Glial glycine transporter 1 function is essential for early postnatal survival but dispensable in adult mice

Volker Eulenburg, Marina Retiounskaia, Theofilos Papadopoulos, Jesús Gomeza, Heinrich Betz

Angaben zur Veröffentlichung / Publication details:

Eulenburg, Volker, Marina Retiounskaia, Theofilos Papadopoulos, Jesús Gomeza, and Heinrich Betz. 2010. "Glial glycine transporter 1 function is essential for early postnatal survival but dispensable in adult mice." *Glia* 58 (9): 1066–73. https://doi.org/10.1002/glia.20987.

Glial Glycine Transporter 1 Function Is Essential for Early Postnatal Survival but Dispensable in Adult Mice

VOLKER EULENBURG,[1]* MARINA RETIOUNSKAIA,[1] THEOFILOS PAPADOPOULOS,[1] JESÚS GOMEZA,[2] AND HEINRICH BETZ[1]
[1]*Department for Neurochemistry, Max-Planck Institute for Brain Research, Deutschordenstrasse 46, 60529 Frankfurt, Germany*
[2]*Institute for Pharmaceutical Biology, University of Bonn, Nussallee 6, 53115 Bonn, Germany*

KEY WORDS
glycine transporter 1; glia; inhibitory neurotransmission; brain stem; spinal cord; synapse function

ABSTRACT
The glycine transporter 1 (GlyT1) is expressed in astrocytes and selected neurons of the mammalian CNS. In newborn mice, GlyT1 is crucial for efficient termination of glycine-mediated inhibitory neurotransmission. Furthermore, GlyT1 has been implicated in the regulation of excitatory N-methyl-D-asparate (NMDA) receptors. To evaluate whether glial and neuronal GlyT1 have distinct roles at inhibitory synapses, we inactivated the GlyT1 gene cell type-specifically using mice carrying floxed GlyT1 alleles $GlyT1^{(+)/+)}$. $GlyT1^{(+)/(+)}$ mice expressing Cre recombinase in glial cells developed severe neuromotor deficits during the first postnatal week, which mimicked the phenotype of conventional GlyT1 knock-out mice and are consistent with glycinergic over-inhibition. In contrast, Cre-mediated inactivation of the GlyT1 gene in neuronal cells did not result in detectable motor impairment. Notably, some animals deficient for glial GlyT1 survived the first postnatal week and did not develop neuromotor deficits throughout adulthood, although GlyT1 expression was efficiently reduced. Thus, glial GlyT1 is critical for the regulation of glycine levels at inhibitory synapses only during early postnatal life.

INTRODUCTION

In caudal regions of the central nervous system (CNS), the amino acid glycine serves as a major inhibitory neurotransmitter by activating strychnine-sensitive glycine receptors (GlyR; see Betz et al., 2000). In addition, glycine is an essential co-agonist of excitatory *N*-methyl-D-aspartate (NMDA) receptors (Johnson and Ascher, 1987). At glycinergic synapses, presynaptically released glycine is rapidly removed by two Na^+/Cl^--dependent glycine transporters, GlyT1 and GlyT2, thus allowing neurotransmission to proceed with high temporal and spatial resolution (Gomeza et al., 2006). GlyT2 is localized in glycinergic nerve terminals, whereas GlyT1 is predominantly expressed by astrocytes (Zafra et al., 1995a,b). In addition, GlyT1 immunoreactivity has been found in retinal, forebrain, and also spinal cord neurons (Cubelos et al., 2005; Pow and Hendrickson, 1999).

Gene inactivation studies in mice have shown that both GlyTs are essential for glycinergic inhibition in the early postnatal CNS. In GlyT1 knock-out (KO) mice, accumulation of glycine in the synaptic cleft results in over-activation of postsynaptic GlyRs and death of the newborn animals due to respiratory and feeding problems (Gomeza et al., 2003a). In contrast, inactivation of both GlyT2 alleles leads to a reduction of presynaptic glycine release and consequently disinhibition of postsynaptic cells, with lethal convulsions developing during the second postnatal week (Gomeza et al., 2003b). Hence, both GlyTs are thought to contribute to glycinergic synapse function in complementary fashions: GlyT2 driven glycine uptake provides for efficient refilling of synaptic vesicles, whereas GlyT1 removes released glycine from the synaptic cleft, thereby accelerating the termination of inhibitory neurotransmission (Gomeza et al., 2003a,b).

The consequences of GlyT1 deletion described above have been attributed to a loss of glial glycine uptake. However, whether neuronal GlyT1 also contributes to inhibitory neurotransmission has not been established. At excitatory synapses in forebrain, conditional inactivation of the GlyT1 gene in neurons (Singer et al., 2007; Yee et al., 2006) has been found to result in increased NMDA receptor currents and significant behavioral changes consistent with altered glutamatergic neurotransmission. Here, we addressed the functions of GlyT1 in neurons and astrocytes by a conditional approach using the Cre/LoxP system and mouse lines expressing Cre recombinase exclusively in either glial or neuronal cells within caudal regions of the rodent CNS. Our data indicate that neuronal GlyT1 does not detectably contribute to the modulation of glycinergic neurotransmission, whereas glial GlyT1 expression is vital during early postnatal development but appears to be dispensable in the adult organism.

Additional Supporting Information may be found in the online version of this article.

Volker Eulenburg is currently at: Institute for Biochemistry and Molecular Medicine, Friedrich-Alexander University Erlangen-Nürnberg, Fahrstrasse 17, 91054 Erlangen, Germany

Grant sponsor: Fond der chemischen Industrie; Grant sponsor: ?Max-Planck Gesellschaft, Deutsche Forschungsgemeinschaft; Grant numbers: ?EU110/1-1, 3, BE718/15-2.

