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Regional differences in GABA and GAD immunoreactivity in rabbit horizontal cells

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Abstract

Mammalian horizontal cells are believed to be GABAergic because, in most species, they contain both GABA and glutamic acid decarboxylase (GAD), and their terminals are presynaptic to GABA receptors. In adult rabbit, however, GABA and GAD immunoreactivity have not been consistently demonstrated in horizontal cells, casting doubts on the assumption that they too are GABAergic. Here we demonstrate that all rabbit horizontal cell terminals—dendritic terminals of type A, and both dendritic and axonal terminals of type B—immunostain for one isoform of GAD, GAD_{67}. In addition, we show that type A horizontal cell somas and primary dendrites in the visual streak (identified by their immunoreactivity to calbindin) are immunoreactive for the other GAD isoform, GAD_{65}. Double-labeling experiments for GAD_{65} and GABA reveal that every cell that stains for GAD_{65} also stains for GABA. The presence of GAD_{67} in horizontal cell terminals suggests that rabbit horizontal cells are GABAergic. The segregation of the two GAD isoforms to different cell compartments suggests that GABA is released at different sites, possibly by two different mechanisms.

Keywords: GAD_{65}, GAD_{67}, Visual streak

Introduction

Most fundamental mechanisms underlying signal processing in vertebrate retina are conserved across species. For example, all vertebrate photoreceptors, bipolar cells, and ganglion cells release glutamate (reviewed by Massey & Maguire, 1995). Similarly, in all mammalian systems tested thus far, rod bipolar and ON cone bipolar cells express the L-AP4 sensitive metabotropic receptor, mGluR6 (Nakajima et al., 1993; Nomura et al., 1994; Masu et al., 1995; Duvoisin & Vardi, 1996; Vardi & Morigiwa, 1997). Another mechanism which seems to be conserved is the generation of a receptive-field surround in photoreceptors and bipolar cells (Werblin & Dowling, 1969; Kaneko, 1970; Baylor et al., 1971; Smith & Sterling, 1990). This is produced by horizontal cell feedback inhibition onto photoreceptors and feedforward inhibition onto bipolar cells (Gerschenfeld & Piccolino, 1980; Murakami et al., 1982; Tachibana & Kaneko, 1984; Yang & Wu, 1991; Mangel, 1991). In cold-blooded vertebrates, feedback and feedforward inhibition is mediated by GABA release (Marc et al., 1978; reviewed by Yazulla, 1995). Consequently, it is expected that mammalian horizontal cells also use GABA as an inhibitory transmitter, but this has been difficult to prove.

In cat and monkey, evidence is accumulating to support this hypothesis. In these species, horizontal cells contain both GABA and its synthesizing enzyme, glutamic acid decarboxylase (GAD) (Agardh et al., 1986, 1987*a*,*b*; Chun & Wässle, 1989; Pourcho & Owczarzak, 1989; Grünert & Wässle, 1990; Vardi et al., 1994). In addition, $GABA_A$ and $GABA_C$ receptors are expressed in the outer plexiform layer (OPL) by bipolar cell dendritic tips (Vardi et al., 1992; Greferath et al., 1993, 1995; Vardi & Sterling, 1994; Enz et al., 1996; Koulen et al., 1997). In monkey retina, the $GABA_A$ receptor is specifically localized to bipolar membranes in apposition to horizontal cells, and not to membranes in apposition to other bipolar cells (Vardi & Sterling, 1994). This strongly suggests that bipolar cells respond to GABA released from horizontal cells.

In contrast to the results from cat and monkey, the claim that horizontal cells in the adult rabbit are GABAergic has been weakened by the fact that most studies failed to find consistent immunoreactivity for GABA and GAD (Brandon, 1985; Mosinger & Yazulla, 1987; Agardh et al., 1986, 1987*a*,*b*; Osborne et al., 1986; reviewed by Redburn, 1992; Perez & Davanger, 1994; Pow et al., 1994; Brandon & Criswell, 1995). Since GABA and GAD were observed to be present in rabbit horizontal cells in postembryonic developmental stages, it was suggested that GABA is not used as a neurotransmitter by these cells, but rather plays a developmental function by acting as a trophic factor during cone synaptogenesis (Messersmith & Redburn, 1992, 1993; Pow et al., 1994; Mitchell & Redburn, 1996). Similar suggestions have been made for developing mouse and human retinas (Schnitzer & Rusoff, 1984; Nag & Wadhwa, 1997).

While GABA undoubtedly plays a role in development, it is also the most likely neurotransmitter candidate in adult horizontal

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cells. This is because GABA receptors are localized to bipolar dendritic tips in the adult rabbit in a pattern similar to that of other mammalian retinas (Greferath et al., 1993, 1994; Enz et al., 1996). Failure to consistently demonstrate immunoreactivity to GABA and GAD may have been due to technical difficulties, use of incorrect antibodies, and/or regional differences in the retina (Messersmith & Redburn, 1992; Rowe-Rendleman & Redburn, 1994; Perez & Davanger, 1994; Nag & Wadhwa, 1997). We have therefore reexamined the issue by carefully studying the immunoreactivity for GABA and for both known isoforms of GAD (GAD_{65} and GAD_{67}) in different retinal regions. We show that GABA is consistently immunoreactive in type A horizontal cells in the visual streak, where it colocalizes with GAD_{65}. In contrast, GAD_{67} is expressed by the terminals of both A and B types of horizontal cells throughout the retina.

