Generation of a Mouse Line Expressing Cre Recombinase in Glycinergic Interneurons

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Summary: In caudal regions of the CNS, glycine constitutes the major inhibitory neurotransmitter. Here, we describe a mouse line that expresses Cre recombinase under the control of a BAC transgenic glycine transporter 2 (GlyT2) promoter fragment. Mating of GlyT2-Cre mice with the Cre reporter mouse lines Rosa26/LacZ and Rosa26/YFP and analysis of double transgenic offsprings revealed strong transgene activity in caudal regions of the central nervous system, i.e., brain stem and spinal cord. Some additional Cre expression was observed in cortical and cerebellar regions. In brain stem and spinal cord, Cre expressing cells were identified as glycinergic interneurons by staining with GlyT2- and glycine-immunoreactive antibodies; here, >80% of the glycine-immunoreactive cells expressed the Cre reporter protein. These data indicate that GlyT2-Cre mice are a useful tool for the genetic manipulation of glycinergic interneurons.

Key words: glycine; Cre recombinase; BAC transgene; interneurons; brain stem; spinal cord

INTRODUCTION

Synaptic inhibition in the central nervous system is mediated by the amino acids γ-aminobutyric acid (GABA) and glycine. These neurotransmitters inhibit postsynaptic cells by activating chloride conducing $GABA_A$ and glycine receptors (GlyRs), thereby producing hyperpolarization or shunting inhibition of the postsynaptic cell. Although GABAergic and glycinergic neurotransmission share several key components like the vesicular inhibitory amino acid transporter (VIAAT, Dumoulin *et al*., 1999) and the postsynaptic scaffolding protein gephyrin (Feng *et al*., 1998), they differ markedly in their anatomical distributions. GABA is the principal inhibitory neurotransmitter in higher brain regions, whereas glycine constitutes the major inhibitory neurotransmitter in brain stem and spinal cord.

The understanding of the how GABAergic inhibition contributes to CNS function has been boosted by the identification of genes that are expressed specifically in GABAergic interneurons (or subpopulations thereof), like the glutamate decarboxylase 67 (GAD67) and parvalbumine genes. Promoter fragments of these genes have been used not only to drive the expression of fluorescent proteins in these neurons (Meyer *et al*., 2002; Tamamaki *et al*., 2003) but also to selectively target genes in a subclass of GABAergic interneurons by the expression of Cre recombinase (Fuchs *et al*., 2007). Similarly, expression of green fluorescent protein (GFP) under the control of a promoter fragment derived from the glycine transporter 2 (GlyT2) gene has been successfully used to specifically label glycinergic interneurons in vivo (Zeilhofer *et al*., 2005).

Here, we have used this GlyT2 promoter fragment to generate a mouse line that shows efficient expression of Cre recombinase in glycinergic interneurons. Our data indicate that this GlyT2-Cre line allows Cre-dependent reporter expression in interneurons that contain high levels of glycine and express GlyT2.

RESULTS AND DISCUSSION

Generation of GlyT2-Cre Mice

To generate mouse lines that allow Cre expression in glycinergic neurons, we made use of a bacterial artificial chromosome (BAC) containing the 5′ UTR as well as all

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Contract grant sponsor: Max-Planck Gesellschaft and Fond der Chemischen Industrie

