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## Guinea Pig Horizontal Cells Express GABA, the GABA-Synthesizing Enzyme GAD<sub>65</sub>, and the GABA Vesicular Transporter

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### Abstract

$\gamma$ -Aminobutyric acid (GABA) is likely expressed in horizontal cells of all species, although conflicting physiological findings have led to considerable controversy regarding its role as a transmitter in the outer retina. This study has evaluated key components of the GABA system in the outer retina of guinea pig, an emerging retinal model system. The presence of GABA, its rate-limiting synthetic enzyme glutamic acid decarboxylase (GAD<sub>65</sub> and GAD<sub>67</sub> isoforms), the plasma membrane GABA transporters (GAT-1 and GAT-3), and the vesicular GABA transporter (VGAT) was evaluated by using immunohistochemistry with well-characterized antibodies. The presence of GAD<sub>65</sub> mRNA was also evaluated by using laser capture microdissection and reverse transcriptase-polymerase chain reaction. Specific GABA, GAD<sub>65</sub>, and VGAT immunostaining was localized to horizontal cell bodies, as well as to their processes and tips in the outer plexiform layer. Furthermore, immunostaining of retinal whole mounts and acutely dissociated retinas showed GAD<sub>65</sub> and VGAT immunoreactivity in both A-type and B-type horizontal cells. However, these cells did not contain GAD<sub>67</sub>, GAT-1, or GAT-3 immunoreactivity. GAD<sub>65</sub> mRNA was detected in horizontal cells, and sequencing of the amplified GAD<sub>65</sub> fragment showed approximately 85% identity with other mammalian GAD<sub>65</sub> mRNAs. These studies demonstrate the presence of GABA, GAD<sub>65</sub>, and VGAT in horizontal cells of the guinea pig retina, and support the idea that GABA is synthesized from GAD<sub>65</sub>, taken up into synaptic vesicles by VGAT, and likely released by a vesicular mechanism from horizontal cells.

### Keywords

GABA; GAD; GAT; VGAT; VIAAT; horizontal cells; retina; visual system

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In the mammalian retina, horizontal cells likely synthesize  $\gamma$ -aminobutyric acid (GABA), although there are inconsistent and conflicting findings regarding both their expression of GABA and its synthetic enzyme, L-glutamic acid decarboxylase (GAD), in different species. Most studies describe GABA- and GAD-immunoreactive horizontal cells in the cat, rabbit, and monkey retina (Mosinger et al., 1986; Osborne et al., 1986; Agardh et al., 1987; Mosinger and Yazulla, 1987; Chun and Wässle, 1989; Pourcho and Owczarzak, 1989; Wässle and Chun, 1989; Vardi et al., 1994; Johnson and Vardi, 1998; Marc et al., 1998). In contrast, in the adult guinea pig retina, it is reported that GABA-containing horizontal cells are absent (Agardh et al., 1986; Osborne et al., 1986; Loeliger and Rees, 2005). There has also been a failure to consistently demonstrate either GABA or GAD immunoreactivity in horizontal cells of both adult mouse and rat retinas. In these species, GABA and GAD immunoreactivities are transiently expressed by horizontal cells during the first several weeks of postnatal retinal development, and immunoreactivity is consistently reported to be absent in adult retinas (Vaughn et al., 1981; Lin et al., 1983; Schnitzer and Rusoff, 1984; Brandon, 1985; Mosinger et al., 1986; Osborne et al., 1986; Versaux-Botteri et al., 1989; Yamasaki et al., 1999; Fletcher and Kalloniatis, 1997; Yamasaki et al., 1999; Dkhissi et al., 2001).

Unlike what is reported for GABA and GAD immunostaining, the vesicular GABA/glycine transporter (VGAT), which mediates the uptake and storage of GABA and/or glycine in synaptic vesicles (for reviews, see Liu and Edwards, 1997; Gasnier, 2004), is reliably expressed in mammalian horizontal cells (Haverkamp et al., 2000; Cueva et al., 2002; Jellali et al., 2002; Johnson et al., 2003; Guo et al., 2009). VGAT immunoreactivity is mainly in horizontal cell processes and their endings located within the photoreceptor synaptic triad (Haverkamp et al., 2000; Cueva et al., 2002; Jellali et al., 2002; Johnson et al., 2003; Guo et al., 2009), suggesting that GABA accumulates into vesicles of horizontal cell endings. Earlier studies showing small, clear-core vesicles in horizontal cell processes and endings are also congruent with the idea that there are synaptic vesicles in these endings (Dowling and Boycott, 1966; Dowling et al., 1966; Gray and Pease, 1971; Fisher and Boycott, 1974; Linberg and Fisher, 1988; Spiwox-Becker et al., 2001).

GABA plasma membrane transporters (GATs) mediate high-affinity GABA uptake from the extracellular space and likely terminate GABA's synaptic action in the central nervous system (CNS) (for reviews, see Chen et al., 2004; Conti et al., 2004; Kanner, 2006). In addition to a role in the clearance of GABA, GATs may also release GABA by a reverse transport mechanism (O'Malley et al., 1992; Dalby, 2003; Richerson and Wu, 2003; Wu et al., 2007). A GAT reportedly mediates GABA release from retinal horizontal cells in fish and amphibians (Schwartz, 1982; Yazulla and Kleinschmidt, 1982, 1983; Schwartz, 1987, 2002; Attwell et al., 1993). However, in the mammalian retina, there is doubt regarding the hypothesis that GABA is released from horizontal cells by a plasma membrane transporter, because currently known GAT isoforms (GAT-1, GAT-2, and GAT-3) are not expressed by horizontal cells (Brecha and Weigmann, 1994; Durkin et al., 1995; Honda et al., 1995; Johnson et al., 1996; Hu et al., 1999; Casini et al., 2006; Guo et al., 2009). Furthermore, mammalian horizontal cells do not accumulate GABA and GABA analogs, as would be expected if a functional GAT were expressed by these cells (Neal and Iversen, 1972; Bruun and Ehinger, 1974; Marshall and Voaden, 1975; Cunningham et al., 1981; Agardh and Ehinger, 1982, 1983; Blanks and Roffler-Tarlov, 1982; Wässle and Chun, 1989).

The aim of the present study was to systematically evaluate the cellular expression of several key components of the GABA system in the outer retina of the guinea pig. These studies found that GABA, GAD<sub>65</sub>, and VGAT are strongly expressed in both A- and B-type horizontal cells. Together, the localization of the GAD<sub>65</sub> and VGAT immunoreactivities to horizontal cell processes and endings suggests the local synthesis and accumulation of

GABA into synaptic vesicles, and the likely release of GABA from these cells by a vesicular mechanism.

## MATERIALS AND METHODS

### Animals

Hartley guinea pigs (Charles River, Wilmington, MA) of either sex at 5–8 weeks age were used for these studies. Guinea pigs were maintained on a 12-hour light/12-hour dark cycle. Animal housing and all experiments were performed in accordance with the guidelines and policies for the welfare of experimental animals issued by the UCLA Animal Research Committee, and U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. Guinea pigs were euthanized with 1–3% isoflurane (Hospira, Lake Forest, IL) before dissection of the eye.

For vertical retinal sections, the eyes were enucleated, the anterior chamber and lens were removed, and the eyecups were immersion-fixed in 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4) for 15–60 minutes at room temperature. Following fixation, the eyes were stored in 30% sucrose for 48 hours at 4°C, briefly washed in 0.1 M PB, embedded in OCT compound (Sakura Finetek, Torrance, CA), and rapidly frozen with dry ice. The eyecup was cut vertically into 12- $\mu$ m-thick sections with a Leica CM3005 cryostat (Leica Microsystems, Bannockburn, IL), and the retinal sections were mounted onto gelatin-coated slides, air-dried, and stored at –20°C. Every 10th section was collected from superior retina to inferior retina. For horizontal sections, the retinas were dissected and immersion-fixed in 4% PFA in 0.1 M PB for 1 hour at 4°C and were transferred to 30% sucrose in 0.1 M PB for 48 hours at 4°C. Retinal whole mounts were then frozen flat on an ice block and cut at 35  $\mu$ m parallel to the retinal layers by using an AO sliding microtome (model 860, American Optical, Buffalo, NY).

### Acute dissociation of retinal cells

The retina was dissected from the eyecup in Hanks' balanced salt solution (HBSS, pH 7.4), cut into pieces, and incubated in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free HBSS containing papain (40–45 U/ml, pH 7.4, Worthington, Lakewood, NJ) for 40 minutes at 37°C. The pieces of retina were transferred to medium containing Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA), 10% fetal bovine serum (Invitrogen), 1X penicillin-streptomycin-glutamine (Invitrogen), and DNase I (100 U/ml, pH 7.4, Worthington), and gently triturated by a glass pipette with the opening pore narrowed by flame to obtain suspensions of isolated cells. Isolated cells were seeded into 12-well cell culture plates onto coverslips coated with concanavalin A (1 mg/ml, Sigma-Aldrich, St. Louis, MO). The cells were allowed to settle for 1–3 hours at 37°C to adhere to the coverslips. The cells were then fixed with 4% PFA for 10 minutes at room temperature and subsequently used for immunocytochemistry.

### Antibodies

Primary antibodies were as follows: rabbit polyclonal antibody to GABA (gift of Dr. David Pow, University of Queensland, Australia), mouse monoclonal antibodies to GAD<sub>65</sub> (MAB531R, clone GAD-6, Millipore, Temecula, CA), GAD<sub>67</sub> (MAB5406, clone 1G10.2, Millipore), and VGAT (131 011; Synaptic Systems, Göttingen, Germany) and affinity-purified rabbit polyclonal antibodies directed to the C-terminus of GAT-1 (346O) and GAT-3 (374D [Johnson et al., 1996]). A mouse monoclonal antibody to the calcium-activated, phospholipid-dependent protein kinase C (K01197M, clone MC5; Biodesign International, Saco, ME) was used to identify rod bipolar cells in mammalian retina (Ghosh et al., 2001). A mouse monoclonal and a rabbit polyclonal antibody to calbindin D-28k (calbindin) were used to identify horizontal cells. Calbindin D-28k is robustly expressed in

axonless A-type and axon-bearing B-type horizontal cells in different mammalian retinas (Röhrenbeck et al., 1987; Celio, 1990; Peichl and González-Soriano, 1994; Massey and Mills, 1996; Johnson and Vardi, 1998; Haverkamp and Wässle, 2000; Raven and Reese, 2002; Hirano et al., 2005; Loeliger and Rees, 2005; Deng et al., 2006; Gargini et al., 2007; Hirano et al., 2007).

### Antibody characterization

All of the antibodies used in this study have been previously characterized in mammalian retina as summarized below. Additional information is provided in Table 1.

