Table 1). After P0, specific-GlyT1 uptake activities in the brain stem membranes decreased steadily, with the lowest activity being observed in membrane fractions from adult mice. On the contrary, GlyT2-mediated [$^3$H]glycine uptake was rather low in P0 brainstem membrane fractions but increased substantially upon further development, with a peak value being observed at P15 (Table 1). In membrane fractions derived from P0 and P5 brain stem, the differences between GlyT1- and GlyT2-mediated transport activities were even more pronounced than in spinal cord. Here GlyT1 contributed roughly 60–70% of the total GlyT uptake activity (Suppl. Fig. S1B). In samples from older animals, however, increased GlyT2 activity resulted in similar contributions of both GlyTs to the total GlyT-mediated glycine uptake activity. Taken together, these results indicate that during postnatal development the relative contributions of GlyT1 and GlyT2 to the total GlyT-driven glycine uptake change more markedly in brain stem than in spinal cord, most likely due to differences in the temporal maturation of both brain regions.

The prevalence of GlyT1-mediated glycine uptake during early postnatal development is consistent with data obtained from GlyT1-deficient mice that display already at birth a strong hypotonic phenotype due to an over-activation of GlyRs [8]. Similarly, glial GlyT1 deficiency produced a comparable phenotype in most of the animals, and hypotonia was always seen prior to P7 [9], i.e. at time-points where most of the GlyT-mediated uptake is carried by GlyT1. After P7, the loss of glial GlyT1 did not produce any symptoms, consistent with compensation by GlyT2. Furthermore, the increase in GlyT2 activity seen in both brain regions between P5 and P15 (Table 1, see also Suppl. Fig. S1) nicely coincides with the appearance of the first symptoms seen in GlyT2 deficient mice, which develop a strong disinhibition phenotype only around P10 [10].

### 3.3. Accumulation of GlyT1 and GlyT2 proteins during development

To investigate whether the changes in GlyT transport activities seen in the uptake experiments correlate with changes in GlyT1 and GlyT2 protein levels, the membrane preparations used for the uptake experiments were subjected to Western blot analyzes. Additionally, membrane fractions were prepared from E12.5 and E15.5 spinal cord, and E15.5 brain stem, respectively. The limited amounts of membrane protein obtained at these embryonic stages precluded their use for glycine uptake measurements. Fig. 2A shows that although similar amount of protein were loaded as indicated by blotting with an antibody against the mitochondrial marker protein GRP75 [19], marked differences in GlyT1 and GlyT2 immunoreactivities were found between membrane fractions isolated at different developmental stages. In spinal cord membranes, GlyT1 immunoreactivity was detected already at E15.5, although a stained band of about 70 kDa [20] was only just visible. Thereafter the intensity of the GlyT1 band increased until P5 followed by a decrease at P10 where the intensity of the GlyT1 immunoreactive band was similar to that observed in samples from adult spinal cord (Fig. 2A, C). In contrast to GlyT1, no GlyT2 immunoreactivity

**Table 1**
Total, GlyT1, GlyT2, total GlyT and non-GlyT mediated [$^3$H]glycine uptake into spinal cord and brain stem membrane fractions prepared at different developmental stages.

| CNS region | Developmental stage | Total uptake (pmol/min/mg protein) | GlyT1-mediated uptake (pmol/min/mg protein) | GlyT2-mediated uptake (pmol/min/mg protein) | Total GlyT-mediated uptake (pmol/min/mg protein) | Non-GlyT-mediated uptake (pmol/min/mg protein) |
|---|---|---|---|---|---|---|
| Spinal cord | P0 | 67.8 ± 5.0 | 24.9 ± 6.6 | 15.9 ± 4.0 | 39.0 ± 6.3 | 28.8 ± 1,4 |
| | P5 | 103.1 ± 11.3 | 32.9 ± 5.2 | 16.4 ± 3.0 | 52.1 ± 6.5 | 51.1 ± 7.7 |
| | P10 | 52.6 ± 3.0 | 17.9 ± 2.1 | 19.3 ± 3.0 | 35.2 ± 3.6 | 17.4 ± 2.0 |
| | P15 | 47.2 ± 4.6 | 11.2 ± 2.2 | 22.2 ± 3.7 | 33.0 ± 3.7 | 14.2 ± 1.4 |
| | Adult | 13.8 ± 2.2 | 5.6 ± 1.2 | 4.8 ± 0.7 | 7.6 ± 1.0 | 6.2 ± 1.5 |
| Brain stem | P0 | 43.6 ± 4.2 | 16.5 ± 5.3 | 7.5 ± 3.0 | 29.5 ± 2.5 | 14.1 ± 2.9 |
| | P5 | 50.2 ± 5.6 | 14.7 ± 3.0 | 8.0 ± 3.2 | 24.6 ± 4.6 | 25.6 ± 1.7 |
| | P10 | 34.0 ± 2.3 | 12.4 ± 1.5 | 8.7 ± 1.6 | 19.8 ± 2.2 | 14.2 ± 1.8 |
| | P15 | 39.0 ± 3.4 | 12.7 ± 1.6 | 15.3 ± 2.0 | 25.9 ± 2.5 | 13.2 ± 1.0 |
| | Adult | 13.1 ± 2.1 | 4.1 ± 1.0 | 3.8 ± 0.5 | 7.5 ± 1.0 | 5.6 ± 1.2 |

