Elimination of the ρ1 Subunit Abolishes GABA$_C$ Receptor Expression and Alters Visual Processing in the Mouse Retina

Maureen A. McCall,[1,2] **Peter D. Lukasiewicz,**[4] **Ronald G. Gregg,**[2,3] **and Neal S. Peachey**[5,6,7]

Departments of [1]Psychological and Brain Sciences, [2]Ophthalmology and Visual Sciences, and [3]Biochemistry and Molecular Biology, University of Louisville, Louisville, Kentucky 40292, [4]Department of Ophthalmology and Visual Sciences, Washington University, St. Louis, Missouri 63110, [5]Cleveland Veterans Affairs Medical Center, Cleveland, Ohio 44106, [6]Cole Eye Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195, and [7]Department of Neurosciences, Case Western Reserve University, Cleveland, Ohio 44106

Inhibition is crucial for normal function in the nervous system. In the CNS, inhibition is mediated primarily by the amino acid GABA via activation of two ionotropic GABA receptors, GABA$_A$ and GABA$_C$. GABA$_A$ receptor composition and function have been well characterized, whereas much less is known about native GABA$_C$ receptors. Differences in molecular composition, anatomical distributions, and physiological properties strongly suggest that GABA$_A$ receptors and GABA$_C$ receptors have distinct functional roles in the CNS. To determine the functional role of GABA$_C$ receptors, we eliminated their expression in mice using a knock-out strategy. Although native rodent GABA$_C$ receptors are composed of ρ1 and ρ2 subunits, we show that after ρ1 subunit expression was selectively eliminated there was no GABA$_C$ receptor expression. We assessed GABA$_C$ receptor function in the retina because GABA$_C$ receptors are highly expressed on the axon terminals of rod bipolar cells and because this site modulates the visual signal to amacrine and ganglion cells. In GABA$_C$$\rho$1 null mice, GABA-evoked responses, normally mediated by GABA$_C$ receptors, were eliminated, and signaling from rod bipolar cells to third order cells was altered. These data demonstrate that elimination of the GABA$_C$$\rho$1 subunit, via gene targeting, results in the absence of GABA$_C$ receptors in the retina and selective alterations in normal visual processing.

Key words: knock-out; rod bipolar cell; electroretinogram; IPSC; whole-cell patch clamp; TPMPA; ionotropic receptor; chloride channel; inhibition

In the CNS, inhibition is mediated primarily by GABA, which activates two ionotropic GABA receptors, GABA$_A$ and GABA$_C$ (Sivilotti and Nistri, 1991). GABA$_A$ and GABA$_C$ receptors are molecularly distinct, differing in both subunit composition and subunit complexity (GABA$_A$: α1–6, β1–3, γ1–3, δ, ϵ, and π subunits; GABA$_C$: ρ1–3 subunits) (for review, see Enz, 2001; Moss and Smart, 2001; Zhang et al., 2001). In addition, each receptor type or subtype forms a chloride channel whose pharmacology, current kinetics, and affinities for GABA differ. Most CNS neurons express GABA$_A$ receptors, and many express more than one subtype (Persohn et al., 1992; Wisden and Seeburg, 1992; Fritschy et al., 1998). In contrast, GABA$_C$ receptors are simpler (Enz and Cutting, 1999a; Zhang et al., 2001), and their expression is more restricted (Johnston, 1994; Boue-Grabot et al., 1998; Rozzo et al., 1999), primarily to the terminals of a class of retinal interneurons, the bipolar cells (Enz et al., 1996; Lukasiewicz, 1996; Koulen et al., 1997; Boue-Grabot et al., 1998; Feigenspan and Bormann, 1998; Fletcher et al., 1998; Wassle et al., 1998).

Both GABA$_A$ and GABA$_C$ receptors modulate inhibition in the retina (Lukasiewicz and Shields, 1998). In particular, the GABA$_C$ receptors are thought to modulate the response properties of retinal ganglion cells, such as their center-surround antagonism, the sustained or transient nature of their responses, and contrast gain (Thibos and Werblin, 1978; Lukasiewicz and Werblin, 1994; Pan and Lipton, 1995; Cook and McReynolds, 1998a,b; Dong and Werblin, 1998; Bloomfield and Xin, 2000; Flores-Herr et al., 2001). The relatively restricted expression of GABA$_C$ receptors and their late expression in postnatal development (Greka et al., 2000) make them particularly amenable for study using gene targeting.

We inactivated the gene encoding the GABA$_C$$\rho$1 subunit in mice and investigated the effects of the elimination of this subunit in the retina. We assessed the specific roles of GABA$_C$ receptors in the inhibitory circuit mediated by the rod bipolar cell. Overall retinal and rod bipolar cells to cell morphology were intact, and both electrophysiological and immunohistochemical experiments showed that eliminating expression of the ρ1 subunit eliminated expression of the GABA$_C$ receptor. Electroretinogram (ERG) measurements showed that only inner retinal function was altered in GABA$_C$$\rho$1 null mice, a result consistent with an alteration in the balance of excitation and inhibition between second and third order retinal neurons. These data show that the GABA$_C$ receptor is not expressed in the retina of GABA$_C$$\rho$1 null mice and represent the first use of gene targeting to alter inhibition in a specific

retinal circuit. These mice will allow us to enhance our understanding of the role of GABA$_C$ receptor-mediated inhibition in visual processing.

MATERIALS AND METHODS

Production of GABA$_C\rho$1 null mice

Targeting vector

A P1 clone was identified using a PCR strategy with two primers within the 3′ end of the cDNA encoding the GABA$_C\rho$1 subunit gene and was purchased from IncyteGenomics. From this genomic clone, two fragments of the GABA$_C\rho$1 subunit gene were isolated: an 18 kbp *Hin*dIII fragment containing the promoter, exon 1, and a portion of intron 1, and a 2.2 kbp *Eco*RI–*Xho*I fragment containing exon 10 and part of the 3′ untranslated region (see Fig. 1*A*). These fragments were used to construct the targeting vector (see Fig. 1*B*), which consisted of four cassettes: a 4.4 kbp 5′ homology unit, which included 4.0 kbp of the GABA$_C\rho$1 promoter, exon 1 (220 bp) and 210 bp of intron 1; a 1.4 kbp 3′ homology unit from the 3′ untranslated region of the gene, and two selectable markers, a neomycin resistance gene (*neo*) and a thymidine kinase gene (*TK*) in pBluescript (Stratagene, La Jolla, CA). When homologous recombination occurs (see Fig. 1*A, dashed Xs*), exons 2–9 are replaced with the *neo* cassette, yielding the modified locus (see Fig. 1*C*).

Production of targeted embryonic stem cells and GABA$_C\rho$1 null mice

The general strategy for the production of targeted embryonic stem (ES) cells has been described previously (McCall et al., 1996a). Five micrograms of the GABA$_C\rho$1 targeting vector was linearized by digestion with *Sal*I and introduced via electroporation into 5×10^6 HM-1 ES cells. Two hundred and thirty-two G418 and 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil-resistant ES cell clones were analyzed by PCR to determine which were correctly targeted on the 3′ end. The primers (indicated by *arrows*, labeled *3F* and *3R*) were located in the *neo* gene and in the 3′ end of the GABA$_C\rho$1 cognate gene (see Fig. 1*C*). Their sequences were 3F: 5′ CGTTGGCTACCCGTGATATT 3′; 3R: 5′ GAATTCCAGACTGACCCGCTTCT 3′. PCRs contained: 1× Gitschier buffer (Kogan et al., 1987) and *Taq* Enhancer, 1 U *Taq* polymerase (Eppendorf, Hamburg, Germany), 0.5 μM of each primer, 0.15 mM dNTP, and 25–50 ng of DNA in a final volume of 25 μl. Cycling conditions for all PCRs were determined empirically, but in general were: 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at the annealing temperature, and 2 min at 72°C, followed by a final incubation for 10 min at 72°C. Amplified samples were analyzed by agarose gel electrophoresis. A 2.2 kbp fragment was amplified in three of the 232 ES cell clones, indicating correct targeting at the 3′ end of the gene (see Fig. 2*B*).

Southern blotting was used to determine which of the three ES cell clones were correctly targeted on the 5′ end (see Fig. 2*A*). Genomic DNA from each ES cell was digested with *Hin*dIII, size separated by agarose gel electrophoresis, denatured, and transferred to a nylon membrane. The membrane was hybridized at 65°C with a ^{32}P radioactively labeled probe from the 5′ end of the gene, outside of the region of homology. One correctly targeted ES cell clone, E11 (see Fig. 2*A*) was identified and contained an 18 kbp *Hin*dIII fragment from the cognate gene [wild-type (WT)] and a 12 kbp *Hin*dIII fragment representing the targeted allele (Target). This clone was expanded and used for blastocyst injections.