1. Mouse monoclonal anti-calbindin D-28K antibody (C9848, clone CB-955; Sigma-Aldrich): This antibody recognizes calbindin D-28K (calbindin) in Western blots of human striatal neurons (Benchoua et al., 2006) and in tissues from other species, including guinea pig (Sigma-Aldrich data sheet). This antibody does not react with other related proteins, including calbindin D-9K, calretinin, and parvalbumin in Western blot (Sigma-Aldrich data sheet). This antibody has been used to immunostain horizontal cells in mouse, rat, ferret, rabbit, and pig retinas (Rossi et al., 2003; Renteria et al., 2005; Deng et al., 2006; Lee et al., 2006; Zhang et al., 2006; Gargini et al., 2007; Hirano et al., 2007; Damiani et al., 2008; Ettaiche et al., 2009; Kyhn et al., 2009).
2. Rabbit polyclonal anti-calbindin D-28K antibody (CB38a; Swant, Bellinzona, Switzerland): This antibody detects a single band of the expected molecular size (~27–28 kDa) for calbindin in Western blots of brain lysates from multiple species, including rat and guinea pig (Katsetos et al., 1994; Veenstra et al., 1997; Swant datasheet). This antibody also detects a band of the expected molecular size (~29–30 kDa) of calretinin at 10-fold higher dilutions used to detect calbindin (Swant datasheet). This antibody shows specific calbindin immunostaining in the cerebellum that matches previous findings (Garcia-Segura et al., 1984; Celio, 1990). This antibody has been used to immunostain horizontal cells in various mammalian retinas, including the retinas of guinea pig (Loeliger and Rees, 2005), rat (Gaillard et al., 2008), and mouse (Haverkamp and Wässle, 2000; Loeliger and Rees, 2005; Gaillard et al., 2008).
3. Rabbit polyclonal anti-GABA antibody: This rabbit polyclonal antibody (GABA) was made to a GABA-porcine thyroglobulin conjugate that was coupled by 4% paraformaldehyde and co-adsorbed with colloidal gold particles (Pow and Crook, 1993). This antibody detects GABA-PFA-bovine serum albumin (BSA) conjugates and does not cross-react with glutamine-, aspartate-, glycine-, and glutamate-PFA-BSA conjugates in immunoblotting assays (Pow and Crook, 1993). Immunohistochemistry experiments show that preadsorption of this antibody with the GABA-PFA-BSA conjugate eliminated specific GABA immunostaining in rabbit retina and cat hypothalamus (Pow and Crook, 1993; Pow et al., 1995). Preadsorption of this antibody with GABA-BSA conjugate eliminated specific GABA immunostaining in guinea pig retina (Fig. 1). This antibody has been used to immunostain retinal cells in different mammalian retinas, including rabbit, rat, mouse, and guinea pig (Pow and Crook, 1993; Pow et al., 1995; Haverkamp and Wässle, 2000; Sullivan et al., 2003; Catalani et al., 2004; Loeliger and Rees, 2005). The overall immunostaining pattern is similar to the GABA immunostaining patterns observed by using other GABA antibodies (Chun and Wässle, 1989; Pourcho and Owczarzak, 1989; Wässle and Chun, 1989; Johnson and Vardi, 1998; Haverkamp and Wässle, 2000).

4. Mouse monoclonal anti-GAD<sub>65</sub> antibody (MAB351R, clone GAD-6; Millipore): This mouse monoclonal antibody (IgG2a isotype) is derived from a hybridoma produced from a mouse immunized by immunoaffinity-purified rat brain GAD from a GAD<sub>65</sub> affinity column (Gottlieb et al., 1986; Chang and Gottlieb, 1988). This antibody detects a single band of the expected molecular size (~59 kDa) of GAD<sub>65</sub> (Erlander et al., 1991) in Western blots of unfractionated homogenates of whole rat brain (Chang and Gottlieb, 1988). This antibody does not cross-react with GAD<sub>67</sub> in Western blots of rat brain homogenates (Chang and Gottlieb, 1988). In addition, a single band corresponding to GAD<sub>65</sub> in Western blots was absent in homogenates of brain from GAD<sub>65</sub> knockout mice (Yamamoto et al., 2004). This antibody has been used to immunostain amacrine and horizontal cells in different mammalian retinas, including mouse, rat, rabbit, cat, and monkey retinas (Johnson and Vardi, 1998; Haverkamp and Wässle, 2000; Lee et al., 2006; Ding and Weinberg, 2007).
5. Mouse monoclonal anti-GAD<sub>67</sub> antibody (MAB5406, clone 1G10.2; Millipore): This antibody recognizes a single band (~67 kDa) at the expected molecular size of GAD<sub>67</sub> (Erlander et al., 1991) in a Western blot of rat cortex, and it does not cross-react with the GAD<sub>65</sub> isoform in a Western blot of rat cortex (Fong et al., 2005). The GAD<sub>67</sub> signal is reduced in a Western blot of the striatum of a heterozygous GAD<sub>67</sub> knockout mouse compared with a wild-type mouse (Heusner et al., 2008). GAD<sub>67</sub> immunostaining is also reduced in the central nervous system of this GAD<sub>67</sub> mutant compared with wild-type mice (Heusner et al., 2008). The immunostaining pattern in the retina and brain (Erlander et al., 1991; Fong et al., 2005; Heusner et al., 2008) using this antibody is similar to the pattern observed by using other polyclonal GAD<sub>67</sub> antibodies (Esclapez et al., 1994; Vardi and Auerbach, 1995; Haverkamp and Wässle, 2000; Fong et al., 2005).
6. Rabbit polyclonal anti-GAT-1 antibody (AB1570, Millipore; Johnson et al., 1996): This antibody recognizes a single band of the expected molecular size (~67 kDa) of GAT-1 (Guastella et al., 1990) in Western blots of mouse brain and retina lysates (Guo et al., 2009). Preadsorption of this antibody with the rat GAT-1<sub>588-599</sub> peptide that was used for immunization at concentrations of 10<sup>-5</sup> to 10<sup>-7</sup> M eliminated GAT-1 immunostaining in mouse, rat, and monkey retina and brain sections (Ikegaki et al., 1994; Honda et al., 1995; Johnson et al., 1996; Conti et al., 1998; Casini et al., 2006; Guo et al., 2009). GAT-1 immunostaining in rat retinal sections was not affected by preadsorption of the GAT-1 antibody with the C-terminus GAT-2<sub>594-602</sub> (RLTELESNC) or GAT-3<sub>607-627</sub> (CEAKVKGDGTISAITEKETHF) peptides at concentrations of 10<sup>-5</sup> to 10<sup>-7</sup> M (Johnson et al., 1996). This antibody has been used to immunostain amacrine and Müller cells in the mouse and rat retina (Johnson et al., 1996; Guo et al., 2009). The immunostaining pattern of this antibody matches the GAT-1 immunostaining pattern obtained by using another GAT-1 antibody in the rat retina (Honda et al., 1995).
7. Rabbit polyclonal anti-GAT-3 antibody (AB1574, Millipore; Johnson et al., 1996): This antibody recognized a single band of the expected molecular size (~71 kDa) of GAT-3 (Lopez-Corcuera et al., 1992) in Western blots of mouse brain and retina lysates (Guo et al., 2009). Preadsorption of this antibody with the rat GAT-3<sub>607-627</sub> peptide that was used for immunization at concentrations of 10<sup>-5</sup> to 10<sup>-7</sup> M eliminated GAT-3 immunostaining in mouse, rat, and monkey retina and brain sections (Johnson et al., 1996; Minelli et al., 1996; Guo et al., 2009). Furthermore, the GAT-3 immunostaining in rat retinal sections was not affected by preadsorption of the GAT-3 antibody with the C-terminus GAT-1<sub>588-599</sub> or GAT-2<sub>594-602</sub>

peptides at concentrations of  $10^{-5}$  to  $10^{-7}$  M (Johnson et al., 1996). This antibody has been used to immunostain amacrine and Müller cells in the rat and mouse retina (Johnson et al., 1996; Guo et al., 2009). The immunostaining pattern of this antibody matches the GAT-3 immunostaining pattern obtained by using another GAT-3 antibody in rat retina (Honda et al., 1995).

8. Mouse monoclonal anti-VGAT antibody (131 011, clone 117G4, Synaptic Systems): This mouse monoclonal antibody (IgG3 isotype) recognizes a single band of the expected molecular size (~57 kDa) of VGAT in Western blots of mouse brain and retina lysates (McIntire et al., 1997; Sagné et al., 1997; Guo et al., 2009). Preadsorption of this antibody with the VGAT N-terminus peptide, VGAT<sub>75-87</sub> (AEPPEVDIHYQR), that was used for immunization, eliminated the VGAT signal in Western blots of a rat brain homogenate (Synaptic Systems, data sheet). This antibody has been used to immunostain amacrine and horizontal cells in mouse retina (Guo et al., 2009). Preadsorption of this antibody with the VGAT N-terminus peptide, VGAT<sub>75-87</sub>, at concentrations of  $10^{-5}$  to  $10^{-7}$  M eliminated VGAT immunostaining in mouse retina sections (data not shown; Guo et al., 2009). The immunostaining pattern of this antibody matches the VGAT immunostaining pattern obtained by using other VGAT antibodies in the rat and mouse retina (Cueva et al., 2002; Jellali et al., 2002; Johnson et al., 2003).
9. Mouse monoclonal anti-PKC (K01197M, clone MC5; Meridian Life Science, Saco, ME): PKC is a well-characterized marker for rod bipolar cells in retina of many species (Haverkamp and Wässle, 2000; Ghosh et al., 2001). This monoclonal antibody (clone MC5) was raised against PKC ( $M_r$  79–80 kDa) purified from bovine brain. The PKC antibody recognized the purified PKC protein, as well as an 80-kDa band from whole-cell extracts of rat glioma and murine NIH3T3 cell lines on Western blots, and specifically immunoprecipitated PKC from cell lysates of 328 glioma and SVK 14 cell lines. The epitope was mapped by enzyme-linked immunosorbent assay (ELISA) by using a peptide corresponding to PKC<sub>304-309</sub> of bovine brain PKC and shorter fragments thereof to the hinge region (PKC<sub>304-309</sub>: KFEKAK), close to or at the trypsin cleavage site of PKC (Young, 1988). Binding of the antibody was completely blocked by the full-length peptide but not blocked by a unrelated peptide (Young, 1988). This antibody reacts with PKC- $\alpha/\beta$ -1/ $\beta$ -2 isoforms (Meridian Life Science datasheet).

### Western blotting

Western blotting with the VGAT antibody was carried out to establish the presence of VGAT in the guinea pig retina, and to further characterize the specificity of this antibody as described in Guo et al. (2009). In brief, the retina and brain were homogenized, and 10–20  $\mu$ g of total protein from brain and retina homogenates were run on a 4–20% polyacrylamide gel. Membranes were probed with the antibody against VGAT (1:1,500) diluted in blocking buffer containing 25 mM Tris-Cl (pH 7.5), 150 mM NaCl, 0.1 mM phenylmethanesulphonylfluoride (PMSF), 2 mM EDTA, 5% nonfat dry milk, and 0.05% Tween-20; then labeled bands were visualized by using horseradish peroxidase (HRP)-conjugated, goat anti-mouse secondary antibody (Pierce, Rockford, IL) diluted in blocking buffer, and detected with enhanced chemiluminescence on Kodak BioMax light film.

