

Deletion of the Mouse Glycine Transporter 2 Results in a Hyperekplexia Phenotype and Postnatal Lethality

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Summary

The glycine transporter subtype 2 (GlyT2) is localized in the axon terminals of glycinergic neurons. Mice deficient in GlyT2 are normal at birth but during the second postnatal week develop a lethal neuromotor deficiency that resembles severe forms of human hyperekplexia (hereditary startle disease) and is characterized by spasticity, tremor, and an inability to right. Histological and immunological analyses failed to reveal anatomical or biochemical abnormalities, but the amplitudes of glycinergic miniature inhibitory currents (mIPSCs) were strikingly reduced in hypoglossal motoneurons and dissociated spinal neurons from GlyT2-deficient mice. Thus, postnatal GlyT2 function is crucial for efficient transmitter loading of synaptic vesicles in glycinergic nerve terminals, and the GlyT2 gene constitutes a candidate disease gene in human hyperekplexia patients.

Introduction

Amino acids serve as neurotransmitters throughout the animal kingdom. Glutamate is the predominant excitatory transmitter, and γ -aminobutyric acid (GABA) and glycine are the major inhibitory transmitters in the mammalian CNS. The postsynaptic actions of these amino acids are terminated by specific ion-driven transporter proteins, which mediate transmitter re-uptake into nerve terminals as well as glial cells (for review, see Amara and Kuhar, 1993; Schloss et al., 1994; Nelson, 1998). Notably, all amino acid transporters exist in different subtypes encoded by different genes, which often show a restricted expression in either glia or neurons. This suggests that the functional roles of glial and neuronal transporters are distinct.

In an attempt to elucidate the *in vivo* functions of glial

and neuronal neurotransmitter transporters, we have employed homologous recombination in mouse to inactivate the transporters known to catalyze the re-uptake of glycine. In the accompanying paper, we reported on the phenotype of mice deficient in the glial glycine transporter 1 (GlyT1). GlyT1^{-/-} mice die on the first postnatal day, due to severe motor and respiratory deficits resulting from overinhibition (Gomeza et al., 2003 [this issue of *Neuron*]). Here, we describe a mouse line that lacks functional glycine transporter 2 (GlyT2), the neuronal GlyT isoform localized in presynaptic terminals of glycinergic interneurons (Zafra, 1995a; Spike et al., 1997). We report that GlyT2-deficient mice display a severe neuromotor disorder characterized by muscular spasticity. The animals die during the second week after birth, revealing a previously unrecognized vital role for GlyT2 in postnatal life. The phenotype of these mice is consistent with GlyT2 being essential for replenishing the cytoplasmic pool of glycine that is needed for transmitter loading of synaptic vesicles in glycinergic nerve terminals. Moreover, it mimics symptoms seen in human hereditary hyperekplexia (or startle disease) and therefore identifies the GlyT2 gene as a candidate disease gene in human patients. Together, our two studies define distinct roles of GlyT1 and GlyT2 in glycine-mediated synaptic transmission.

Results

Generation of GlyT2^{-/-} Mice

The GlyT2 gene was inactivated in mouse E14 (129/OLA) embryonic stem cells by replacing a segment of 2.1 kb, which contains the exon encoding the fourth transmembrane domain of the transporter, with a neomycin-resistance cassette (Figure 1A). By using standard transgenic and mouse breeding techniques, chimeric mice were obtained and used to generate F1 offspring heterozygous for GlyT2. These animals appeared phenotypically normal and showed undisturbed development and fertility. Intercrossing of the heterozygous mice generated wild-type (+/+) as well as heterozygous (+/-) and homozygous (-/-) GlyT2 mutant mice, as determined by Southern blot analysis (Figure 1B).

GlyT2^{-/-} newborn pups were obtained with the expected Mendelian frequency, indicating that there was no increased embryonic mortality in the mutant mice. RT-PCR experiments showed that GlyT2 mRNA was present in caudal regions of the CNS in wild-type mice, whereas no PCR product was obtained with mRNA extracted from GlyT2^{-/-} brain stem (Figure 1C). Thus, GlyT2 transcripts were absent in the homozygous mutants. Similarly, Western blot analysis with an antiserum raised in rabbits against the N-terminal cytoplasmic domain of the rat GlyT2 protein failed to reveal immunoreactive protein in the GlyT2-deficient mice (Figure 1D). We therefore conclude that GlyT2 was not expressed in homozygous animals.

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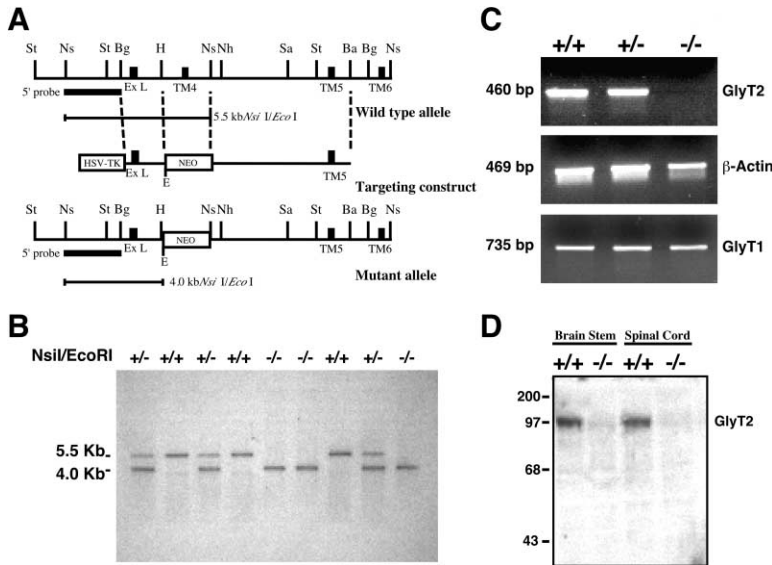


Figure 1. Targeted Disruption of the Mouse GlyT2 Gene

(A) Knockout strategy showing restriction maps of the wild-type GlyT2 locus, the targeting construct, and the targeted allele. Exons are represented as black boxes. The probe used for Southern analysis and the sizes of the restriction fragments detected with this probe are indicated. Only relevant restriction sites are shown. St, *Stul*; Ns, *NsiI*; Bg, *BglII*; H, *HindIII*; Nh, *NheI*; Sa, *SacI*; Ba, *BamHI*; E, *EcoRI*.