Methods

Tissue preparation and section orientation

Eyes were obtained from 11 adult Dutch-belted rabbits under deep anesthesia (10 mg/kg xylazine, followed after 15 min by 0.7 mg/kg acepromazine and 40 mg/kg ketamine). Animal treatment was in accordance with NIH guidelines and University of Pennsylvania policies. After the eyes were removed, the animal was sacrificed by an overdose of sodium pentobarbital (Nembutal). The eyes were quickly hemisected, the eyecup everted, and the vitreous removed. The eyecups were fixed in a solution of 4% paraformaldehyde and 0.01% glutaraldehyde in phosphate buffer (PB) for 1 h at room temperature. They were then washed repeatedly with phosphate buffer containing 5% sucrose (SPB), cryoprotected in 30% sucrose in PB overnight at 4°C, and dissected as shown in Fig. 1. The first two retinas were cut into nine pieces and sections from each piece were processed (Fig. 1A). These experiments showed that the staining pattern in the visual streak was different from regions elsewhere, but that there was no difference between peripheral pieces. Therefore, in later experiments, we processed long sections through the visual streak, and included the medullary rays for orientation (Fig. 1B). All data shown here are from these later experiments. Each immunohistochemistry experiment was performed on retinal sections from a minimum of three different animals, and each experiment was repeated at least four times.

For Vibratome sections, retinal pieces were embedded in a solution of 4% agarose in PB and cut into 70-μm radial sections. The sections were processed free-floating. For frozen sections, retinal pieces were incubated in a 2:1 mixture of Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC) and 30% sucrose for 20 min, then placed in a fresh mixture of the same medium, slowly immersed in liquid nitrogen, and stored at −80°C until use. 12-μm radial sections were collected on gelatin-coated slides and allowed to dry. They were then either used immediately for immunolabeling, or stored at −80°C for later use.

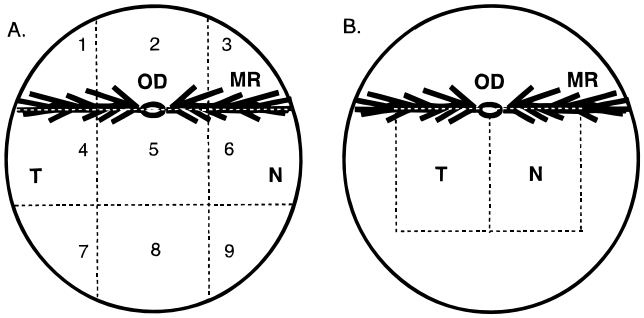

Fig. 1. Retinal regions used for Vibratome and cryostat sectioning. A: Schematic showing how the first two retinas were cut. Care was taken to maintain the orientation throughout sectioning and staining. These sections were used to determine the distribution of GAD_{65} and GAD_{67}. B: For all subsequent experiments, the retina was cut horizontally along the center of the medullary rays (MR) to a width of about 2 cm, and vertically to a length of 1–1.5 cm (dashed lines). Both nasal (N) and temporal (T) pieces were used to cut radial sections. No difference was observed between nasal and temporal retina. OD: optic disc.

Immunolabeling

For single labeling, sections were preincubated for 1 h at room temperature (RT) in diluent (10% normal goat serum, 0.5% Triton X-100, and 0.01% NaN_3 in SPB). They were then incubated in primary antibody for 1–3 days at RT, rinsed, and incubated in secondary antibody conjugated either to a fluorescent marker or to horseradish peroxidase (HRP) for 2–3 h at RT. Sections developed for fluorescence were then rinsed and mounted in Vectashield (Vector Laboratories, Burlingame, CA), coverslipped, and sealed with nailpolish. Sections incubated in HRP conjugated secondary antibodies were preincubated for 10 min in diazoaminobenzadine (DAB), followed by DAB and hydrogen peroxide (0.03%) for 15 min. *For double labeling with mouse anti-GAD_{65} and rabbit anti-GAD_{67}*, staining for the first primary and secondary antibodies proceeded as before. Sections were then rinsed and incubated in the second primary antibody for 1–3 days at RT, rinsed, and then incubated in the second secondary antibody for 2–3 h at RT. Simultaneous incubation in the two primary antibodies also worked, but sequential incubation gave clearer results with less background. *For double labeling with mouse anti-GAD_{65} and mouse anti-Calbindin*, we used two different methods. In the first, sections were incubated in anti-GAD_{65}, rinsed, incubated in an excess of anti-mouse F(ab) fragments conjugated to indocarbocyanine (Cy3) or lissamine rhodamine, rinsed, incubated in anti-calbindin, rinsed, and incubated in anti-mouse conjugated to Fluoresceine isothiocyanate (FITC). It was important to start with the antibody that gave a weaker, less intense, staining (as judged by single labeling) because this reduced binding of the second secondary antibody to the first primary antibody. To control for this binding, the second primary antibody, anti-calbindin, was omitted in some experiments. This revealed residual weak staining of the inner plexiform layer (IPL) with green fluorescence, but the horizontal cells were not labeled. In the second method, tissue was incubated in anti-GAD_{65}, rinsed, incubated in anti-mouse HRP, and then developed with DAB. The antibodies were then eluted with glycine buffer (pH = 2.2), the tissue was incubated in anti-calbindin, rinsed, incubated in anti-mouse conjugated to Oregon Green, rinsed, and mounted. Control experiments involved the omission of anti-calbindin. This method yielded similar results, although the DAB-reaction product masked the Oregon Green fluorescence, making it more difficult to distinguish cell types labeled with anti-calbindin.