### Immunohistochemistry

Immunostaining was carried out with the indirect immunofluorescence method (Hirano et al., 2007) by using retinal sections from central and peripheral retina, whole mounts, and acutely dissociated cells. Retinal sections were incubated in 10% normal goat serum (NGS), 1% BSA, 0.5% Triton X-100, and 0.05% sodium azide in 0.1 M phosphate-buffered saline

(PBS), pH 7.4, for 1 hour at room temperature. The blocking solution was replaced by the primary antibody diluted in 3% NGS, 1% BSA, 0.5% Triton X-100, and 0.05% sodium azide in PBS and incubated overnight at 4°C in a humidified chamber. Following three washes of 0.1 M PB for 10 minutes each, the sections were incubated for 30–60 minutes at room temperature in secondary antibodies conjugated to either Alexa 488 or Alexa 568 at a dilution of 1:1,000 (Invitrogen), diluted in the same solution as the primary antibody, except that sodium azide was omitted. Following washes in 0.1 M PB, the sections were air-dried, and mounted in Aqua Poly/Mount (Polysciences, Warrington, PA).

Horizontal retinal sections were incubated free floating with primary antibodies for 7 days and secondary antibodies for 3 days at 4°C on a shaker. The primary antibody/antigen complex was detected by using the appropriate secondary antibodies conjugated to either Alexa 488 or Alexa 568 at a dilution of 1:1,000 (Invitrogen). Following washes in 0.1 M PB, the sections were mounted in Aqua Poly/Mount.

Acutely dissociated retinal cultures were incubated in primary and secondary antibodies for overnight and 1 hour, respectively, in 12-well cell culture plates. Antibody dilutions were the same as used for the vertical sections. The washing steps were performed by briefly dipping coverslips into 0.1 M PB for three times, and coverslips were air-dried and mounted on gelatin-coated slides.

For immunohistochemistry controls, all antibodies were tested on guinea pig retina as single-labeling experiments at least three times to confirm specificity and optimize concentration prior to performing any double-labeling experiments. Preadsorption experiments using GABA-BSA eliminated the specific immunostaining GABA antibody (Fig. 1). For double-labeling controls, one of the two primary antibodies used for double labeling was omitted during the primary incubation step. In this case, only the immunostaining by the remaining primary antibody was detected.

### Confocal imaging

Images of retinal sections, whole mounts, and acutely dissociated retinal cells were acquired by using a Zeiss Laser Scanning Microscope 510 META (Carl Zeiss Microimaging, Thornwood, NY). Samples were imaged with either a Plan Neofluar 40× 1.3 NA oil objective, or a C-Apochromat 40× 1.2 NA water objective. To identify fluorescent signals, different lasers were used for excitation: for Alexa 488, the 488-nm argon laser line was used, and for Alexa 568, the 543-nm HeNe laser line was used. During acquisition of signals from double-labeled specimens, the scans were collected sequentially to prevent spectral bleed-through. Specific laser excitation and filters were used to achieve proper separation of signals (for single labeling with Alexa 488: 505–530 BP; Alexa 568: 560 LP; for double labeling with Alexa 488 and Alexa 568, 505–530 BP and 560–LP were used, respectively). All images were acquired at a resolution of 2,048 × 2,048 as 12-bit signals. To increase the signal-to-noise, images were averaged online (e.g.,  $n = 4$ ), and the scan speed and photomultiplier detector gain were decreased. Most confocal images were acquired at an approximate optical thickness of 1.0 μm, usually 0.7–1.0 Airy unit. For projections, typically 5–6 optical sections, but as many as 12, as noted, were acquired and compressed for viewing. Digital confocal images were saved as Zeiss .LSM files, and final publication quality images were exported in the .TIFF format by using Zeiss LSM 510 Meta software version 3.2 (Carl Zeiss Microimaging). All images were processed, adjusted for brightness and contrast, and resized to 300 dpi by using Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA).

### Laser capture microdissection (LCM) of horizontal cells

The eyecups were cryoprotected in 30% sucrose at 4°C for 4 hours, embedded into OCT, and fresh frozen with dry ice. Vertical retinal sections were made at 10 µm by using a Leica CM3005 cryostat, mounted onto polyethylene-naphthalate (PEN) membrane-based slides (Leica Microsystems), and stored at -80°C. Sections were fixed with ice-cold 70% ethanol for 1 minute, serially dehydrated by dipping into 75%, 95%, and 100% ethanol for 1 minute each, and air-dried for 10–30 minutes inside a laminar flow hood. Horizontal cells were identified in the vertical section viewed with differential interference contrast (DIC) optics in the Leica AS LMD system (Leica Microsystems) based on their cell morphology, size, and the localization of the cell bodies close to the outer plexiform layer (OPL). A circular boundary was defined within the contour of the cell body for laser capture of a single horizontal cell in a retinal section, to ensure minimal contamination from surrounding area. Horizontal cells were collected by using the Leica AS LMD system following the manufacturer's operation manual. The following settings of the Leica AS LMD system were employed: laser beam intensity = 11, aperture = 9–11, apertureDiff = 45, bridge = medium.

### Degenerate RT-PCR

Forty horizontal cells were collected by using LCM, and total RNA was isolated by using the Absolutely RNA nanoprep kit (Stratagene, La Jolla, CA). RNA preparations were reverse-transcribed by using oligo-dT primers and the SuperScript III First-Strand Synthesis System (Invitrogen), according to the manufacturer's instructions. Platinum PCR SuperMix (Invitrogen) was used for polymerase chain reaction (PCR) reactions. Degenerate primers complementary to highly conserved regions of mouse GAD<sub>65</sub> were used with the first-strand cDNA as template to amplify a 166-bp DNA product. The conserved domains of the GAD<sub>65</sub> protein was screened by using National Center for Biotechnology Information (NCBI) protein blast; the designed primers recognize the mRNA sequence corresponding to the middle of the pyridoxal-dependent decarboxylase conserved domain of the GAD<sub>65</sub> protein: the sense primer (5'-TAYAAARATHHTGGATGCA-3') and the antisense primer (5'-CATCATYTTTRTGNGGRTTCC-3'). For sequencing purposes, T7 and T3 sequences were tagged to the 5' ends of the sense and antisense primers, respectively. PCR was performed by using degenerate primers at a concentration of 12 µM and 40 cycles of the following amplification profile: template denaturation and enzyme activation for 1 minute at 94°C, denaturation for 30 second at 94°C, annealing for 30 seconds at 55°C, extension for 45 seconds at 72°C. DNA amplification products (amplicons) were separated on 1.8% agarose gels, extracted by using QIAEX II Gel Extraction Kit (QIAGEN, Valencia, CA) and sent for sequencing at the UCLA Genotyping and Sequencing Core ([http://www.genoseq.ucla.edu/action/view/Main\\_Page](http://www.genoseq.ucla.edu/action/view/Main_Page)).

## RESULTS

Horizontal cells were identified on the basis of their soma size, position in the distal inner nuclear layer (INL), and distribution of their processes in the OPL. Guinea pig horizontal cells also contain calbindin immunoreactivity (Hamano et al., 1990; Peichl and González-Soriano, 1994; Loeliger and Rees, 2005). The present study included an analysis of both central and peripheral retinal regions.

### GABA immunoreactivity

Specific GABA immunostaining was in both central and peripheral retina, and was localized to amacrine and horizontal cells (Fig. 2). In the inner retina, strong GABA immunostaining was found in many amacrine cell somata, in displaced amacrine cell somata, and in their processes distributed to all laminae of the inner plexiform layer (IPL; Fig. 2A). In the outer retina, GABA immunostaining was strongest in horizontal cell somata, and immunostaining



was weaker in horizontal cell processes and endings (Fig. 2D & G). All calbindin-immunoreactive horizontal cell bodies contained GABA immunoreactivity, implying that both A- and B-type horizontal cells have GABA.

Previous findings in adult guinea pig retina reported little or no GABA immunoreactivity in horizontal cells (Agardh et al., 1986; Osborne et al., 1986; Loeliger and Rees, 2005). We found that fixation duration markedly influenced the levels of GABA immunostaining in the outer retina. Short periods of fixation from 15 to 30 minutes gave better immunostaining of horizontal cells and their processes; the GABA immunolabeling was generally strong and specific. However, longer fixation periods (> 60 minutes) resulted in GABA immunostaining at levels at or just above background, and often immunoreactivity was undetectable (data not shown).

### **GAD<sub>65</sub> and GAD<sub>67</sub> immunoreactivity**

GAD is the key synthetic enzyme that converts glutamic acid into GABA. Both GAD isoforms, GAD<sub>65</sub> and GAD<sub>67</sub>, are found in horizontal cells of some mammalian retinas (Agardh et al., 1987; Vardi et al., 1994; Johnson and Vardi, 1998; Calaza et al., 2006). In guinea pig retina, GAD<sub>65</sub> immunoreactivity was in amacrine and displaced amacrine cell bodies, and in their processes distributed in the IPL (Kao and Sterling, 2006), as well as horizontal cell bodies and their processes and endings (Fig. 3). GAD<sub>65</sub>, and calbindin immunoreactivity was co-localized in horizontal cell bodies and processes in vertical sections (Fig. 3F,I). The intensity of GAD<sub>65</sub> immunostaining in horizontal somata was weaker than in processes and terminals. Interestingly, there was a polarization of the distribution of the GAD<sub>65</sub> immunostaining in horizontal cell somata: high levels of GAD<sub>65</sub> immunoreactivity were concentrated in the somatic region adjacent to the nucleus closest to the OPL (Fig. 3D,G). Strong GAD<sub>65</sub> immunostaining was also observed along the processes and at their endings with a punctate staining pattern (Fig. 3G).

Similar to GABA immunostaining, GAD<sub>65</sub> immunoreactivity was in all the horizontal cells that were calbindin immunoreactive in vertical sections (Fig. 3F & I), suggesting that GAD<sub>65</sub> immunoreactivity was in both A- and B-type horizontal cells. However, double labeling for calbindin and GAD<sub>65</sub> in horizontal sections revealed that about 80% (six fields [225 × 225 μm] from five horizontally sectioned retinas) of the calbindin-expressing horizontal cells contain GAD<sub>65</sub> immunoreactivity (Fig. 4). The white dots in Figure 4 indicate the position of somata containing both GAD<sub>65</sub> and calbindin immunoreactivity. There were fewer GAD<sub>65</sub>-immunoreactive horizontal cell bodies in the horizontal sections; the lack of GAD<sub>65</sub> immunostaining is likely due to technical issues including the plane of section through the horizontal cell bodies, tissue fixation, and incomplete GAD<sub>65</sub> antibody penetration of the tissue.