(B) Genotyping of F2 offspring by Southern blot analysis of *NsiI/EcoRI*-digested mouse tail DNA. The 5.5 kb and 4.0 kb bands represent the wild-type and mutant GlyT2 alleles, respectively.

(C) RT-PCR analysis of brain stem expression of GlyT2, β -actin, and GlyT1 in wild-type and GlyT2^{+/-} and GlyT2^{-/-} mice. Note the absence of GlyT2 transcripts in homozygous mutant animals.

(D) Western blot analysis of brain stem and spinal cord fractions (30 μ g protein/lane) using the GlyT2 antibody. Note that the GlyT2 polypeptide is absent in the homozygous mutant mice.

GlyT2^{-/-} Mice Display Low Glycine Transport Activity in Caudal Regions of the CNS

In order to reveal the functional consequences of GlyT2 gene deletion, [³H]glycine uptake assays were performed with P2 membrane fractions prepared from different regions of the mouse CNS at the tenth postnatal day (P10). As shown in Figure 2, [³H]glycine transport was reduced by about 65% and 73% in brain stem and spinal cord membrane preparations from GlyT2^{-/-} mice, respectively, as compared to membrane samples obtained from wild-type animals ($p < 0.001$). This result is consistent with the known expression profiles of GlyT1 and GlyT2 during postnatal development (Zafra et al., 1995b; Friauf et al., 1999). Also, a more modest (~25%) but significant reduction was found with membranes from homo-

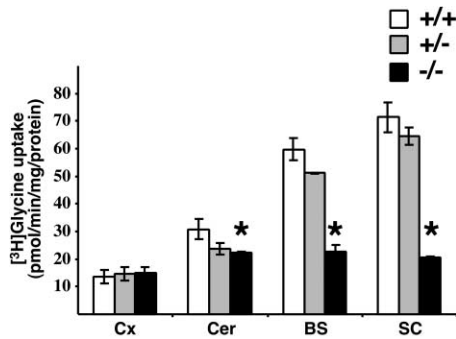


Figure 2. High-Affinity Glycine Uptake in Different CNS Regions of Wild-Type, Heterozygous, and Homozygous GlyT2-Deficient Mice Membranes were prepared from the indicated tissues of 10-day-old mice and preincubated for 1 min at 37°C. Transport was initiated by the addition of 2 μ M [³H]glycine in Krebs-Henseleit buffer for 1 min. Cx, cortex; Cer, cerebellum; BS, brainstem; SC, spinal cord. Data are given as means \pm SD ($n = 3-4$ for each genotype). Asterisk indicates significantly different from wild-type mice, $p < 0.001$ (Student's *t* test).

zygous mutant cerebellum, a region known to contain few glycinergic interneurons. In contrast, all genotypes analyzed showed comparable [³H]glycine uptake values with membrane fractions prepared from frontal cortex, where GlyT2 is not expressed and glial GlyT1 constitutes the only GlyT isoform that catalyzes the clearance of extracellular glycine (Zafra et al., 1995a, 1995b). These results are consistent with a complete lack of GlyT2-mediated glycine uptake in the homozygous mutant mice.

GlyT2^{-/-} Mice Show a Strong Neuromotor Phenotype and Die during the Second Postnatal Week

Body weight and general appearance of the homozygous GlyT2^{-/-} pups were normal at birth. However, GlyT2 mutant animals gained weight more slowly than their wild-type littermates (Table 1). Unexpectedly, homozygous GlyT2^{-/-} mice died prematurely around the end of the second postnatal week after displaying a complex neuromotor phenotype characterized by spasticity, a rigid muscle tone, strong tremor, and a severely impaired righting response. In most homozygous animals, these symptoms were clearly seen at P10; usually, first signs of an increased muscle tone were already detectable at P8. Only an extremely low number of pups already displayed spasticity and tremor at P9, or after P10, i.e., at P11 only. Homozygous animals therefore were routinely killed at P10 for analysis.

In an attempt to monitor the phenotypic properties of these mutant mice more closely, simple handling assays were performed at P10. First, littermates were screened for the development of tremors. Tremor could be easily induced and properly recorded when animals were picked up and fixed by their tails onto an electromechanical transducer, as described previously (Becker et al., 2000). Vibrations produced by tremor-derived movements were recorded over time as apparent weight changes. In contrast to wild-type and heterozygous littermates,

Table 1. Phenotype Characteristics of Littermates

Genotype (n)	+/+ (20)	+/- (18)	-/- (13)
Body weight (g)	7.91 ± 1.09	8.05 ± 1.13	4.53 ± 0.99 ^a
Hind feet claspings (%)	0	0	100
Tremor (%)	0	0	100
Righting time ≤1 s (%)	100	100	0

Data are expressed as means ± SD.

^aSignificantly different from the wild-type value, $p < 0.001$ (Student's *t* test).

all GlyT2^{-/-} mice developed strong spontaneous tremor, as demonstrated by high amplitudes of the electromechanical tracings, which reflect strong tremor-induced movements (Figure 3A). As a second criterion to evaluate neuromotor performance, we scored the so-called "hind feet claspings" behavior that characterizes mice carrying mutations in GlyR structural genes (Hartenstein et al., 1996; Becker et al., 2000). When picked up by their tail, GlyT2^{+/+} and GlyT2^{+/-} mice tended to move heavily while spreading out their legs. In contrast, GlyT2 mutant animals showed an abnormal behavior, claspings their hind feet between episodes of tremor, as shown in Figure 3B. Finally, a third parameter measured to monitor animal motor performance was the righting response reflex (Hartenstein et al., 1996). Mice were turned

on their back, and the time required to regain the upright position was determined. Wild-type and heterozygous animals immediately turned themselves over, righting themselves onto all four legs. Notably, GlyT2^{-/-} mice were unable to perform this task and never righted themselves, as indicated in Table 1. In summary, GlyT2-deficient mice displayed a lethal neuromotor disorder that developed after the first postnatal week and was characterized by muscular spasticity, tremor, and an inability to right. Death presumably occurred as a consequence of the inability to feed, desiccation, and continued convulsions.

GlyT2^{-/-} Mice Show Normal Histology and Synaptic Protein Expression

To exclude the possibility that the lethal phenotype of GlyT2^{-/-} mice is due to developmental abnormalities, a general histological analysis was performed. No defects in skeletal muscles or visceral organs were found in 10-day-old mutant animals (data not shown). Similarly, no differences between GlyT2^{-/-} and GlyT2^{+/+} mice were detected when examining brain stem and spinal cord sections (Figure 4A). Thus, homozygous GlyT2^{-/-} animals appeared histologically indistinguishable from GlyT2^{+/+} littermates.