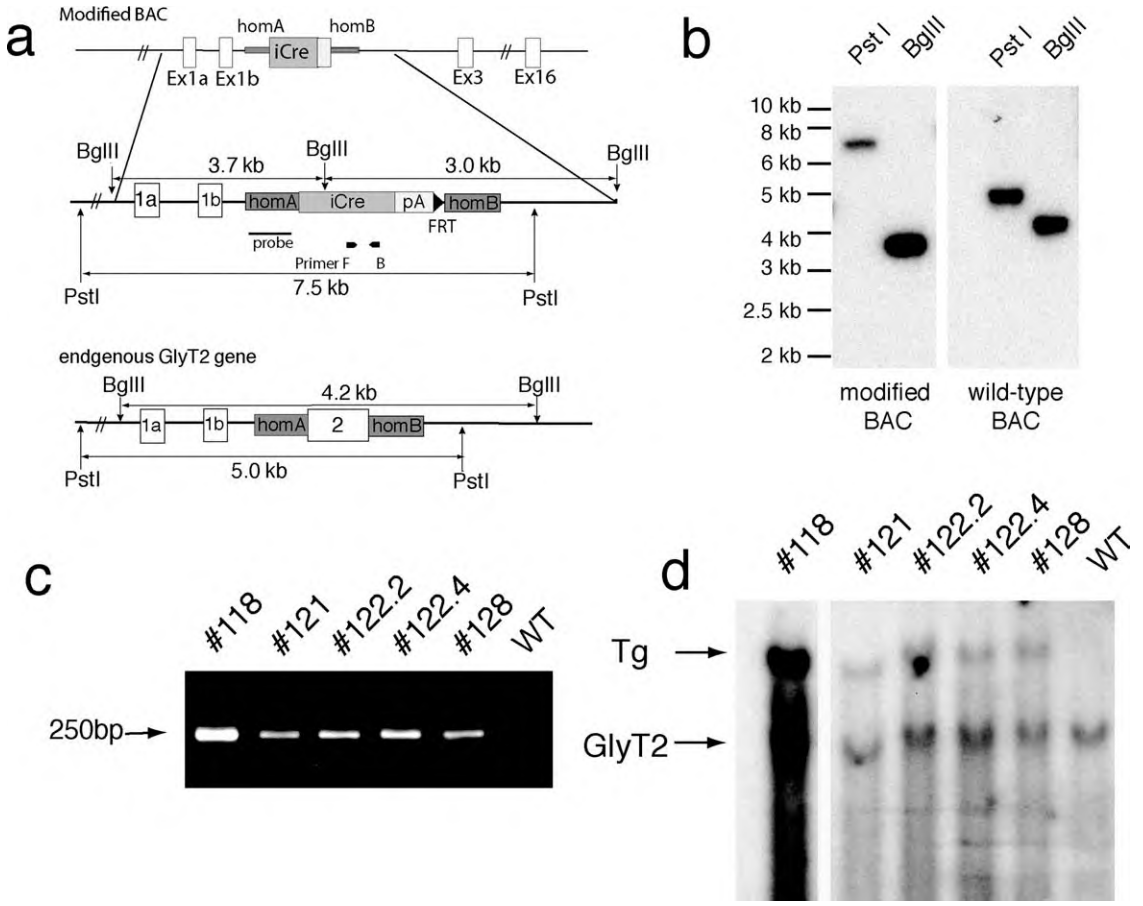

FIG. 1. Generation of GlyT2-Cre mice expressing Cre-recombinase in glycinergic neurons. (**a**) Schematic representation of the modified BAC RPCI 23-365E4 containing the iCre cassette (top). Details of the central region are shown in the middle; BglII and PstI restriction sites, the homology regions used for reconbination (homA and homB), the polyadenylation signal (pA), the Flippase Recognition Target (FRT) site as well as the probe used for Southern blotting and the positions of the primers used for genotyping are indicated. Exons are shown as open squares. The corresponding region of the endogenous GlyT2 gene is shown at the bottom. (**b**) Southern blot analysis of the modified and the wild-type (WT) BACs after PstI and BglII digestion, using the probe indicated in (a). (**c**) Genotyping of the transgenic mice by PCR using primers F and B as indicated in (a). A 250 bp iCre fragment was amplified from genomic DNA isolated from the indicated transgenic mouse lines but not from WT animals. (**d**) Southern blot of PstI-digested genomic DNA obtained from transgenic animals of the indicated mouse lines and from WT littermates, respectively; hybridization probe as indicated in (a).

coding exons (exons 1–16) of the GlyT2 gene. This BAC has been previously shown to contain major regions of the GlyT2 promoter, since the expression pattern of GFP driven by this BAC was very similar to that of endogenous GlyT2 (Zeilhofer *et al.*, 2005). Here, we inserted the coding sequence for a codon-optimized Cre recombinase including the nuclear localization signal (iCre see Shimshek *et al.*, 2002) in frame into exon 2 by using a two-step homologous recombination procdure (Fig. 1a, and Yang *et al.*, 1997). Proper targeting was verified by PCR and Southern blotting (Fig. 1b). The modified BAC was linearized and used for pronucleus injection into B6D2/F1 one-cell zygotes. After implantation into pseudopregnant foster mothers, 80 mice were born, of which seven had integrated the transgene into their genome and transduced it to their progeny, as shown by PCR on DNA samples derived from tail biopsies (data not shown, and Fig. 1c). From these lines, five were subsequently analyzed. Genomic DNAs derived from animals of these GlyT2-Cre lines (lines 118, 121, 122.2, 122.4, 128) were subjected to Southern blot analysis to obtain an estimate of how many copies of the transgene had been incorporated (Fig. 1d). The probe used in these experiments did not only hybridize to the transgene but also to the endogenous GlyT2 gene, thus allowing a quantitative assessment of transgene copy numbers. With the exception of lines 118 and 128 that contained five and two transgene copies, respectively, all other lines carried single copy integrations of the transgene (data not shown, but see Fig. 1d).

Characterization of Transgene Expression Patterns

To determine the expression patterns of Cre recombinase in the GlyT2-Cre transgenic mouse lines, mice from the GlyT2-Cre lines were mated with ROSA26/LacZ re-

porter mice (Soriano, 1999). In these mice, β-galactosidase expression is induced upon Cre expression. When cryosections of GlyT2-cre//ROSA26/LacZ double transgenic mice were stained for β-galactosidase, distinct cell populations of β-galactosidase-positive cells were found in particular in brain stem and spinal cord (Fig. 2a–c and data not shown). More specifically, in sections derived from double transgenic mice derived from the lines 121, 122.2, and 122.4, numerous LacZ-positive cells were found in all major brain regions, and the distribution of these cells was comparable in all three mouse lines. These results demonstrate that a single copy of the GlyT2-Cre transgene is sufficient for reliable recombination efficiency. In brain sections derived from Cre reporter positive mice derived from matings with GlyT2-Cre transgenic mouse lines 118, and 128, however, only a low number of Cre positive cells were detected in both brainstem and spinal cord, possibly due to unfavorable integration sites of the transgene. All GlyT2-Cre negative animals were devoid of detectable β-galactosidase activity within brain tissue (Fig. 2e), whereas control mice showing ubiquitous LacZ expression (Z/AP mice, Lobe *et al*., 1999) displayed strong labeling throughout the central nervous system (Fig. 2d). Interestingly, mating of GlyT2-Cre mice with other Cre reporter mice, like the Z/AP mice which drive expression of a double reporter system under the control of a CMV-enhanced chicken β-actin promoter, revealed only a very low number of Cre reporter-positive cells in all lines analyzed (data not shown). This might reflect an inefficient expression of the reporter in Cre expressing cells or a reduced efficiency of Cre-mediated excision in specific cell populations at the integration site of the Z/AP transgene (Hebert and McConnell, 2000).

