Biochemical and Biophysical Research Communications 423 (2012) 661–666

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Deepti Lall [a,b], Anja Armbruster [b], Karelia Ruffert [a,1], Heinrich Betz [a,*], Volker Eulenburg [a,b,*]

[a] Department of Neurochemistry, Max-Planck Institute for Brain Research, Frankfurt 60528, Germany
[b] Institute for Biochemistry and Molecular Medicine, University Erlangen-Nürnberg, Erlangen 91054, Germany

ARTICLE INFO

Article history:
Received 29 May 2012
Available online 10 June 2012

Keywords:
Glycinergic neurotransmission
Glycine transporters
Spinal cord
Brain stem
Glycine uptake activity
Expression

ABSTRACT

Glycine serves as a neurotransmitter in spinal cord and brain stem, where it activates inhibitory glycine receptors. In addition, it serves as an essential co-agonist of excitatory N-methyl-D-aspartate receptors. In the central nervous system, extracellular glycine concentrations are regulated by two specific glycine transporters (GlyTs), GlyT1 and GlyT2. Here, we determined the relative transport activities and protein levels of GlyT1 and GlyT2 in membrane preparations from mouse brain stem and spinal cord at different developmental stages. We report that early postnatally (up to postnatal day P5) GlyT1 is the predominant transporter isoform responsible for a major fraction of the GlyT-mediated [^{3}H]glycine uptake. At later stages (⩾P10), however, the transport activity and expression of GlyT2 increases, and in membrane fractions from adult mice both GlyTs contribute about equally to glycine uptake. These alterations in the activities and expression profiles of the GlyTs suggest that the contributions of GlyT1 and GlyT2 to the regulation of extracellular glycine concentrations at glycinergic synapses changes during development.

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.

* Corresponding authors. Addresses: Max-Planck Institute for Medical Research, Jahnstrasse 29, Heidelberg 69120, Germany (H. Betz); Institute for Biochemistry and Molecular Medicine, University Erlangen-Nürnberg, Fahrstrasse 17, Erlangen 91054, Germany (V. Eulenburg).

E-mail addresses: Heinrich.Betz@mpimf-heidelberg.mpg.de (H. Betz), Volker.Eulenburg@biochem.uni-erlangen.de (V. Eulenburg).

[1] Present address: Faculté des Sciences Pharmaceutiques et Biologiques, Université Paris Descartes, Paris 75006, France.

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.06.007

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