Depression of glycine uptake, due to inactivation of GlyT2, might cause compensatory changes in the expression of synaptic proteins. Western blot analysis performed on spinal cord membranes prepared from P10 wild-type and GlyT2-deficient mice as described (Gomez et al., 2003) showed that the immunoreactivities of the postsynaptic GlyR clustering protein gephyrin (Pfeiffer et al., 1984; Prior et al., 1992), the NMDA receptor subunit NR1 (Moriyoshi et al., 1991), the NMDA receptor anchoring protein PSD-95 (Cho et al., 1992), the vesicular inhibitory amino acid transporter (VIAAT) (Dumoulin et al., 1999), the presynaptic membrane protein syntaxin (Bennett et al., 1992), GlyR α subunits (GlyR α ; Pfeiffer et al., 1984), and GlyT1 (Zafra et al., 1995a) all were rather similar in all genotypes studied (Figure 4B). Quantitation of the immunoreactive bands in 3–4 independent membrane samples each corroborated that average protein levels were unchanged upon GlyT2 deficiency (Table 2). Similarly, immunocytochemical stainings of brain stem and spinal cord sections from 10-day-old littermates revealed a lack of GlyT2 immunoreactivity in brain stem from homozygous mutant animals (Figure 4C), but a VIAAT-specific antibody produced comparable patterns of immunoreactivity on parallel GlyT2^{-/-} and wild-type sections (Figure 4C). Thus, a loss of inhibitory presynaptic terminals can be excluded. Also, no differences in the postsynaptic punctate staining of GlyR

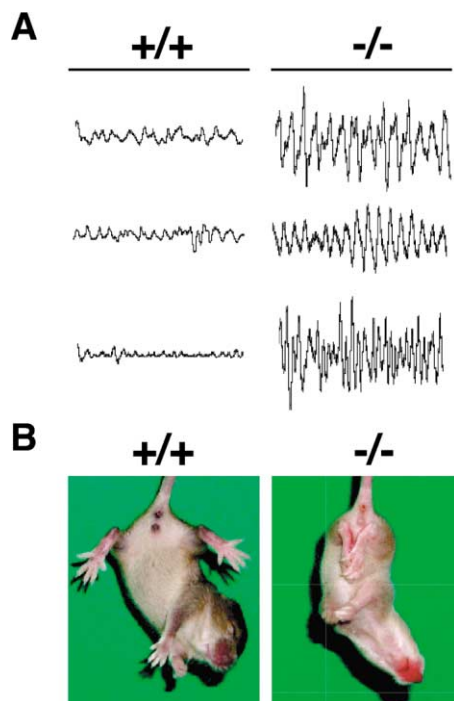


Figure 3. Neuromotor Behavior of P10 GlyT2^{-/-} Mice

(A) Electromechanical tracings of tremor-derived movement recorded from three wild-type (+/+) and three GlyT2 mutant (-/-) animals. All traces represent 1 s recordings. The amplitudes are proportional to the strength of the movement recorded over time. Note the appearance of spontaneous strong tremor in GlyT2^{-/-} mice.

(B) "Hind feet claspings" phenotype in GlyT2^{-/-} mice. When picked up by their tail, wild-type mice (+/+) tend to move heavily, spreading out their legs (left). GlyT2 homozygous animals (-/-) in this situation clasp their hind feet (right) between episodes of tremor.

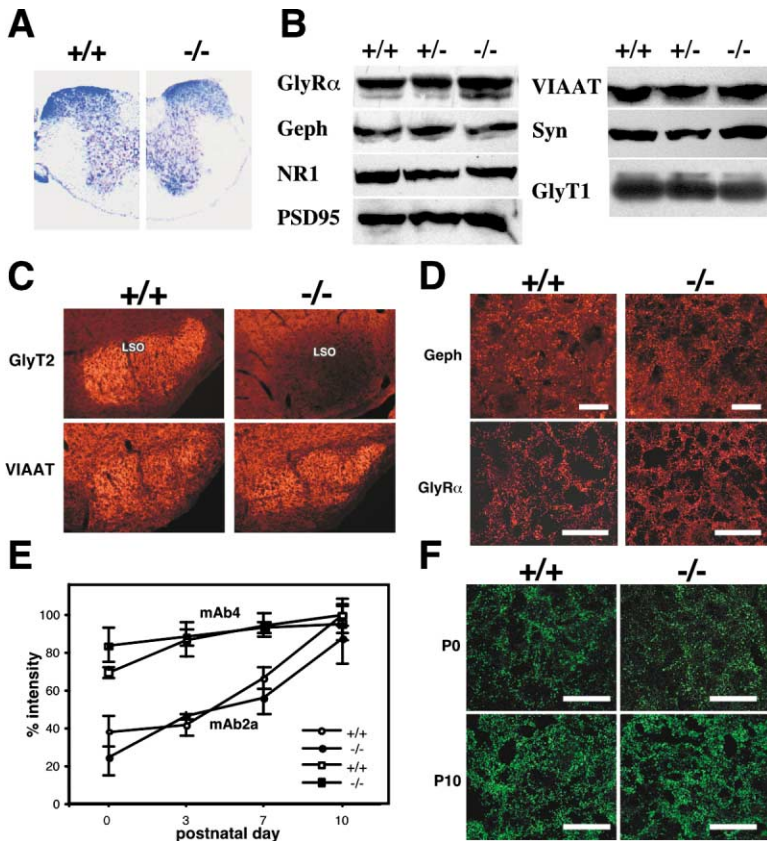


Figure 4. Histological and Immunohistochemical Analyses of Wild-Type (+/+) and GlyT2^{-/-} Mice

(A) Cresyl violet-stained spinal cord sections from P10 wild-type (+/+) and GlyT2 mutant (-/-) mice are shown. No defects were detected in the homozygous mutants, and the overall anatomy appeared normal in all animals analyzed.

(B) Western blot analysis of spinal cord fractions prepared from P10 wild-type (+/+), heterozygous (+/-), and homozygous (-/-) GlyT2 mutant animals. Equal amounts of protein (30 μg/lane) were loaded and probed with the indicated antisera. Note that the expression levels of all proteins tested were similar in the different genotypes.

(C) Immunostaining of brainstem sections with antibodies specific for GlyT2 and VIAAT. No differences in staining were detected when comparing sections from P10 GlyT2 mutant (-/-) and wild-type (+/+) animals except for GlyT2 immunoreactivity, which was absent in the mutant sections. LSO, lateral superior olivary complex.