Subsequently, the GlyT2-Cre line 121 was analyzed in more detail. In spinal cord sections from GlyT2-Cre//Rosa26/LacZ double transgenic mice of this line, β-galactosidase positive cells were found predominantly in grey matter, whereas the surrounding white matter was largely devoid of labeling. An accumulation of labeled cells was found in the dorsal and ventral horns of the spinal cord (Fig. 2a1,a2); both regions are known to be rich in glycinergic neurons (Legendre, 2001). In brain stem, β-galactosidase positive cells were found in all areas, with highest densities in the medial vestibular nucleus, the spinal trigeminal tract and the region of the lateral paragigantocellular nucleus (Fig. 2b1–b3). Additionally, reporter-positive cells were found in the cerebellum (Fig. 2b4). Based on the size and localization of these cells, mainly subpopulations of Purkinje cells and some basket and stellate cells appeared to be β-galactosidase positive. In the forebrain, some stained cells were found in cortex whereas the hippocampus was devoid of β-galactosidase activity (Fig. 2c).

Cre Recombinase is Only Expressed in Neurons of GlyT2-Cre Mice

To establish the cellular identity of the cells that were Cre reporter-positive, GlyT2-Cre mice were mated with ROSA26/YFP mice, which express yellow fluorescent protein (YFP) in a Cre-dependent manner (Srinivas *et al*., 2001). Brain sections from mice carrying both transgenes were analyzed using a YFP-crossreactive GFP antibody and antibodies for the pan-neuronal marker NeuN (see Fig. 3). In sections derived from animals carrying the ROSA26/YFP transgene but not the GlyT2-Cre transgene, no YFP-positive cells were found in all brain regions analyzed (Fig. 3a, Vh neg). In double transgenic animals, however, YFP expressing cells were seen in all major brain regions. With the exception of the cerebellum (Fig. 3a, Cbx), only NeuN-positive cell bodies were found to express the Cre reporter, indicating that in most brain regions Cre activity was, as expected, restricted to neurons (Fig. 3a). Additionally, YFP immunoreactivity was found in areas surrounding the cell bodies, which most likely corresponded to processes of reporter-positive cells. In the dorsal and ventral horns of the spinal cord, 50% and 40%, respectively, of all NeuN-positive cells were found to be reporter-positive (Fig. 3a Vh, Dh, Fig. 3b). In the area around the central canal, >80% of all NeuN-positive cells expressed YFP (Fig. 3b). In contrast to β-galactosidase activity that was strictly restricted to grey matter, YFP immunoreactivity was also found in white matter of the spinal cord (data not shown but see Fig 4b, WM). Here, YFP was never found to be associated with nuclei, suggesting that only projecting fibers from neurons were stained (data not shown, but see inset in Fig. 4b WM). Consistent with this view, structures immunoreactive for YFP were not associated with glial cells identified by co-labeling with antibodies against the glial marker proteins GFAP or S100β (data not shown).

In brain stem, about 30–40% of all neurons were positive for the Cre reporter (Fig. 3b). In contrast, in cerebral-cortical regions ≤10% of all neurons were labeled with YFP (Fig. 3b). In the cerebellum, only granular cells were found to be NeuN-positive, whereas Purkinje cells, Golgi cells as well as stellate and basket cells were not labeled by this marker protein under the conditions used. Consistent with granule cells not expressing the Cre reporter, we failed to detect NeuN positive cells that stained for YFP. Similar to our findings in spinal cord, reporter expression was not observed in glial cells of this brain region (data not shown). These findings are consistent with previous results on GlyT2-GFP mice (Simat *et al*., 2007). There, GFP expressing cells localized in the cerebellum were identified as GABAergic neurons, which apparently display ectopic expression of the transgene.

A Major Fraction of Glycinergic Neurons Shows Cre Reporter Expression in GlyT2-Cre//ROSA26/YFP Double Transgenic Mice

To analyze whether the reporter-positive cells in GlyT2-cre//ROSA26/YFP double-transgenic mice indeed represent glycinergic neurons, we performed immunostaining for other glycinergic markers. Glycinergic neu-