*Correspondence to: V. Eulenburg, Institute for Biochemistry and Molecular Medicine, Friedrich-Alexander University Erlangen-Nürnberg, Fahrstrasse 17, 91054 Erlangen, Germany. E-mail: Volker.Eulenburg@biochem.uni-erlangen.de

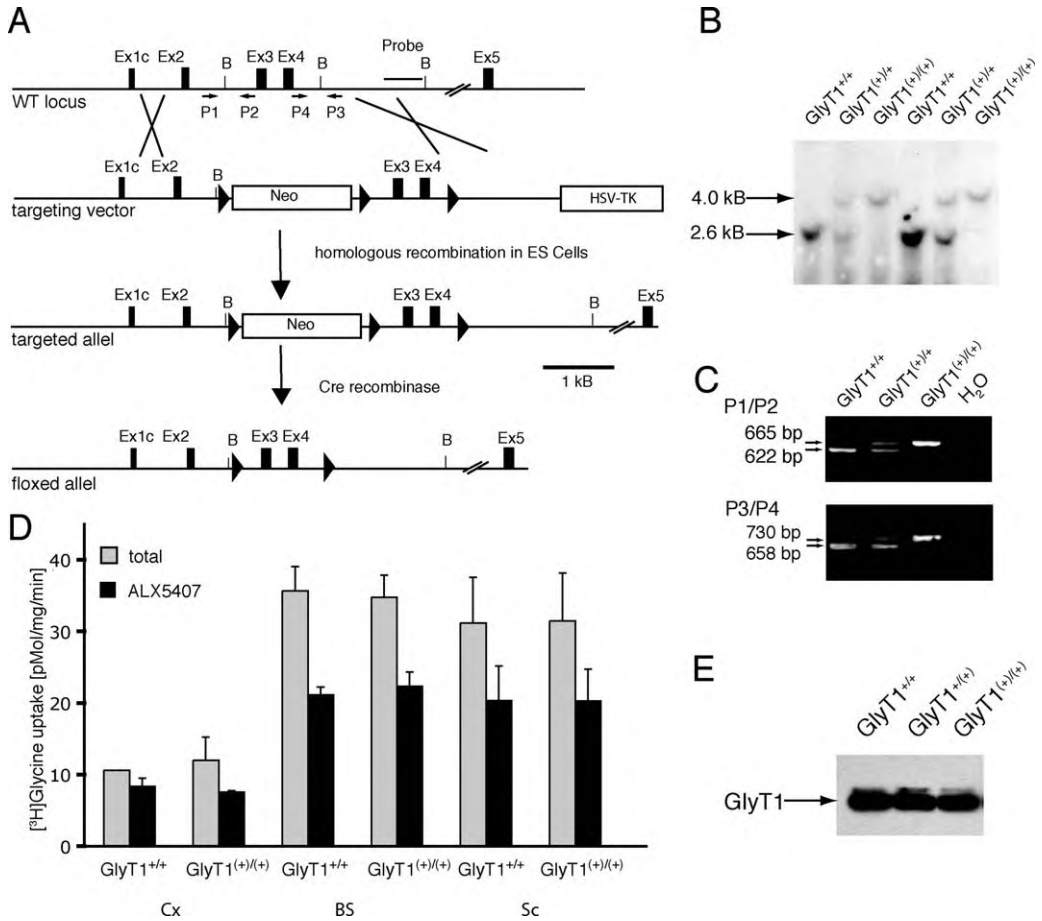

Fig. 1. Generation of mice with a floxed GlyT1 allel. **A**: Schematic representation of the targeting strategy showing the wt GlyT1 gene, the targeting vector, the targeted locus and the recombined allele. Exons are indicated as black boxes, and the neomycin resistance cassette (Neo) and the Herpes simplex thymidine kinase cassette (HSV-TK) as white boxes. The hybridization sites of the probe used for Southern blot analysis and of the primers used for genotyping (arrows P1-4) as well as BamH1 restriction sites (**B**) are shown. B: Southern blot analysis of BamH1-digested tail DNA from $GlyT1^{+/+}$, $GlyT1^{+/(+)}$, and $GlyT1^{(+)/(+)}$ mice. The 2.6 kB and 4.0 kB bands correspond to the wt and floxed GlyT1 alleles, respectively. **C**: PCR-based genotyping of tail DNA from mice of the indicated genotypes. Amplicons of 622 bp or 658 bp correspond to the wt and 665 bp or 730 to the floxed allele for the primer combination P1/P2 or P3/P4, respectively. **D**: [^{3}H]Glycine uptake into P2 membrane preparations from cortex (Cx), brain stem (BS), and spinal cord (SC) of adult $GlyT1^{+/+}$ and $GlyT1^{(+)/(+)}$ mice in the absence (total) or presence of the GlyT1 inhibitor ALX5407 (ALX5407). **E**: Western blot analysis of crude membrane preparations derived from spinal cord using a GlyT1-specific antibody. Note that GlyT1 protein expression in homozygous $GlyT1^{(+)/(+)}$ mice is similar to that seen in heterozygous and $GlyT1^{+/+}$ animals.

MATERIALS AND METHODS

Generation of Conditional GlyT1 Deficient Mice

Genomic fragments of the mouse GlyT1 gene were obtained from a BAC clone containing the first five exons of the GlyT1 gene. These genomic fragments were used to generate a replacement targeting vector in pEasyFlox (kindly provided by Dr. K. Rajewsky, University of Cologne, Germany), in which a 5 kB fragment that contained exons 1C and 2 derived from a BamH1 digest of the BAC DNA was used as a long arm, and a 1.5 kB BamH1 XbaI fragment 5′ of exon 4 as a short arm, respectively. The neomycine resistance cassette as well as exons 3 and 4 were flanked by three LoxP sites (see Fig. 1A). This targeting vector was linearized by a unique Not I site and introduced into Sv129Ola mouse embryonic stem cells (E14.1) by electroporation. Successfully targeted embryonic stem cell clones were identified by double selection with G418 and 2′deoxy-2′fluoro-β-D-arabinofuranosyl-5-iodouracil (FIAU). Totally, 400 surviving clones were isolated and screened by PCR and Southern blot analysis using a 5′ external probe to demonstrate proper integration. Five clones were identified that contained a properly targeted GlyT1 allele (data not shown). Two of these clones were transiently transfected with a plasmid encoding Cre recombinase (kindly provided by Dr. K. Rajewsky). After 48 h, the cells were seeded at low density, and individual clones were isolated six days later. Clones that had lost the neomycine selection cassette but contained the floxed exons 3 and 4 were identified by PCR and Southern blotting (data not shown). Two of these clones were selected, expanded, and used for blastocyte injection.