(D) Immunostainings of P10 spinal cord sections with antibodies specific for GlyRα and gephyrin. Note similar punctate staining patterns in the different sections.

(E) Postnatal changes in GlyRα (mAb 4b) and GlyRα1 (mAb 2a) immunoreactivities in spinal cord sections prepared from wild-type and GlyT2^{-/-} animals. At birth, α1 was expressed at a comparatively low level but increased about 3-fold until P10 in both genotypes, whereas GlyRα values changed only modestly.

(F) At both birth (P0) and P10, the punctate synaptic distributions of GlyRα1 staining were comparable in GlyT2^{-/-} and wild-type sections. Scale bar equals 20 μm.

α subunits were detected when comparing GlyT2 mutant and wild-type spinal cord preparations (Figure 4D). Likewise, the density of gephyrin immunoreactive puncta was unaltered in spinal cord sections of GlyT2^{-/-} mice. Taken together, these results indicate that no compensatory changes occur in the expression and synaptic localization of glycine transmission-related synaptic proteins upon GlyT2 gene inactivation.

Since the onset of neurological symptoms in GlyT2^{-/-} mice coincides with the postnatal replacement of the neonatal GlyR α2 by the adult GlyR α1 subunit (Becker et al., 1988; Malosio et al., 1991), we examined GlyRα1

Table 2. Quantification of Synaptic Protein Immunoreactivities

Genotype	GlyT2 ^{+/+}	GlyT2 ^{+/-}	GlyT2 ^{-/-}
GlyRα	100 ± 14	96 ± 11	111 ± 12
Gephyrin	100 ± 18	86 ± 14	110 ± 7
NR1	100 ± 7	109 ± 9	93 ± 7
PSD95	100 ± 12	77 ± 19	107 ± 13
VIAAT	100 ± 7	123 ± 21	109 ± 15
Syntaxin	100 ± 20	75 ± 16	91 ± 11
GlyT1	100 ± 14	96 ± 9	90 ± 9

Values were determined by scanning immunoreactive bands on Western blots and represent means ± SD (n ≥ 3). None of the mutant values differs significantly from wild-type levels.

expression during postnatal development. Spinal cord sections were stained using the antibody mAb 2a that specifically detects the α1 subunit of the GlyR (Pfeiffer et al., 1984; Schroder et al., 1991). Consistent with previous reports (Becker et al., 1988, 1992), in wild-type sections the intensity of GlyRα1 immunoreactivity visualized by mAb 2a was low at birth but increased about 3-fold until P10, although total GlyRα amounts as quantified by mAb 4b staining changed only little (Figure 4E). This postnatal increase in GlyRα1 expression was unaltered in spinal cord sections of GlyT2 mutant animals. Moreover, the punctate distribution of mAb 2a staining indicative of postsynaptically clustered GlyRs also was indistinguishable in GlyT2^{+/+} and GlyT2^{-/-} sections at both at P0 and P10 (Figure 4F). We therefore conclude that the absence of GlyT2 does not affect postnatal GlyR α subunit expression and localization in developing spinal neurons.

Reduction of Glycinergic IPSCs in GlyT2^{-/-} Hypoglossal Motoneurons

To examine the effects of GlyT2 gene deletion on glycinergic neurotransmission, spontaneous glycinergic postsynaptic currents (IPSCs) were recorded in hypoglossal motoneurons in acutely isolated brainstem slices from 8- to 9-day-old wild-type and GlyT2^{-/-} mice. At this

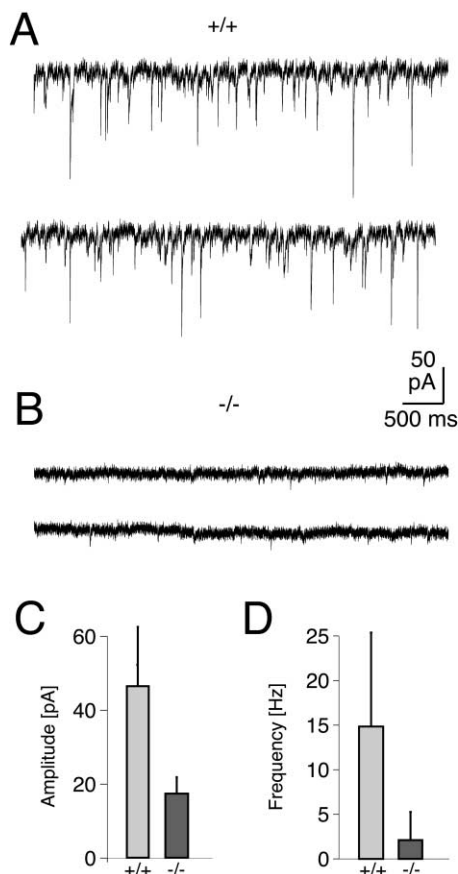


Figure 5. Amplitudes and Frequencies of Spontaneous IPSCs Recorded from Hypoglossal Motoneurons In Situ at Postnatal Days 8 and 9

(A and B) Chart recordings of typical spontaneous synaptic activity in hypoglossal motoneurons in slices from wild-type (+/+, A) and GlyT2-deficient (-/-, B) mice. The mean IPSC amplitudes shown in these representative traces were 45.0 ± 36.7 pA (control) and 16.7 ± 3.1 pA (GlyT2^{-/-}).

(C and D) Summary graphs showing the IPSC mean amplitudes (C) and frequencies (D) found in wild-type (+/+, n = 6) and GlyT2^{-/-} (-/-; n = 11) hypoglossal motoneurons.

stage, the first neuromotor deficits become apparent. Hypoglossal motoneurons from wild-type animals generated glycinergic IPSCs with a mean amplitude of 46.5 ± 16.5 pA (n = 6; Figures 5A and 5C). In GlyT2^{-/-} neurons, mean IPSC amplitudes were significantly reduced (17.4 ± 4.4 pA; n = 11, p < 0.001; Figures 5B and 5C). Mean IPSC frequencies in wild-type neurons were 14.9 ± 10.5 Hz (n = 6) and in GlyT2^{-/-} cells 2.1 ± 3.2 Hz (n = 11, p = 0.001) (Figure 5D). We attribute this reduction in IPSC frequency to small events that were not detectable in the comparatively high noise of these recordings (Figure 5B).