Electron microscopy

Vibratome sections for electron microscopy (EM) were processed as above with DAB. In some sections Triton X-100 was omitted. The DAB reaction product was intensified by the gold-substituted

silver-intensified peroxidase method (after Sassoè-Pognetto et al., 1994; modified from Van den Pol, 1988): tissue was postfixed in 2.5% glutaraldehyde, washed overnight in 0.1 M cacodylate buffer, rinsed in deionized water (dH_2O), incubated for 10 min at 60°C in a solution of 2.6% hexamethylene tetramine, 0.2% silver nitrate, and 0.2% disodium tetra-borate in dH_2O, rinsed in dH_2O, incubated in 0.05% gold chloride in dH_2O, rinsed in dH_2O, incubated in 2.5% sodium thiosulfate in dH_2O, rinsed in dH_2O, and rinsed in cacodylate buffer. Following silver intensification, the tissue was osmicated (4% osmium tetroxide in 0.2 M cacodylate buffer, 60 min), dehydrated in 50% and 70% ethanol, stained with 2% uranyl acetate in 70% ethanol (60 min), dehydrated in 90% and 100% ethanol, cleared in propylene oxide, and embedded in Epon 812. Ultrathin sections (silver-gold) were mounted on formvar-coated slot grids and stained lightly with lead citrate and uranyl acetate.

Antibodies

Primary antibodies used: monoclonal antibody to GAD_{65} raised in mouse (supernatant from Developmental Studies Hybridoma Bank, University of Iowa, Department of Biological Sciences, 436 BB, Iowa City, IA; 1:5); polyclonal antibody to GAD_{67} raised in rabbit (Chemicon, Temecula, CA; 1:1000); polyclonal antibody to GABA raised in guinea pig (Chemicon; 1:1000); monoclonal antibody to Calbindin-D raised in mouse (Sigma, St. Louis, MO; 1:250, 1:500). Secondary antibodies used: anti-guinea pig conjugated to Fluorescein isothiocyanate (FITC) (Jackson Immunoresearch, West Grove, PA; 1:1000); anti-mouse F(ab) fragments conjugated to indocarbocyanine (Cy3) (Jackson; 1:1000); anti-mouse F(ab) fragments conjugated to lissamine rhodamine (Jackson; 1:100); anti-mouse conjugated to FITC (Jackson; 1:1000); anti-mouse conjugated to HRP (Protos Immunoresearch, San Francisco, CA; 1:100); anti-mouse conjugated to Oregon Green (Molecular Probes, Eugene, OR; 1:100); anti-rabbit F(ab) fragments conjugated to FITC (Jackson; 1:100); anti-rabbit conjugated to HRP (Protos Immunoresearch; 1:100); anti-rabbit conjugated to Cy3 (Jackson; 1:1000).

Image acquisition and processing

Sections stained with DAB reaction product were viewed using Differential Interference Contrast microscopy (Polyvar II). Sections stained with fluorescent markers were viewed with the following filter sets: for FITC and Oregon Green, excitation 450-495, barrier 520-560; for Cy3 and rhodamine, excitation 530-585, barrier L615. Photomicrographs of stained sections were digitized with a Sprintscan35 scanner at about 1012 dots/inch. Some images were captured using a cooled CCD camera (Princeton Inst., Trenton, NJ). Images were imported to Adobe Photoshop, cropped, contrast enhanced, and imported to Adobe Illustrator for labeling. Figures were printed on either a dye sublimation or Itocho 320 printer at final resolution of about 300 dots/inch.

Results

Immunoreactivity for GAD_{67}

Staining for GAD_{67} revealed many amacrine cell somas, both in the inner nuclear layer and in the ganglion cell layer (Fig. 2; see also Fig. 6). In the inner plexiform layer, the stain was stratified in a pattern consistent with Brandon and Criswell (1995). Staining was also present in the outer plexiform layer where it was homogeneous throughout the retina. In all locations, the stain was punctate and restricted to the outer (top) region of the outer plexiform layer. The stain was difficult to see at low magnification in cryosections (as in Fig. 6) but it was clear and punctate under higher magnification (Fig. 2). At the EM level, the stain was localized to the lateral elements of the triad at the ribbon synapse, indicating that these are horizontal cell terminals. In rabbit, there are three types of horizontal cell terminals: the axon terminals of type B cells, which invaginate the rod spherules, the dendritic terminals of type A cells, and the dendritic terminals of type B cells, both of which invaginate cone pedicles (Dowling et al., 1966; Bloomfield & Miller, 1982; Dacheux & Raviola, 1982). Staining was clearly present in axon terminals invaginating the rod spherule and in dendritic terminals invaginating the cone pedicle (Fig. 3).