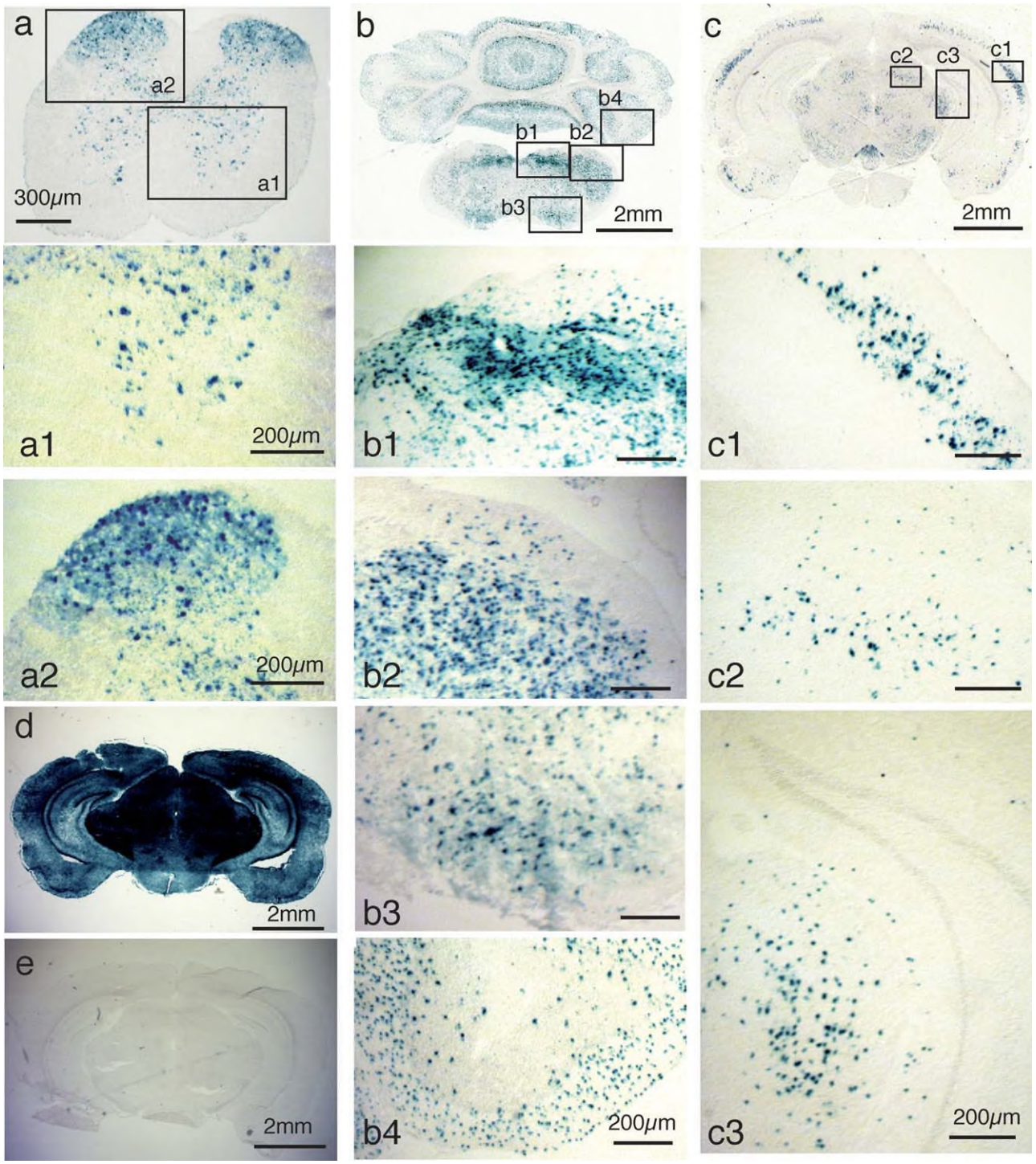

FIG. 2. GlyT2-Cre mice display Cre activity in brain stem, spinal cord, and other brain regions. Cryosections of different brain regions derived from GlyT2-Cre #121//ROSA26/LacZ double transgenic mice (**a**–**c**), Z/AP mice (d) or ROSA26/LacZ mice (**e**) were analyzed for β-galactosidase expression by X-gal staining. Boxes in a–c indicate the regions displayed at higher magnification in a1-2, b1–4, and c1–3. (a) β-Galactosidase expression in a spinal cord section from GlyT2-Cre #121//ROSA26/LacZ double transgenic animals. Prominent labeling was observed in the ventral horn (a1) and the dorsal horn (a2). (b) Brain stem and cerebellar cryosections of GlyT2-Cre #121//ROSA26/LacZ double transgenic animals. β-Galactosidase staining was found in the solitary tract and the medial vestibular nucleus (b1), the spinal trigeminal tract (b2), the pyramidal tract and the lateral paragigantocellular nucleus (b3); additional labeling was seen within the cerebellar cortex (b4). (c) In forebrain and midbrain sections, positive cells were mainly seen in cortex (c1) and midbrain (c2 and c3). Note that no β-galactosidase positive cells were found in the hippocampal layers (c3). Sections derived from Z/AP mice (**d**) and Rosa26/LacZ mice (e) were used as positive and negative controls for LacZ staining.