We also analyzed action potential-independent IPSCs (mIPSCs) in the presence of 0.5 μ M tetrodotoxin and 100 mM sucrose (data not shown). Under these conditions, mIPSCs in hypoglossal motoneurons from wild-type animals showed an amplitude of 28.8 ± 7.9 pA (n = 4), whereas the mIPSC amplitudes in GlyT2^{-/-} neurons were significantly reduced, with a mean amplitude of

15.9 ± 3.9 pA (n = 6, p < 0.001). The mean mIPSC frequency in wild-type neurons was 2.5 ± 1.1 Hz (n = 4). In GlyT2^{-/-} mice, the frequency of mIPSCs was reduced to 0.4 ± 0.4 Hz (n = 6, p = 0.001). The decay of mIPSC was not significantly changed in the mutant (wild-type, 14.4 ± 2.7 ms versus GlyT2^{-/-}, 11.9 ± 5.9 ms).

Reduced Glycinergic mIPSCs in Cultured GlyT2^{-/-} Spinal Neurons

As viable brain stem slices were difficult to prepare at stages later than P9, glycinergic mIPSCs were in addition recorded from dissociated cell cultures prepared from wild-type and GlyT2^{-/-} E13 embryonic mouse spinal cord after 21 days of in vitro differentiation, i.e., a time period corresponding to the end of the second postnatal week (Figure 6A). Cultured neurons from wild-type animals displayed a regular burst pattern, with a frequency of 26.4 ± 6.6 Hz (mean \pm SD; n = 5; Figure 6B). No significant change in inter-mIPSC intervals was observed in preparations from GlyT2^{-/-} mutant mice, which displayed a burst frequency of 23.2 ± 5.6 Hz (n = 5). Notably, however, mIPSC amplitudes were significantly reduced in the GlyT2^{-/-} neurons (Figure 6A). Compared to wild-type cells, a mean decrease of $42\% \pm 15\%$ (p < 0.01) in the peak amplitude of the average mIPSC was observed in GlyT2^{-/-} spinal cord neurons (Figure 6B). Application of the GlyR antagonist strychnine abolished mIPSC events in both wild-type and mutant cultures, thereby confirming the glycinergic nature of the recorded mIPSC (Figure 6A). No differences were found in the kinetics of mIPSC decay between wild-type and GlyT2^{-/-} neurons. Averaged mIPSCs (50 consecutive events) decayed with a time constant of 9–12 ms in cultures from wild-type mice and 9–14 ms in those from GlyT2^{-/-} mice (n = 3, each).

To exclude a possible postsynaptic origin of the reduced mIPSC amplitudes found in the mutant cultures, the synaptic localization of GlyR α was examined in the cultured neurons (Figure 6C). In wild-type cells, a punctate staining was produced by mAb 4b that was predominantly membranous, as revealed by confocal sectioning. Confirming the results obtained from spinal cord sections, mAb 4b staining of GlyT2^{-/-} neurons did not differ from that of wild-type cells. Again, a clear punctate distribution of GlyR α immunoreactivity was found. Also, the GlyR α 1 subunit-specific antibody mAb 2a produced a very similar punctate pattern in cultures prepared from both genotypes (data not shown). As no alterations in synaptic GlyR staining could be detected under both in vivo and in vitro conditions, we conclude that the GlyT2 deficiency exerts exclusively presynaptic effects. Our data indicate that the absence of GlyT2 reduces the amplitude but not the frequency of glycinergic mIPSCs, a finding that accounts for the reduced glycinergic activity of the mutant animals.

Discussion

GlyT2 Is Essential for Vital Postnatal Functions

Here, we report a genetic analysis of the in vivo function of the neuronal GlyT subtype, GlyT2, in the mouse central nervous system. As demonstrated by Southern blotting, RT-PCR, Western blot analysis, and glycine uptake

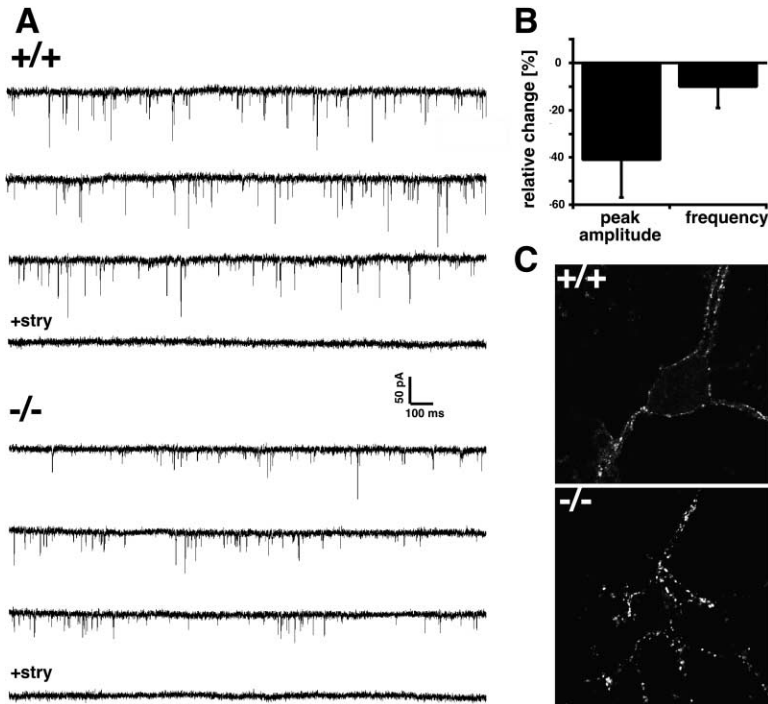


Figure 6. Amplitudes and Frequencies of mIPSCs Recorded from Cultured Spinal Neurons after 21 Days In Vitro

(A) Chart recordings of typical spontaneous synaptic activity in spinal cord cells isolated from wild-type (+/+) and GlyT2-deficient (-/-) embryos in the presence of tetrodotoxin (0.5 μ M). The mean mIPSC amplitudes shown in these representative traces were 54.4 ± 15.4 pA (wt) and 28.8 ± 10.1 pA (GlyT2^{-/-}). The lower traces (+Stry) were recorded in the presence of 200 nM strychnine. (B) Summary graph showing the relative changes in mIPSC mean amplitudes and frequencies found in GlyT2^{-/-} neurons. Values are normalized to control values obtained with wild-type cells (n = 5, each). (C) Immunohistochemical localization of GlyR clusters on cultured mouse spinal neurons stained with mAb 4b. The anti-GlyR antibody was visualized with FITC-labeled secondary antibody (green). Scale bar equals 20 μ m.

assays, inactivation of the GlyT2 gene by homologous recombination caused a complete loss of transporter expression in caudal regions of the CNS. Our results confirm the authenticity of the GlyT2^{-/-} line and exclude the possibility that loss of GlyT2 may lead to compensatory upregulation of the functionally closely related glial transporter, GlyT1. The severe phenotype observed in the homozygous knockout mice thus can be confidently attributed to GlyT2 deletion.