Production of GABA$_C\rho$1 null mice

All procedures using animals were approved by the Institutional Animal Care and Use Committees at each institution. The expanded ES cell clone was injected into C57BL/6J blastocysts at the Transgenic Facility at the University of Wisconsin. Three male chimeric founders transmitted the GABA$_C\rho$1 targeted allele through the germline and were crossed to C57BL/6J females to produce mice heterozygous for the targeted allele. The heterozygotes were intercrossed, and their offspring were genotyped by PCR for the deleted (WT) and targeted (Target) alleles (see Fig. 2*C*). DNA for the PCRs was isolated from tail biopsies of each offspring using Chelex 100 resin (Bio-Rad, Hercules, CA) (McCall et al., 1996b). The location of the primers (indicated by *arrows*) for the WT allele are shown in Figure 1*A* and for the targeted allele (Target) in Figure 1*C*. Their sequences were WT F: 5′ CAGGGACAATCGGCTGTAGG 3′ and R: 5′TTGTTGGAGCTGGGGAAAGA3′ and Target F: 5′CCACAT-GAAGCAGCACGA 3′ and R: 5′AGGATGTTGCCGTCCTCCTTT3′.

The composition of the PCR solution and cycling conditions are described above.

Histology

Light microscopy

Retinal tissue was prepared as described previously (McCall et al., 1996b). Mice were killed by anesthetic overdose, and their eyes were removed and fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 18 min, and the fixed eyecups were rinsed (three times for 10 min each) in 0.1 M PB. For plastic sections, eyecups were dehydrated through a graded series of alcohols (70, 80, 90, and 95%) for 1 hr each and infiltrated overnight in JB-4 (Electron Microscopy Sciences, Fort Washington, PA). The eyecups were then submerged in JB-4 plus polymerizer in BEEM capsules. After the plastic had hardened, 1 μm transverse sections were cut on an ultramicrotome, stained with cresyl violet, and coverslipped. Images were captured on a Nikon Optiphot-2 with a cooled CCD camera and associated software (Spot 2; Diagnostic Instruments, Sterling Heights, MI).

Immunohistochemistry

Eyecups were prepared and fixed as described above. The retinas were dissected out of the eyecup, cryoprotected through a graded series of sucrose, and frozen in OCT–20% sucrose (at a ratio of 2:1) (Barthel and Raymond, 1990). For most experiments the retinas from WT and GABA$_C\rho$1 null littermates were sandwiched together, embedded in the same mold, and when cut and mounted onto slides were separated at most by 150 μm. Sixteen micrometer sections were cut on a cryostat and mounted onto slides and stored at -70°C. Before immunohistochemistry the slides were warmed to 37°C and washed in 0.1 M PB three times for 15 min. Sections were incubated in 0.5% Triton X-100 in 0.1 M PB (PBX), followed by incubation at room temperature (RT) for 1 hr in a blocking solution consisting of 10% normal goat serum in PBX. Sections were incubated overnight at RT in one of the following primary antibodies diluted in blocking solution: anti-PKC [1:1000 (rabbit polyclonal antibody to α isoenzymes (Amersham Biosciences, Arlington Heights, IL) and anti-GABA$_C$ [1:100 (rabbit polyclonal antibody to GABA$_C\rho$1, ρ2, and ρ3 subunits of the receptor (Enz et al., 1995)]. The sections were rinsed in 0.5% PBX for 1 hr at RT before incubation with fluorescent secondary antibody (either anti-rabbit Alexa 488 or 546; Molecular Probes, Eugene, OR) diluted 1:1000 in blocking solution for 1 hr at RT. The sections were rinsed three times for 5 min in PB and then coverslipped with Immumount (Shandon, Pittsburgh, PA). As a control, primary antibodies were omitted from sections on one slide. The protocols for double labeling were similar to those described above, with each primary and secondary antibody incubated sequentially.

Confocal images were acquired using the Zeiss (Oberkochen, Germany) LSM 510 laser-scanning microscope equipped with an Argon and a HeNe laser. Images were captured using a Plan-Apochromat 63×/1.4 water immersion objective. Laser lines and emission filters were optimized with the Zeiss LSM510 software. High-resolution scanning was performed at 1024 × 1024 pixels. Confocal images were analyzed, and brightness and contrast were adjusted using the LSM510 analysis package. Serial optical sections ($n = 5$), collapsed into a single plane, are shown in Figure 4*A–C* (z-axis step size, 0.6 μm).

Electrophysiology

Patch-clamp experiments

Preparation of mouse retinal slices. The experimental techniques were identical to those described for studies in ferret retinal slices (Lukasiewicz and Wong, 1997; Shields et al., 2000). Briefly, mice aged postnatal day 28 to adult were killed using carbon dioxide, and their eyes were enucleated. The cornea, lens, and the vitreous were removed, and the retina was dissected in cold, oxygenated extracellular medium (see Electrode and bath solutions). Slices were prepared as previously described and maintained in oxygenated media at room temperature (Werblin, 1978; Lukasiewicz and Wong, 1997).

Whole-cell patch recordings. Whole-cell patch recordings were obtained from rod bipolar cells in the mouse retinal slices. IPSCs were recorded when rod bipolar cells were voltage clamped to 0 mV, the reversal potential for nonspecific cation currents. Rod bipolar cells were identified by their characteristic morphology after filling them with Lucifer yellow (0.015%), which was included in the recording electrode (Euler and Wassle, 1998; Shields et al., 2000) (see Fig. 4*D,E*). The recording procedures and microscope system have been described in detail previously
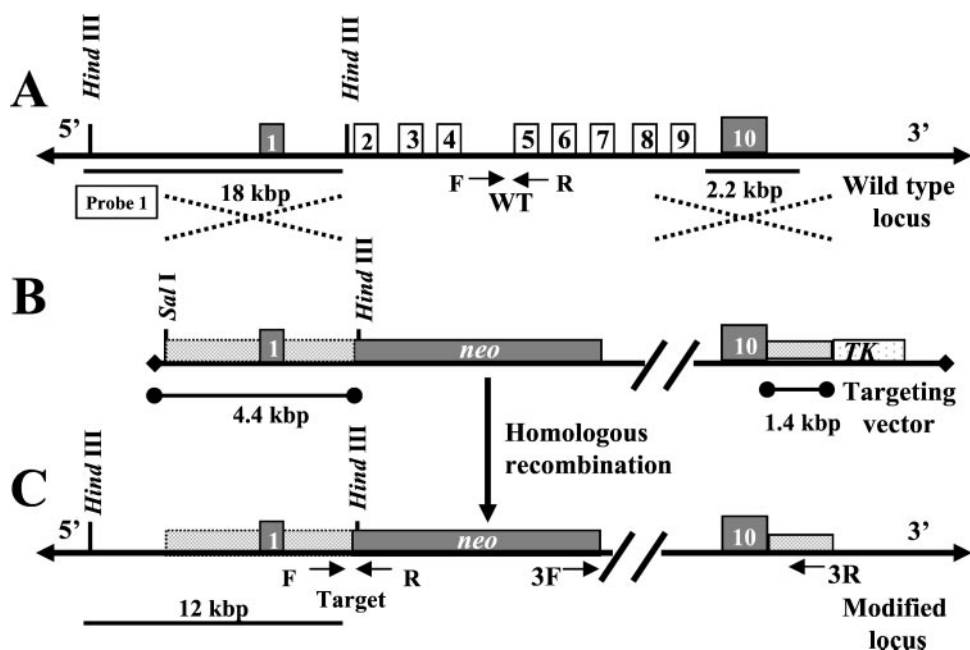
Figure 1. The GABA$_C\rho1$ subunit gene structure, targeting vector design, and the GABA$_C\rho1$ subunit gene modified by homologous recombination. Schematic representations of the wild-type locus of the GABA$_C\rho1$ subunit gene (*A*), the targeting vector used to inactivate the GABA$_C\rho1$ subunit gene (*B*), and the modified locus for this gene that results from homologous recombination between the targeting vector and the cognate GABA$_C\rho1$ subunit gene (*C*). (Diagrams are not drawn to scale.) The targeting vector (*B*) consists of two selection cassettes: the neomycin gene (*neo*) and the thymidine kinase gene (*TK*) as well as a 4.4 kbp 5′ homology unit that included the GABA$_C\rho1$ subunit promoter, exon 1, and a portion of intron 1 and a 1.4 kbp 3′ homology unit that included the 3′UTR of the gene. When homologous recombination occurs between the targeting vector and the cognate gene (*large Xs*) exons 2–9 are replaced by the neo cassette resulting in the modified allele (*C*).

(Lukasiewicz and Roeder, 1995). Electrodes were pulled from borosilicate glass (1B150F-4; World Precision Instruments, Sarasota, FL) on a P97 Flaming–Brown puller (Sutter Instruments, Novato, CA) and had measured resistances of <5 MΩ. Patchit software (White Perch Software, Somerville, MA) was used to generate voltage command outputs, acquire data, gate the drug perfusion valves, and trigger the Picospritzer (General Valve, Fairfield, NH). The data were digitized and stored with a Pentium personal computer using a Labmaster DMA data acquisition board (Scientific Solutions, Solon, OH). Responses were filtered at 1 kHz with the four-pole Bessel low-pass filter on the Axopatch 200B (Axon Instruments, Foster City, CA) and sampled at 1–2 kHz.