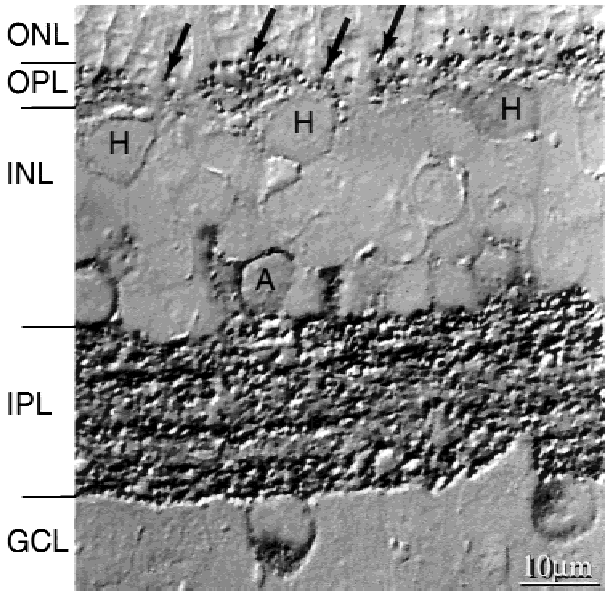

Fig. 2. GAD_{67} immunoreactivity is punctate in the OPL (light micrograph of 1-μm Epon section visualized with DAB reaction product). The stained puncta in the OPL (arrows) represent horizontal cell terminals. Staining in the IPL is intense and stratified into four distinct bands. Amacrine cell somas (A) in the INL are stained. Horizontal cell somas (H) can be identified due to their large size by differential interference contrast, but they are either unstained (middle soma) or stained very weakly relative to the stained amacrine cells and the other stained cells in the GCL. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; and GCL: ganglion cell layer.

Although not every horizontal cell terminal in all the cone pedicles examined was stained, we propose that both type A and type B dendritic terminals express GAD_{67} for the following reasons: (1) It has been suggested that the two lateral elements of a cone synaptic triad are composed of type A and type B terminals (Sterling, 1983). In most triads where both lateral elements could be seen, both were stained. (2) In cat, a ratio of 59% type A to 41% type B terminals can be computed from data presented in Wässle et al. (1978). Given the similarity between rabbit and cat horizontal cell coverage factors and density ratios (Mills & Massey, 1994), it is likely that the ratio between the two types of horizontal cell terminals in rabbit is similar between these two species. Thus, if only one type were expressing GAD_{65}, we would expect about 50% of the profiles to stain. We counted the number of stained and unstained profiles of horizontal cell terminals in several cone ter-

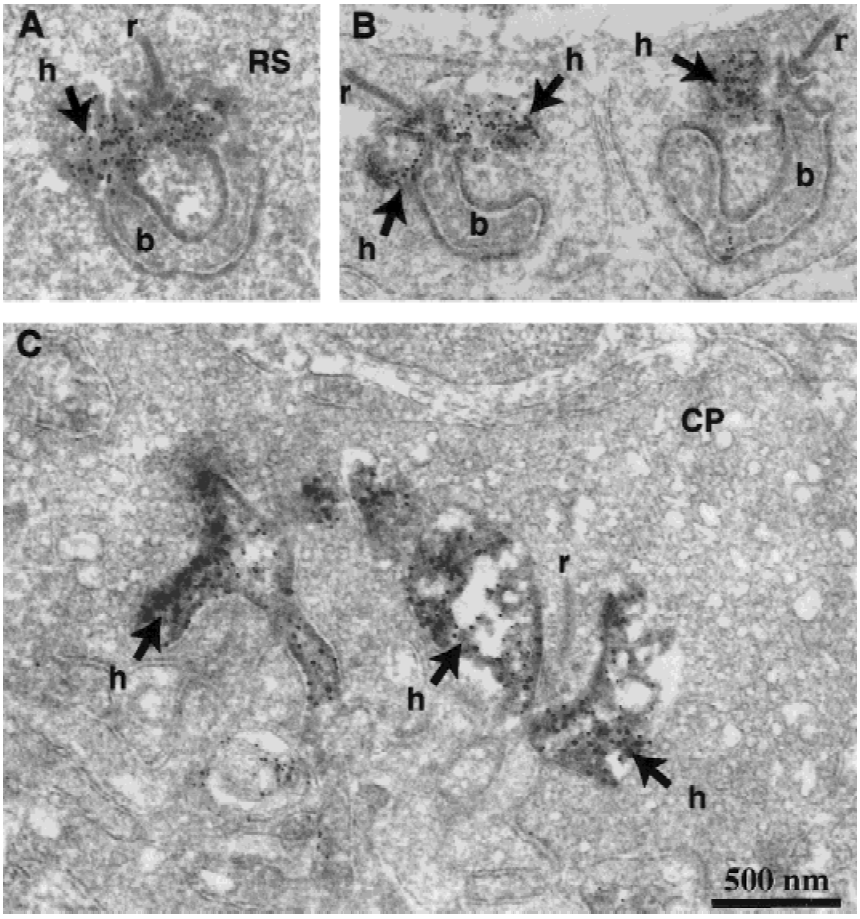

Fig. 3. All horizontal cell terminals express GAD_{67} (electron micrographs taken from a region near the medullary rays). A, B: Rod spherules (RS). Stain (gold deposits) is present in both lateral elements, i.e. invaginating axon terminals of type B horizontal cells (h). Bipolar dendrites (b) are unstained. C: Cone pedicle (CP). Stain is present in all invaginating terminals. In the triad on the right, both lateral elements stain. h: horizontal cell terminal; r: ribbon.