[^{3}H]Glycine Uptake Assays

Crude P2 membrane fractions were prepared from different CNS regions as described previously (Gomeza et al., 2003a). 20 μl aliquots of the membrane fractions

(30–50 μg total protein) were mixed with 80 μl prewarmed Krebs-Henseleit buffer (KHB; in mM: 125 NaCl; 2.7 $CaCl_2$; 1.3 $MgCl_2$, 25 HEPES/Tris, pH 7.4) containing either 12.5 μM of the GlyT1 inhibitor ALX5407 dissolved in DMSO, or DMSO alone (final concentration <0.1%), and incubated for 4 min at 37°C. Then 100 μL uptake solution containing 4 μM glycine and 100 nM [^{3}H]glycine with or without 10 μM ALX5407 were added. After 1 min, uptake was stopped by the addition of 5 mL ice-cold KHB, and membranes were separated by rapid filtration onto cellulose acetate filters (0.45 μM, Whatman) followed by two washes with 5 mL ice-cold KHB, each. [^{3}H]Glycine radioactivity was determined in a liquid scintillation counter (Beckmann Coulter). GlyT1-specific uptake corresponded to the ALX5407-sensitive fraction, and non-GlyT1 mediated uptake to the ALX5407-resistant fraction, of total uptake. Data represent means ± SD. Statistical significance was evaluated with the Student's *t*-test (*$P < 0.01$; **$P < 0.001$).

Immunohistochemistry

For immunohistochemical analysis, mice were killed by cervical dislocation; the brains were removed quickly and fixed overnight in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS; in mM: 138 NaCl, 2.7 KCl, 1.5 KH_2PO_4; 8 NaH_2PO_4). Then the tissue was cryoprotected in PBS containing 30% (w/v) sucrose. Cryosections (12 μM) were briefly fixed with 4% (w/v) paraformaldehyde in PBS, followed by incubation with blocking solution (2% (v/v) normal goat serum, 2% (w/v) bovine serum albumine, 0.3% (w/v) Triton X-100 in PBS). The sections were incubated overnight with the indicated antibodies (rabbit anti-EGFP, 1:500, Invitrogen; mouse anti-S100β, 1:2,000, Sigma; mouse anti-GFAP, 1:2,000, and mouse anti-NeuN, 1:1,000, both Millipore; all diluted in blocking solution). After washing, bound antibodies were visualized by incubation with fluorescently conjugated secondary antibodies (goat anti-rabbit Alexa-488 and goat anti-mouse Alexa-546, Invitrogen). Images were obtained using an AxioImager microscope equipped with an apotome (Zeiss, Goettingen, Germany) by collecting optical sections.

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 1:2,000, Gomeza et al., 2003a), vesicular inhibitory amino acid transporter (VIAAT, rabbit 1:2,000, Synaptic Systems), the α subunits of the GlyR (GlyRα; mouse, mAb4, 1:200, Synaptic Systems) and a newly generated antibody raised against a fusion protein of GST and the intracellular C-terminal domain of mouse GlyT1 (rabbit 1:2,000).

Behavioural Analysis

Motor behavior in the open field and rota-rod paradigms was examined as described previously (Papadopoulos et al., 2007).

RESULTS

Generation of a Conditional GlyT1 Mouse Line

To generate a mouse line allowing for conditional inactivation of the GlyT1 gene, we constructed the targeting vector shown in Fig. 1A (for details see Material and Methods). This vector was designed to enable Cre recombinase-mediated inactivation of the GlyT1 gene through deletion of exons 3 and 4, which encode the second and third transmembrane domains as well as the N-terminal region of the large second extracellular loop of the GlyT1 protein.

Two independent ES cell clones that carried a correctly targeted GlyT1 allele as revealed by PCR and Southern blot analysis (data not shown) were selected for blastocyte injection. Resulting male chimeras were mated with wild-type (wt) C57BL6J mice to establish germline transmission of the modified gene. F1 animals heterozygous for the conditional allele ($GlyT1^{+/(+)}$) were subsequently mated to obtain homozygous mice. Correct modification of the GlyT1 locus was confirmed by Southern blot analysis of genomic DNA isolated from tail biopsies (Fig. 1B) and by PCR-based genotyping using primers flanking each of the LoxP sites (Fig. 1C). Mice homozygous for the modified allele ($GlyT1^{(+)/(+)}$) were born at the expected Mendelian ratio, displayed normal body weights and lifespans, were fertile and did not show any motor deficits in the RotaRod and open field tests (data not shown).

To assess whether the engineered GlyT1 locus was fully functional, GlyT1 activity was analyzed by determining [^{3}H]glycine uptake in membrane fractions that were prepared from spinal cord, brain stem and cortex of both adult $GlyT1^{(+)/(+)}$ animals and their wt ($GlyT1^{+/+}$) littermates. GlyT1-specific uptake as well as the total [^{3}H]glycine uptake measured in membrane preparations from adult $GlyT1^{(+)/(+)}$ mice were indistinguishable from those obtained with membranes from $GlyT1^{+/+}$ animals (Fig. 1D). Additionally, Western blot analysis of the brain stem and spinal cord membrane preparations with a GlyT1-specific antibody revealed no differences in GlyT1 protein expression between $GlyT1^{(+)/(+)}$ mice and their wt littermates (Fig. 1E). These data indicate that the floxed GlyT1 allele was fully functional.

To show that the floxed GlyT1 allel is accessible to Cre-mediated inactivation, $GlyT1^{(+)/+}$ mice were mated with mice carrying a EIIa-Cre transgene allowing ubiquitous Cre recombination (Lakso et al., 1996). This resulted in efficient recombination of the modified GlyT1 gene (data not shown). After intercrossing of these mice, animals carrying the inactivated allele homozygously ($GlyT1^{(+)/(+)}$/EIIa-Cre) were obtained that displayed all hallmarks of the phenotype seen in conventional GlyT1 KO mice and died within 8 h after birth (see Supp. Info.