GAD<sub>65</sub> immunoreactivity was also in cells in acutely dissociated preparations from adult retina. Horizontal cells were identified on the basis of the co-localization of GAD<sub>65</sub> and calbindin immunoreactivity in large somata having the appearance of horizontal cells (Fig. 5). Putative A-type horizontal cells were identified on the basis of a symmetrical distribution of dendrites and an absence of an axon-like process (Fig. 5A–D). Putative B-type horizontal cells were identified on the basis of multiple fine-caliber dendrites (Fig. 5E–H). Some of these cells had a thicker, axon-like process characteristic of guinea pig axons (Peichl and González-Soriano, 1994) that often ended with finer branches (Fig. 5E–H). In some cases, this putative axon and terminal system was absent, likely lost during the dissociation procedure.

GAD<sub>65</sub> immunoreactivity was distributed to the putative A- and B-type horizontal cells in all cellular compartments, including their soma, processes, and endings, in the dissociated

preparations (Fig. 5). Immunostaining was absent in the nucleus. The distribution of GAD<sub>65</sub> immunoreactivity in dissociated horizontal cells differed from the asymmetric distribution of GAD<sub>65</sub> immunoreactivity observed in horizontal cells from retinal sections and whole mounts.

### GAD<sub>65</sub> mRNA in horizontal cells

Degenerate oligonucleotide primers were designed to highly conserved sequences of the known GAD<sub>65</sub> proteins to screen for GAD<sub>65</sub> expression in guinea pig horizontal cells. A 166-bp PCR fragment was amplified from first-strand cDNA synthesized from guinea pig brain (Fig. 6B, lane 2), retina (Fig. 6B, lane 3), and 40 horizontal cells (Fig. 6C, lane 2) collected by LCM (Fig. 6A). Both DNA strands of the PCR products from retina were sequenced at least twice; sequencing results indicated that it had about 86% identity (84/97) to mouse (*Mus musculus*) GAD<sub>65</sub> mRNA, and about 83% identity (81/97) to *Homo sapiens* GAD<sub>65</sub> mRNA sequence (Fig. 6D sequence overlay). As expected, the deduced amino acid sequence appears to be conserved between guinea pig and mouse, with most of the nucleotide differences occurring in the third position of the codon.

Strong GAD<sub>67</sub> immunostaining was found in many amacrine cells, displaced amacrine cells, and their processes in the IPL (Fig. 7A). In contrast, GAD<sub>67</sub> immunostaining was absent from the outer retina (Fig. 7A,D), indicating that guinea pig horizontal cells do not contain GAD<sub>67</sub>. To test whether low levels of GAD<sub>67</sub> immunoreactivity were present in horizontal cells, some sections were immunostained with high concentrations (up to 1:100) of GAD<sub>67</sub> antibodies; however, no specific immunostaining was observed in the OPL.

### VGAT immunoreactivity

VGAT accumulates GABA into synaptic vesicles (Chaudhry et al., 1998; Dumoulin et al., 1999a; Takamori et al., 2000). In the retina, VGAT is prominently expressed in horizontal cell endings in several mammalian species (Haverkamp et al., 2000; Cueva et al., 2002; Jellali et al., 2002; Johnson et al., 2003; Guo et al., 2009).

VGAT immunoreactivity was detected in extracts of the guinea pig retina and brain by Western blotting (Fig. 8). A single band of  $M_r \sim 57$  kDa was detected for guinea pig retina and brain. This is the predicted molecular weight of VGAT in mouse and rat brain and retina (McIntire et al., 1997; Sagné et al., 1997; Chaudhry et al., 1998; Takamori et al., 2000; Bragina et al., 2007; Guo et al., 2009). These studies also indicated the specificity of the VGAT antibody in guinea pig tissues, because it detected a single band in these preparations.

VGAT immunostaining was expressed by both amacrine and horizontal cells in the guinea pig retina (Fig. 9). Many, but not all, amacrine and displaced amacrine cells were immunolabeled for VGAT. Amacrine and displaced amacrine cell somata were characterized by weak immunostaining of their cytoplasm. In contrast, very strong VGAT-immunostained processes were distributed to all laminae of the IPL (Fig. 9A). Very weak VGAT immunoreactivity was also in horizontal cell somata (Fig. 9D). In the outer retina, VGAT immunoreactivity was principally distributed to horizontal cell processes and endings, with the strongest immunostaining in the tips producing a punctate staining pattern (Fig. 9D,G).

VGAT immunoreactivity was in putative horizontal cells in acute retinal dissociation preparations (Fig. 10A,E). Double immunostaining with VGAT (Fig. 10A,E) and calbindin (Fig. 10B,F) antibodies revealed co-localization of the two signals (Fig. 10C,G); putative A-type horizontal cells (Fig. 10A–D) and B-type horizontal cells (Fig. 10E–H) were seen in these preparations. VGAT immunoreactivity was distributed to all cellular compartments of

dissociated horizontal cells, similar to the distribution of GAD<sub>65</sub> in dissociated horizontal cells.

### **GAT-1 and GAT-3 immunoreactivity**

A proposed mechanism of GABA release from horizontal cells in nonmammalian retinas is based on the action of a plasmalemmal GAT that mediates GABA secretion (for review, see Schwartz, 2002). We therefore tested for the expression of the two GABA plasma membrane transporters GAT-1 and GAT-3 found in the mammalian retina (Brecha and Weigmann, 1994; Honda et al., 1995; Johnson et al., 1996; Guo et al., 2009).

The pattern of GAT-1 (Fig. 11B) and GAT-3 (Fig. 12B) immunostaining in guinea pig retina was similar to the immunostaining pattern in mouse and rat retina (Honda et al., 1995; Johnson et al., 1996; Guo et al., 2009). GAT-1 immunoreactivity (Fig. 11B) was robust in the inner retina, with numerous, well-stained amacrine cell bodies, and their processes in all laminae of the IPL. GAT-1 immunoreactivity (Fig. 11B) was very weak in Müller cell bodies and processes, which were distributed across the retina. GAT-3 labeling (Fig. 12B) showed a diffuse staining pattern of Müller cell bodies in the INL and their processes extending from the outer limiting membrane to the inner limiting membrane. In addition, GAT-3 immunoreactivity (Fig. 12B) was localized to some amacrine cell bodies and processes in the IPL.

In the OPL, GAT-1 immunoreactivity (Fig. 11E) had a punctate appearance, and GAT-3 immunoreactivity (Fig. 12E) had a honeycomb-like appearance. Neither GAT-1 (Fig. 11F,I) nor GAT-3 (Fig. 12F,I) immunoreactive structures were co-localized with the VGAT-immunoreactive horizontal cell processes and terminals. In addition, double-labeling experiments using antibodies to GAT-1 and calbindin (Fig. 13A–C) or PKC (Fig. 13D–F) showed that GAT-1 immunoreactivity in the OPL was not distributed to horizontal cell processes and terminals labeled by calbindin (Fig. 12C), nor was it distributed to rod bipolar cell dendrites labeled by PKC (Fig. 13F). Finally, GAT-1 or GAT-3 immunoreactivity was not localized to isolated horizontal cells in dissociated retinal preparations (data not shown).

## **DISCUSSION**

These studies form a comprehensive examination of the presence of GABA, its synthetic enzyme GAD, and its transporters, GAT and VGAT, in the outer retina of the guinea pig, which revealed that both A- and B-type horizontal cells contain GABA, and express GAD<sub>65</sub> and VGAT. These findings are in contrast to previous reports that GABA immunoreactivity was absent in the outer retina or present at low levels in a small number of horizontal cells in the adult guinea pig retina (Agardh et al., 1986; Osborne et al., 1986; Loeliger and Rees, 2005). Together, these findings fulfill three of the classical requirements for a neurotransmitter candidate in a neuron: 1) it is located at the presynaptic terminal; 2) it is synthesized by the neuron; and 3) mechanisms exist for removal or inactivation of the transmitter (Cooper et al., 2003). Findings from this study support the proposition that GABA is a horizontal cell transmitter.

### **GABA is in both A-type and B-type horizontal cells**

GABA immunoreactivity is expressed in both A- and B-type horizontal cells in the guinea pig retina based on the co-localization of calbindin and GABA immunoreactivities in horizontal cell bodies in our preparations. This finding is in contrast to previous studies reporting that GABA immunoreactivity was absent in guinea pig horizontal cells (Agardh et al., 1986; Osborne et al., 1986). In addition, a more recent study by Loeliger and Rees (2005) indicated that GABA immunoreactivity was strong in horizontal cells during the

postnatal development period, but by day 60, GABA immunostaining was faint and detected in only a few horizontal cells, using the same rabbit anti-GABA polyclonal antibody that was used for this study. Loeliger and Rees (2005) speculated that only one type of horizontal cell contained GABA in adult guinea pig retina. However, our study suggests that both types of horizontal cells contain GABA (Fig. 2), which is consistent with the presence of GAD<sub>65</sub> immunoreactivity in both types of horizontal cells (Figs. 3–5). The discrepancy between our findings and those of others may be due to tissue preparation and fixation conditions.

For example, we found that the GABA immunostaining was best achieved by using retinas that were fixed for 15 minutes, which contrasts with previous studies that used fixation times greater than 2 hours (Agardh et al., 1986; Osborne et al., 1986; Loeliger and Rees, 2005). We determined that the longer fixation times have a deleterious effect on GABA immunostaining. In the present study, we observed GABA-containing horizontal cells distributed to all retinal regions of the guinea pig retina. This finding is similar to the distribution of GABA-containing horizontal cells in the cat retina (Chun and Wässle, 1989; Pourcho and Owczarzak, 1989; Wässle and Chun, 1989).

### **GAD<sub>65</sub> is expressed in horizontal cells**

GABA is synthesized from glutamic acid by the rate-limiting enzyme GAD, which has two well-characterized isoforms, GAD<sub>65</sub> and GAD<sub>67</sub>. Only the GAD<sub>65</sub> isoform was detected in guinea pig horizontal cells using GAD isoform-specific antibodies (Figs. 3–5). The suggestion that GAD<sub>65</sub> is expressed in these cells was further strengthened by the detection of GAD<sub>65</sub> mRNA in laser-captured horizontal cells using degenerate RT-PCR (Fig. 6).

All calbindin-immunoreactive horizontal cells appeared to contain GAD<sub>65</sub> immunoreactivity in a series of serial vertical sections (~100 sections). However, double labeling of GAD<sub>65</sub> and calbindin of horizontally sectioned retinas showed that about 80% of the horizontal cells contained GAD<sub>65</sub>. The difference in the co-localization between the vertical and horizontal sectioned retinas is likely due to technical reasons including tissue preparation, poor antibody penetration, and access to the antigen. Consistent with these findings, GABA and GAD immunostaining in horizontal cells has been reported in cat, rabbit, opossum, and primate retina (Mosinger et al., 1986; Osborne et al., 1986; Agardh et al., 1987; Mosinger and Yazulla, 1987; Chun and Wässle, 1989; Pourcho and Owczarzak, 1989; Wässle and Chun, 1989; Grünert and Wässle, 1990; Vardi et al., 1994; Johnson and Vardi, 1998; Marc et al., 1998; Calaza et al., 2006). Rabbit horizontal cells express both isoforms (GAD<sub>65</sub> and GAD<sub>67</sub>) (Johnson and Vardi, 1998); whereas cat A- and B-type horizontal cells, identified with antibodies to parvalbumin (Röhrenbeck et al., 1987), express GAD<sub>67</sub> mRNA (Sarthy and Fu, 1989a), consistent with GAD<sub>67</sub> immunoreactivity in cat horizontal cells (Vardi et al., 1994). In contrast, monkey H1 and H2 horizontal cells express GAD<sub>65</sub>, but not GAD<sub>67</sub> immunoreactivity (Vardi et al., 1994), also consistent with the lack of GAD<sub>67</sub> mRNA in horizontal cells of the monkey (macaque) or human retina (Sarthy and Fu, 1989b). Together, these findings support the idea that the expression of GAD isoforms in horizontal cells is species dependent.