GlyT2^{-/-} mice appeared normal at birth but died during the second postnatal week after developing a severe neuromotor disorder, whose symptoms were entirely different from those seen upon GlyT1 deletion (Gomez et al., 2003). As in GlyT1^{-/-} mice, no histological defects were detected in peripheral organs or in caudal regions of the CNS, where GlyT2 is mainly expressed. We therefore conclude that GlyT2, like GlyT1, is not required for organogenesis during embryonic development but is essential for postnatal CNS functions.

GlyT2 Deficiency Results in a Complex Neuromotor Disorder

The neuromotor phenotype of GlyT2 mutant animals develops during the second postnatal week and is characterized by rigidity, tremor, and an impaired righting response. Interestingly, alterations in glycinergic neurotransmission are known to underlie the pathogenesis of neurological dysfunctions displaying rather similar symptoms (Kuhse et al., 1995; Rajendra and Schofield, 1995; Breiting and Becker, 1998). Spastic (spa), spasmodic (spd), and oscillator (spd⁰) mutant mice are normal at birth but develop an exaggerated startle reflex, inducible rapid tremor, rigidity, and impaired righting reflexes at the end of the second postnatal week. The mutations spd and spd⁰ represent partial or complete loss-of-function mutations of the GlyR α 1 subunit (Buck-

walter et al., 1994; Ryan et al., 1994; Saul et al., 1994; Kling et al., 1997). In contrast, the mutant spa has an intronic insertion of a transposable LINE-1 element in the GlyR β subunit gene that leads to a strong reduction in the levels of mature β subunit mRNA and, consequently, low levels of GlyR at the synaptic plasma membrane (Kingsmore et al., 1994; Mulhardt et al., 1994; Hartenstein et al., 1996). Similarly, loss of the GlyR anchoring protein gephyrin in gephyrin knockout mice prevents synaptic accumulation of GlyRs and leads to a lethal phenotype that mimics symptoms seen upon strychnine poisoning (Feng et al., 1998). In conclusion, impaired postsynaptic GlyR function or clustering causes a phenotype that closely resembles that of GlyT2^{-/-} animals.

Because of these similarities, we considered the possibility that the neurological symptoms of GlyT2^{-/-} mice might be due to changes in GlyR expression or postsynaptic clustering. Up- or downregulation of distinct components of synapses has been observed previously in monoamine transporter knockout mice (Bengel et al., 1998; Gainetdinov et al., 1998; Jones et al., 1998, 1999). However, ablation of the GlyT2 gene did not lead to detectable changes in the expression levels of GlyR α subunits, as revealed by Western blot analysis. Likewise, our immunofluorescence studies showed that the distribution and density of GlyR α subunit puncta were unaltered in spinal cord sections derived from GlyT2-deficient mice. Previous studies on brain stem neurons have revealed an excellent correlation of punctate GlyR and/or gephyrin immunoreactivities (Triller et al., 1985) with the size of the postsynaptic response as defined by mIPSC amplitude measurements (Lim et al., 1999). Hence, our results imply that the density of postsynaptic GlyRs is unchanged. Similar findings were obtained when the expression levels and localization of other

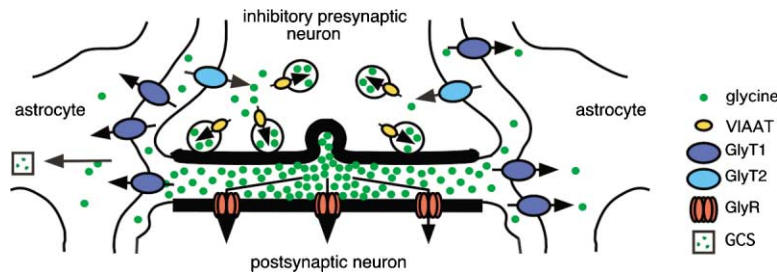


Figure 7. Model of GlyT1 and GlyT2 Functions at Glycinergic Synapses

The different localizations of the two GlyTs are depicted. The neuronal GlyT isoform GlyT2 is essential for glycine uptake into the nerve-terminal cytosol and thereby provides substrate for VIAAT-mediated refilling of re-endocytosed synaptic vesicles, whereas the glial isoform GlyT1 removes released glycine from postsynaptic GlyRs and allows for its degradation by the glial glycine cleavage system (GCS). Consequently, the two transporters cooperate functionally at different steps of glycine neurotransmitter life cycle. For details, see text.

pre- and postsynaptic components were analyzed. Furthermore, GlyR cluster immunofluorescence in cultured spinal neurons prepared from the knockout mice was indistinguishable from that seen in wild-type cells. Together with our results on GlyT1^{-/-} mice (Gomez et al., 2003), these data indicate that GlyT proteins are not required for the formation of pre- and postsynaptic membrane specializations. Furthermore, ablation of the corresponding genes does not cause adaptive alterations in synapse biochemistry.

GlyR α Isoform Switching Proceeds Normally in GlyT2-Deficient Mice

Expression of the different GlyR α subunits is developmentally regulated throughout the mammalian CNS (reviewed in Kuhse et al., 1995; Laube et al., 2002). GlyR α 2 is the fetal subunit isoform predominant at birth (Becker et al., 1988; Malosio et al., 1991), whereas α 1 is the major adult subunit that replaces α 2 during the first two weeks of postnatal life (Grenningloh et al., 1990; Becker et al., 1992; Singer et al., 1998). Consequently, the duration of postsynaptic glycine currents is gradually reduced postsynaptically because of a progressive transition from the kinetically slow α 2 to the fast α 1 GlyR (Takahashi et al., 1992; Singer et al., 1998). At intermediate developmental stages, glycinergic synapses express variable proportions of α 2 and α 1 subunits, which result in postsynaptic currents of intermediate duration (Singer et al., 1998). Thus, alterations in the expression levels or localization of these GlyR α subunits might account for the abnormal phenotype seen in GlyT2^{-/-} mice. Therefore, we examined the postnatal expression of the GlyR α 1 subunit in spinal cord sections of GlyT2^{-/-} mice and wild-type littermates. In the latter, low levels of the α 1 subunit were present at birth, which increased about 3-fold during the first 10 postnatal days. No differences were found during this developmental period in the fluorescence intensity and localization of α 1-immunoreactive puncta between wild-type and GlyT2 knockout sections, indicating that the postnatal switch of GlyR α subunit isoforms is not affected in GlyT2^{-/-} animals. Hence, the severe phenotype observed in GlyT2^{-/-} mice cannot be attributed to an alteration in GlyR subunit composition.