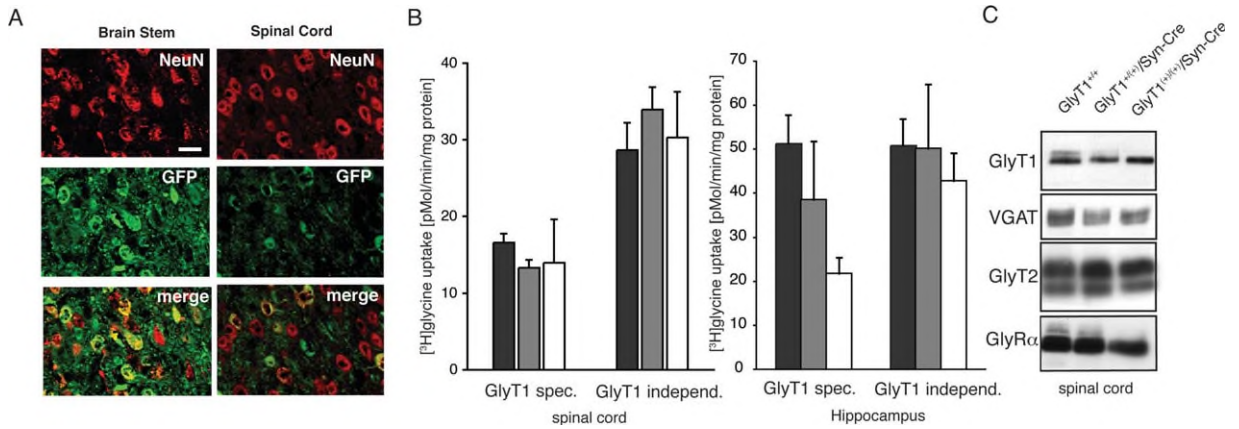

Fig. 2. Inactivation of the GlyT1 gene in neurons. **A**: Immunohistochemical analysis of brain stem and spinal cord sections derived from of Syn-Cre/ZEG double transgenic mice using GFP (green) and NeuN (red) specific antibodies. Note that only NeuN positive cells are labeled by the GFP antibody. Scale bar, 40 μm. **B**: GlyT1-specific and GlyT1-independent [^{3}H]glycine uptake in P2 membrane fractions prepared from spinal cord and hippocampus of GlyT1$^{+/+}$ (■), GlyT1$^{+/(+)}$/Syn-Cre (■), and GlyT1$^{(+)/(+)}$/Syn-Cre (□) mice. Data represent mean ± SD ($n \geq 6$ per genotype). **C**: Western blot analysis of P2 membrane fractions prepared from spinal cords or hippocampus of GlyT1$^{+/+}$, GlyT1$^{(+)/+}$/Syn-Cre and GlyT1$^{(+)/(+)}$/Syn-Cre mice using the indicated antibodies. Note lack of difference between genotypes in (B) and (C).

Fig. 1A). PCR-based analysis of DNA derived from tail biopsies of these mice revealed that in GlyT1$^{(+)/(+)}$/EIIa-Cre mice only the inactivated GlyT1 allele but not the floxed gene was present (Supp. Info. Fig. 1B). Thus, the LoxP sites introduced into the GlyT1 gene were accessible for Cre recombinase, thereby allowing efficient GlyT1 gene inactivation *in vivo*.

Neuronal GlyT1 Does Not Contribute to the Phenotype of GlyT1-Deficient Mice

To examine whether neuronal expression of GlyT1 may contribute to the phenotype seen in GlyT1 deficient mice, we used transgenic mice expressing Cre recombinase under the control of the synapsin 1 promoter, which has been reported to be neuron-specific (Zhu et al., 2001). On mating Synapsin-Cre (Syn-Cre) mice with ZEG reporter mice (Novak et al., 2000), the resulting double transgenic animals displayed neuronal GFP expression as monitored by colocalization of GFP and NeuN immunoreactivity in the majority of forebrain, brainstem and spinal cord neurons (data not shown, and Fig 2A; GFP in >60% of all NeuN-positive cells). This confirmed that in Syn-Cre mice recombinase expression is restricted to neurons.

Next, GlyT1$^{+/(+)}$ mice were mated with Synapsin-Cre mice to obtain animals positive for the GlyT1 floxed allele and Cre expression (GlyT1$^{+/(+)}$/Syn-Cre). Upon mating GlyT1$^{+/(+)}$/Syn-Cre with GlyT1$^{(+)/(+)}$ mice, animals with neuronally inactivated GlyT1 alleles (GlyT1$^{(+)/(+)}$/Syn-Cre) were obtained at the expected Mendelian ratio. GlyT1$^{(+)/(+)}$/Syn-Cre mice survived, were fertile and did not show any of the behavioural symptoms characteristic of GlyT1 deficient animals, such as hypotonia (Gomeza et al., 2003a). Also, no motor deficits were found in the open field and RotaRod tests (data not shown). [^{3}H]glycine uptake activities measured in membrane preparations from brain stem and spinal cord of GlyT1$^{(+)/(+)}$/Syn-Cre mice were indistinguishable from those obtained with membranes from GlyT1$^{+/+}$ animals. Both the GlyT1-specific fraction of [^{3}H]glycine uptake and the ALX5407-resistent fraction, which largely represents GlyT2-mediated uptake, were not altered (Fig 2B). In contrast, GlyT1-specific uptake was reduced by more than 50% in membrane preparation derived from hippocampus from GlyT1$^{(+)/(+)}$/Syn-Cre mice, as compared to samples from wt littermates. These data demonstrate that in neurons from GlyT1$^{(+)/(+)}$/Syn-Cre the GlyT1 gene was efficiently inactivated. In Western blots of P2 membrane fractions prepared from spinal cord of GlyT1$^{(+)/(+)}$/Syn-Cre mice, the intensity of the major GlyT1 immunoreactive band was not altered. Notably, however, a second band recognized by the GlyT1 antibody displaying an apparent molecular weight slightly higher than that of the major GlyT1 immunoreactive band was strongly decreased (Fig. 2*C*) In parallel immunoblot experiments with antibodies against VIAAT, GlyT2, and GlyRα, we found no upregulation of other components of glycinergic neurotransmission (Fig. 2C). We also analyzed membrane preparations from the hippocampus of these mice. In wild-type samples from this brain region, three distinct GlyT1 immunreactive bands were observed that were not seen when analyzing samples from GlyT1$^{(+)/(+)}$/Syn-Cre mice (V.E. unpublished data). This indicates that the expression of this fraction of GlyT1 protein was efficiently abolished upon neuron-specific GlyT1 gene inactivation. Taken together, these data indicate that in caudal regions of the CNS neuronal GlyT1 does not contribute significantly to the regulation of inhibitory glycinergic neurotransmission.