The subcellular localization of GAD also varies depending upon the species. In rabbit horizontal cells, GAD<sub>65</sub> immunoreactivity was localized at the A-type horizontal cell somata and primary dendrites in the visual streak, and GAD<sub>67</sub> was localized to the terminals of A- and B-type horizontal cells (Johnson and Vardi, 1998). In guinea pig horizontal cells, GAD<sub>65</sub> was distributed predominantly to the dendritic terminals of A-type cells, and to the dendritic and axonal terminals of B-type cells, as well as in the region of the cell body closest to the OPL (Fig. 3D,G). The distribution of GAD<sub>65</sub> in horizontal cell endings, where VGAT immunostaining is strongest, suggests that GABA is synthesized and packaged into the vesicles locally at the endings.

### **VGAT is expressed in horizontal cells**

Specific VGAT immunoreactivity was detected primarily in horizontal cell processes and terminals in the OPL (Fig. 9D), supporting the idea that this vesicular transporter mediates GABA uptake into vesicles in guinea pig horizontal cells. The distribution of VGAT immunoreactivity to horizontal cell processes and terminals correlates well with the preferential localization of GAD<sub>65</sub> to these subcellular compartments, further supporting the hypothesis that GABA is synthesized and packaged locally near or within the presynaptic terminals. An earlier study has shown that GABA synthesized by synaptic vesicle-associated GAD is preferentially transported into synaptic vesicles by VGAT (Jin et al., 2003), and a similar mechanism is likely for guinea pig horizontal cells based on the localization of GAD and VGAT to horizontal cell endings. Finally, the co-localization of GAD and VGAT to the tips of horizontal cells implies that GABA is released by a vesicular mechanism from these endings.

### **Plasmalemmal GABA transporters are expressed by glia but not by horizontal cells in the outer retina**

GAT-1 and GAT-3 immunoreactivities were absent from guinea pig horizontal cells (Figs. 11–13), similar to macaque horizontal cells (Haverkamp et al., 2000). These findings are consistent with the failure of guinea pig horizontal cells to accumulate GABA and GABA analogs (Bruun and Ehinger, 1974; Marshall and Voaden, 1975; Bauer and Ehinger, 1978; Cunningham et al., 1981; Agardh and Ehinger, 1982, 1983), which are transported by these plasmalemmal transporters (Guastella et al., 1990; Borden et al., 1992; Clark et al., 1992). Instead, GAT-1 and GAT-3 are expressed by Müller cells of the guinea pig retina, based on their overall immunostaining patterns (Figs. 11, 12) and their Na<sup>+</sup>- and Cl<sup>-</sup>-dependent GABA uptake properties, which are similar to the properties of GAT-1 and GAT-3 in cell expression systems (Biedermann et al., 2002). These findings are thus in agreement with previous studies indicating the expression of GAT-1 and GAT-3 in Müller cells in the mammalian retina (Honda et al., 1995; Johnson et al., 1996; Biedermann et al., 2002; Guo et al., 2009). The action of GATs expressed on Müller cells is likely to involve the uptake of GABA released into the extracellular space (Neal and Iversen, 1972; Bruun and Ehinger, 1974; Blanks and Roffler-Tarlov, 1982), thus favoring a role for Müller cells in the rapid removal and metabolism of GABA in the mammalian retina (Biedermann et al., 2002).

### **Physiological significance**

The discovery that most, if not all, horizontal cells express GABA and GAD<sub>65</sub>, as well as VGAT in guinea pig retina, suggests that mammalian horizontal cells in this species likely utilize GABA as a neurotransmitter. Moreover, the presence of VGAT strongly supports a vesicular release mechanism (for reviews, see Liu and Edwards, 1997; Gasnier, 2004). In the CNS, the expression of VGAT is localized to synaptic vesicles (Chaudhry et al., 1998; Dumoulin et al., 1999b; Takamori et al., 2000). VGAT expression in horizontal cell endings also suggests that GABA is packaged into synaptic vesicles in these cells. This finding is consistent with ultrastructural observations showing small, clear-core vesicles in horizontal cell processes and endings in the photoreceptor triad (Dowling and Boycott, 1966; Dowling et al., 1966; Gray and Pease, 1971; Fisher and Boycott, 1974; Brandon and Lam, 1983; Linberg and Fisher, 1988; Spiwox-Becker et al., 2001), as well as the findings demonstrating that critical components (SNAP-25, complexins, syntaxin-1 and -4) of the soluble N-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) complex are present in mammalian horizontal cells (Hirano et al., 2005; Sherry et al., 2006; Hirano et al., 2007). In addition, the absence of plasmalemmal GABA transporters (GAT-1 and GAT-3) in guinea pig horizontal cells (Figs. 11, 12) argues against a plasma membrane transporter-mediated release mechanism for GABA.

GABA may act at multiple sites in the outer retina. Ionotropic GABA receptors have been localized to bipolar cell dendrites and horizontal cells in various species (Vardi et al., 1992; Greferath et al., 1993, 1994; Vardi and Sterling, 1994; Enz et al., 1996; Koulen et al., 1997; Wässle et al., 1998; Haverkamp et al., 2000). Ionotropic GABA receptors flux  $\text{Cl}^-$  (Macdonald and Olsen, 1994) and can mediate hyperpolarizations as well as depolarizations (Ben-Ari et al., 1994) depending on the reversal potential for  $\text{Cl}^-$  (Leinekugel et al., 1999). This mechanism may contribute in part to the formation of surround inhibition at bipolar cell dendrites of ON and OFF bipolar cells (Miller and Dacheux, 1983; Billups and Attwell, 2002; Varela et al., 2005; Duebel et al., 2006). Indeed, there is evidence that differential  $\text{Cl}^-$  gradients exist in the two functional types of bipolar cells (Satoh et al., 2001; Duebel et al., 2006). In salamander OFF bipolar cells, GABA selectively blocked the surround response while leaving the center response unaltered (Wu, 1986). Finally, GABA is reported to modulate horizontal cell light responses via an autocrine action on horizontal cells (Blanco et al., 1996; Feigenspan and Weiler, 2004; Varela et al., 2005).

There are also a few studies reporting ionotropic GABA receptor immunostaining of mammalian cone photoreceptor terminals (Greferath et al., 1995; Haverkamp and Wässle, 2000; Pattnaik et al., 2000) suggesting GABA influences surround inhibition of photoreceptors. In fish retina, GABA hyperpolarized cone photoreceptors (Wu and Dowling, 1980). GABA evoked chloride currents or chloride-dependent membrane voltage changes in turtle, frog, mouse and pig cone photoreceptors (Kaneko and Tachibana, 1986; Picaud et al., 1998; Pattnaik et al., 2000; Liu et al., 2005, 2006; Tatsukawa et al., 2005; Liu and Yang, 2006). GABA elicited a conductance increase with a reversal potential consistent with a chloride conductance in salamander cones (Wu, 1986). At bullfrog cone terminals, GABA appears to affect calcium currents via  $\text{GABA}_B$  receptor activation (Liu et al., 2005). However, other studies suggest that surround inhibition at macaque cone photoreceptors is not mediated by GABA (Verweij et al., 2003; McMahon et al., 2004).

Although it is generally accepted that horizontal cells provide inhibitory feedback onto photoreceptor terminals, the synaptic and cellular mechanisms underlying this process remain poorly understood. In addition to the evidence for a GABAergic mechanism presented here, there are several proposed mechanisms of synaptic transmission from horizontal cells to photoreceptors. One alternative mechanism for horizontal cell to photoreceptor feedback in both mammalian and nonmammalian retinas is a proton-dependent mechanism whereby a decrease in extracellular pH in the photoreceptor triad shifts the voltage activation of photoreceptor  $\text{Ca}^{2+}$  channels, which in turn regulates photoreceptor transmitter release (Barnes et al., 1993; DeVries, 2001; Barnes, 2003; Hirasawa and Kaneko, 2003; Vessey et al., 2005; Jonz and Barnes, 2007; Davenport et al., 2008; Thoreson et al., 2008; Babai and Thoreson, 2009). Finally, an ephaptic model of feedback onto cone terminals was originally conceived by Byzov and colleagues (Byzov and Shura-Bura, 1986), and later elaborated to involve hemichannels as current sinks near the presynaptic calcium channels (Kamerlings et al., 2001).

The presence of high levels of GABA,  $\text{GAD}_{65}$ , and VGAT in adult guinea pig horizontal cells suggests that the guinea pig retina may serve as a useful model system for future studies of GABA signaling mechanisms in mammalian horizontal cells and the outer retina.