GlyT1- and GlyT2-mediated [$^3$H]glycine uptake were determined as fractions of the total [$^3$H]glycine uptake sensitive to 1 μM ALX 5407 or 300 nM Org 25543, respectively. Total GlyT mediated uptake was determined as the fraction of the total uptake sensitive to a combination of both inhibitors, whereas the non-GlyT-mediated uptake was defined as the inhibitor resistant fraction of [$^3$H]glycine uptake. Values represent means ± SEM; $n$ = 3–6.

was found in spinal cord membranes prepared from embryonic tissue. A faint GlyT2 immunoreactive band running at ca. 100 kDa [20] was detected first only at P0 (Fig. 2A, C). At subsequent postnatal stages, the intensity of the GlyT2 immunoreactive bands increased, reaching a maximal level at P10 that was maintained until adulthood (Fig. 2A, C).

In brain stem membrane fractions, GlyT1 immunoreactivity was found already at E15.5 (Fig. 2B). Albeit being lower as compared to that in age-matched spinal cord membranes, GlyT1 immunoreactivity increased in membrane fractions isolated from P0 animals (Fig. 2B, D) and plateaued at P5 until adulthood. Similar to our findings with spinal cord samples, an anti-GlyT2 reactive band was found only postnatally in brain stem membranes, with rather weak GlyT2 immunoreactivity being observed at P0 (Fig. 2B). The intensity of the GlyT2 band increased markedly at P5 and P10 and then remained constant up to adult stages (Fig. 2B, D).

The data presented here are consistent with previous studies in rats showing that the GlyT1 mRNA accumulates earlier than that of GlyT2 but contrasts Western blot results showing GlyT2 immunoreactivity already in embryonic tissue before GlyT1 immunoreactivity is detected [11]. Although species differences cannot be ruled out, these discrepancies most likely result from differences in antibody specificity and sensitivity. Here, comparison of the GlyT transport activities and protein expression profiles revealed a good correlation during early postnatal development (P0–P10). However, in membrane preparations from adult animals, the activities of both GlyTs appear to decrease, although the protein levels of both GlyT1 and GlyT2 do not change much after P10. The