Glial GlyT1 Is Important for Early Postnatal Survival

To interfere with the function of glially expressed GlyT1, we crossed GlyT1$^{+/(+)}$ mice with GFAP-Cre mice,

which express Cre recombinase under the control of the mouse glial fibrillary acidic protein (GFAP) promoter (Marino et al., 2000). Again, we first examined the expression pattern of Cre recombinase in GFAP-Cre/ZEG double transgenic mice. Co-labelling of GFP with the pan-neuronal marker NeuN revealed that in brain stem and spinal cord of GFAP-Cre/ZEG mice, most of the neurons (>95%) were clearly devoid of EGFP immunoreactivity. The numerous GFP-positive cells found were identified as astrocytes by immunostaining with the astrocytic markers GFAP and S100β (Fig. 3A), consistent with glial expression of Cre recombinase in brain stem and spinal cord.

Mice carrying a floxed GlyT1 allele and the GFAP-Cre transgene ($GlyT1^{(+)/+}$/GFAP-Cre) were viable and did not show any motor impairment. After crossing these animals with $GlyT1^{(+)/(+)}$ mice, 80% of the resulting $GlyT1^{(+)/(+)}$/GFAP-Cre progeny developed a strong hypotonic phenotype, which finally resulted in premature death (Fig. 3B). Notably, the onset of the phenotype varied between earliest the first postnatal day (P1) and latest P10, with lethality showing a peak around P3 (Fig. 3C). [^{3}H]Glycine uptake assays revealed a >60–70% reduction in GlyT1-specific uptake activity in membrane fractions prepared from brain stem and spinal cord of affected animals (Fig. 3D, $P < 0.001$). In unaffected $GlyT1^{(+)/(+)}$/GFAP-Cre animals, the reduction of GlyT1-specific uptake activity was less pronounced as compared with $GlyT1^{+/+}$ animals, although still statistically significant (Fig. 3D, $P < 0.01$). In contrast, the GlyT1-independent fraction of [^{3}H]glycine uptake was not altered in all animals analyzed (data not shown). In conclusion, our data obtained with $GlyT1^{(+)/(+)}$/GFAP-Cre mice, together with those from the $GlyT1^{(+)/(+)}$/Syn-Cre animals, support the view that it is solely the loss of glial GlyT1 which causes the phenotype previously observed in conventional GlyT1 KO mice (Gomeza et al., 2003a).