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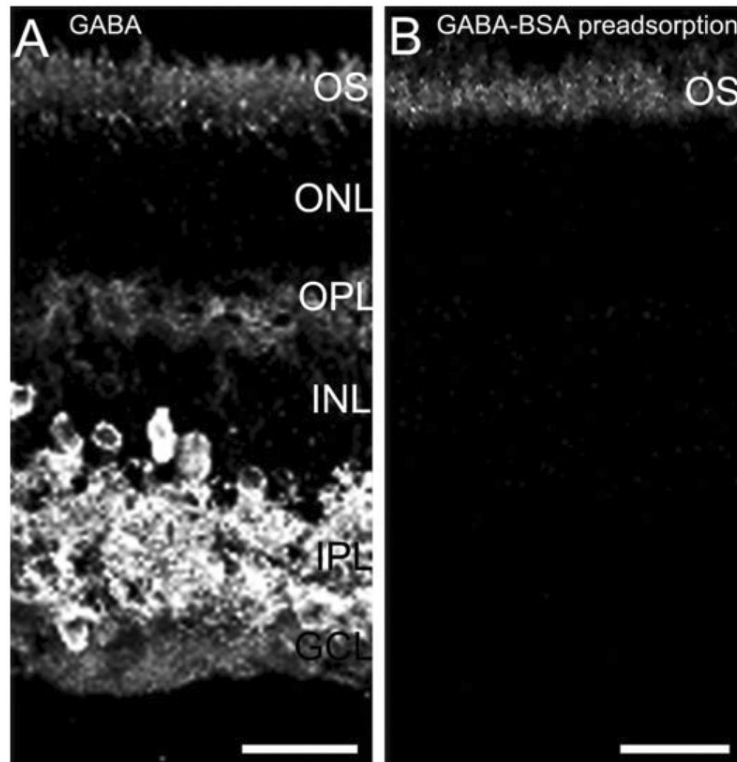
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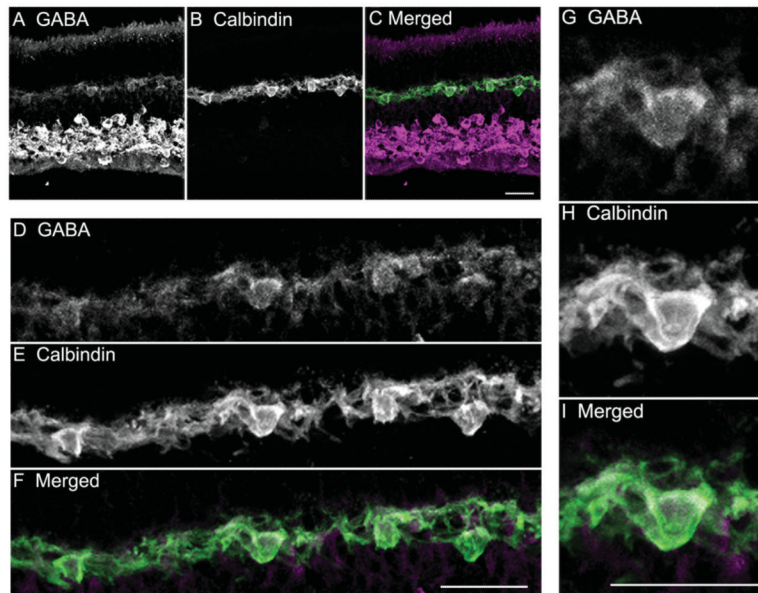
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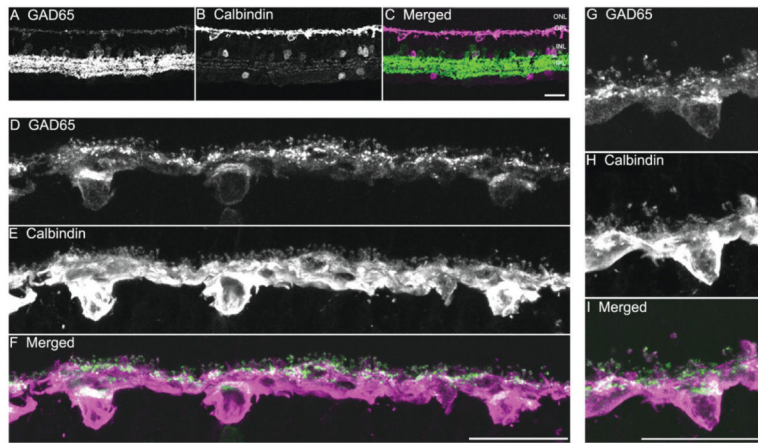


**Figure 1.** Specific GABA immunostaining is in the guinea pig retina. GABA immunostaining is absent following preadsorption of the GABA antibody with a GABA-BSA conjugate. Guinea pig retinal vertical sections were immunostained with GABA antibody (A) or GABA antibody preadsorbed with GABA-BSA conjugate (B). **A:** Robust GABA immunostaining is distributed to amacrine cell and displaced amacrine cell somata, as well as their processes in the IPL in the inner retina, and weak GABA immunostaining is in the outer retina in horizontal cell bodies and processes. **B:** Retina vertical section immunostained with GABA antibody preadsorbed by GABA-BSA conjugate. GABA immunoreactivity in the outer and inner retina was eliminated by GABA-BSA preadsorption; the outer segments (OS) of photoreceptors showed nonspecific staining that was not blocked by GABA-BSA conjugates. Confocal images were scanned at 1- $\mu\text{m}$  intervals, and six optical sections were obtained and compressed for viewing. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar = 20  $\mu\text{m}$  in A,B.



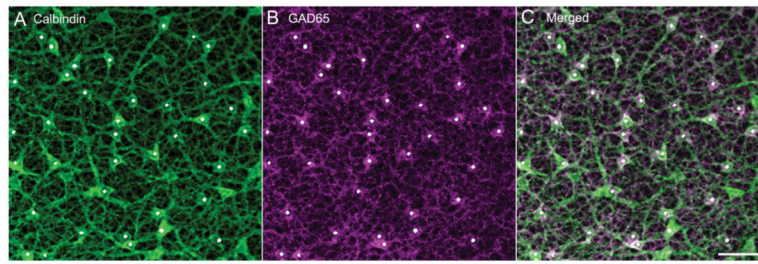
**Figure 2.** GABA immunoreactivity is localized to horizontal cell bodies and their processes. A vertical section through the guinea pig retina was double labeled with antibodies to GABA and calbindin-28K (calbindin). **A:** Weak GABA immunostaining is in the outer retina, and robust GABA immunostaining is distributed to amacrine cell and displaced amacrine cell somata, and their processes in the IPL in the inner retina. **B:** Strong calbindin immunoreactivity occurred in horizontal cell somata and processes in the outer retina. **C:** Merged image shows the co-localization of GABA and calbindin immunoreactivities in the outer retina. **D–F:** Enlarged images of the OPL show the co-localization of GABA and calbindin immunoreactivities. **D:** GABA immunoreactivity in horizontal cell somata and processes. **E:** Calbindin immunoreactivity in horizontal cell somata and processes in the OPL. **F:** Merged image. Co-localization of GABA and calbindin immunoreactivity is in horizontal cell somata and processes. **G–I:** Enlarged images showing distribution of GABA and calbindin immunoreactivities in a horizontal cell. **G:** GABA immunoreactivity. **H:** Calbindin immunoreactivity is in the horizontal cell body, processes, and endings. **I:** Merged image show that the GABA immunoreactivity is in the horizontal cell body and processes. Confocal images were scanned at 1- $\mu\text{m}$  intervals, and six optical sections were obtained and compressed for viewing. Scale bar = 20  $\mu\text{m}$  in C (applies to A–C), F (applies to D–F), and I (applies to G–I).



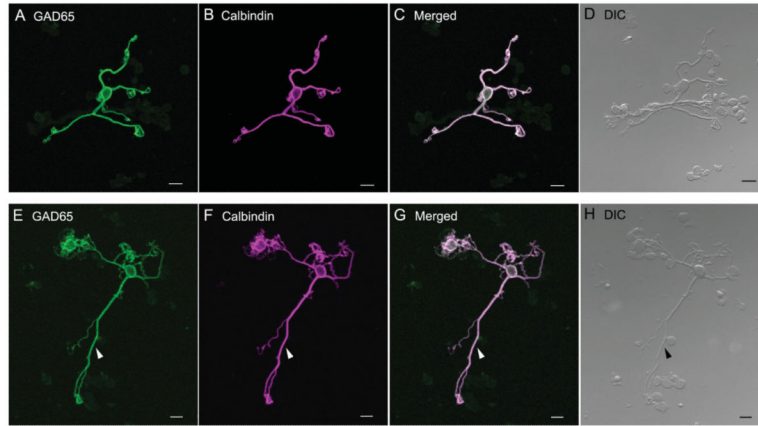


**Figure 3.**

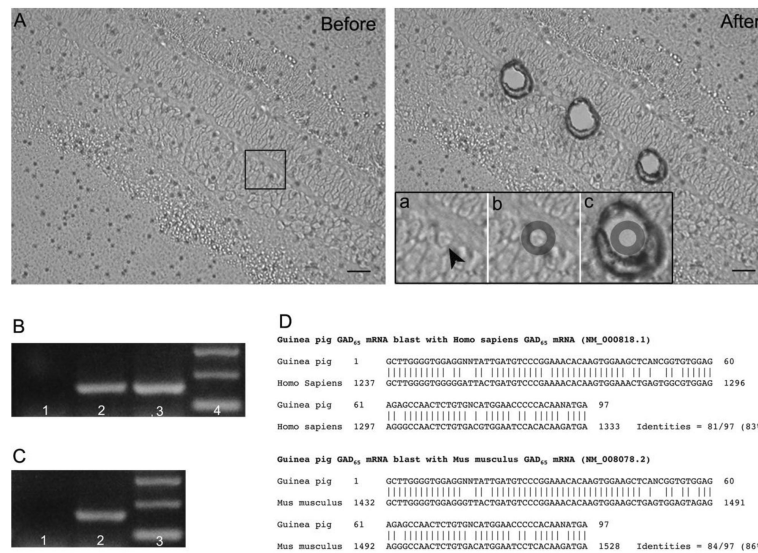
GAD<sub>65</sub> immunoreactivity is localized to horizontal cells. A vertical section through a guinea pig retina was double labeled with antibodies to GAD<sub>65</sub> and calbindin. **A:** Weak GAD<sub>65</sub> immunostaining is present in the cell bodies and processes in the OPL; strong GAD<sub>65</sub> immunoreactivity is distributed to amacrine cell somata and their processes in the IPL. **B:** Calbindin is expressed in horizontal cell somata and processes in the outer retina, and some amacrine and ganglion cell somata and their processes in the inner retina. **C:** Merged image shows the co-localization of GAD<sub>65</sub> and calbindin immunoreactivities in the outer retina. **D–F:** Enlarged images of the OPL show the distribution of GAD<sub>65</sub> and calbindin immunoreactivities. **D:** GAD<sub>65</sub>-immunoreactive cell bodies, processes, and tips in the OPL. **E:** Calbindin-immunoreactive horizontal cell bodies, processes, and tips. **F:** Merged image shows the co-expression of GAD<sub>65</sub> and calbindin immunoreactivities in the OPL, indicating that GAD<sub>65</sub> immunoreactivity is localized to horizontal cell bodies, processes, and tips. **G–I:** Enlarged images show distribution of GAD<sub>65</sub> and calbindin immunoreactivities in a horizontal cell. **G:** GAD<sub>65</sub> immunoreactivity. **H:** Calbindin immunoreactivity is in horizontal cell body, processes, and tips. **I:** Merged image shows that GAD<sub>65</sub> immunoreactivity is concentrated in a subcellular region of the soma close to the OPL, and in processes and terminals. Confocal images were scanned at 1- $\mu$ m intervals, and six optical sections were obtained and compressed for viewing. Scale bar = 20  $\mu$ m in C (applies to A–C), F (applies to D–F), and I (applies to G–I).



**Figure 4.** Calbindin and GAD<sub>65</sub> double staining of a horizontal section through the outer retina showing the presence of calbindin-immunostained horizontal cells containing GAD<sub>65</sub> immunoreactivity. Horizontal cells that contain calbindin and GAD<sub>65</sub> are marked by a white dot. **A:** Horizontal cell somata and processes containing calbindin immunoreactivity. **B:** Horizontal cell somata and processes containing GAD<sub>65</sub> immunoreactivity. **C:** Merged image shows that most calbindin-immunostained horizontal cells contained GAD<sub>65</sub> immunoreactivity. Confocal images were scanned at 1- $\mu$ m intervals, and 12 optical sections were obtained and compressed for viewing. Scale bar = 20  $\mu$ m in C (applies to A–C).