Data analysis. Tack software (White Perch Software) was used to average records and to measure the peak amplitude, decay time, and charge transfer for each cell. The decay time was measured by computing the D_{37}, the time at which the current has declined to 37% of its peak amplitude. Student's *t* tests (two-tailed, unequal variance) were used to compare these aspects of the current from WT and GABA$_C\rho1$ null rod bipolar cells. To obtain the mean current for each cell, three to five leak-subtracted responses were averaged. Data in the text and figure legends are expressed as mean ± SE. Measures of the current from individual cells obtained in the presence of GABA$_A$ and GABA$_C$ receptor antagonists and after drug wash out were normalized to the predrug, control current.

Solutions and drugs. The standard bathing medium (normal mouse Ringer's solution) contained (in mM): 137 NaCl, 5 KCl, 1 MgCl$_2$, 2.5 CaCl$_2$, 28 glucose, and 10 HEPES. The standard intracellular electrode solution for puff experiments consisted of (in mM): 120 Cs gluconate, 1 CaCl$_2$, 2 MgCl$_2$, 10 Na HEPES, 11 EGTA, and 30 glucose, adjusted to a pH of 7.2 with CsOH. Unless otherwise indicated, all chemicals were obtained from Sigma (St. Louis, MO).

The control bathing solution used in these slice experiments was formulated to pharmacologically isolate rod bipolar cell responses to GABA. In all experiments, glycine receptors were antagonized with strychnine (10 μM), AMPA–kainate (AMPA–KA) receptors were blocked with 6-cyano-7-nitroquinoxaline-2,3-dione (10 μM) (NBQX), and NMDA receptors were blocked with D-2-amino-5-phosphonopentenoic acid (D-AP5) (50 μM). NBQX and D-AP5 were both obtained from Precision Biochemicals (Vancouver, British Columbia, Canada). GABA$_A$ receptors were antagonized with bicuculline methbromide (either 200 or 500 μM), and bicuculline-resistant GABA responses were blocked with (1,2,5,6-tetrahydropyridine-4yl) methyphosphinic acid (50 or 100 μM) (TPMPA; RBI, Natick, MA). Antagonists were applied to a region of the slice under study (several millimeters in width) by a gravity-driven superfusion system described previously (Lukasiewicz and Roeder, 1995).

Puffing agonist onto bipolar cell terminals. GABA (100–300 μM) was puffed onto the terminals of bipolar cells in the inner plexiform layer (IPL) in the slice preparation with a Picospritzer at 45 or 60 sec intervals. The pipette was positioned near the Lucifer yellow-labeled axon terminal to optimize the response of the cell to the puff, by minimizing response rise time and maximizing response amplitude. The puff pressure and duration (typically 10–30 msec) were then adjusted to give no larger than a half-maximal response. Because the slice was continuously superfused, which diluted the puff, and because puff duration and pressure were submaximal, the GABA concentration at the receptors was most likely much lower than the pipette concentration.

Electroretinography. The methods used to prepare the mice for electroretinography are similar to those described in detail previously (Pardue et al., 1998; Xu et al., 2000). After overnight dark adaptation, anesthesia was induced with an intramuscular injection of a mixture of ketamine–xylazine in saline (80 mg/kg; 16 mg/kg, respectively). Eye drops were used to dilate the pupils (2.5% phenylephrine HCl, 1% mydriacyl, 1% cyclopentolate). For ERG recordings, body temperature was maintained using a heating pad. Retinal responses were recorded using a stainless steel wire contacting the corneal surface through a layer of 1% methylcellulose, and electrodes were placed in the cheek and tail to serve as reference and ground leads, respectively. ERG responses were obtained to strobe flash stimuli presented in the dark. Flash intensities ranged from −4.4 to 5.0 log cd sec/m^2 and interstimulus intervals increased from 1.4 sec at lower intensities to 45 sec at the highest stimulus levels. Stimuli were presented in order of increasing intensity, and at least two responses were averaged at each flash intensity. ERG responses were differentially amplified using a frequency bandpass setting of 0.5–1500 Hz, and the frequency bandpass was changed to 30–1500 Hz to isolate the oscillatory potentials (OPs). Responses were averaged and stored using an LKC UTAS E-2000 (Gaithersburg, MD) signal averaging system. The amplitude of the a-wave was measured from the prestimulus baseline to the a-wave trough. The amplitude of the b-wave was measured from the a-wave trough to the peak of the b-wave. Time-to-peak (implicit time) was measured from the time of flash onset to the a-wave trough or the b-wave peak. Individual OP wavelets were measured from the initial negative trough to the positive peak. An ANOVA with repeated measures was used to determine statistical significance between these measures in the ERGs of WT and GABA$_C\rho1$ null mice.

RESULTS

Homologous recombination disrupts the expression of the GABA$_C\rho1$ subunit

Figure 1 illustrates the targeting scheme used to eliminate the expression of the GABA$_C\rho1$ subunit gene. After homologous

Figure 2. Characterization of embryonic stem cells and mice that carry the GABA$_C\rho$1 targeted allele. *A,* Scanned images of Southern blots of genomic DNA isolated from WT and from WT ES cells (*HM1*), a targeted ES cell line (*E11*), a wild-type (*WT*), and a GABA$_C\rho$1 null (*Null*) mouse. Genomic DNAs were digested with *Hin*dIII and separated by size using agarose gel electrophoresis. DNA was transferred to nylon membrane and hybridized to radiolabeled Probe 1 (Fig. 1*A*). The probe hybridizes to an 18 kbp *Hin*dIII fragment from the WT locus in DNA from untransfected ES cells and the WT mouse. The probe hybridizes to both the 18 kbp WT allele and a 12 kbp fragment in DNA from ES cell clone (*E11*) representing the presence of a targeted allele. Thus, E11 is correctly targeted on the 5′ end of the gene. A single 12 kbp fragment in the DNA from the GABA$_C\rho$1 null mouse indicates the presence of only targeted alleles. *B,* PCR results indicating that ES cell clone, E11, is correctly targeted on the 3′ end of the GABA$_C\rho$1 subunit gene. *Arrows* on the drawing of the modified locus (Fig. 1*C*) indicate the positions of the primers 3F/3R for this PCR, which amplified a 2.2 kbp fragment from within the *neo* gene through

the 3′ UTR of the GABA$_C\rho$1 subunit gene, indicative of correct targeting on the 3′ end. *mm,* Molecular weight marker; *A10,* non-targeted ES cell clone; *E11,* targeted ES cell clone; −, negative control, no DNA added to the reaction. *C,* PCR results indicating the genotypes of five littermates resulting from a cross between two mice heterozygous for the targeted allele. The primers for these PCRs are indicated in Figure 1*A* (WT F/R: located in intron 4 and exon 5) and Figure 1*C* (Target F/R: located in exon 1 and the neo gene) and amplify only wild-type (*WT*) fragments in WT mice, only targeted fragments (*Target*) in GABA$_C\rho$1 null (*Null*) mice, and both fragments in heterozygous (*Het*) mice.
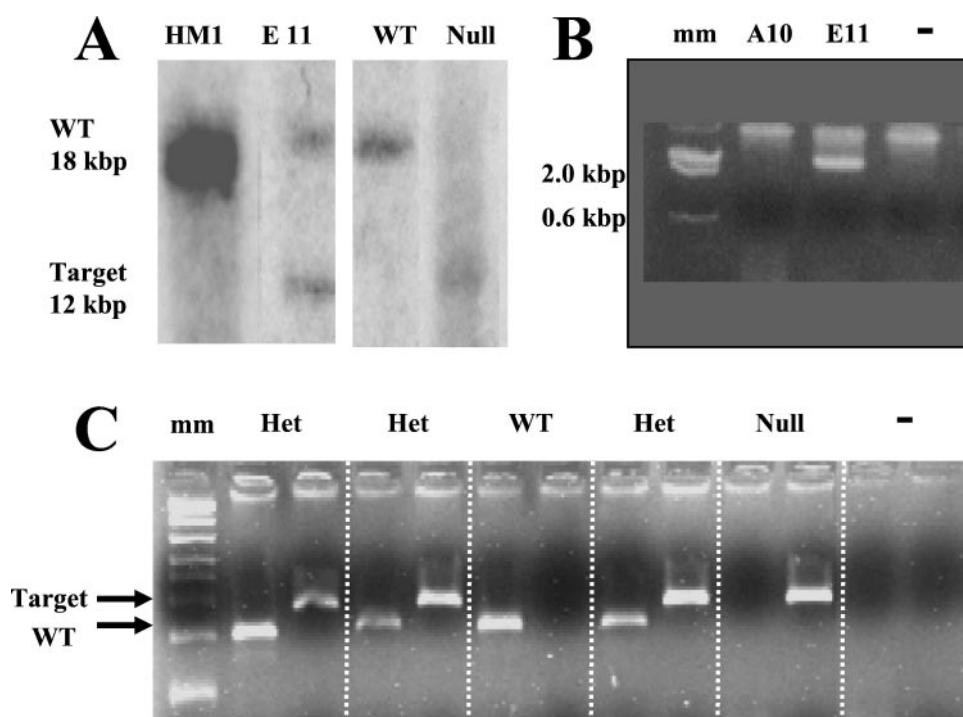
recombination, ES cells that were heterozygous for the modified locus were identified, using Southern blotting, for the 5′ homology unit (Fig. 2*A*) and PCR for the 3′ homology unit (Fig. 2*B*). Injections of the expanded ES clone into C57BL/6J blastocysts generated three male germline chimeras that were crossed to C57BL/6J mice and produced heterozygous offspring. These heterozygous mice were intercrossed, and their progeny were genotyped by PCR (Fig. 2*C*). The GABA$_C\rho$1 null offspring resulting from these crosses were viable and fertile. Because the genetic background of these mice is a mixture of C57BL/6J and SV129 Olah, all comparisons are made between GABA$_C\rho$1 null mice and their WT littermates.