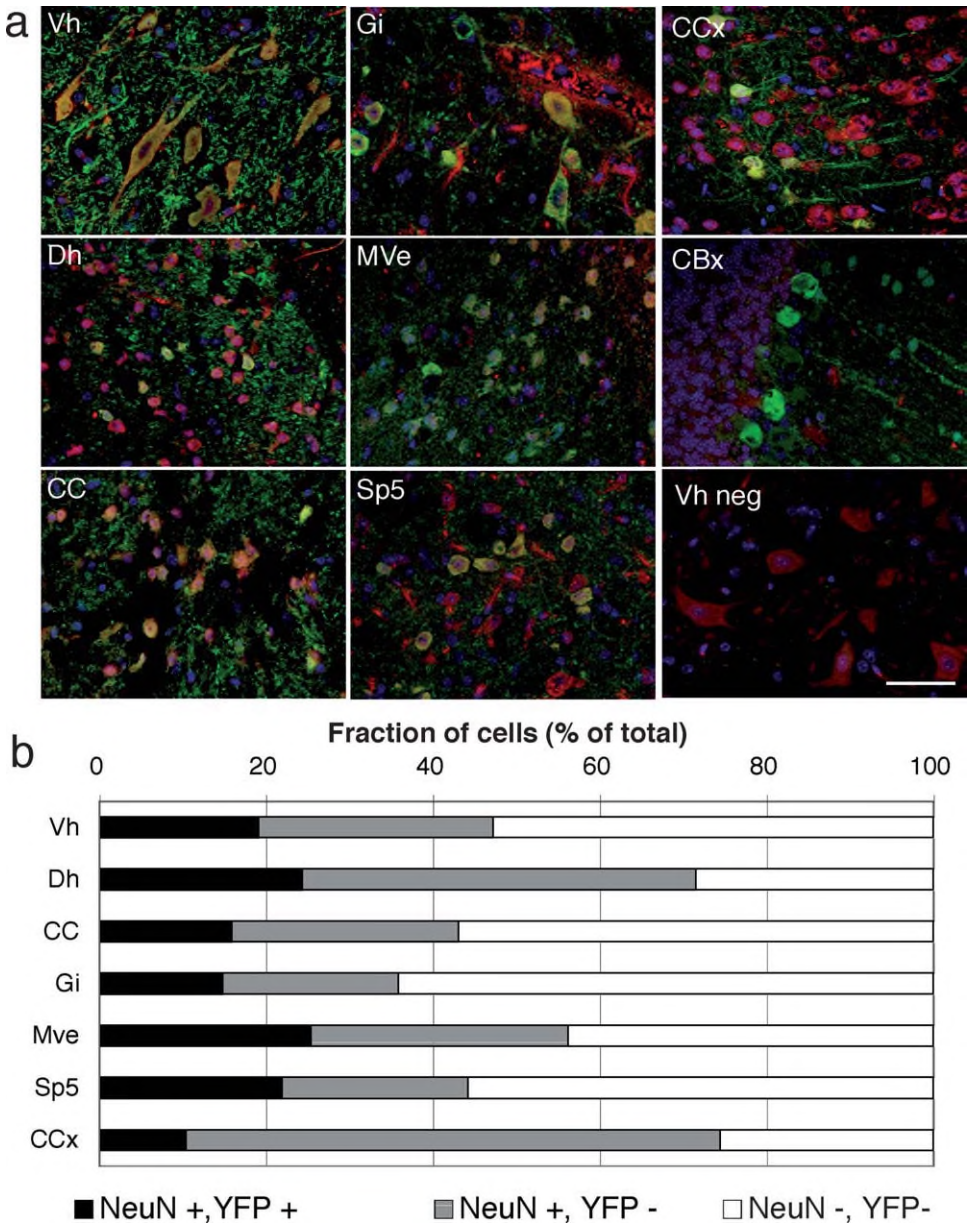

FIG. 3. Cre activity in GlyT2-Cre mice is restricted to neurons in brain stem, spinal cord, and cerebral cortex. (**a**) Cyrosections of GlyT2-Cre #121//ROSA26/YFP double-transgenic animals were stained with anti-GFP (green) and anti-NeuN (red) antibodies; nuclei were visualized with DAPI (blue). Merged images showing all three fluorescences for the brain regions indicated: left, ventral horn (Vh), dorsal horn (Dh), and central canal (CC) of the spinal cord; middle, the brain stem regions of the gigantocellular reticular nucleus (Gi), the medial vestibular nucleus (MVe), and the spinal trigeminal tract (Sp5); right, cerebral cortex (CCx), cerebellar cortex (CBx), and the negative control without cre expression (Vh neg). Note that NeuN immunoreactivity is predominantly seen in cell bodies, whereas YFP is additionally present in surrounding fibers. With the exception of the cerebellum, YFP immunreactive cells in all other brain regions also showed NeuN expression. In cerebellum, Purkinje cells as well as stelate and basket cells (not shown) were positive for the Cre reporter, but displayed only weak NeuN immunoreactivity. Scale bar, 50 μm. (**b**) Quantification of YFP and NeuN double-positive (NeuN +, YFP +), NeuN-positive (NeuN +, YFP −), and unlabeled (NeuN −, YFP −) cells in the brain regions indicated (n = 3). Note that, in the regions shown, all YFP-positive cells were NeuN-positive.

rons contain high intracellular glycine concentrations, most likely due to efficient accumulation of the neurotransmitter by GlyT2-driven glycine uptake. Double labeling experiments with YFP-specific antibodies and antibodies raised against a paraformaldehyde conjugate of glycine revealed hardly any glycine-positive cells in forebrain neurons (data not shown). This is consistent with previous reports showing that this brain region is nearly devoid of glycinergic synapses (Rampon *et al.*, 1996; van den Pol and Gorcs, 1988). Similar results were obtained for the cerebellum (Fig. 4a). In brain stem and spinal cord, numerous glycine-immunoreactive cells were revealed by the antibody used; ≥80% of these cells were also positive for the Cre reporter (see lower panels