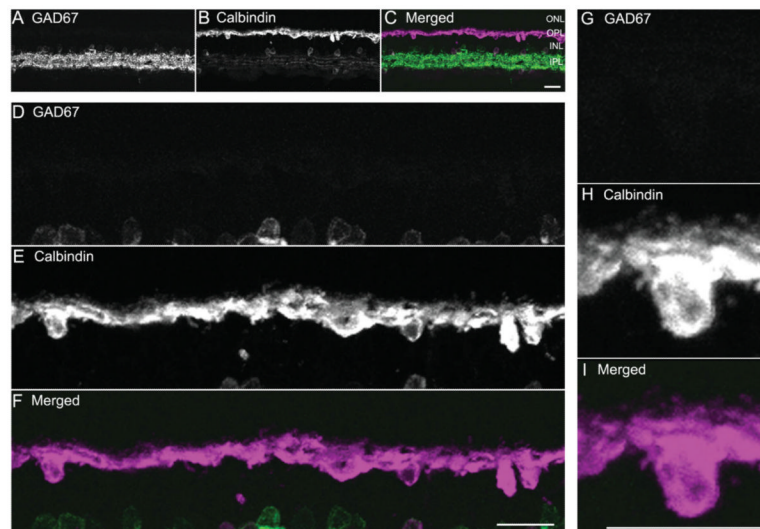


**Figure 5.** GAD<sub>65</sub> and calbindin double staining of acutely dissociated horizontal cells. **A–D:** Putative axonless A-type horizontal cell double labeled with GAD<sub>65</sub> and calbindin antibodies. **A:** GAD<sub>65</sub> immunoreactivity is localized throughout the horizontal cell cytoplasm. **B:** Calbindin immunoreactivity is in horizontal cell body, processes, and tips. **C:** Merged image shows co-localization of GAD<sub>65</sub> and calbindin immunoreactivities. **D:** DIC image of the field of view. **E–H:** Putative axon-bearing B-type horizontal cell double labeled with GAD<sub>65</sub> and calbindin antibodies. **E:** GAD<sub>65</sub> immunoreactivity is localized throughout the horizontal cell. **F:** Calbindin immunoreactivity is in horizontal cell body, processes, and tips. **G:** Merged image shows co-localization of GAD<sub>65</sub> and calbindin immunoreactivities. Arrowhead indicates an axon-like process. **H:** DIC image of the field of view. Confocal images were scanned at 1- $\mu$ m intervals, and five optical sections were obtained and compressed for viewing. Scale bar = 10  $\mu$ m in A–H.

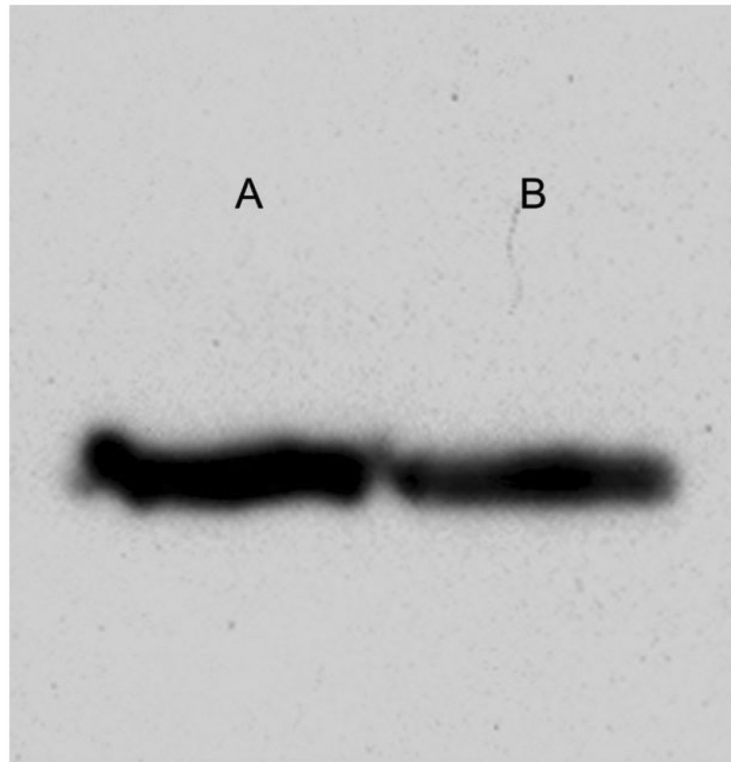


**Figure 6.**

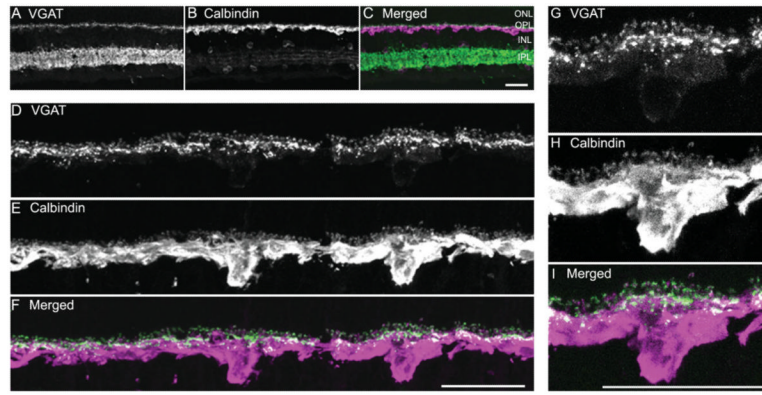
Laser capture microdissection and degenerate RT-PCR. **A:** Images of a vertical section through an adult guinea pig retina before (left) and after (right) laser capture microdissection. Inset (a–c) shows the steps of a horizontal cell being laser captured. **a:** The horizontal cell body was identified based on its morphology and its location next to the OPL (arrowhead). **b:** A circular boundary was defined around the cell contour for laser microdissection, as indicated by the dark gray circle. **c:** After laser microdissection. **B:** Degenerate RT-PCR amplified a 166-bp product (including 40 bp from tagged T3 and T7 sequences) from oligo(dT)-primed cDNA synthesized from both adult guinea pig brain and retina total RNA. Lanes: 1. Negative control in which reverse-transcribed cDNA was omitted. 2. PCR product amplified from brain cDNA. 3. PCR product amplified from retina cDNA. 4. 100-bp DNA ladder. **C:** Degenerate RT-PCR using cDNA synthesized from total RNA prepared from 40 laser-capture-microdissected horizontal cells amplified a 166-bp product. Lanes: 1. Negative control in which reverse-transcribed cDNA was omitted. 2. PCR product amplified from horizontal cell cDNA. 3. 100-bp DNA ladder. **D:** Nucleotide sequence alignment between the amplified guinea pig *GAD<sub>65</sub>* mRNA sequence and that of human (*Homo sapiens*, top) and mouse (*Mus musculus*, bottom). Scale bar = 20  $\mu$ m in A (applies to A,B).



**Figure 7.** GAD<sub>67</sub> immunoreactivity was not present in horizontal cell bodies or processes. A vertical section through a guinea pig retina was double labeled with antibodies to GAD<sub>67</sub> and calbindin. **A:** GAD<sub>67</sub> is distributed to amacrine cell somata and their processes in the IPL. No GAD<sub>67</sub> immunoreactivity was observed in the OPL. **B:** Calbindin is expressed in horizontal cell somata and processes in the outer retina, and some amacrine and ganglion cell somata and their processes in the inner retina. **C:** Merged image shows the localization of GAD<sub>67</sub> and calbindin immunoreactivity in amacrine cell bodies. **D–F:** Enlarged images of the outer retina show GAD<sub>67</sub> and calbindin immunoreactivities in the OPL. **D:** No GAD<sub>67</sub> immunoreactivity is observed in the OPL. **E:** Calbindin-immunoreactive horizontal cell bodies and processes. **F:** Merged image shows no co-localization. Confocal images were scanned at 1- $\mu$ m intervals, and six optical sections were obtained and compressed for viewing. Scale bar = 20  $\mu$ m in C (applies to A–C), F (applies to D–F), and I (applies to G–I).

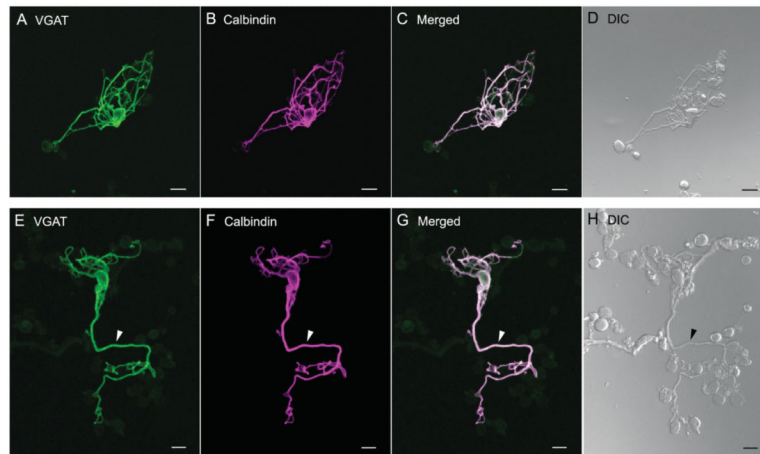


**Figure 8.** Immunoblot of VGAT in adult guinea pig retina (lane A) and brain (lane B) homogenates. The VGAT (Synaptic Systems #131 011, 1:1,500) monoclonal antibody detected a single band at  $M_r$  ~57 kDa in both retina and brain. Retina and brain total protein: 8–10  $\mu$ g per lane.