Retinal morphology is normal in GABA$_C\rho$1 null mice

Experimental manipulations that eliminate a particular cell class or signaling pathway frequently cause neuronal loss and/or developmental abnormalities (McCall et al., 1996b; Peachey et al., 1997; Jiang et al., 1998; Rohrer et al., 1999). To determine if elimination of the GABA$_C\rho$1 subunit altered retinal anatomy, we examined transverse sections of the retina of WT (Fig. 3*A*) and GABA$_C\rho$1 null (Fig. 3*B*) mice at the light microscopic level. We found no differences in the overall morphology or laminar thickness between of the retinas of these mice, indicating that eliminating the expression of the GABA$_C\rho$1 subunit did not alter retinal neurons or their processes.

Elimination of the GABA$_C\rho$1 subunit results in the absence of retinal GABA$_C$ receptors

The GABA$_C$ receptor in rodent retina is thought to be a heterooligomeric channel, comprised of both ρ1 and ρ2 subunits, and is expressed primarily on the axon terminals of the rod bipolar cells (Enz et al., 1995; Zhang et al., 1995). To confirm that gene targeting eliminated expression of the ρ1 subunit and to deter-

mine if expression of any other ρ subunits was present, we compared the expression of the GABA$_C$ receptors in the retinas of GABA$_C\rho$1 null and WT mice. Rod bipolar cells were identified using an antibody to protein kinase C (PKC) (Wassle et al., 1991), and the expression of GABA$_C$ receptor was characterized using a rabbit polyclonal antibody that recognizes all three of the GABA$_C\rho$ subunits (Enz et al., 1995). Figure 4*A* is a confocal image of a transverse section through a WT mouse retina. PKC immunoreactivity (green) outlines the entire rod bipolar cell and GABA$_C$ receptor-immunoreactivity (red) produces a characteristic punctate staining pattern in the IPL. These puncta decorate the axon terminals of rod bipolar cells in the IPL, and to a lesser extent, their dendritic processes in the outer plexiform layer (OPL) (Enz et al., 1995; Koulen et al., 1998; Haverkamp and Wassle, 2000). The presence of GABA$_C$ receptors on the dendrites of the bipolar cells in the OPL, evident in the WT mice, also has been observed previously (Picaud et al., 1998; Haverkamp and Wassle, 2000; Vitanova et al., 2001). The rod bipolar cells in GABA$_C\rho$1 null mice also are immunoreactive for PKC and have a similar morphology to that seen in WT mice. In contrast, no GABA$_C$ receptor immunoreactivity was evident in the GABA$_C\rho$1 null mouse retina (Fig. 4*B*). Figure 4*C* shows that there is little nonspecific GABA$_C$ receptor immunoreactivity in WT control sections when the GABA$_C$ receptor primary antibody is omitted from the reaction. These data indicate that the absence of the GABA$_C\rho$1 subunit eliminates the expression of the ρ2 subunit and the GABA$_C$ receptor in both plexiform layers of the retina (IPL and OPL), but does not alter the gross morphology or PKC expression of rod bipolar cells. Similar observations were made in retinas of seven other WT and eight other GABA$_C\rho$1 null mice.
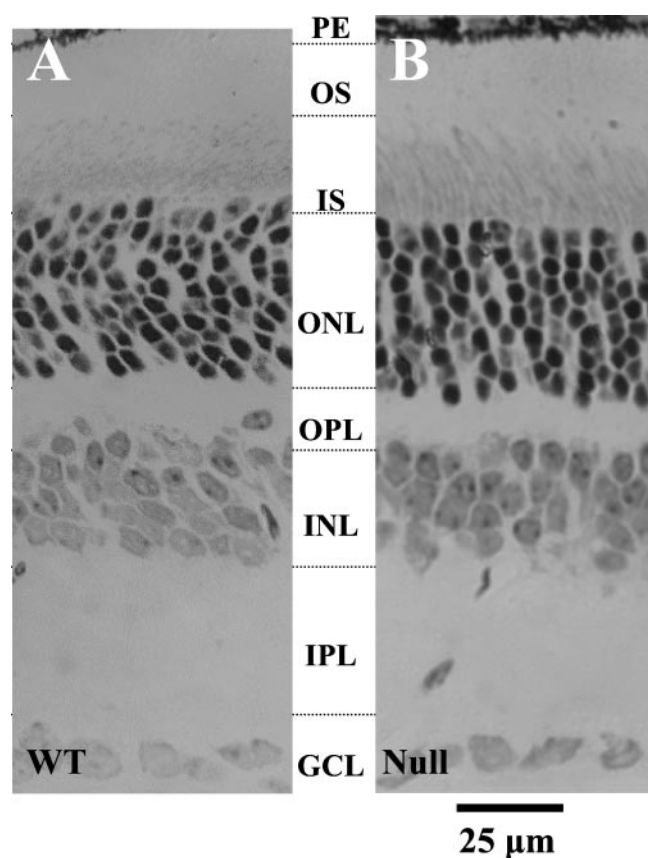
Figure 3. Retinal morphology in GABA$_C\rho$1 null mice is normal. Representative photomicrographs of 1 μm transverse, plastic sections of retina stained with cresyl violet from a WT littermate (*A*) and a GABA$_C\rho$1 null mouse (*B*). No obvious differences are seen in the overall structure of any of the retinal layers or in the gross morphology of the cell bodies within the retinal layers. *PE,* Pigment epithelium; *IS/OS,* inner segments and outer segments of the photoreceptors; *ONL,* outer nuclear layer; *OPL,* outer plexiform layer; *INL,* inner nuclear layer; *IPL,* inner plexiform layer; *GCL,* ganglion cell layer.

GABA responses from GABA$_C\rho$1 null rod bipolar cells lack a GABA$_C$ receptor component

GABA current kinetics

Rod bipolar cells have the largest GABA$_C$ receptor-mediated currents in the retina (Euler and Wassle, 1998; Shields et al., 2000). Therefore, we examined the effects of eliminating the expression of the GABA$_C\rho$1 subunit on GABA evoked responses in these cells. We targeted rod bipolar cells, using the locations of their somas in the INL near its border with the OPL. After each cell was filled with Lucifer yellow, its morphological identity was verified by the location of its cell body in the INL and its axon terminals in substratum 5 of the IPL, near the border with the ganglion cells (Euler and Wassle, 1998; Shields et al., 2000). Fluorescence images of filled cells from both groups of mice were examined under the light microscope, and no morphological differences in the dendritic or axonal arbors were found between WT and GABA$_C\rho$1 null rod bipolar cells (see Fig. 4*D,E,* for representative cells), which is consistent with our light microscopic observations described above.

In retinal slices of GABA$_C\rho$1 null and WT mice, we recorded and averaged the responses of rod bipolar cells to focally applied GABA at their axon terminals in control bath solution. From the averaged response of each cell, we measured three aspects of current kinetics: charge transfer (measured by integrating the current response), peak amplitude, and decay time (time to decline to 37% of peak amplitude) and obtained mean values for WT and GABA$_C\rho$1 null cells. In WT slices, rod bipolar cells responded to a brief puff (30 msec) of GABA with a rapidly rising but sustained current (Fig. 5*A*). In GABA$_C\rho$1 null retinas, rod bipolar cells responded to the GABA puff, but the duration of the GABA evoked current was significantly briefer, and their charge transfer and peak amplitudes were significantly smaller (Fig. 5*B*). The mean values for these parameters and the *p* values from the *t* tests comparing the means between the two groups of mice are shown in Table 1. The kinetics of the current evoked in the rod bipolar cells in the GABA$_C\rho$1 null mice were identical to the GABA responses, which are mediated by only GABA$_A$ receptors in ganglion cells of ferret (Lukasiewicz and Shields, 1998) and rat (Euler and Wassle, 1998; Euler and Masland, 2000), and in ganglion cells that we recorded in both WT and null retinas (data not shown). Each of the changes in current kinetics is consistent with the interpretation that a slow, sustained GABA$_C$-mediated response is absent in GABA$_C\rho$1 null cells.

