Characterization of Mice with Targeted Deletion of Glycine Receptor Alpha 2

T. L. Young-Pearse, 1 L. Ivic, 2 A. R. Kriegstein, 3 and C. L. Cepko 1*

Department of Genetics and Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts 02115; Department of Neurology, Mount Sinai School of Medicine, New York, New York 10029; and Department of Neurology and Program in Developmental and Stem Cell Biology, University of California, San Francisco, California 94143

Received 8 February 2006/Returned for modification 7 April 2006/Accepted 22 May 2006

Glycine receptors are ligand-gated chloride channels that mediate inhibitory neurotransmission in the adult nervous system. During development, glycine receptor alpha 2 (GlyRα2) is expressed in the retina, in the spinal cord, and throughout the brain. Within the cortex, GlyRα2 is expressed in immature cells and these receptors have been shown to be active and excitatory. In the developing retina, inhibition of glycine receptor activity prevents proper rod photoreceptor development. These data suggest that GlyRα2, the developmentally expressed glycine receptor, may play an important role in neuronal development. We have generated mice with a targeted deletion of glycine receptor alpha 2 (Glrα2). Although these mice lack expression of GlyRα2, no gross morphological or molecular alterations were observed in the nervous system. In addition, the cerebral cortex does not appear to require glycine receptor activity for proper development, as Glrα2 knockout mice did not show any electrophysiological responses to glycine.

In the adult nervous system, inhibitory neurotransmission is mediated by γ-aminobutyric acid (GABA) and glycine. Glycine receptors are ligand-gated chloride channels that are composed of pentamers of alpha and beta subunits. Four alpha subunits (α1, α2, α3, and α4) and one beta (β) subunit are present in the murine genome. In the mature nervous system, glycine receptors are heteropentamers composed of alpha subunits and beta subunits. Glycine receptors were initially described as being composed of three alpha subunits, which contained the ligand binding domain, and two beta subunits (5, 13). However, a recent study provides evidence that glycine receptor heteropentamers are composed of two alpha subunits and three beta subunits, with both the alpha and beta subunits contributing to the ligand binding domain (10). Homopentamers of alpha subunits also have been identified, and these forms were shown to be expressed extrasynaptically (28, 30). In the mature nervous system, glycine receptors are expressed at high levels in the spinal cord and brain stem and at lower levels in the thalamus and hypothalamus (reviewed in reference 17). These mutations lead to either a reduction in levels of glycine receptors or impairment of glycine receptor chloride conductance. Two recessive mutations also have been identified in Glrα1 that, when homozygous, lead to complete loss of GlyRα1 expression (4, 25). Interestingly, these mutations are no more severe than the dominant point mutations.

In addition to human disease, there are three naturally occurring mouse startle disease mutants: spastic, spasmodic, and oscillator. The spastic mutant contains a transposon insertion in Glrb, which results in aberrant splicing and a severe reduction in GlyRβ protein levels (11, 21). Spasmodic mice harbor a point mutation in Glrα1 that results in a sixfold decrease in glycine sensitivity (26, 27). The oscillator mutant harbors a frameshift mutation in Glrα1 that results in a drastic reduction in GlyRα1 cell surface expression (12). This is the only glycine receptor mutation that leads to a lethal phenotype. Each of these three mouse mutants appears phenotypically normal up to 2 weeks after birth. Phenotypic startle responses appear at 3 weeks, corresponding to the developmental switch in subunit expression from GlyRα2 to GlyRα1, leading to the suggestion that developmental GlyRα2 expression is responsible for the delay in phenotypic onset (2).

Ligand binding to glycine receptors induces channel pore opening, followed by chloride ion diffusion through the pore. In mature adult neurons, this results in chloride ion influx and hyperpolarization of the membrane. In contrast, during embryonic development, the chloride ion concentration is higher intracellularly than in the extracellular environment. Therefore, glycine receptor activation embryonically leads to chloride ion efflux from the cell and calcium ion influx (8). Embryonically, both GlyRα2 homopentamers and GlyRα2 and GlyRβ heteropentamers are expressed (1, 14). GlyRα2 is expressed in the cortex before functional synapses are formed.