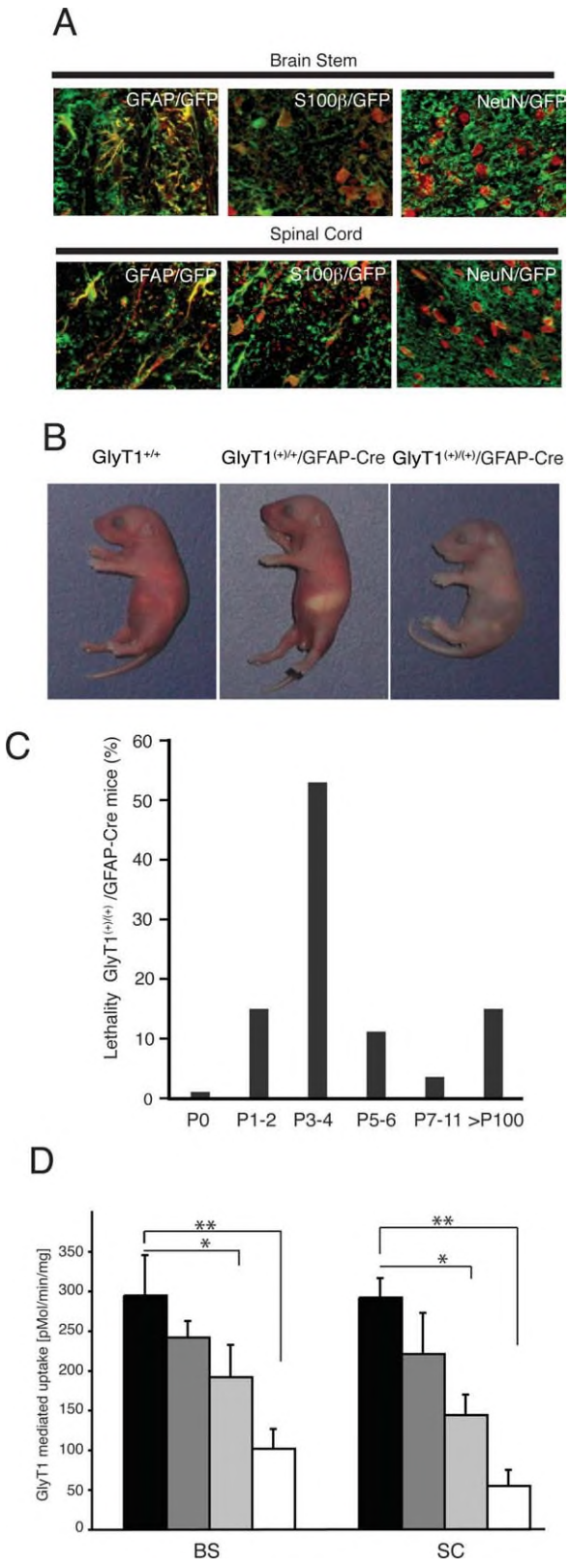

Fig. 3. Inactivation of GlyT1 in glial cells. **A**: Immunohistochemical analysis of brain stem and spinal cord sections derived from GFAP-Cre/ZEG double transgenic mice using GFP (green) and GFAP, S100β or NeuN (all red) specific antibodies. In both regions, GFP immunofluorescence was only detected in cells positive for the glial markers GFAP (upper panels) and S100β (middle panels) but not in cells positive for the neuronal marker NeuN (lower panels). **B**: Phenotype of mice carrying different GlyT1 alleles. In contrast to the $GlyT1^{+/+}$ and $GlyT1^{(+)/+}$/GFAP-Cre animals, the majority of the newborn $GlyT1^{(+)/(+)}$/GFAP-Cre littermates displayed a marked lethargic phenotype after birth as discernible by limb position and lack of milk in their belly, and died between P1 and P10. **C**: Lethality of $GlyT1^{(+)/(+)}$/GFAP-Cre mice. Postnatal death of $GlyT1^{(+)/(+)}$/GFAP-Cre mice was preceded by hypotonia for at least 3 h. Animals older than P11 survived to adulthood and never developed a detectable neuromotor phenotype. **D**: GlyT1-specific [^{3}H]glycine uptake in P2 membrane fractions prepared from brain stem (BS) and spinal cord (SC) from $GlyT1^{+/+}$ (■), $GlyT1^{+/(+)}$/GFAP-Cre (■), unaffected $GlyT1^{(+)/(+)}$/GFAP-Cre (■) and hypotonic $GlyT1^{(+)/(+)}$/GFAP-Cre (□) mice. Data represent means ± SD ($n \geq 5$ per genotype). *$P < 0.01$; **$P < 0.001$ (Student's t-test).

Glial GlyT1 Expression Is Dispensable in Adult Mice

Interestingly, about 20% of the $GlyT1^{(+)/(+)}$/GFAP-Cre mice developed no detectable phenotype and exhibited a normal lifespan (>P100; Fig. 3C). However, [^{3}H]glycine

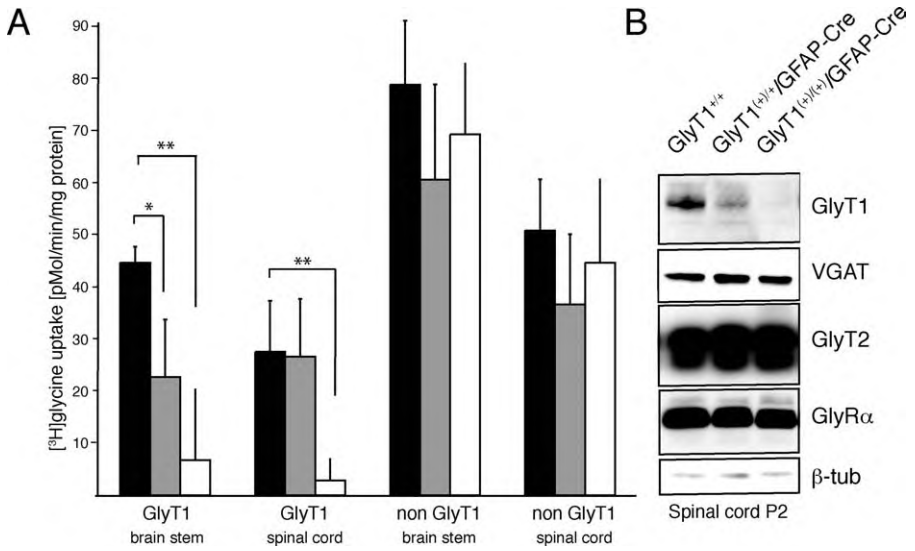

Fig. 4. GlyT1 protein expression and GlyT1-mediated glycine uptake in membrane fractions prepared from adult GlyT1$^{(+)/(+)}$/GFAP-Cre mice. **A**: GlyT1-specific and GlyT1-independent [^{3}H]glycine uptake into P2 membrane fractions prepared from brain stem (BS) and spinal cord (SC) of adult GlyT1$^{+/+}$ (■), GlyT1$^{(+)/+}$/GFAP-Cre (■), and GlyT1$^{(+)/(+)}$/GFAP-Cre (□) mice. Data represent means ± SD ($n \geq 8$ per genotype). *$P < 0.01$; **$P < 0.001$ (Student's t-test). **B**: Western blot analysis of spinal cord (SC) membrane fractions for the antigens indicated. Note that although GlyT1 protein was hardly detectable in GlyT1$^{(+)/(+)}$/GFAP-Cre mice, the expression of none of the other synaptic proteins analyzed was significantly altered.

uptake assays on membrane preparations derived from brain stem and spinal cord of such adult GlyT1$^{(+)/(+)}$/GFAP-Cre mice showed a marked reduction (>80%, $P < 0.001$) of GlyT1-specific uptake activity as compared with membranes prepared from wt littermates, whereas the GlyT1-independent fraction of [^{3}H]glycine uptake was not altered (Fig. 4A). To clarify whether this reduction in GlyT1 uptake activity was due to reduced GlyT1 expression, we subjected aliquots of these membrane preparations to Western blot analysis. This revealed a >80% reduction in GlyT1 immunoreactive protein in samples from the adult GlyT1$^{(+)/(+)}$/GFAP-Cre animals as compared to wt animals, whereas membranes from adult heterozygous mice contained intermediate GlyT1 levels (Fig. 4B). Notably, again no compensatory changes in other glycinergic proteins were observed. VIAAT, GlyT2 and GlyRα all were expressed at levels comparable with those seen in wt animals (Fig. 4B). We attribute the lack of hypotonic symptoms observed in these surviving GlyT1$^{(+)/(+)}$/GFAP-Cre animals to a delayed inactivation of the GlyT1 gene. Apparently GlyT1 function is dispensable in adult animals.