minals from two retinal regions. A terminal was determined to be stained by the presence of three or more gold particles. Sections used in these experiments were treated with 0.5% triton X-100, to achieve as complete staining as possible. Of all the horizontal cell profiles observed, about 92% were stained (Table 1). This is significantly higher than the 59% expected, and thus is inconsistent with the hypothesis that only one cell type stained. This hypothesis would be supported by our data only if the cell that expresses GAD_{67} contributes 84% or more of the terminals invaginating a cone. This was computed from the equation $\chi^2 = 4d^2/n$, where χ^2 is the chi-square value, d is the deviation [observed number of stained (or unstained) minus expected], and n is the total number of horizontal cell terminal profiles (Snedecor & Cochran, 1974). In summary, it is highly likely that both types of horizontal cell dendritic terminals stain for GAD_{67}, and any unstained terminals in our experiments were probably due to incomplete immunostaining.

Table 1. *Number of stained and unstained horizontal cell (HC) terminals in the visual streak and just below the medullary rays*

	Visual streak	Medullary rays	Total
No. of cone pedicles	9	14	23
No. of stained HC terminals	72	41	113
No. of unstained HC terminals	8	1	9
% stained	90%	~98%	~92%
Expected % stained	59%	59%	59%
χ^2	29.91	25.90	52.02
P[a]	<0.005	<0.005	<0.005

[a]The P value indicates that the obtained ratio is significantly different from that expected, based on the hypothesis that only one type expresses GAD_{67}.

Immunoreactivity for GAD_{65}

Staining for GAD_{65} was evident in horizontal cells in the visual streak but absent elsewhere (Fig. 4). Vardi and Auerbach (1995) used the antibody for GAD_{65} diluted 1:20. We used higher concentration and allowed longer incubation times in order to intensify the stain. This improved the staining of horizontal cells in the visual streak without increasing background level. Horizontal cells outside the visual streak, however, were not stained. The transition between the stained region and unstained regions was gradual.