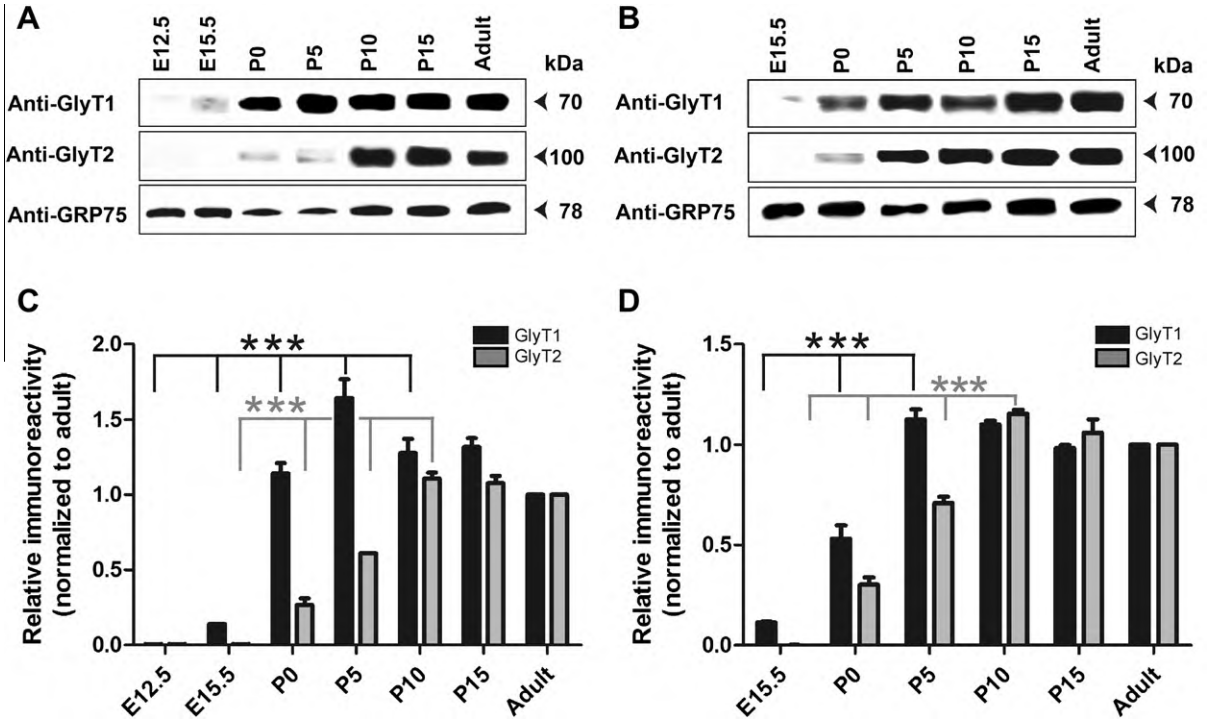


**Fig. 2.** Membrane fractions isolated from spinal cord (A) and brain stem (B) at the indicated ages were subjected to Western blot analysis with antibodies against GlyT1, GlyT2, and GRP75. (C and D) Densitometric analysis of GlyT1 and GlyT2 immunoreactive bands. The ratios of GlyT1, GlyT2 immunoreactivities normalized to GRP75 are shown as fractions of the band intensities observed in samples from adult spinal cord and brain stem membrane preparations. All values represent means ± SEM ($n$ = 3). *** $p < 0.01$ (one-way ANOVA followed by Bonferroni's Multiple Comparison Test).