**Figure 9.**

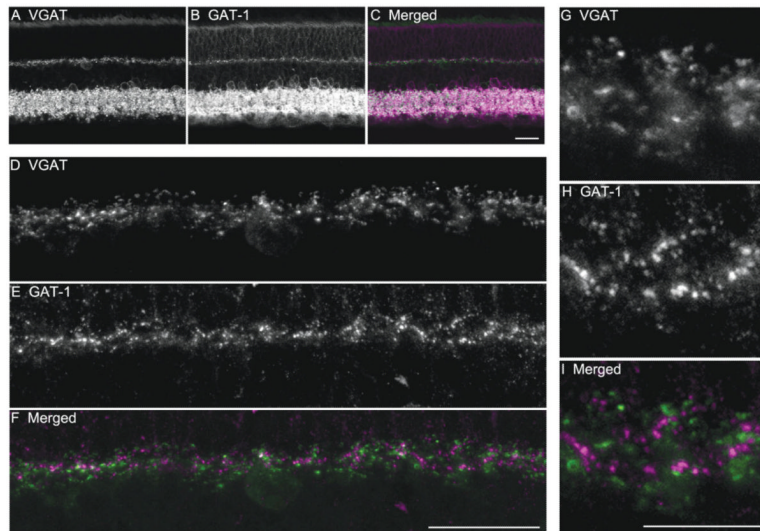
VGAT immunoreactivity is localized to horizontal cell bodies and processes. A vertical section through an adult guinea pig retina was double immunostained with antibodies to VGAT and calbindin. **A:** VGAT immunoreactivity is present in the cell bodies and processes in the OPL; VGAT immunoreactivity is distributed to amacrine cell and displaced amacrine cell somata and their processes in the IPL. **B:** Calbindin is expressed by horizontal cell somata and processes in the outer retina, and some amacrine and ganglion cell somata and their processes in the inner retina. **C:** Merged image shows the localization of VGAT and calbindin immunoreactivities in the retina. **D–F:** Enlarged images of the OPL show the co-localization of VGAT and calbindin immunoreactivities in horizontal cells. **D:** VGAT-immunoreactive processes and tips in the OPL. **E:** Calbindin-immunoreactive horizontal cell bodies and processes. **F:** Merged image shows the co-localization of VGAT and calbindin immunoreactivities in the OPL, indicating that VGAT immunoreactivity is localized to horizontal cell bodies and processes. **G–I:** Enlarged images show distribution of VGAT and calbindin immunoreactivities in a horizontal cell. **G:** Weak VGAT immunoreactivity is localized at the cell body, whereas strong VGAT immunoreactivity is distributed to processes and tips. **H:** Calbindin immunoreactivity is in horizontal cell body, processes, and tips. **I:** Merged image indicating that VGAT immunoreactivity is in horizontal cell body and processes, with more intense immunoreactivity in the tips. Confocal images were scanned at 1- $\mu\text{m}$  intervals, and six optical sections were obtained and compressed for viewing. Scale bar = 20  $\mu\text{m}$  in C (applies to A–C), F (applies to D–F), and I (applies to G–I).



**Figure 10.**

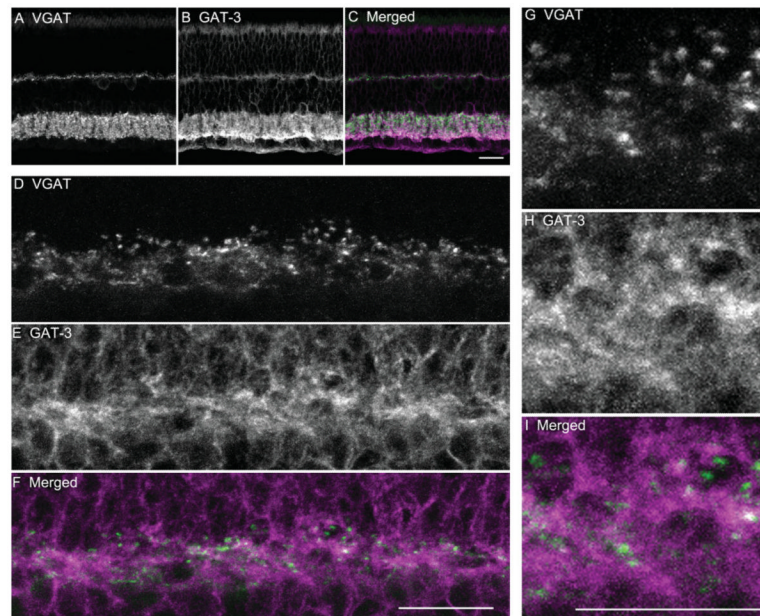
VGAT and calbindin double staining of dissociated horizontal cells. **A–D:** Putative axonless A-type horizontal cell double labeled with VGAT and calbindin antibodies. **A:** VGAT immunoreactivity is present throughout the horizontal cell. **B:** Calbindin immunoreactivity is in the horizontal cell body, processes, and tips. **C:** Merged image shows the co-localization of VGAT and calbindin immunoreactivities. **D:** DIC image of the field of view. **E–H:** Putative axon-bearing B-type horizontal cell double labeled with VGAT and calbindin antibodies. **E:** VGAT immunoreactivity is localized at horizontal cell body and processes and tips. **F:** Calbindin immunoreactivity is in the horizontal cell body, processes, and tips. **G:** Merged image shows the co-localization of VGAT and calbindin immunoreactivities. **H:** DIC image of the field of view. Arrowheads point to the likely axonal process. Confocal images were scanned at 1- $\mu\text{m}$  intervals, and six optical sections were obtained and compressed for viewing. Scale bar = 10  $\mu\text{m}$  in A–H.



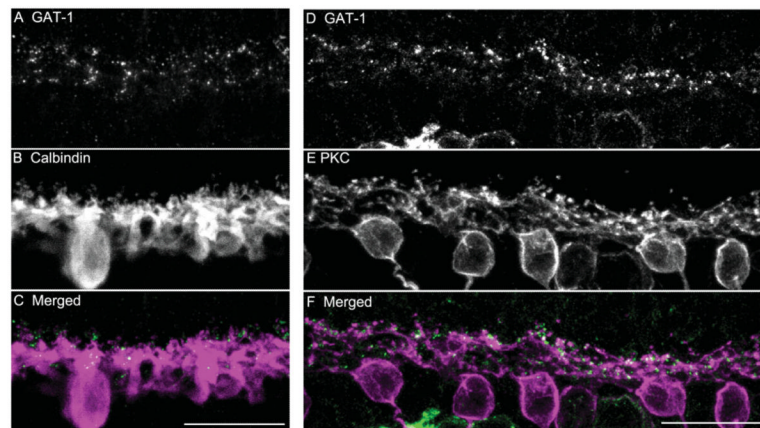


**Figure 11.**

VGAT and GAT-1 immunostaining showed that GAT-1 immunoreactivity is not localized to horizontal cell processes in the outer retina. Images are of a vertical section through an adult guinea pig retina double labeled with antibodies to VGAT and GAT-1. **A:** VGAT immunostaining is distributed to horizontal cells and amacrine cells with strong signals mainly distributed to the processes and terminals. **B:** GAT-1 immunostaining occurs primarily in the plexiform layers as well as some amacrine cell bodies. **C:** Merged image shows the localization of VGAT and GAT-1 immunostaining in the retina. **D–F:** Enlarged images of the OPL show the lack of co-localization of VGAT and GAT-1 immunoreactivities. **D:** VGAT immunoreactivity is localized in horizontal cell processes and terminals in the OPL. **E:** GAT-1-immunostained processes in the OPL. **F:** Merged image shows that the GAT-1 and VGAT immunoreactivities do not co-localize in the OPL. **G–I:** High-magnification images show the distribution of VGAT (**G**) and GAT-1 (**H**) immunoreactivities in the OPL. **I:** Merged image shows that the VGAT and GAT-1 immunoreactivities do not co-localize. Confocal images were scanned at 1- $\mu\text{m}$  intervals, and six optical sections were obtained and compressed for viewing. Scale bar = 20  $\mu\text{m}$  in **C** (applies to **A–C**) and **F** (applies to **D–F**); 10  $\mu\text{m}$  in **I** (applies to **G–I**).



**Figure 12.** VGAT and GAT-3 immunolabeling showed that GAT-3 immunoreactivity was not distributed to horizontal cells or their processes in the outer retina. Images are of a vertical section through an adult guinea pig retina double labeled with antibodies to VGAT and GAT-3. **A:** VGAT immunostaining is present primarily in the plexiform layers with strong signals mainly distributed to the processes and terminals. **B:** GAT-3 immunostaining is localized to Müller cells and their processes, and at some amacrine cells and their processes in the IPL. **C:** Merged image shows the localization of VGAT and GAT-3 immunoreactivities in the retina. **D–F:** Enlarged images of the OPL show the lack of VGAT and GAT-3 co-localization. **D:** VGAT immunoreactivity is localized to horizontal cell processes and terminals in the OPL. **E:** GAT-3 labeling shows a typical honeycomb-like staining pattern of Müller cell processes in the OPL. **F:** Merged image shows that VGAT and GAT-3 immunoreactivities do not co-localize in the OPL. **G–I:** High-magnification images show the distribution of VGAT and GAT-3 in the OPL. **G:** VGAT immunoreactivity occurs in the horizontal cell processes and endings. **H:** GAT-3 immunoreactivity in the Müller cells processes. **I:** Merged image indicates that GAT-3 immunoreactivity is not distributed to horizontal cells. Confocal images were scanned at 1- $\mu\text{m}$  intervals, and six optical sections were obtained and compressed for viewing. Scale bar = 20  $\mu\text{m}$  in C (applies to A–C), F (applies to D–F), and I (applies to G–I).



**Figure 13.** Double labeling of GAT-1 with calbindin (A–C) or PKC (D–E) on vertical sections through an adult guinea pig retina. Images were taken at the OPL. **A:** GAT-1 immunoreactivity in the OPL. **B:** Calbindin immunolabeling of horizontal cell bodies, processes, and terminals in the OPL. **C:** Merged image shows that the punctate GAT-1 immunoreactivity is not co-localized with the calbindin immunoreactivity in the OPL, indicating that GAT-1 immunoreactivity is not distributed to the horizontal cell processes and terminals. **D:** GAT-1 immunoreactivity in the OPL. **E:** PKC immunoreactivity in the rod bipolar cell bodies and dendrites. **F:** Merged image shows that GAT-1 immunoreactivity is not co-localized with PKC immunoreactivity in the OPL, indicating that GAT-1-immunoreactivity is not distributed to rod bipolar cell dendritic terminals. Confocal images were scanned at 1- $\mu\text{m}$  intervals, and six optical sections were obtained and compressed for viewing. Scale bar = 20  $\mu\text{m}$  in C (applies to A–C) and F (applies to D–F).

TABLE 1

Antibodies Used in This Study<sup>1</sup>

Antibody	Host	Antigen
GABA	Rabbit polyclonal	GABA conjugated to a carrier protein thyroglobulin using paraformaldehyde
GAD <sub>65</sub>	Mouse monoclonal	Purified rat brain GAD, clone-6
GAD <sub>67</sub>	Mouse monoclonal	A recombinant human GAD <sub>67</sub> -his tag containing the his tag and the unique N terminal region of GAD <sub>67</sub> (GAD <sub>67</sub> 4-101: STPSSSATSSNAGADPNTTTLRPTTYDTWCGVAHGCTRKLGLKICGFLQRTNSLEEKSRLLVSAFKERQSSKNLLSCENS DRDARFR... Information provided by Millipore technical support)
VGAT	Mouse monoclonal	Synthetic peptide AEPPVEGDIHYQR (VGAT <sub>75-87</sub> in rat) coupled to KLH
Calbindin	Mouse monoclonal	Bovine kidney calbindin-D
Calbindin	Rabbit polyclonal	Recombinant rat calbindin D-28k
GAT-1	Rabbit polyclonal	C-terminus of rat GAT-1 (aa 588-599) coupled to KLH
GAT-3	Rabbit polyclonal	C-terminus of rat GAT-3 (aa 607-627) coupled to KLH.
PKC	Mouse monoclonal	PKC from bovine brain, clone MC5

<sup>1</sup>Information supplied by the vendor unless otherwise shown. Abbreviations: GABA,  $\gamma$ -aminobutyric acid; GAD, glutamic acid decarboxylase; GAT, GABA transporter; KLH, keyhole limpet hemocyanin; PKC, protein kinase C; VGAT, vesicular GABA transporter.