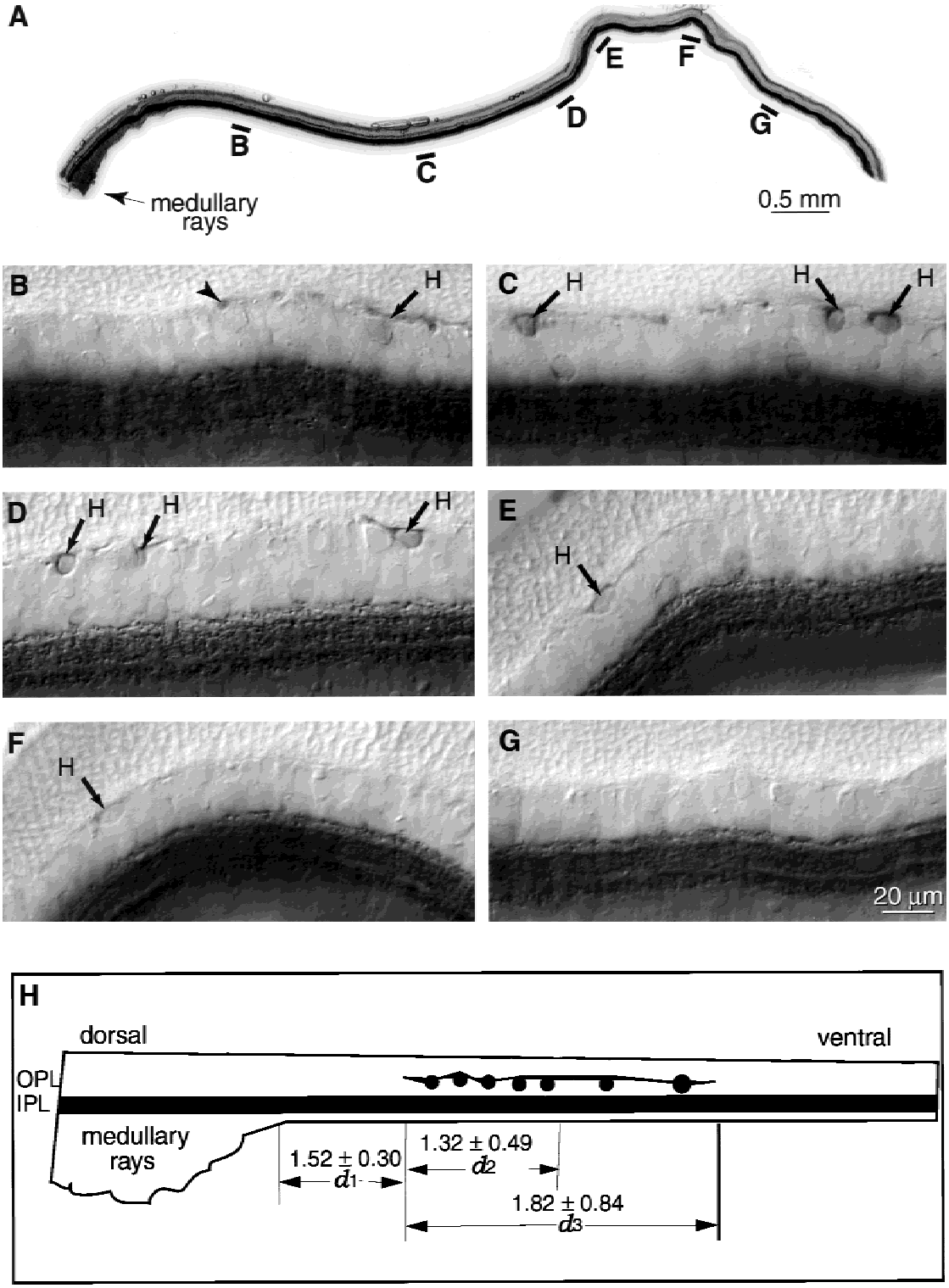

Fig. 4. GAD_{65} immunoreactivity is restricted to the visual streak. A: Low-magnification light micrograph of a 70-μm-thick retinal section stained for GAD_{65} and visualized with DAB reaction product. The B–G letters designate regions shown under higher magnification. B: A dorsal region in which stain in horizontal cells starts to appear (arrowhead). C, D: Visual streak (region *d*2). Staining in horizontal cells is intense. E: Ventral to the visual streak, staining in horizontal cells is declining (region *d*3). This horizontal cell was counted as stained. F: This horizontal cell can be identified by DIC, and was considered unstained. G: Periphery. Horizontal cells are never stained. H: A schematic of a typical cryostat section; measurements (in millimeters) were taken from 14 sections of nine retinas from five different animals. *d*1: distance from the edge of the medullary rays to the first stained processes of horizontal cells; *d*2: Length of region with strongly stained horizontal cells; and *d*3: Length of region with strongly and weakly stained horizontal cells.

Cells were determined to be stained if the level of stain in the soma was clearly above the background stain (see examples in Fig. 4). The region in which horizontal cells stained most intensely for GAD_{65} extended for 1.32 ± 0.49 mm (region *d*2 in Fig. 4H). Occasionally, scattered somas were very weakly stained ventral to this region. Consequently, when measured to the very last, weakly stained soma, the GAD_{65}-immunoreactive region was longer (1.82 ± 0.84 mm). These measurements correspond well with the width of the visual streak as measured by Vaney (1980) and Vaney and Hughes (1976). In regions where the horizontal cell somas did not stain, a few fine dendrites, strongly stained, were occasionally observed (data not shown). These are probably interplexiform cell processes (Mosinger et al., 1986; Redburn, 1992; reviewed by Marc, 1995).

Although the stain for GAD_{65} in somas and primary dendrites of horizontal cells was strong, it was weak or absent in invaginating horizontal cell terminals (Fig. 5). Thus, GAD_{65} and GAD_{67} seem to localize to different compartments of the cells. This can be illustrated by double labeling for GAD_{65} and GAD_{67}, as shown in Fig. 6. To reveal whether both types of horizontal cell somas were immunoreactive for GAD_{65}, we used an antibody for calbindin which stains type A horizontal cells strongly and type B either weakly or not at all (Lyser et al., 1994; Mills & Massey, 1994; Mitchell et al., 1995). In the visual streak, every soma that was brightly stained for calbindin was also stained for GAD_{65} (Figs. 7A and 7B). This was quantified in one experiment in which a total of 80 horizontal cell somas (8 cryosections, 12 μm thick) were labeled for GAD_{65}; they were all also brightly stained for calbindin. Outside of the visual streak, horizontal cells brightly stained for calbindin were not stained for GAD_{65} (Figs. 7C and 7D). This indicates that the bright FITC label for calbindin did not bleed through the rhodamine filter, thus the stain observed under the rhodamine filter truly represents GAD_{65}. The weak labeling of the IPL seen under the FITC filter is not consistent with tissue singly labeled for calbindin. This implies that the second secondary antibody reacted with the anti-GAD_{65}. This, however, does not interfere with the labeling of type A horizontal cells by the antibody for calbindin because the stain is very intense. Double labeling for GAD_{65}, visualized with DAB reaction product, and calbindin, visualized with Oregon Green, produced similar results (data not shown).

Immunoreactivity for GABA

Immunoreactivity for GABA was observed in somas and primary dendrites of horizontal cells in the visual streak (Fig. 8). This immunoreactivity was weaker than that in the strongly stained amacrine cells, but at the same level or stronger than the weakly stained amacrine cells. Staining for GABA with the antibody used here was tricky because, on the one hand a good fixation (with gluteraldehyde) promoted antigen recognition, but on the other hand, such fixation prevented antibody penetration, so only structures close to the surface were stained. Double labeling for GABA and GAD_{65} showed that every horizontal cell which stained for GAD_{65} also stained for GABA (Fig. 9). The colocalization of GABA with GAD_{65} is consistent with Mosinger and Yazulla (1987), who reported that although not every horizontal cell stained for GAD, every cell that did also immunoreacted for GABA. We were unable to detect GABA in horizontal cells outside of the visual streak. GABA was also difficult to localize to horizontal cell terminals, but processes of Müller cells were often stained (Fig. 9).