GABA current pharmacology

We characterized the pharmacology of the GABA response using GABA$_A$ and GABA$_C$ antagonists for all 13 WT and 9/18 GABA$_C\rho$1 null rod bipolar cells, whose current kinetics were evaluated in control solution. To quantify changes in the current, we measured the charge transfer in the presence of antagonist and normalized this response to the charge transfer obtained in the control bath solution. When the GABA$_A$ receptor antagonist, bicuculline, was included in the bath, the peak amplitude of the GABA response in WT cells was smaller (Fig. 5*A*), although its decay was not significantly altered (control, 1394.5 $\pm$ 111.55 msec vs bicuculline, 1359.0 $\pm$ 112.92 msec). The charge transfer of the GABA response in WT bipolar cells in the presence of bicuculline was reduced to 86% of control (Fig. 5*C*). This indicates that the GABA$_A$ receptor contribution to the total GABA response is ~14%, which is similar to results in rat (Euler and Wassle, 1995; Euler and Masland, 2000), rabbit (McGillem et al., 2000), and ferret rod bipolar cells (Shields et al., 2000). We found that when the GABA$_C$ antagonist, TPMPA, was administered alone to WT mouse ganglion cells, which express GABA$_A$ receptors but do not express GABA$_C$ receptors (M. A. McCall, unpublished observations), the drug reduced GABA responses by ~15% of control. Therefore, we only assessed the effects of TPMPA on the bicuculline-insensitive component (GABA$_C$-mediated component) of the GABA current in rod bipolar cells. When TPMPA was added in combination with bicuculline, the GABA response in WT rod bipolar cells was almost completely eliminated (Fig. 5*A*). On average, only 6% of the control current remained in the presence of both bicuculline and TPMPA (Fig. 5*C*), in agreement with findings in rabbit rod bipolar cells (McGillem et al., 2000). GABA responses recovered to their control levels (79 $\pm$ 5%) after wash out of the antagonists (data not shown). These data indicate that in WT mouse rod bipolar cells, the response to GABA at the axon terminal is composed primarily of a GABA$_C$ receptor component with a small GABA$_A$ receptor component and a third component that is neither GABA$_A$ or GABA$_C$ receptor-mediated.

In GABA$_C\rho$1 null retinas, when the GABA$_A$ receptor antagonist, bicuculline, was added to the bath, the GABA response was almost completely eliminated, with the charge transfer reduced by 88% of control values (Fig. 5*B,D*). Addition of TPMPA to the

Figure 4. Eliminating the expression of the GABA$_C\rho$1 subunit results in the absence of expression of the GABA$_C$ receptor. Confocal images of transverse frozen sections of retina from a WT (*A*) and a GABA$_C\rho$1 null (*B*) mouse reacted with antibodies to protein kinase C (*green fluorescence*) and all GABA$_C$ receptor subunits (*red fluorescence*). In retinas from both the WT and GABA$_C\rho$1 null mice, protein kinase C immunoreactivity outlines the cell bodies of the rod bipolar cells, their dendritic processes in the OPL, and their axons and axon terminals in the IPL. In the WT animal, the punctate labeling that is characteristic of the GABA$_C$ receptors is distributed throughout the IPL and also found on the dendrites in the OPL. This immunoreactivity is completely absent in the retinas of the GABA$_C\rho$1 null mice (*B*). This observation is the same in the other seven WT and eight GABA$_C\rho$1 null retinas that we analyzed. A control WT retina (*C*) was reacted as described for the retinas shown in *A* and *B*, but the GABA$_C$ receptor primary antibody was omitted. The labeling pattern of this control is virtually identical to the GABA$_C\rho$1 null mouse reacted with antibodies to both PKC and the GABA$_C$ receptor. *D, E,* Fluorescence photomicrographs of two Lucifer yellow-filled rod bipolar cells whose responses to GABA puffs were recorded intracellularly. These cells were classified as rod bipolars because their cell bodies were located in the top part of the INL, and their axon terminals were found in the IPL near its border with the ganglion cell bodies. (*D*, GABA$_C\rho$1 null; *E,* WT rod bipolar cells.)
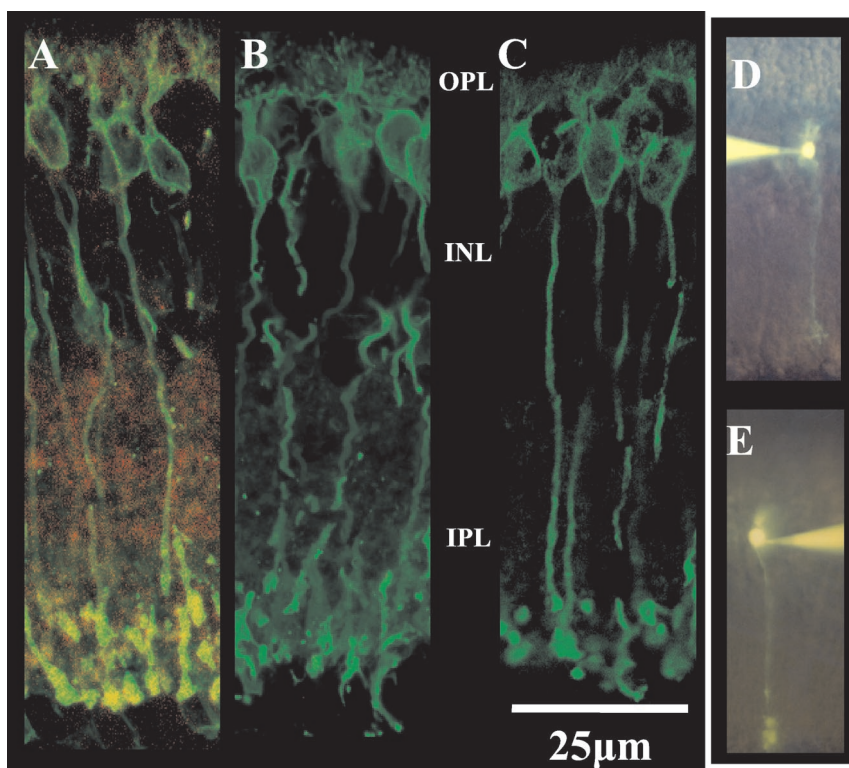
bath had no additional effect on this current (reduced by 90% of control), indicating that this small remaining current also was not mediated by GABA$_A$ or GABA$_C$ receptors. These responses recovered to their pretreatment levels after wash out of the antagonists from the bathing solution (81% of control). Thus, the pharmacology of the current in the GABA$_C\rho$1 null cells also is identical to the GABA responses mediated by GABA$_A$ receptors in ferret (Lukasiewicz and Shields, 1998), rat (Euler and Wassle, 1998; Euler and Masland, 2000), and in ganglion cells that we recorded in both WT and null retinas (data not shown). These data indicate that in GABA$_C\rho$1 null rod bipolar cells the response to GABA at the axon terminal is composed primarily of a GABA$_A$ receptor component.

Whether the GABA$_C$ receptor contributes to the GABA response is best illustrated by comparing the effects of TPMPA on the bicuculline-resistant component of the response from WT and GABA$_C\rho$1 null rod bipolar cells. In WT cells, the bicuculline-resistant response was virtually eliminated by TPMPA (Fig. 5C) (reduced by $92 \pm 3\%$ of control values). In contrast, the very small bicuculline-resistant component that was observed in some of the GABA$_C\rho$1 null rod bipolar cells was unaffected by TPMPA (Fig. 5D) (reduced by $4 \pm 9\%$ of control values). These data also indicate that in GABA$_C\rho$1 null rod bipolar cells, the response to GABA is comprised almost exclusively of a GABA$_A$ receptor-mediated current. The very small bicuculline and TPMPA-resistant current found in cells in both groups of mice could be mediated by a GABA transporter (Yang et al., 1997).

There is little evidence of interactions between the GABA$_A$ and GABA$_C$ receptors. However, whether there is a compensatory upregulation by GABA$_A$ receptors in the absence of GABA$_C$ receptors remains an important question. Our data indicate that the GABA current in WT rod bipolar cells is mediated by a combination of GABA$_A$ and GABA$_C$ receptors. Our results also demonstrate that the GABA$_C$ receptors are absent in rod

bipolar cells of GABA$_C\rho$1 null mice and that their GABA response is almost exclusively mediated by GABA$_A$ receptors. There is no direct way to determine the absolute numbers of GABA$_A$ receptors on rod bipolar cells from our data. However, we can use the charge transfer of the GABA response from WT and GABA$_C\rho$1 null bipolar cells in the control bath solution and in the presence of bicuculline to estimate the relative contribution of the GABA$_A$ receptors to the total GABA current. The GABA$_A$ receptor contribution in WT bipolar cells can be estimated from the percentage reduction in the mean charge transfer in the presence of bicuculline relative to control. In the presence of bicuculline, WT charge transfer is reduced to 86% of the control (Fig. 5C), indicating that GABA$_A$ receptors contribute ~14% of the total current. The GABA$_A$ receptor contribution in GABA$_C\rho$1 null bipolar cells, which only express GABA$_A$ receptors, can be estimated from the percent reduction in the mean charge transfer in control solution relative to WT bipolar cells in control bath solution (Table 1). This ratio yields an estimate of 18% of the total WT current contributed by GABA$_A$ receptors in GABA$_C\rho$1 null bipolar cells. Thus, these comparisons suggest that the GABA$_A$ receptor contribution to the total GABA current in bipolar cells is similar in the two groups of mice and that no compensation by GABA$_A$ receptors occurs in the absence of GABA$_C$ receptors.