* Corresponding author. Mailing address: Department of Genetics and Howard Hughes Medical Institute, NRB 360, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115. Phone: (617) 432-7618. Fax: (617) 432-7595. E-mail: cepko@receptor.med.harvard.edu.
(18). Flint et al. have shown that these extrasynaptic glycine receptors are excitatory (8). The functional implications of the developmental physiology of glycine receptors have not been established.

Previously, we showed that transient knockdown of GlyRa2 at postnatal day 0 (P0) with short hairpin RNA in the rat retina inhibits the production of rod photoreceptors (34). At P0, ~85% of the cells that exit mitosis become rod photoreceptors (24). As GlyRa2 is expressed throughout the central nervous system during development, we wanted to determine whether it is involved more generally in neuronal development. In order to further examine the role of GlyRa2, we generated mice that have a targeted deletion within the Glra2 genomic locus that results in a complete loss of GlyRa2 expression. Glra2−/− mice are viable and fertile. Morphological and molecular analyses of developing and adult retinae and brains reveal no profound phenotype. In the retina and spinal cord, expression of other glycine receptor subunits may compensate for the lack of a profound phenotype. However, electrophysiology of the embryonic cortex showed no responses to glycine in Glra2−/− animals.

MATERIALS AND METHODS

Generation of Glra2 knockout (KO) mice. Mice were housed and cared for under the guidelines set up by Harvard University in compliance with federal standards.

Bacterial artificial chromosome (BAC) filters containing 129/Sv mouse genomic DNA inserts were purchased from Genome Systems Inc. and hybridized with a probe encoding exons 6 and 7 of mouse glycine receptor alpha 2 (GlyRa2). BAC end sequencing and Southern blot restriction mapping revealed a 160-kb insert that contained genomic DNA that included GlyRa2 exons 3 to 8. In order to replace exons 6 and 7 with an FLP recombination target (FRT)-flanked neomycin cassette within the BAC, the methodology for bacterial recombination developed by Stewart et al. was employed (22). A PGK-neo cassette was PCR amplified with primers that added 62-bp homology arms to each side of the PGK-neo cassette. The sequences of these homology arms are TACATTTTGCCTTTTCTCAAGAAAAAGTACATTGGTTGTGACTCCTTTT and CGGAA GTTGTGCCTTCCGACAAGAACCCTTGAAAGCCTGAGGCTACCTG. The amplified PGK-neo cassette with the homology arms was electroporated into bacteria containing the BAC and recombination machinery. Homologous recombination within the BAC resulted in the deletion of a 2.3-kb fragment containing both exons 6 and 7 and replacement with the PGK-neo cassette. The vector sequence was removed from the BAC by cleavage with NotI, and the modified BAC insert was purified. This fragment was electroporated into 129/SvJ embryonic stem (ES) cells. Homologously recombined ES cell clones were analyzed by PCR and Southern blotting for the presence of Glra2 exon 9 and the absence of Glra2 exons 6 and 7. The Glra2 exon 6/7 probe was generated and sequence verified by PCR amplification with the primers TATGGACAAAGCATTACAAACA and CATTITACCTTCTTATTITTTTG.

Homologously recombined ES cells were injected into blastocysts to generate chimeric animals. F1 animals were genotyped by Southern blot analysis with probe 1 (Fig. 1). Probe 1 was generated by PCR amplification of genomic DNA with primers TCATTGGGTTCTTTGCTTGTAG and AATGAGCCTTGGTTGTGTCTCATTAG and was subcloned into pBluescript and sequenced verified.

Brain slice preparation. Timed pregnant Glra2 KO mice and control C57/BL6 mice (Taconic, New York) at embryonic day 17 (E17) were used for all experiments, except where indicated otherwise. Animals were anesthetized (with ketamine at 90 mg/kg and xylazine at 10 mg/kg), and embryos were removed for further dissection. Following rapid decapitation, the brain was removed in chilled artificial cerebrospinal fluid (ACSF; 125 mM NaCl, 5 mM KCl, 1.25 mM NaH2PO4, 1 mM MgSO4, 2 mM CaCl2, 25 mM NaHCO3, 25 mM glucose, pH 7.4, 310 mosM/liter). The dissected brain was placed in 4% low-melting-point agarose (Fisher Scientific) in ASCF. Agarose was cooled on ice and allowed to solidify, and the embedded brain was sliced into coronal sections (400 μm) in ice-cold ACSF with a VT1005 vibratome-blade microtome (Leica, Nussloch, Germany). Slices were allowed to recover at room temperature in oxygenated (95% O2 and 5% CO2) ACSF. They were subsequently used for electrophysiological recording or loaded for calcium imaging.