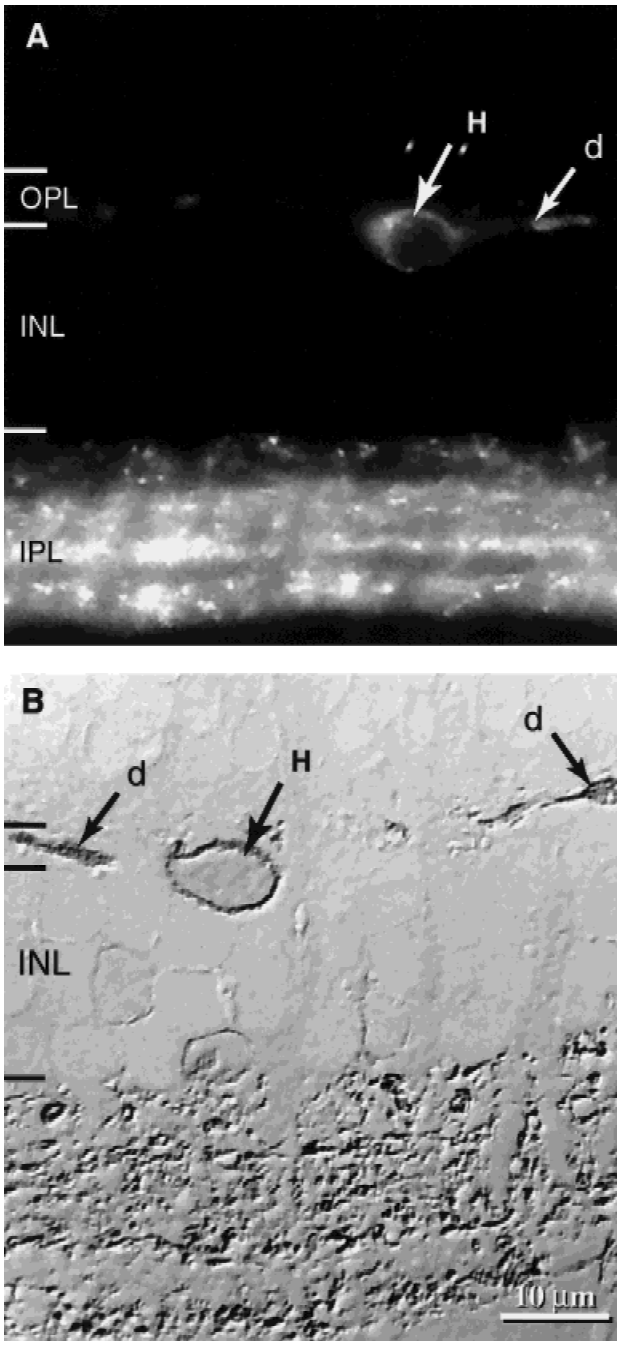

Fig. 5. GAD_{65} in the visual streak is not present in horizontal cell terminals. Type A horizontal cell somas (H) and primary dendrites are stained (d); horizontal cell terminals are unstained. A: 10-μm cryostat section visualized with Cy3. B: 1-μm Epon section visualized with intensified DAB reaction product (different retina than A). Compare this stain to Fig. 2.

Discussion

The pattern of GAD immunoreactivity in rabbit horizontal cells is unique in several respects. (1) Rabbit horizontal cells express both isoforms of GAD (GAD_{65} and GAD_{67}), whereas cat and monkey horizontal cells express only one isoform. Cat horizontal cells express GAD_{65}, and monkey horizontal cells express GAD_{67} (Vardi et al., 1994). (2) In rabbit retina, the GAD enzymes are restricted