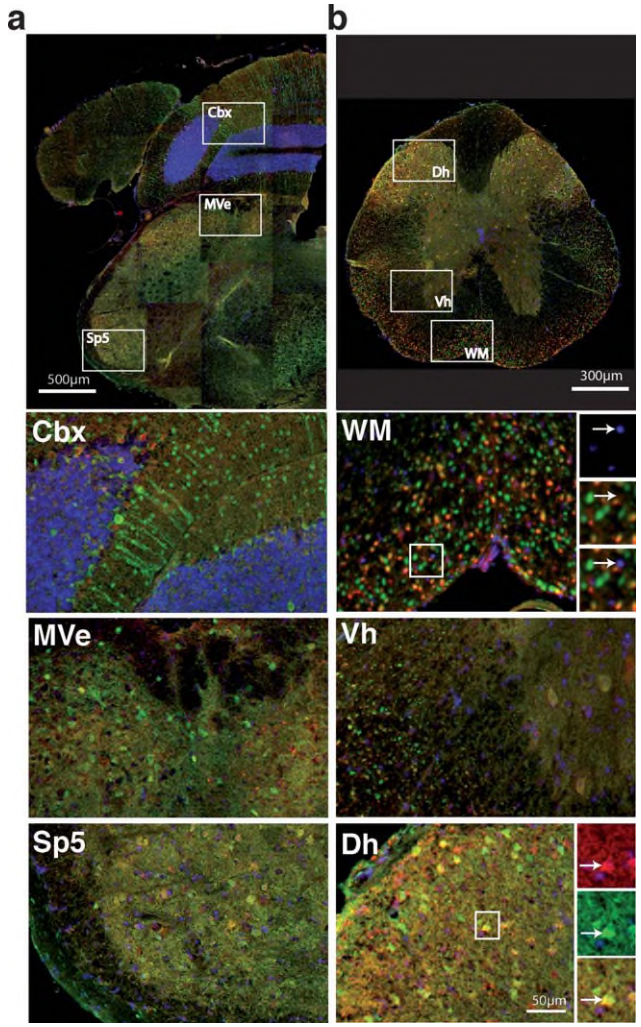

FIG. 4. Cre expressing cells in GlyT2-Cre mice are glycinergic. Cryosections of brain stem (**a**) and spinal cord (**b**) derived from GlyT2-Cre #121//ROSA26/YFP double transgenic mice were probed with anti-GFP (green) and anti-glycine (red) antibodies; nuclei are visualized by DAPI staining. (a,b) Composite images of brain stem/cerebellum (a) and spinal cord (b). Boxes in a and b indicate regions displayed at higher magnification in the lower panels; cerebellar cortex (Cbx); medial vestibular nucleus (MVe); spinal trigeminal tract (Sp5); white matter (WM); ventral horn (Vh); dorsal horn (Dh). Insets in WM and Dh show higher magnification as well as channel separation of the boxed area. Arrows indicate the position of a presumptive glial cell and glycinergic neuron, respectively. Scale bar for CbX, MVe, Sp5, and Vh is presented in Dh. Note that glycine immunoreactivity was not only seen in YFP-positive cell bodies, but also in processes emerging from them.

in Fig 4a,b). Quantitative evaluations revealed that in the dorsal and ventral horns of the spinal cord 84% of all glycine positive cells were labeled by the YFP antibody ($n \geq 50$ cells for each region), whereas in the medial vestibular nucleus (MVe) of the brainstem 93%, and in the spinal trigeminal tract (Sp5) roughly 90%, of all glycine-positive cells expressed YFP ($n \geq 150$ cells for each region). This result indicates that in these brain regions Cre recombinase is expressed in most glycinergic interneurons. Additionally, strong YFP immunoreactivity was observed in the white matter of spinal cord (Fig. 4b, WM). Here, YFP fluorescence was never found to be associated with nuclei as indicated by DAPI fluorescence, but partial colocalization of glycine and YFP immunoreactivity was observed (Fig. 4b, inset; WM), suggesting that the YFP-positive structures were indeed extensions of glycinergic neurons, most likely axonal specializations.

To more directly show that the cells that were found to be Cre reporter-positive were indeed GlyT2 expressing cells, we performed colabeling experiments with GFP- and GlyT2-specific antibodies (see Fig. 5). GlyT2 immunoreactivity was found to be widely distributed in brain stem and in the grey matter of spinal cord, however, it was absent from cell bodies in these brain regions (Fig. 5a2,b2,c2). This is consistent with GlyT2 being enriched at presynaptic terminals. Additional GlyT2 immunoreactivity was found in small structures within the white matter of spinal cord (Fig. 5b). Although no colocalization of YFP- and GlyT2 immunoreactivity was found in the YFP-labeled cell bodies of presumptive glycinergic neurons, in surrounding nerve fibers a partial overlap of both fluorescences was observed. A similar apposition of both fluorescences was noted in the white matter. Here, ring-like GlyT2 immunoreactivity was found surrounding YFP-positive structures, consistent with labeling of an integral plasma membrane protein. Together these data are consistent with Cre recombinase being present in GlyT2 expressing cells.

CONCLUSIONS

Taken together, the data presented in this study indicate that the GlyT2-Cre mice generated here are suitable for the selective targeting of floxed genes and transgenes in glycinergic interneurons of brain stem and spinal cord. Additional expression of Cre activity was found in the cerebral cortex, midbrain and cerebellum. Future work is required to clarify whether the Cre expression seen in these regions is caused by missing or dysfunctional regulatory elements within the BAC transgene used or due to the expression of glycinergic marker genes in a subpopulation of GABAergic neurons or their precursor cells.