Calcium imaging. Brain slices were bath loaded with the acetoxymethyl ester form of the calcium indicator dye fluo-3 (10 to 15 μM; Molecular Probes, Eugene, OR). Loading was performed in the dark at room temperature for 1 to 3 h. Loaded slices were placed in an imaging chamber on the stage of an upright compound microscope (BX50WI; Olympus, Tokyo, Japan) and continuously perfused with oxygenated ACSF. Epi-PH imaging of fluo-3 was performed with a 100-W mercury light source and a low-light charge-coupled device camera (300-T; Dage-MTI, Michigan City, IN). For fluo-3 imaging, we used the following fluorescence filters (Chroma Technology, Brattleboro, VT): excitation filter, 480 ± 20 nm; dichroic mirror, 505 nm long pass; emission filter, 535 ± 25 nm. Cells were imaged with a 10× water immersion objective, and photobleaching was minimized by controlling a shutter positioned in the excitation light path (Uniblitz S25; Vincent Associates, Rochester, NY). Time-lapse images were acquired every 2 s by the Scion Image program on a Macintosh G3 computer equipped with a video frame grabber (Scion LG-3; Scion Corp., Frederick, MD). Fluorescence changes were measured in selected cells by Scion Image.

FIG. 1. Targeted mutation at the Glra2 locus. (A) Strategy for targeted deletion of Glra2 exons 6 and 7. Homologous recombination within a BAC containing exons 6 and 7 was used to replace exons 6 and 7 and the intervening intron with a PGK-neo cassette flanked by FRT sites. Shaded boxes labeled 1 represent probes used in Southern blot assays to screen for homologous recombination and deletion of exons 6 and 7. Wild-type (WT) genomic DNA digested with EcoRI and probed with probe 1 yields a fragment of 4.9 kb. In KO alleles, an endogenous EcoRI site present in the intron between exons 6 and 7 is deleted, yielding a 5.8-kb fragment when probed with probe 1. R, EcoRI. (B) Southern blot analysis of genomic DNA isolated from mouse tails. Southern blot assays of EcoRI-digested DNA probed with probe 1 presents a 4.9-kb band for the wild-type allele and a 3.5-kb band for the deletion-containing allele. (C) Diagram of GlyRa2 protein showing its four transmembrane domains (M1 to M4) with the approximate region of the protein encoded by exons 6 and 7 shaded in gray. Alternate splicing of exon 5 to exon 8 would result in a premature stop codon four amino acids into exon 8.
Pharmacological agents. Glycine, taurine (Sigma, St. Louis, MO), and GABA (RBI, Natick, MA) were prepared as stock solutions in double-distilled H2O and diluted in ACSF to final concentration. All drugs were applied focally with the DAD-12 Superfusion System (ALA Scientific Instruments, Westbury, NY).

Electrophysiology. Whole-cell patch clamp recordings were performed with an EPC-9 patch clamp amplifier (HEKA Electronic, Lambrecht, Germany). Borosilicate glass (Warner Instrument Corp., Hamden, CT) electrode pipettes (5 to 8 MΩ) were filled with 130 mM CsCl, 2 mM CaCl2, 10 mM HEPES, and 11 mM EGTA (pH 7.4 at 25°C, 265 to 275 mosM/liter). Unless otherwise indicated, neurons were voltage clamped at −60 mV.