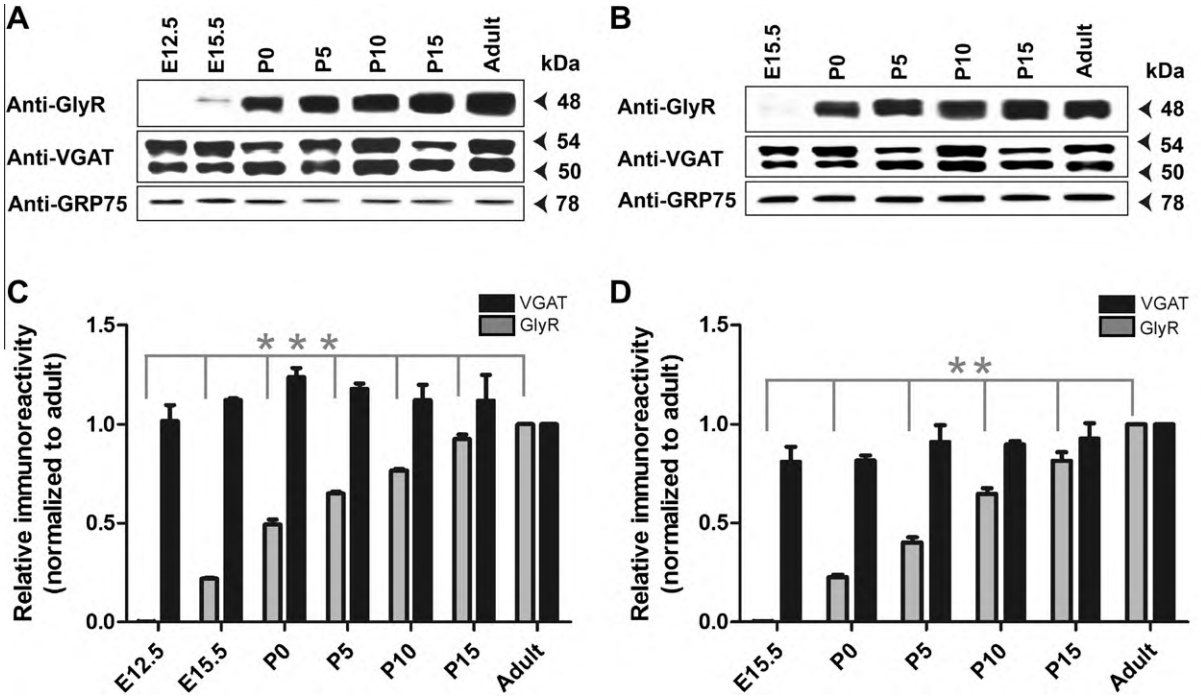


**Fig. 3.** Membrane fractions isolated from spinal cord (A) and brain stem (B) at the indicated ages were subjected to Western-Blot analysis with the indicated antibodies. (C and D) Densitometric analysis of GlyRα and VGAT immunoreactive bands. The ratios of GlyRα, VGAT immunoreactivities normalized to GRP75 are presented as fractions of the band intensities observed in samples from adult membrane preparations. All values represent means ± SEM ($n = 3$). *** $p < 0.01$; ** $p < 0.05$ (one-way ANOVA followed by Bonferroni's Multiple Comparison Test).

apparent decrease in GlyT uptake activities might reflect increases in the intracellular fractions of the transporters. In synaptosomal preparations from adult rat spinal cord, it has been reported that only a minor fraction of GlyT2 resides in the plasma membrane, and that this fraction is dynamically regulated [21]. Furthermore, changes in the composition of the membrane preparations might affect apparent GlyT activities; in particular the comparatively high content of myelinated axons in mature CNS tissue might cause a dilution of the neuronal plasma membrane fragments or/and affect average vesicle size in membrane preparations isolated at adult stages, and thus reduce the activity or/and capacity of glycine uptake.

To examine whether the expression of the GlyTs correlates with inhibitory synapse formation, Western blots of both spinal cord and brain stem membranes were re-probed with antibodies against the inhibitory synapse marker proteins VGAT and an antibody (mAb4a) recognizing all postsynaptic GlyRα-subunits (Fig. 3). In both brain regions, similar VGAT immunoreactivities were observed at all developmental stages analyzed, consistent with previous findings describing VGAT as an early marker of inhibitory interneuron neurogenesis [22] (Fig. 3A, B). In contrast, GlyR immunoreactivity was first detected only in membranes prepared at E15.5 from spinal cord, and at P0 from brain stem. In both CNS regions, intense GlyRα immunoreactivity was seen at P0 and/or thereafter, showing that the onset of GlyT1 expression parallels that of GlyRα, whereas the expression of GlyT2 starts only later (compare Figs. 2 and 3A, B). This is in agreement with the idea that only GlyT1 but not GlyT2 is essential for the regulation of glycinergic transmission at birth and during early postnatal development [8]. At later developmental stages, GlyRα immunoreactivity increased further, with the strongest signals being observed in samples from adult mice. This is paralleled by a strong up-regulation of GlyT2 activity and expression during the second postnatal week. Interestingly, this up-regulation of GlyT2 coincides with the replacement of the embryonic GlyRα2 by the adult GlyRα1 isoform (between P7 and P14) [23]. Moreover, in brain stem and spinal cord, the activities of the chloride co-transporters KCC2 and NKCC1 have been shown to also change during this time window, resulting in a lowering of intracellular $Cl^-$ concentrations [24,25]. These ionic changes are thought to be a prerequisite for the inhibitory nature of glycinergic and GABAergic neurotransmission characteristic of the mature mammalian CNS [26], thus putting the changes in GlyT2 expression and activity described here into the context of major functional changes in glycinergic neurotransmission.

Taken together, our data demonstrate that in the neonatal mouse spinal cord and brain stem GlyT1 is the transporter responsible for the majority of GlyT activity. At later developmental stages, GlyT2 expression and uptake increase significantly, and in mature brain stem and spinal cord membranes, both transporters contribute similarly to glycine uptake. This is consistent with synergistic and possibly redundant functions of both GlyTs in the adult animal. Further experiments will be required to determine how the changes in the expression and activity of the individual GlyTs contribute to the regulation of glycinergic neurotransmission.

## Acknowledgments

We thank Prof. Dr. C.M. Becker for his continuous support, Prof. Dr. C. Korbmacher for providing *Xenopus laevis* oocytes and Organon Ltd., Cambridgeshire, UK, for generously supplying ORG 25543. This work was supported by the Max-Planck-Gesellschaft (MPG) and grants from Deutsche Forschungsgemeinschaft (DFG) and Interdisziplinäres Zentrum für Klinische Forschung (IZKF), Universitätsklinikum, Erlangen.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.007.

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