MATERIALS AND METHODS

Modification of the BAC Clone and Generation of GlyT2-Cre Mice

The BAC clone RPCI-23 365E4 isolated from a mouse C57/Bl6 BAC library (Research Genetics Inc., Huntsville, AL) containing the 5′ region and all coding exons of the GlyT2 (slc6A5) gene has been shown to efficiently drive expression in glycinergic neurons (Zeilhofer *et al.*, 2005). Since GlyT2 is expressed in different splice variants which differ in exon 1 (Ponce *et al.*, 1998), a codon-optimized cDNA encoding Cre recombinase (iCre) was cloned in frame into the first codon of exon 2 of the BAC-GlyT2 gene (Fig 1a) by using a two-step in vivo recombination procedure (Yang *et al.*, 1997). The targeting vector

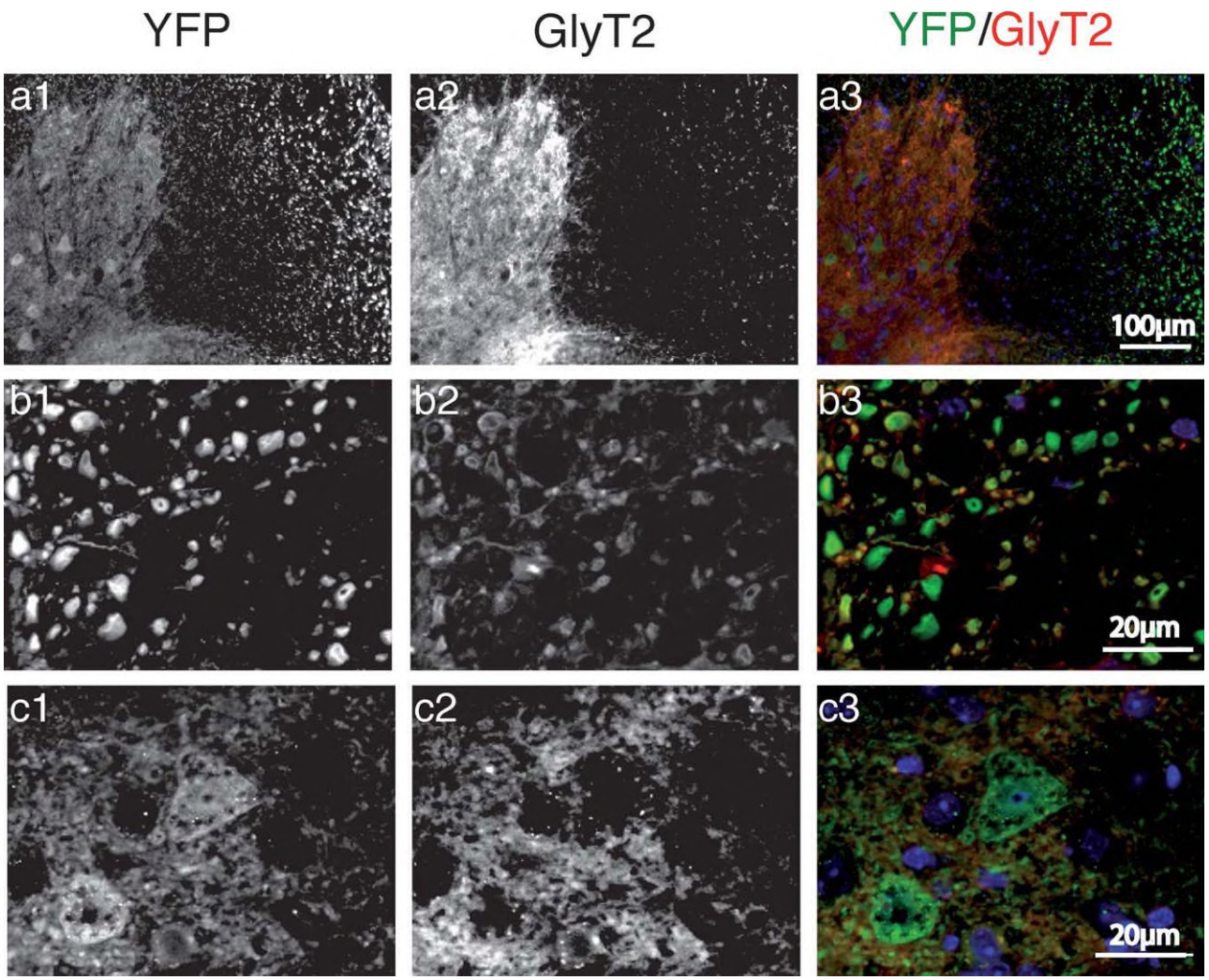

FIG. 5. GlyT2 immunoreactive processes contain the Cre reporter YFP. Cryosections of spinal cord from GlyT2-cre//ROSA26/YFP double transgenic animals were stained with anti-GFP and anti-GlyT2 antibodies; nuclei are visualized by DAPI (blue). Panels a1–a3, overview images of the ventral horn and the surrounding white matter; b1–b3, white matter; and c1–c3, grey matter including cell bodies of neurons in the ventral horn. Note that although YFP and GlyT2 immunoreactivities did not co-localize on cell bodies within the ventral horn, many processes within both grey and white matter were double-positive.

used for BAC modification contained the iCre cDNA followed by the bovine growth hormone polyadenylation (polyA) signal derived from the plasmid pcDNA3.1 (Invitrogen, Carlsbad, USA, see also Goodwin and Rottman, 1992) and a FRT site. The iCre-polyA-FRT insert was flanked by short DNA fragments corresponding to the 526 bp upstream and 561 bp downstream regions flanking the 5′ and 3′ ends of exon 2 of the GlyT2 gene, respectively. Proper recombination after each round of homologous recombination was verified by Southern blotting with a probe corresponding to the 5′ homology strand (HomA, Fig 1b). The successfully modified BAC was linearized using NotI. The purified linearized DNA was then used for pronuclear injection into B6D2/F1 1-cell zygotes. Out of 303 injected zygotes, 293 were transferred into pseudopregnant foster mice. Mice carrying the transgene were identified by PCR and Southern blot analysis of genomic DNA isolated from tail biopsies.