Essential Function of GlyT2 in Glycinergic Synaptic Neurotransmission

Immunocytochemical studies have shown that, similar to other neurotransmitter transporters, GlyT2 is local-

ized in some distance from the glycine-releasing active zones confined to the synaptic cleft region (Zafra et al., 1995a; Poyatos et al., 1997; Spike et al., 1997; Mahendrasingam et al., 2000). This location suggests that the primary role of GlyT2 might not be clearance of glycine from the synaptic cleft but replenishment of neurotransmitter into presynaptic boutons. Here, recording of spontaneous IPSCs and/or mIPSCs from hypoglossal motoneurons in situ and from spinal cord cultures demonstrated that the amplitudes of GlyR-mediated mIPSCs are reduced upon GlyT2 deficiency. As discussed above, synaptic GlyR immunoreactivities in both sections and cultured neurons from spinal cord were indistinguishable between wild-type and GlyT2^{-/-} samples. Thus, changes in receptor number cannot account for the reduced mIPSC amplitude. At GABAergic synapses, inhibition of GABA transport has been shown to result in a reduction in IPSC and, to some extent, mIPSC amplitudes (Overstreet et al., 2000). This has been attributed to postsynaptic receptor desensitization by elevated levels of extracellular GABA. However, desensitization of postsynaptic GlyRs is unlikely to cause the changes seen in GlyT2^{-/-} neurons, since (1) in our spinal cord cultures, released glycine should be rapidly diluted into the culture medium, and (2) GlyRs desensitize poorly even in the presence of high agonist concentrations. Furthermore, in the accompanying manuscript we show that the amplitude of glycinergic IPSC is not reduced upon increasing extracellular glycine levels by GlyT1 deletion (Gomez et al., 2003). Together, these observations argue against a postsynaptic origin of the observed reduction in mIPSC amplitudes and suggest that the consequences of GlyT2 deficiency are exclusively presynaptic. In slice preparations, a decrease in IPSC and mIPSC frequencies was additionally seen, which was not apparent in recordings from cultured cells. We attribute this difference to a loss of small events resulting from the relatively high noise levels present in slices. In summary, our results support the view that GlyT2 function is required for the maintenance of normal quantal glycine contents in synaptic vesicles. Apparently, glycinergic neurotransmission critically depends on an appropriately sized pool of glycine in the presynaptic terminal, which cannot be maintained by intraneuronal glycine synthesis alone. We therefore conclude that the primary function of GlyT2 is to provide substrate for the VIAAT-catalyzed transport of glycine into synaptic vesicles rather than to shape the time course of glycinergic neurotransmission. Our inability to detect differences in

mIPSC decay kinetics between wild-type and GlyT2^{-/-} neurons lends further support to this conclusion.

The interpretation given above is also consistent with the “spastic” phenotype of the GlyT2^{-/-} mice and contrasts the overinhibition seen in mutant mice deficient in GlyT1, the glial transporter isoform that is responsible for the control of extracellular glycine levels (Gomez et al., 2003). Taken together, the available knockout data suggest that GlyT1 and GlyT2 have rather distinct functions at glycinergic synapses (Figure 7). The glial transporter GlyT1 is crucial for lowering glycine concentrations in the synaptic cleft and thereby ensures efficient silencing of GlyR activation (Gomez et al., 2003). Within the glial cytosol, glycine is then degraded by the glycine cleavage system (Sakata et al., 2001). In contrast, GlyT2-driven glycine uptake is essential for the refilling of synaptic vesicles by VIAAT. Thus, the two mammalian GlyTs control different steps in glycine turnover at inhibitory synapses. The late manifestation of the neuromotor phenotype observed with GlyT2^{-/-} animals may relate to the postnatal GlyR isotype switch that proceeds normally in the mutants. At “slow” neonatal $\alpha 2$ GlyRs characterized by long mean channel open times (Takahashi et al., 1992; Singer et al., 1998), even release events of reduced quantal content may produce sufficient postsynaptic inhibition, whereas a higher number of receptors must be activated at postsynapses containing predominantly “fast” adult $\alpha 1$ GlyRs, which exhibit short mean channel open times.

GlyT2^{-/-} Mice, a New Model for Human Hyperekplexia?

The results presented in this study reveal a previously unrecognized vital role for GlyT2 in postnatal life. Moreover, they provide strong evidence that the major function of GlyT2 in the CNS is to transport extracellular glycine into presynaptic boutons. In addition, GlyT2^{-/-} mice display a severe neuromotor disorder characterized by spasticity, muscular rigidity, tremor, and impaired righting response. These symptoms are similar to those associated with a group of human hereditary motor diseases that develop in early postnatal life, named startle disease or hyperekplexia (Rajendra and Schofield, 1995; Andrew and Owen, 1997; Tijssen et al., 1997). Some dominant and recessive forms of this disease are known to be associated with missense mutations in the $\alpha 1$ subunit gene of the inhibitory GlyR (Langosch et al., 1994; Rees et al., 1994; Shiang et al., 1995; Milani et al., 1996; Lewis et al., 1998). Because of their molecularly defined defects and their graded phenotypes, GlyR mouse mutants so far constitute the only experimental models of the human hyperekplexia syndrome. The data presented here suggests that some yet unclassified forms of hyperekplexia may involve mutations in the human GlyT2 gene, especially in cases where no GlyR deficits could be identified (Vergouwe et al., 1997). Thus, the GlyT2 gene is a candidate disease gene, and our GlyT2^{-/-} mice might constitute a new valuable model for human diseases.