Retinal function is altered in GABA$_C\rho$1 null mice

Overall function of the rod pathway in the retina can be assessed by evaluating the dark-adapted ERG (Robson and Frishman, 1995). To assess the impact of the elimination of the GABA$_C$ receptor and its inhibitory modulation on visual signaling through the rod bipolar cells, we compared dark-adapted ERGs obtained from GABA$_C\rho$1 null and WT mice. Figure 6A shows representative dark-adapted ERGs recorded from a WT and a GABA$_C\rho$1 null mouse to full-field, flash stimuli at nine different stimulus
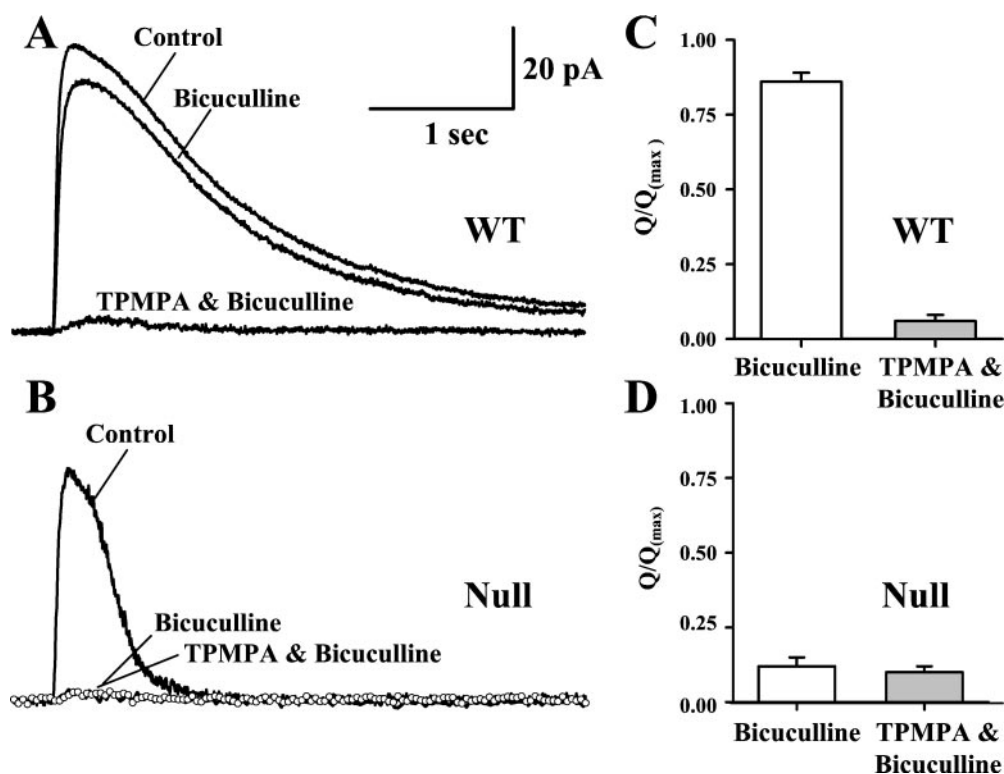
Figure 5. GABA currents are mediated by only GABA$_A$ receptors in GABA$_C$ρ1 null mice. *A,* Representative WT current responses to brief puffs (30 msec) of GABA delivered focally onto the axon terminals of rod bipolar cells (*Control*) and in the presence of antagonists to GABA$_A$ receptors (*Bicuculline*) or both GABA$_C$ and GABA$_A$ receptors (*TPMPA & Bicuculline*). Bicuculline (500 μM) reduced the amplitude of the WT GABA current slightly and delayed its time to peak. TPMPA (50 μM) combined with bicuculline eliminated this GABA current, indicating that both GABA$_A$ and GABA$_C$ receptors mediate the WT GABA response in the mouse. *B,* Representative GABA$_C$ρ1 null current responses to brief GABA puffs delivered focally onto the axon terminals of rod bipolar cells in the presence of bicuculline or bicuculline and TPMPA. The response to GABA in GABA$_C$ρ1 null bipolar cells is different in its kinetics and pharmacology compared with WT. In GABA$_C$ρ1 null rod bipolar cells, the decay of the GABA response was much more rapid than in WT (compare *Control* traces, *A, B*). Addition of bicuculline (500 μM) eliminated the GABA current (−) and the addition of TPMPA (100 μM) had no further effect (○), indicating that GABA$_C$ receptors did not contribute to the response. *C, D,* Responses to GABA in WT and GABA$_C$ρ1 null rod bipolar cells were quantified by measuring charge transfer and changes in these responses in the presence of bicuculline or bicuculline + TPMPA expressed as a percentage of the response in the control bath solution (Q/Q_{max}). *C,* WT rod bipolar cell responses were reduced slightly by addition of bicuculline to the bath (86 ± 3%; $n = 13$). WT responses were almost completely eliminated when both TPMPA and bicuculline were added to the bath (6 ± 2%; $n = 13$). *D,* In contrast, GABA$_C$ρ1 null responses were substantially reduced in the presence of only bicuculline (12 ± 3%; $n = 9$) and the addition of TPMPA had no further effect (10 ± 2%; $n = 9$), indicating that the residual current was not mediated by GABA$_C$ receptors.

Table 1. Comparison of rod bipolar cell GABA current kinetics in control bathing solution

	Peak amplitude (pA)	Decay - D$_{37}$ (msec)	Charge transfer (pA)
WT ($n = 13$)			
Mean (SE)	70.8 (8.9)	1394.5 (111.6)	114,593.7 (13,029.2)
GABA$_C$ρ1 null ($n = 18$)			
Mean (SE)	48.7 (4.2)	353.9 (16.9)	20,941.4 (1,825.0)
$p <$	0.04	0.000001	0.00001

intensities. The ERGs from seven other WT and six other GABA$_C$ρ1 null mice showed the same general shape. Under these stimulus conditions, the responses reflect rod-driven activity, with little or no contribution from cone photoreceptors (Xu et al., 2000). The responses to low-intensity stimuli (−4.4 to −1.9 log cd sec/m^2) were dominated by the cornea-positive b-wave, which reflects depolarizing (ON) rod bipolar cell function (Masu et al., 1995; Robson and Frishman, 1995). Oscillatory potentials, seen as rhythmic oscillations superimposed on the b-wave, were present in ERGs in both groups of mice. The OPs are thought to reflect the feedback loops involving bipolar cell axon terminals, amacrine cell processes, and ganglion cell dendrites in the IPL (Wachtmeister, 1998). As flash intensity increased (−1.4 to 0.5

log cd sec/m^2), the amplitudes of both the b-wave and the OPs increased, and a negative polarity a-wave appeared in advance of the b-wave. The a-wave reflects rod outer segment function (Hood and Birch, 1996; Lamb, 1996). We measured several aspects of the ERG waveform to determine if there were differences in signal processing between the WT and GABA$_C$ρ1 null retinas. Figure 6, *B* and *C,* plots intensity response functions for peak amplitude and time-to-peak (implicit time) for the ERG a- and b-waves in the WT and GABA$_C$ρ1 null mice. At all flash intensities, both measures of the a-wave were identical between the two groups of mice (Fig. 6*B,C*) ($p = 0.99$ and $p = 0.24$, respectively), as is the peak amplitude of their b-waves (Fig. 6*C*) ($p = 0.42$). In contrast, the time-to-peak of the b-wave was significantly faster in GABA$_C$ρ1 null mice ($p = 0.001$).

We isolated the OPs from the rest of the ERG using a high pass filter, and Figure 7*A* shows the resulting waveforms recorded at the highest flash intensity from eight WT and seven GABA$_C$ρ1 null mice. Two aspects of this response were different between the WT and null mice. As shown in Figure 7*B*, at all flash intensities we found a significant increase in the number of OP wavelets in the ERGs of GABA$_C$ρ1 null mice compared to WT mice ($p < 0.0001$). In addition, with the exception of the first wavelet (OP1), the mean peak amplitudes of the remaining wavelets were significantly larger in GABA$_C$ρ1 null mice (Fig. 7*C*) ($p < 0.0001$). These results indicate that elimination of the GABA$_C$ receptor does not compromise rod photoreceptor signal transduction (a-wave), the transmission of this signal to the rod bipolar cells or the amplification of this signal by the rod bipolar cells (b-wave peak amplitude). However, the elimination of the