Genotyping

Genomic DNA from tail biopsies was isolated by phenol-chloroform extraction, precipitated with ethanol and subjected to PCR analysis using primers complementary to the iCre sequence (Fig. 1a; Primer F: 5′-ATTGCCTA CAACACCCTGCTGC-3′; Primer B: 5′-CCACACCATTCTTT CTGACCCG-3′, expected band size 250 bp) and the HotStarTaq Master Mix Kit (QIAGEN, Hilden, Germany). Genotyping of ROSA26/YFP mice and ROSA26/LacZ mice was performed as described (Soriano, 1999).

Southern Blot Analysis

For Southern blot analysis, genomic DNA was digested with BglII or PstI, separated on a 0.8% agarose gel, transferred to a nylon membrane (Hybond N+, GE-Healthcare, Uppsala, Sweden) and immobilized by UV-mediated crosslinking. Hybridization probes corresponding to HomA (Fig. 1a) were amplified by PCR from BAC RPCI-23 365E4 and labeled with ^{32}P-dCTP using a random labeling kit (Roche, Basel, Switzerland). Hybridization was performed using the QuickHyb Hybridization Solution (Stratagene, La Jolla, USA) following the manufacturer's protocol.

Tissue Sections

Adult mice of the respective genotypes were anesthetized with isoflurane, killed by rapid cervical dislocation, and both brain and spinal cord were removed and briefly rinsed in ice-cold phosphate buffered saline (PBS; 137 mM NaCl, 4.3 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4). Then the tissue was fixed in 4% (w/v) paraformaldehyde in PBS at 4°C overnight. The tissue was washed three times for 15–30 min in ice-cold PBS, cryoprotected in 30% (w/v) sucrose in PBS, and embedded in Tissu-Tek OCT (Sakura, Zoeterwoude, Nederlands) at 4°C for 1 h prior to freezing on dry ice.

For LacZ staining, tissue was fixed with LacZ fix (PBS containing 0.2% (v/v) glutaraldehyde, 50 mM EGTA, 100 mM $MgCl_2$, 0.02% (v/v) Nonidet-P40, 0.01% (w/v) sodium desoxycholate, pH 7.3) at 4°C for 1 h. After washing with ice-cold PBS and cryoprotection in 30% (w/v) sucrose in PBS overnight at 4°C, the tissue was embedded in Tissue-Tek OCT.

Tissue blocks of the respective brain regions were cryosectioned at 12 μm, mounted onto SuperFrost plus glass slides (Menzel, Braunschweig, Germany) and dried for 1–4 h at room temperature before storage at −80°C.

LacZ Staining

Frozen sections were briefly postfixed in ice-cold PBS containing 0.2% (v/v) glutaraldehyde for 10 min. After washing three times with LacZ buffer (PBS containing 2 mM $MgCl_2$, 0.02% (v/v) Nonidet-P40, 0.01% (w/v) sodium desoxycholate, pH 7.3) at room temperature, sections were incubated with LacZ staining solution (LacZ buffer containing 0.5 mg/ml 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-gal), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide) for 24 h at 37°C. Slides were then washed in PBS, dehydrated through an ethanol series, and mounted onto coverslips with Entellan (Merck, Darmstadt, Germany). Pictures were taken with a digital camera mounted on a binocular (both Leica, Wetzlar, Germany). High-resolution images were acquired using an AxioPhot microscope (Zeiss, Goettingen, Germany) equipped with a Spot digital camera (Diagnostics Instruments, Sterling Heights, USA). All images were processed with MetaMorph software (Molecular Devices, Sunnyvale, USA).

Immunostaining

Sections were briefly postfixed in ice-cold PBS containing 4% (w/v) paraformaldehyde for 10 min. After washing with either 0.1 M glycine in PBS, or 10 mM triethanolamin (for anti-glycine staining), for 10 min and blocking with PBS containing 1% (v/v) goat serum, 2.5% (w/v) bovine serum albumine and 0.3% (v/v) Nonident-P40 (for anti-glycine staining), or Triton X 100 (for anti-GlyT2), at room temperature for 1 h, sections were incubated with the indicated antibodies (rabbit anti-GFP, Molecular Probes, 1:500; rat anti-glycine, Immuno Solution, Australia, 1:1,000; guinea pig anti-GlyT2, Millipore 1:2,000; mouse anti-NeuN, Millipore, 1:1,000; all diluted in blocking solution) at 4°C overnight. Then the sections were washed thrice with PBS for 10 min at room temperature, and the bound primary antibodies were visualized with goat fluorophore-conjugated secondary antibodies (Alexa 488 anti-rabbit, Alexa 546 anti-mouse, Alexa 546 anti-rat, Alexa 546 anti-guinea pig, all diluted 1:1,000 in blocking solution, Invitrogen, Carlsbad, USA). After washing with PBS, cell nuclei were visualized by incubation with DAPI (0.5 μg/ml in PBS) at room temperature for 10 min, followed by three further washes with PBS, and mounted onto coverslips with Aquamount (Polyscience, Warrington, USA). Images were aquired using an AxioImager microscope equipped with an Apotome (Zeiss) to obtain optical sections. Images were processed with the AxioVision software (Zeiss).

ACKNOWLEDGMENTS

The authors thank Dr. Hans-Ulrich Zeilhofer for providing a BAC containing a GlyT2 promoter fragment, Dr. Rolf Sprengel for an iCre containing plasmid, Dr. Nathaniel Heintz for providing plasmids allowing for in vivo recombineering of BAC DNA, and Maren Krause for excellent technical assistance.

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