DISCUSSION

Here, we report a genetic analysis of the *in vivo* functions of GlyT1 in caudal regions of the mature and immature rodent CNS by generating a mouse line that allows Cre-mediated inactivation of the GlyT1 gene. The conditional GlyT1$^{(+)}$ allele created here was found to function normally, as deduced from Western blot analysis and [^{3}H]glycine uptake assays with membrane preparations derived from various CNS regions. Furthermore, ubiquitous expression of Cre recombinase in GlyT1$^{(+)/(+)}$ mice resulted in an efficient removal of the floxed exons, as demonstrated by both genetic analysis and the lethal phenotype of the resulting GlyT1$^{(+)/(+)}$/EIIA-Cre animals.

The contribution to CNS function of glial vs. neuronal GlyT1 was then analysed by mating GlyT1$^{+/(+)}$ animals with mice expressing Cre recombinase under either the control of the neuronal synapsin 1 or the glial GFAP promoter, respectively. Neuronal inactivation of the GlyT1 gene in GlyT1$^{(+)/(+)}$/Syn-Cre mice did not result in the development of neuromotor symptoms, such as hypotonia, nor in a significant reduction of GlyT1-mediated [^{3}H]glycine uptake into brain stem and spinal cord membranes. In forebrain regions like the hippocampus, however, a strong reduction in GlyT1 uptake activity was observed. Moreover, the behavioral analysis of these animals revealed deficits in forebrain-associated learning and memory (M. R. and V.E., unpublished data) that are consistent with changes in the function of glutamatergic synapses. Our findings are in agreement with previous *in situ* hybridisation and immunohistochemical data showing that GlyT1 is predominantly expressed in glial cells in brain stem and spinal cord (Zafra et al., 1995a,b). This contrasts findings from forebrain regions, including the hippocampus, where a major fraction (>50%) of GlyT1 protein and GlyT1 driven [^{3}H]glycine uptake is provided by neuronally expressed transporters (V. E and M. R. unpublished data; see also Yee et al., 2006 and Fig. 2B).

The majority of the mice carrying a glia-specific disruption of the GlyT1 gene displayed the strong hypotonic phenotype previously seen in GlyT1$^{-/-}$ mice (Gomeza et al., 2003a). However, the onset of disease symptoms was more variable in GlyT1$^{(+)/(+)}$/GFAP-Cre mice than in GlyT1$^{-/-}$ animals. These variations most likely reflect differences in the rates of recombination of the modified GlyT1 allele caused by interanimal

differences. Consistent with this interpretation, animals displaying the hypotonic phenotype showed large reductions in GlyT1-specific uptake activity (V. E. unpublished data; and see also Fig. 3D). The lack of glial GlyT1 in young GlyT1$^{(+)/(+)}$/GFAP-Cre mice should result in an increase in extracellular glycine concentrations causing prolonged activation of GlyRs, as reported for conventional GlyT1$^{-/-}$ mice (Gomeza et al., 2003a). The hypotonic phenotype seen in GlyT1$^{(+)/(+)}$/GFAP-Cre mice can thus be attributed to over-inhibition in caudal regions of the CNS.

Importantly, the phenotype seen in GlyT1$^{(+)/(+)}$/GFAP-Cre mice did not show full penetrance. About 20% of these animals survived until adulthood without detectable symptoms. This might in part reflect incomplete recombination in glial cells of brain stem and spinal cord. In the majority of the adult GlyT1$^{(+)/(+)}$/GFAP-Cre mice analyzed (17/20), however, the GlyT1 gene was efficiently inactivated, as deduced from both the reduction of GlyT1-specific uptake and Western blot analysis. GlyT1 protein expression was reduced by >80%, and in some animals (n = 6) even below detection limits. In contrast to young animals, where a >60–70% decrease in GlyT1-specific uptake was accompanied by symptoms of hypotonia, this reduction did not result in a detectable phenotype in the older animals. Our findings differ from previous studies, in which acute pharmacological inhibition of GlyT1 function by ALX5407 or LY2365109 produced respiratory distress in adult rats and mice (Perry et al., 2008). This difference might reflect a relatively slow decline in GlyT1 activity after genetic inactivation as compared with rapid pharmacological inhibition, which might not allow adaptive compensation to occur. By Western blot analyses, we found similar expression levels of the GlyRα subunit, VIAAT and GlyT2 in GlyT1$^{(+)/(+)}$/GFAP-Cre and GlyT1$^{+/+}$ mice, suggesting that inactivation of the GlyT1 gene does not alter synaptic protein accumulation at glycinergic synapses. Although we cannot exclude that residual GlyT1 protein, which displays activity too low to be detected by the assays used here, may be sufficient to maintain low synaptic glycine levels, the simplest explanation of our data is that the development of an over-inhibition phenotype is prevented by the increase in GlyT2 expression occurring after birth (Friauf et al., 1999). This implies that at later postnatal stages the activity of GlyT2, or of other nonselective uptake systems such as system A (Mackenzie et al., 2003), would be sufficient to remove released glycine from glycinergic synapses. This interpretation is consistent with pharmacological studies, which indicate a significant role of GlyT2 in controlling synaptic glycine levels. In spinal cord slice preparations, inhibition of GlyT2 resulted in increased GlyR-dependent membrane current noise and prolonged decay kinetics of glycinergic mIPSCs (Bradaia et al., 2004). Furthermore, in adult animals, spinal application of GlyT2-specific antagonists leads to respiratory depression (Hermanns et al., 2008). Both findings are consistent with high levels of glycine accumulating at postsynaptic GlyRs in the absense of GlyT2 function. In addition, adaptive changes such as alterations in the agonist sensitivity of the GlyRs (Meier et al., 2005) or in glycinergic vs. GABAergic inhibition ratio (Aubrey et al., 2007) may contribute to phenotypic compensation.