In situ hybridization and immunofluorescence. Littermates were decapitated, and their heads were immediately fixed in 4% paraformaldehyde overnight at 4°C. Tissues were washed two times in phosphate-buffered saline (PBS), equilibrated in 30% sucrose in PBS overnight, equilibrated in a 1:1 mixture of 30% sucrose in PBS–optimal cutting temperature embedding medium, and embedded in optimal cutting temperature. Sections in situ hybridizations were performed with total RNA (10 μg) isolated from P0 mouse brain. The Glra2 probe was a 32P-labeled, full-length GlyRα2 cDNA. A probe for β-actin was used as a loading control. (C) In situ hybridization analysis of glycine receptor subunits in wild-type (WT) or Glra2 KO retinae at P0. The probes used recognized RNA species encoding glycine receptors alpha 1 (Glra1), alpha 2 (Glra2), alpha 3 (Glra3), alpha 4 (Glra4), and beta (Glrb).

RESULTS

Generation of GlyRα2 KO mice. In order to generate a Glra2 targeting construct, a BAC containing exons 3 to 8 of the Glra2 locus was identified through hybridization of a BAC library (Genome Systems) with a probe containing exons 6 and 7 of GlyRα2. BAC end sequencing revealed that the BAC contained ~160 kb of sequence, 56 kb upstream of exon 6 and 103 kb downstream of exon 7. Bacterial recombination was utilized to replace exons 6 and 7 in the BAC with an FRT-flanked PGK-neomycin cassette by the system developed by Stewart et al. (22). This targeting construct was used to delete exons 6 and 7 in ES cells. As the Glra2 locus is present on the X chromosome and the ES cells used contained only one X chromosome, correctly targeted ES cells were identified by the loss of exons 6 and 7 by Southern blotting and PCR analysis. One correctly targeted ES cell clone out of 390 clones screened was identified. A higher frequency of homologous recombination was expected because of the large homology arms of this construct (33), suggesting that the genomic location of Glra2 may be refractory to recombination. Blastocyst injection of this positive ES clone generated 22 founder mice. Five of these lines were further expanded for analysis. Genomic deletion of exons 6 and 7 in mice was confirmed by Southern blotting with a probe 3′ to the deleted region and within an EcoRI fragment.
This probe recognizes a 4.9-kb fragment in the wild-type allele and a 5.8-kb fragment in the KO allele (Fig. 1A and B).

Exons 6 and 7 encode two transmembrane domains of GlyRα2 and a large portion of its ligand binding domain (Fig. 1C). Alternative splicing of exon 5 to exon 8 would result in an immediate premature stop codon and should lead to nonsense-mediated degradation of the mRNA (7). In order to confirm the lack of GlyRα2 mRNA, reverse transcription-PCR, Northern blotting, and in situ hybridization analyses were performed with P0 wild-type and Glra2−/− brain RNAs (Fig. 2).

Glycine receptor KO animals exhibit normal spinal cord development. GlyRα2−/− animals were born in normal ratios and were healthy and fertile. No gross behavioral or morphological phenotypes were apparent in newborn or adult mice, as they suckled normally, had a normal gait, and bred and ate as their wild-type littermates did.

GlyRα2 is the predominant subunit expressed in the developing spinal cord (1, 18). Although Glra2−/− animals do not express GlyRα2 RNA in the P0 spinal cord (Fig. 3A and B), the developmental morphology of the spinal cord appeared normal; the diameter and cell density within the spinal cord were unchanged in Glra2−/− and wild-type littermates throughout the anterior-posterior axis (Fig. 3C and D).

Glycine receptor activity is not necessary for normal cortical development. GlyRα2 is expressed and active in the developing cerebral cortex (8, 18). Patch clamp recordings of neonatal wild-type neurons show that these cells respond to the application of glycine and taurine by activating endogenously expressed glycine receptors (8). In addition, wild-type neonatal (8) and late embryonic cerebral cortex slices loaded with a calcium indicator showed an increase in intracellular calcium levels in an entire field of imaged cortical neurons in response to glycine application (Fig. 4D). This glycine-evoked calcium influx was reversibly inhibited by preincubation with strychnine, a specific inhibitor of glycine receptors (Fig. 4D). In contrast, standard whole-cell patch
clamp recordings of individual cortical neurons in brain slices from Glra2−/− animals did not show responses to a low (500 μM) or a high (1 mM) concentration of glycine or taurine in the presence or absence of bicuculline at E17 (Fig. 4A and B). The results obtained with individual neurons were further confirmed by calcium imaging of a population of neurons in Glra2−/− cortical slices (Fig. 4E). However, Glra2−/− neurons did respond normally to GABA (30 μM) (Fig. 4B), and that response induced a calcium increase in slices loaded with a calcium indicator (Fig. 4E). At postnatal day 7, glycine responses were recovered in Glra2−/− cortical cells (Fig. 4C), presumably because of neonatal expression of other glycine receptor subunits.