Figure 6. Dark-adapted photoreceptor function and synaptic transmission to bipolar cells are normal in GABA$_C$ρ1 null mice. *A,* Representative dark-adapted ERG responses recorded from one wild-type (*WT*) and one GABA$_C$ρ1 null (*Null*) mouse to brief full-field light flashes. The traces from *top* to *bottom* represent the responses of each mouse from the dimmest (-4.4 log cd sec/m^2) to the brightest (0.5 log cd sec/m^2) flash. At all intensities, the number and amplitude of the OPs are larger in GABA$_C$ρ1 null mice than in WT mice (see Fig. 7 for quantification). The ERGs from seven other WT and six other GABA$_C$ρ1 null mice were similar. *B,* The mean peak amplitudes ($\pm$ 1 SEM) of the a- and b-waves of the dark-adapted ERG are plotted as a function of flash intensity for all WT (○) and GABA$_C$ρ1 null (●) mice. No significant difference in the peak amplitude of either wave was observed when WT and GABA$_C$ρ1 null data were compared. *C,* The mean time-to-peak (implicit time) for the a- and b-waves of the dark-adapted ERG are plotted as a function of flash intensity for all WT and GABA$_C$ρ1 null mice. Conventions are the same as in *B*. Although no significant difference in the implicit time of the a-wave was observed, the implicit times of the b-wave in GABA$_C$ρ1 null mice were significantly more rapid than in WT mice.
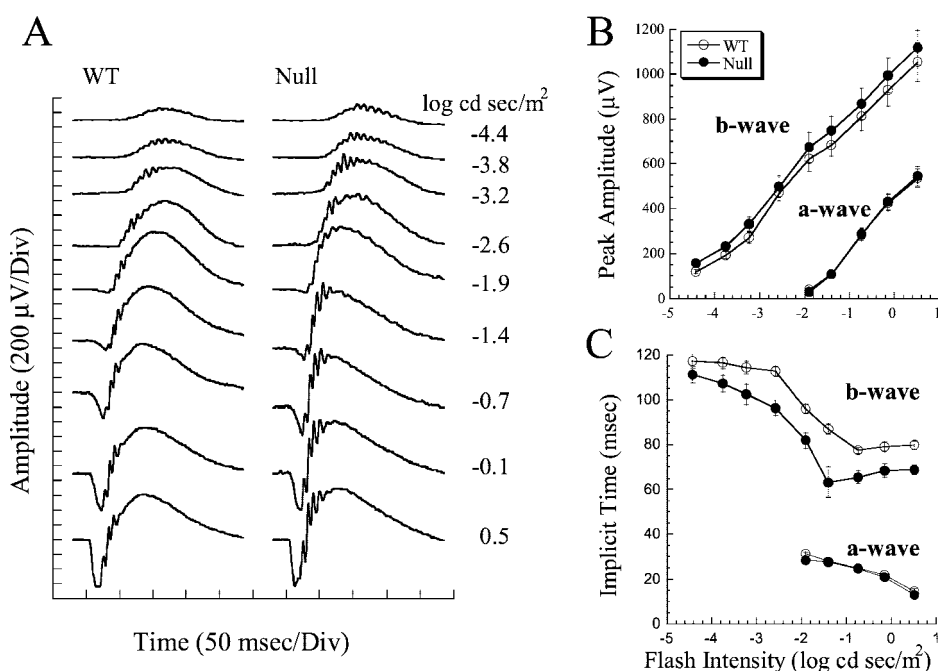

Figure 7. Dark-adapted ERG components that reflect bipolar cell output are altered in GABA$_C$ρ1 null mice. *A,* Dark-adapted ERGs to the brightest flash stimulus, filtered to isolate the OPs, in eight WT and seven GABA$_C$ρ1 null mice. In the filtered responses from every GABA$_C$ρ1 null mouse, there were more OPs, and their amplitude was larger than the number and amplitude of the response in WT mice. *B,* Mean number of OP wavelets ($\pm$ 1 SEM) as a function of stimulus intensity in WT (*open bars*) and in GABA$_C$ρ1 null mice (*filled bars*). At all stimulus intensities, significantly more OPs are observed in responses of GABA$_C$ρ1 null mice. *C,* Mean peak amplitude of each of the individual OPs from WT and GABA$_C$ρ1 null mice elicited by the brightest flash (0.5 log cd sec/m^2). Conventions are the same as in *B*. Note that OP5 is present in all GABA$_C$ρ1 null mice but absent in all WT mice.
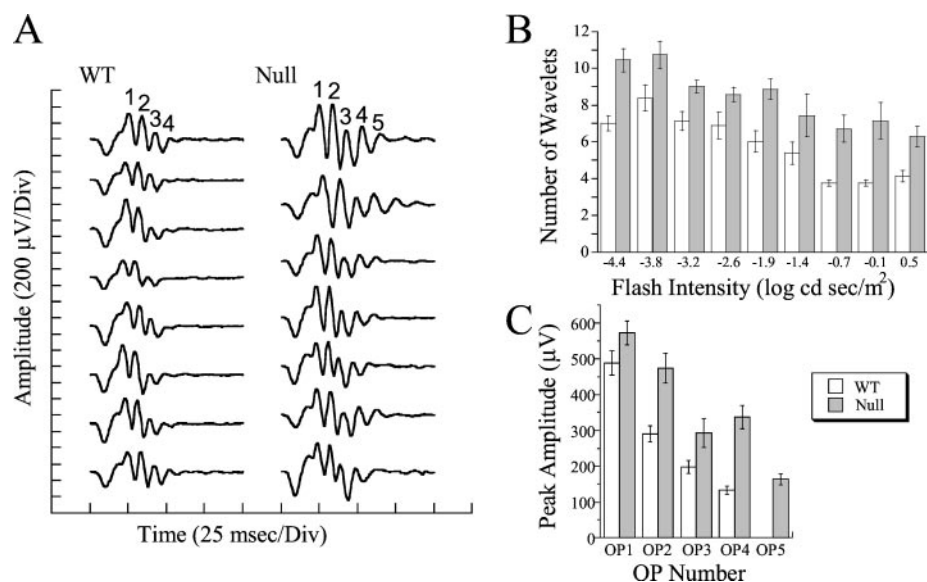
GABA$_C$ receptor does alter transmission of the visual signal from the rod bipolar cells to third order neurons in the retina.

In summary, although rodent GABA$_C$ receptors are thought to be comprised of ρ1 and ρ2 subunits, we found that in GABA$_C$ρ1 null mice, deletion of exons 2 through 9 of the GABA$_C$ρ1 subunit gene eliminated the GABA$_C$ receptors in the retina. Using an antibody that recognizes all of the GABA$_C$ρ subunits (Enz et al., 1995), we showed that there was no ρ subunit labeling in the retinas of GABA$_C$ρ1 null mice. Our patch-clamp recordings showed that the normally large GABA$_C$ receptor current present in WT rod bipolar cells was completely absent in GABA$_C$ρ1 null rod bipolar cells. Although a GABA$_A$ receptor current was present in the GABA$_C$ρ1 null rod bipolar cells, this did not compensate for the loss of GABA$_C$ receptors because our ERG recordings showed that inner retinal function was altered in GABA$_C$ρ1 null mice.

DISCUSSION

Is expression of the GABA$_C$ρ1 subunit required for expression of GABA$_C$ receptors?

Although initial studies indicated that native retinal GABA$_C$ receptors were homomeric channels, more recent results suggest that they are heteromeric channels, composed of ρ1 and ρ2 subunits (Yeh et al., 1990; Shimada et al., 1992; Enz et al., 1995; Zhang et al., 1995; Shingai et al., 1996; Boue-Grabot et al., 1998; Wegelius et al., 1998; Qian and Ripps, 1999; Wotring et al., 1999). Thus, the absence of GABA$_C$ receptor expression in GABA$_C$ρ1 null mice is probably not caused by the loss of expression of homomeric ρ1 channels, but to one of several other possibilities. Our gene-targeting strategy deleted ~30 kbp between intron 1 and exon 10 of the ρ1 subunit gene. It is possible that this change alters ρ2 expression, because it is located ~40 kbp upstream of ρ1

(McCall, unpublished observations) on chromosome 4 (Cutting et al., 1992; Greka et al., 2000). Such an alteration in neighboring gene expression has been observed for both the $GABA_A\alpha1$ and $\beta2$ subunits after gene targeting of the $GABA_A\alpha6$ subunit, which are all clustered on chromosome 11 (Garrett et al., 1997; Uusi-Oukari et al., 2000). The absence of $GABA_C$ receptor expression also could occur if a domain within the $GABA_C\rho1$ subunit protein is required for the correct assembly or targeting of functional $GABA_C$ receptors into the bipolar cell terminals *in vivo*. Although little is known about the control of $GABA_C$ receptor targeting *in vivo*, *in vitro*, separate domains on $\rho1$ and $\rho2$ subunits have been found to control homomeric and heteromeric receptor assembly (Enz and Cutting, 1999b). Thus, the exact nature of the interactions that form and target the $GABA_C$ receptor both between the $\rho1$ and $\rho2$ subunits and with other proteins remains to be determined. Regardless of the mechanism of receptor loss, the $GABA_C\rho1$ null mice lack the $GABA_C$ receptor and they provide a powerful background on which to express recombinant ρ subunits to study the structure and function of these receptors *in vivo*.

The GABA_C receptor does not play a role in retinal development

Alterations in the expression of genes sometimes change either the development or maintenance of CNS structures. In the retina, there is evidence that GABA, via $GABA_A$ receptors, plays a trophic role during development (Redburn, 1992). Because the $GABA_C\rho1$ subunit is not expressed until postnatal day 9 (Greka et al., 2000; Wu and Cutting, 2001), we hypothesized that its elimination would not effect overall retinal development. We compared the gross anatomy of the retina and Lucifer yellow-labeled rod bipolar cells of $GABA_C\rho1$ null and WT mice at the light microscopic level and found no qualitative differences. The overall lamination pattern and thickness of each lamina are comparable and the morphology of $GABA_C\rho1$ null rod bipolar cell processes appear normal. These data strongly suggest that the absence of the $GABA_C\rho1$ subunit does not alter general retinal development.