Experimental Procedures

Generation of GlyT2^{-/-} Mice

The mouse gene encoding GlyT2 was isolated from a mouse genomic library prepared from 129SvJ mouse DNA (Genome Systems,

St. Louis, MO) by hybridization with a PCR fragment corresponding to the region of the GlyT2 cDNA that encodes the fourth up to the seventh transmembrane domain. The targeting vector consisted of a 9.5 kb genomic sequence, in which a 1.9 kb fragment encompassing the exon encoding the fourth transmembrane region was replaced with the 1.8 kb PGK-neomycin resistance gene (PGK-neo). A 2.8 kb *Herpes simplex* virus thymidine kinase gene fragment was attached to the 5' end of the construct for negative selection. All subsequent procedures were identical to those described in the accompanying paper (Gomez et al., 2003), except that a 5' external probe was used for Southern blot analysis. From a total of 198 clones screened, two had been targeted properly. These were used for generating chimeric mice with an inactivated GlyT2 allele.

Behavioral Characterization of GlyT2^{-/-} Mice

The methods used for monitoring the neuromotor performance of GlyT2^{-/-} mice have been described (Hartenstein et al., 1996; Becker et al., 2000). Briefly, hind feet clasping behavior was induced by lifting the animals by their tails. For tremor recordings, mice were fixed by their tail to a F30 force transducer (Type 372) connected to a bridge amplifier (Type 336, both from Hugo Sachs Elektronik, 79229 March-Hugstetten, Germany) and a conventional recorder. For righting response quantification, mice were brought into a supine position by twitching the tail, and the time that elapsed after release of the tail until resuming an upright position was determined.

Preparation of Spinal Cord Cultures

Spinal cords were removed from 13-day-old mouse embryos that had been killed by decapitation, dissected in phosphate-buffered saline (PBS) supplemented with 33 mM glucose, and dissociated by trituration as described (Kirsch and Betz, 1995). The cells were plated into a 24-well cluster onto poly-L-lysine-coated coverslips and kept in primary culture for 3 weeks.

Immunocytochemistry and Confocal Microscopy

Coverslips carrying spinal neurons were fixed in 95% (v/v) methanol and 5% (v/v) acetic acid and air-dried. Cells were then permeabilized in 0.2% (w/v) Triton X-100 for 5 min followed by incubation in 5% (v/v) goat serum for 20 min prior to processing for immunofluorescence as described (Kirsch and Betz, 1995; Laube, 2002). To obtain spinal cord and brain stem sections, tissue was cut in blocks of 5 mm and immediately frozen. Cryostat sections were fixed for 2 min in 4% (w/v) paraformaldehyde and processed for immunofluorescence. Confocal microscopy was performed using a confocal laser-scanning microscope Leica TCS-SP (Wetzlar, Germany) equipped with the image software Leica-TCS-NT version 1.6.551.

Electrophysiological Recordings from Acutely Isolated Hypoglossal Slices

200–300 μ m slices of the caudal medulla containing the hypoglossal nucleus from P8–P9 mice were prepared as described (Hülsmann et al., 2000).

Whole-cell recordings were obtained with an L/M-PCA patch clamp amplifier (E.S.F., Göttingen, Germany). Patch electrodes were pulled from borosilicate glass capillaries (Biomedical Instruments, Germany) on a programmable pipette-puller (Zeitz Instruments, Augsburg, Germany) and had resistances ranging from 2 to 6 M Ω when filled with intracellular solution. Series resistance compensation was more than 50%. Hypoglossal motoneurons were voltage clamped at a holding potential of -70 mV. Currents were filtered at 3 kHz with a four-pole Bessel filter and digitized at 10 kHz using a PowerLab/8s recording unit and Chart 4.2 software (ADInstruments Pty. Ltd., Mountain View, CA) and stored on hard disk for offline analysis. Spontaneous and miniature IPSCs were analyzed using the Mini Analysis program (Jaejin Software, Leonia, NJ) as described in the accompanying paper (Gomez et al., 2003). The aCSF contained (in mM): 118 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgCl₂, 1 NaH₂PO₄, 25 NaHCO₃, and 30 D-glucose (pH 7.4). The osmolarity of the aCSF was 310 mosm/l. Experiments were performed at room temperature. During IPSC recordings, 20 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 100 μ M DL-2-amino-5-phosphonopentanoate (AP5, Alexis), and 20 μ M bicuculline were added to the external solution. For mIPSC recordings, tetrodotoxin (TTX, 0.5 μ M) was added to the

aCSF to block synaptic transmission. To increase the apparently low frequency of mIPSCs, sucrose (100 mM) was added to increase the osmolarity of the solution. The electrode solution contained (in mM): 110 CsCl, 30 TEA-Cl, 1 CaCl₂, 2 MgCl₂, 4 Na₂ATP, 10 HEPES, 10 EGTA (pH adjusted to 7.2 with KOH). The equilibrium potential for chloride was at about 0 mV. Therefore, with the holding potential of -70 mV, we observed IPSCs as inward currents. Drugs were obtained from Sigma (St. Louis, MO), Alomone Labs (Israel), and Tocris Cookson Ltd. (Bristol, UK).

Electrophysiological Recording of mIPSCs in Cell Culture

The whole-cell configuration of the patch-clamp technique was used to record mIPSCs at room temperature (20°C–25°C) in the presence of tetrodotoxin (0.5 μM), bicuculline (30 μM), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) as described previously (Laube, 2002). The holding potential was -70 mV. Electrodes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) with a Zeitz DMZ Universal Puller (Zeitz Instruments, Augsburg, Germany) to yield tip resistances of 2–6 MΩ. Pipettes were filled with a solution containing (in mM): 120 CsCl, 20 tetraethylammonium chloride, 1 CaCl₂, 2 MgCl₂, 11 EGTA, and 10 HEPES (pH 7.2). The bathing Ringer solution consisted of (in mM): 137 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 20 glucose, and 5 HEPES (pH 7.4). Current recordings were obtained with an EPC-9 amplifier (HEKA Elektronik, Lambrecht, Germany) controlled by HEKA software. mIPSCs were acquired for 10 min, sampled at 10 kHz, stored on disk, and evaluated offline as described (Laube, 2002). Drugs were applied using a microcapillary application system (DAD-12; Adams and List, Westbury, NY) and purchased from Sigma (Deisenhofen, Germany) with the exception of CNQX, which was from Tocris Cookson (Bristol, UK). Differences in mean amplitudes and frequencies of mIPSCs were examined by Student's two-tailed t test and considered to be statistically significant at $p < 0.05$.

Miscellaneous Methods

RNA isolation, RT-PCR analysis, the preparation of brain membranes, [³H]glycine transport assays, Western blotting, and the histological analysis of tissue sections were performed as described in the accompanying paper (Gomez et al., 2003). Antibodies specific for PSD-95 (1:1000) and syntaxin (1:1000) were obtained from Chemicon (Hofheim, Germany).

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