In spite of a lack of developmental electrophysiological responses to glycine or taurine in the cortex, wild-type and Glra2−/− cortices at P0 (Fig. 5A to D) and adult ages (Fig. 5E and F) were morphologically indistinguishable. At P0, there appeared to be no obvious change in the expression pattern of Notch1, Id2, or GABA(A) subunit 6 (Fig. 5C and D and data not shown). In addition, the cerebella of Glra2−/− animals also appeared normal by hematoxylin-eosin staining (Fig. 5G and H).

Retinal development and function appear normal in Glra2−/− animals. GlyRα2 is expressed in the developing rodent retina (34). Section in situ hybridization of P0 retinae from wild-type and Glra2−/− animals revealed no detectable morphological or molecular difference (Fig. 2C). In addition to GlyRα2, other glycine receptor subunits also are expressed in the P0 retina. However, no differences in α1, α3, α4, or β expression could be detected between wild-type and Glra2 KO retinae (Fig. 2C).

At P0, the majority of retinal cells are mitotic progenitor cells or postmitotic undifferentiated cells, with smaller numbers of differentiated ganglion, amacrine, and cone photoreceptor cells. Notch1, Id2, Big2, TUG1, and GABA(A) subunit 6 are each expressed in subsets of progenitor and/or precursor cells. Nrl is expressed in photoreceptor precursor cells (19). The expression of these genes appeared unchanged in Glra2−/− retinae (Fig. 6).

Hematoxylin-eosin staining of adult retinae from wild-type and Glra2−/− littermates showed no obvious morphological difference; outer nuclear and plexiform layers, inner nuclear and plexiform layers, and ganglion cell layers were present and of similar thicknesses and cell densities (Fig. 7). In addition, the inner and outer segments of photoreceptors appeared normal. Electroretinogram analyses revealed no appreciable difference between adult retinae from wild-type and Glra2−/− animals (B. Pawlyk and T. Li, personal communication).
Glycine receptors mediate inhibitory neurotransmission in the mature brain stem and spinal cord. However, glycine receptors, particularly the \( \alpha_2 \) subunit, also are expressed throughout the developing nervous system (18). During development, functionally active glycine receptors are expressed on migrating and differentiating cortical neurons (8). In addition, a ligand for these receptors, taurine, is present at abundant levels in the developing cortex (29). In contrast to glycine, there is a higher intracellular chloride concentration, the channel opening results in chloride ions flowing out of the cell and the subsequent depolarization leads to an influx of calcium ions. Thus, it is possible that GABA activity may compensate for the loss of glycine activity in the adult, as binding of these ligands to their respective receptors results in opening of the chloride-permeable channel (8, 15). In both cases, since embryonic neurons have a higher intracellular chloride concentration, the channel opening results in chloride ions flowing out of the cell and the subsequent depolarization leads to an influx of calcium ions. Therefore, the effectiveness of clonazepam is proposed to be due to its potentiation of GABA(A) receptors.

One possibility that this Glra2\(^{-/-}\) mouse raises is that, in spite of the widespread expression of glycine receptors and their ligands embryonically, glycine receptor signaling may not be critical for neuronal development. In the future, interbreeding of Glra2\(^{-/-}\) mice with previously established Glr1, Glra3, and Glrb null mice, and perhaps with GABA receptor-deficient animals, could provide valuable insights into the roles of glycine receptors in the nervous system.

ACKNOWLEDGMENTS

We thank J. Munson and J. Trimarchi for technical assistance and B. Pawlyk and T. Li for providing electroretinogram data.

The mice described in this study were generated within the Mental Retardation Research Center (MRRC)/Children’s Hospital Gene Manipulation Facility (NIH P30-HD 18655) and the Brigham and Women's Hospital Transgenic Mouse Facility. This work was supported by...
funds from NIH (R01EY009076). C.L.C. is an investigator of the Howard Hughes Medical Institute.

REFERENCES