The absence of the GABA_C receptor alters inhibitory processing in the retina

Rod bipolar cells

The $GABA_C$ receptors are localized primarily on the axon terminals of the bipolar cells in the IPL (Enz et al., 1996; Euler and Wassle, 1998; Koulen et al., 1998; Euler and Masland, 2000) where they mediate a chloride current in response to focally applied GABA (Shields et al., 2000) or light stimulation of the retina (Roska et al., 1998; Euler and Masland, 2000). Pharmacological characterizations have established that this GABA current is a combination of $GABA_A$ and $GABA_C$ receptor-mediated responses, with $GABA_C$ dominating the combined current (Euler and Wassle, 1998; McGillem et al., 2000; Shields et al., 2000). In $GABA_C\rho1$ null rod bipolar cells, GABA evoked a current that exhibited channel kinetics and pharmacology typical of a $GABA_A$ receptor channel (Feigenspan et al., 1993; Lukasiewicz et al., 1994; Lukasiewicz and Werblin, 1994; Lukasiewicz and Wong, 1997; Lukasiewicz and Shields, 1998). Thus, these data also clearly demonstrate that the $\rho1$ subunit eliminates expression of the $GABA_C$ receptors in rod bipolar cells.

In $GABA_C\rho1$ null mice, the kinetics and pharmacology of the response to GABA are very different from WT responses. The total charge transfer of the GABA response in $GABA_C\rho1$ null

cells is significantly reduced to ~20% of the WT response, which is mediated by both $GABA_A$ and $GABA_C$ receptors. In addition, the peak amplitude of the response in $GABA_C\rho1$ null cells is significantly reduced. These data indicate that the inhibitory signal is reduced in $GABA_C\rho1$ null rod bipolar cells by approximately the same proportion that we estimate for the $GABA_C$ receptor contribution to the WT GABA response (Fig. 5A–D, Table 1). As a consequence, these estimates suggest that compensation by $GABA_A$ receptors in the absence of $GABA_C$ receptors is unlikely. Results from other studies support this conclusion. First, there is no evidence of interactions between any $GABA_A$ and $GABA_C$ receptors. Native $GABA_A$ and $GABA_C\rho$ subunits do not combine in mammals either *in vivo* or *in vitro* (Shimada et al., 1992; Hackam et al., 1998; Koulen et al., 1998), although *in vitro* mutant rat $\rho1$ subunits (Pan et al., 2000) and variants of a perch ρ subunit, $\rho1B$, can assemble with the $GABA_A\gamma2$ subunit (Qian and Ripps, 1999). Our preliminary immunohistochemistry experiments in the retinas of $GABA_C\rho1$ null and WT mice indicate that the location and expression levels of the most common $GABA_A$ subunits, $\alpha1$ and $\gamma2$, are normal (data not shown). Finally, $GABA_A$ and $GABA_C$ receptors form distinct clusters in the IPL (Koulen et al., 1998), which suggests that these receptors may use different membrane localization mechanisms (Moss and Smart, 2001).

GABA-mediated inhibition is critical to normal processing of the visual signal through the bipolar cells to the amacrine and ganglion cells in the retina of most vertebrates (for review, see Lukasiewicz, 1996). For example, bipolar cell $GABA_C$ receptors reduce the depolarization-induced influx of Ca^{2+} ions through voltage-dependent calcium channels (Pan and Lipton, 1995), which in turn reduces both neurotransmitter release (Lukasiewicz and Werblin, 1994) and postsynaptic activity (Wellis and Werblin, 1995). The inhibitory feedback mediated by the $GABA_C$ receptors also has been suggested to modify the temporal tuning of this visual signal, making it more transient (Zhang et al., 1997; Dong and Werblin, 1998), and these cellular mechanisms may account for a significant proportion of surround inhibition (Cook and McReynolds, 1998a; Bloomfield and Xin, 2000; Flores-Herr et al., 2001).

The electroretinogram

Although the ERG does not pinpoint the functional role of the $GABA_C$ receptor in vision, we used this technique because it is the best tool to assess alterations in overall retinal processing. The ERG evaluates phototransduction and transmission of this signal to the bipolar cells. It also assesses the G-protein-mediated amplification of the signal that occurs in the bipolar cells and aspects of inner retinal function (Robson and Frishman, 1998). Dark-adapted conditions were chosen because they limited our assessment of retinal function to the rod pathway (Xu et al., 2000), where our patch-clamp experiments also had assessed the GABA currents in rod bipolar cells and where the role of the $GABA_C$ receptor has been most thoroughly characterized (Euler and Wassle, 1998; Fletcher et al., 1998; Euler and Masland, 2000). Specifically, its role at the feedback synapse between the A17 amacrine and the rod bipolar cell terminal has been examined (Kolb and Nelson, 1983; Fletcher and Wassle, 1999) and has been proposed to create the robust surround inhibition that is found in dark-adapted AII amacrine cells (Bloomfield and Xin, 2000).

Our results are consistent with previous evidence that the photoreceptor response is unaffected by exogenous GABA application (Wachtmeister and Dowling, 1978; Wachtmeister, 1980;

Dick and Miller, 1985; Naarendorp and Sieving, 1991; Verweij et al., 1996; Arnarsson and Eysteinsson, 1997; Vitanova et al., 2001). The amplitude of the b-wave, which corresponds to the transmission of the visual signal from the photoreceptors to the depolarizing bipolar cells, also was unaffected. This result was somewhat unexpected because b-wave changes have been reported after application of exogenous GABA or GABAergic agents (Wachtmeister and Dowling, 1978; Wachtmeister, 1980; Dick and Miller, 1985; Naarendorp and Sieving, 1991; Arnarsson and Eysteinsson, 1997; Vitanova et al., 2001). These reports on the effects of GABA are however, variable and probably reflect differences in species, recording techniques, and concentrations of pharmacological agents. Of particular importance is the lack of a selective GABA$_C$ receptor antagonist, one that does not also act as a weak agonist or antagonist of GABA$_A$ receptors and GABA$_B$ receptors (Ragozzino et al., 1996; Flores-Herr et al., 2001).

The decreased time-to-peak (implicit time) of the b-wave is our most unexpected ERG result, particularly in light of its normal amplitude. The explanation for this result is unclear and requires further investigation, but could reflect a functional role for the GABA$_C$ receptor in the OPL where we, and others, have observed its presence (Wassle et al., 1998; Picaud et al., 1998; Pattnaik et al., 2000; Du and Yang, 2000; Vitanova et al., 2001). It is possible that the GABA$_C$ receptors provide tonic inhibition that contributes to setting the light adaptation level in the outer retina. In the absence of the GABA$_C$ receptor, the functional equivalent of a more light-adapted retina could occur and would shorten the b-wave time-to-peak.

In GABA$_C$ρ1 null mice, both the amplitude and number of OPs are increased. These data confirm a role for the GABA$_C$ receptors in inner retinal circuitry of the rod pathway that generates or modulates the OPs (Wachtmeister and Dowling, 1978; Dick and Miller, 1985; Green and Kapousta-Bruneau, 1999). Evidence suggests that some of the OPs are generated by a negative feedback loop in the IPL between the A17 GABAergic amacrine cells and the axon terminals of the rod bipolar cells (Kolb and Nelson, 1981; Wachtmeister, 1998). Consistent with our results are increases in OP amplitude that also were noted in the rat ERG in the presence of GABA$_C$ receptor antagonists (Wachtmeister, 1998; Kapousta-Bruneau, 2000) and increases in both bipolar cell synaptic drive to this circuit (Lukasiewicz and Werblin, 1990) and the sustained nature of this drive when GABA$_C$ receptors are blocked (Dong and Werblin, 1998). A similar increase in the amplitude of an oscillatory response was observed when GABA$_A$β3 expression in an olfactory bulb interneuron was eliminated (Nusser et al., 2001). Thus, similar functions may be performed by these inhibitory receptors in different sensory systems (Hartveit, 1999). *Post hoc* comparisons of individual OP amplitudes between the GABA$_C$ρ1 null mice and controls suggest that OP1 is not altered, which is compatible with data indicating that independent mechanisms may generate some of the OPs (Wachtmeister, 1998).

The data presented here establish that the retinal expression of the GABA$_C$ receptor is eliminated in GABA$_C$ρ1 null mice. The absence of the GABA$_C$ receptor does not affect the development of the retina or the rod bipolar cells, but it does produce a discrete effect on retinal circuitry, which appears to be limited to GABA$_C$ receptor-mediated inhibition. Thus, these results firmly establish that the GABA$_C$ receptor modulates inner retinal circuitry, particularly with respect to the generation or modulation of the OPs. Previous studies have suggested that the GABA$_C$ receptor plays one of several roles in synaptic transmission from bipolar cells to third order cells (Dong and Werblin, 1998; Hartveit, 1999; Cook et al., 2000; Bloomfield and Xin, 2000; Flores-Herr et al., 2001). However, its exact role in shaping this signal remains to be resolved. Because GABA$_C$ρ1 null mice lack the GABA$_C$ receptor, they represent a useful model system in which to tackle these important questions. In addition, they represent a system in which the separate roles of the GABA$_C$ receptor and GABA$_A$ receptor-mediated inhibition in receptive field organization, visual sensitivity, and response properties of amacrine and ganglion cells can be further defined.

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