In conclusion, our data show that the loss of glial GlyT1 expression causes the lethal phenotype previously found in newborn GlyT1$^{-/-}$ mice. Furthermore, upon maturation of the CNS, there is a clear reduction in the dependence on glial GlyT1 for regulating synaptic glycine concentrations. Most likely the increased expression of GlyT2 occuring after birth compensates for the loss of the glial transporter at later developmental stages. Clearly further experiments are needed to understand how glial and neuronal glycine uptakes cooperate in the regulation of glycinergic neurotransmission in caudal regions of the adult CNS.

ACKNOWLEDGMENTS

The authors thank Dr. Ulrike Müller for 129/Ola ES cells and Dr. Klaus Rajewsky for providing the pEasy-Flox vector. The technical assistance of Maren Krause is gratefully acknowledged.

REFERENCES

Aubrey KR, Rossi FM, Ruivo R, Alboni S, Bellenchi GC, Le Goff A, Gasnier B, Supplisson S. 2007. The transporters GlyT2 and VIAAT cooperate to determine the vesicular glycinergic phenotype. J Neurosci 27:6273–6281.

Betz H, Harvey RJ, Schloss P. 2000. Structures, diversity and pharmacology of glycine receptors and transporters. In: Möhler H, editor. Pharmacology of GABA and glycine neurotransmission. Berlin: Springer-Verlag. pp 375–401.

Bradaia A, Schlichter R, Trouslard J. 2004. Role of glial and neuronal glycine transporters in the control of glycinergic and glutamatergic synaptic transmission in lamina X of the rat spinal cord. J Physiol 559(Part 1):169–186.

Cubelos B, Gimenez C, Zafra F. 2005. Localization of the GLYT1 glycine transporter at glutamatergic synapses in the rat brain. Cereb Cortex 15:448–459.

Friauf E, Aragon C, Lohrke S, Westenfelder B, Zafra F. 1999. Developmental expression of the glycine transporter GLYT2 in the auditory system of rats suggests involvement in synapse maturation. J Comp Neurol 412:17–37.

Gomeza J, Armsen W, Betz H, Eulenburg V. 2006. Lessons from the knocked-out glycine transporters. Handb Exp Pharmacol 175:457–483.

Gomeza J, Hulsmann S, Ohno K, Eulenburg V, Szoke K, Richter D, Betz H. 2003a. Inactivation of the glycine transporter 1 gene discloses vital role of glial glycine uptake in glycinergic inhibition. Neuron 40:785–796.

Gomeza J, Ohno K, Hulsmann S, Armsen W, Eulenburg V, Richter DW, Laube B, Betz H. 2003b. Deletion of the mouse glycine transporter 2 results in a hyperekplexia phenotype and postnatal lethality. Neuron 40:797–806.

Hermanns H, Muth-Selbach U, Williams R, Krug S, Lipfert P, Werdehausen R, Braun S, Bauer I. 2008. Differential effects of spinally applied glycine transporter inhibitors on nociception in a rat model of neuropathic pain. Neurosci Lett 445:214–219.

Johnson JW, Ascher P. 1987. Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature 325:529–531.

Lakso M, Pichel JG, Gorman JR, Sauer B, Okamoto Y, Lee E, Alt FW, Westphal H. 1996. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. Proc Natl Acad Sci USA 93:5860–5865.

Mackenzie B, Schafer MK, Erickson JD, Hediger MA, Weihe E, Varoqui H. 2003. Functional properties and cellular distribution of the system A glutamine transporter SNAT1 support specialized roles in central neurons. J Biol Chem 278:23720–23730.

Marino S, Vooijs M, van Der Gulden H, Jonkers J, Berns A. 2000. Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. Genes Dev 14:994–1004.

Meier JC, Henneberger C, Melnick I, Racca C, Harvey RJ, Heinemann U, Schmieden V, Grantyn R. 2005. RNA editing produces glycine receptor alpha3(P185L), resulting in high agonist potency. Nat Neurosci 8:736–744.

Novak A, Guo C, Yang W, Nagy A, Lobe CG. 2000. Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. Genesis 28(3–4):147–155.

Papadopoulos T, Korte M, Eulenburg V, Kubota H, Retiounskaia M, Harvey RJ, Harvey K, O'sullivan GA, Laube B, Hulsmann S, Geiger JR, Betz H. 2007. Impaired GABAergic transmission and altered hippocampal synaptic plasticity in collybistin-deficient mice. Embo J 26:3888–3899.

Perry KW, Falcone JF, Fell MJ, Ryder JW, Yu H, Love PL, Katner J, Gordon KD, Wade MR, Man T, et al. 2008. Neurochemical and behavioral profiling of the selective GlyT1 inhibitors ALX5407 and LY2365109 indicate a preferential action in caudal vs. cortical brain areas. Neuropharmacology 55:743–754.

Pow DV, Hendrickson AE. 1999. Distribution of the glycine transporter glyt-1 in mammalian and nonmammalian retinae. Vis Neurosci 16:231–239.

Singer P, Boison D, Mohler H, Feldon J, Yee BK. 2007. Enhanced recognition memory following glycine transporter 1 deletion in forebrain neurons. Behav Neurosci 121:815–825.

Yee BK, Balic E, Singer P, Schwerdel C, Grampp T, Gabernet L, Knuesel I, Benke D, Feldon J, Mohler H, Boison D. 2006. Disruption of glycine transporter 1 restricted to forebrain neurons is associated with a procognitive and antipsychotic phenotypic profile. J Neurosci 26:3169–3181.

Zafra F, Aragon C, Olivares L, Danbolt NC, Gimenez C, Storm-Mathisen J. 1995a. Glycine transporters are differentially expressed among CNS cells. J Neurosci 15(Part 2):3952–3969.

Zafra F, Gomeza J, Olivares L, Aragon C, Gimenez C. 1995b. Regional distribution and developmental variation of the glycine transporters GLYT1 and GLYT2 in the rat CNS. Eur J Neurosci 7:1342–1352.

Zhu Y, Romero MI, Ghosh P, Ye Z, Charnay P, Rushing EJ, Marth JD, Parada LF. 2001. Ablation of NF1 function in neurons induces abnormal development of cerebral cortex and reactive gliosis in the brain. Genes Dev 15:859–876.