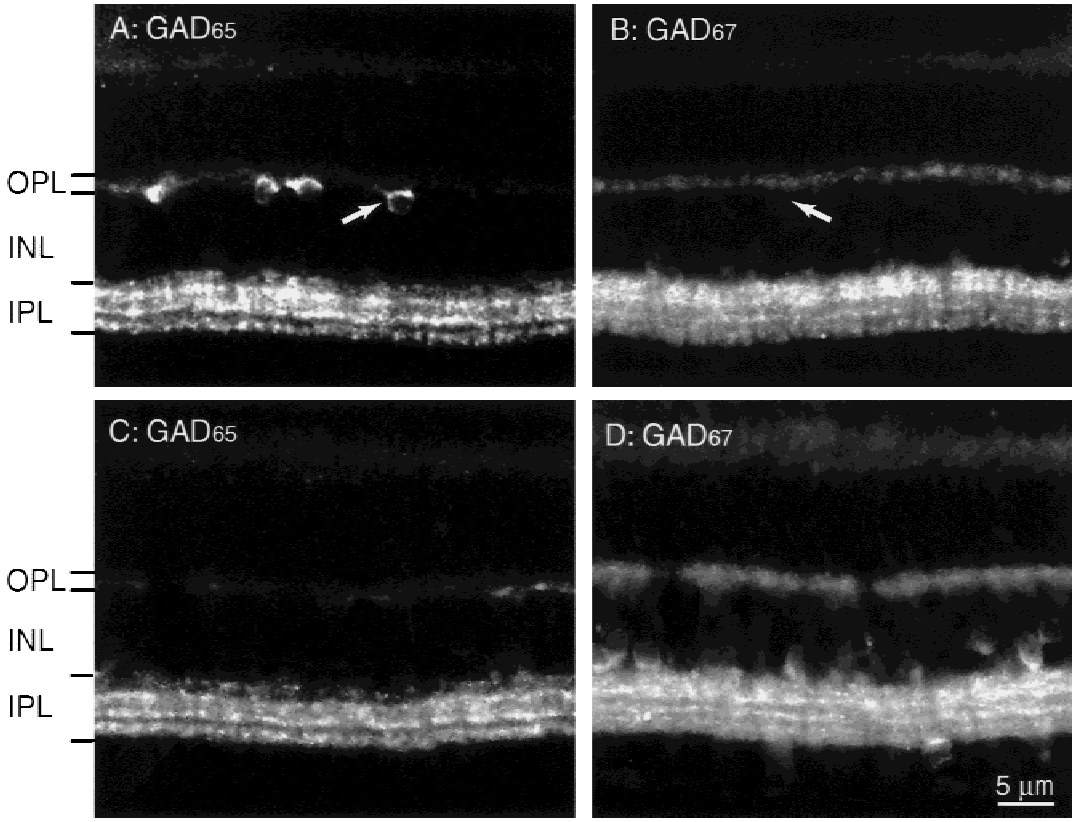

Fig. 6. Double labeling for GAD_{65} (visualized with Cy3), and GAD_{67} (visualized with FITC) (cryosections). B and D are mirror images of A and C, respectively. A, B: Visual streak. The soma and primary dendrite of a GAD_{65} stained horizontal cell (arrow in A) is unstained for GAD_{67} (arrow in B). C, D: 1.68 mm ventral to A, B. Note that the two isoforms do not colocalize in the OPL. GAD_{67} is expressed in the upper region of the OPL in invaginating terminals, while GAD_{65} is expressed in the lower region of OPL in horizontal cell primary dendrites.

to certain compartments in the cells; GAD_{67} is limited to the terminals and GAD_{65} is localized to somas and dendrites. In cat and monkey, the enzymes are localized to both the somas and the terminals. (3) In rabbit, there is a regional difference in the localization of GAD_{65}. Different localization of GAD has not yet been reported in adult mammalian horizontal cells, but it has been suggested in developing rabbit and human retina (Rowe-Rendleman & Redburn, 1994; Nag & Wadhwa, 1997). Regional differences in GABA content were also suggested (Agardh et al., 1987*a*; Grünert & Wässle, 1990).

The unique expression pattern of GAD in rabbit might explain why there has been so much confusion regarding its localization in this species. The restricted localization of GAD_{67} to the invaginating terminals may explain why it has been missed in previous studies. The restricted localization of GAD_{65} to a small population of type A horizontal cells may explain why previous studies, using an antibody which recognized primarily the GAD_{65} isoform, found only occasional immunoreactivity in the outer plexiform layer. The same explanation also applies to GABA localization, since GABA is detected only in horizontal cells expressing GAD_{65}, i.e. in type A horizontal cells in the visual streak.

Are horizontal cells in the adult rabbit GABAergic?

The main argument against the notion that rabbit horizontal cells are GABAergic is that GABA is down-regulated in postembryonic development, and could not be consistently localized to adult horizontal cells (reviewed by Redburn, 1992; Pow et al., 1994). However, two lines of evidence suggest that horizontal cells probably are GABAergic. (1) All horizontal cells in adult retina express GAD_{67} in their terminals. We are confident that the immunoreactivity of GAD_{67} is specific because specificity has been demonstrated with the same antibody in previous studies in the central nervous system (Erlander et al., 1991; Kaufman et al., 1991; Esclapez et al., 1994). It is likely that the presence of GAD_{67} indicates GABA synthesis in the terminals because, when it is expressed by Rat-1 cells, GABA is in fact synthesized (Ruppert et al., 1993). Furthermore, in cat, all GAD_{67}-expressing amacrine cells and horizontal cells also contain GABA, suggesting that the enzyme actually synthesizes GABA (Vardi et al., 1994; Vardi & Auerbach, 1995). (2) $GABA_A$ receptors are localized to cone bipolar dendrites just beneath cones, and $GABA_C$ receptors are localized to rod bipolar dendrites (Greferath et al., 1994; Enz et al., 1996). This suggests that there should be a source of GABA in this region of the outer plexiform layer. This region is occupied mainly by bipolar dendrites and horizontal cell processes. Since most or all bipolar cells are glutamatergic (Massey & Maguire, 1995), and probably cannot release GABA from their dendrites, horizontal cells remain the most reasonable source of GABA. Processes of GABAergic interplexiform cells are also found in the outer plexiform layer, but they are very scarce (Oyster & Takahashi, 1977; Brandon, 1985; Perez & Davanger, 1994; Marc, 1995; this study).

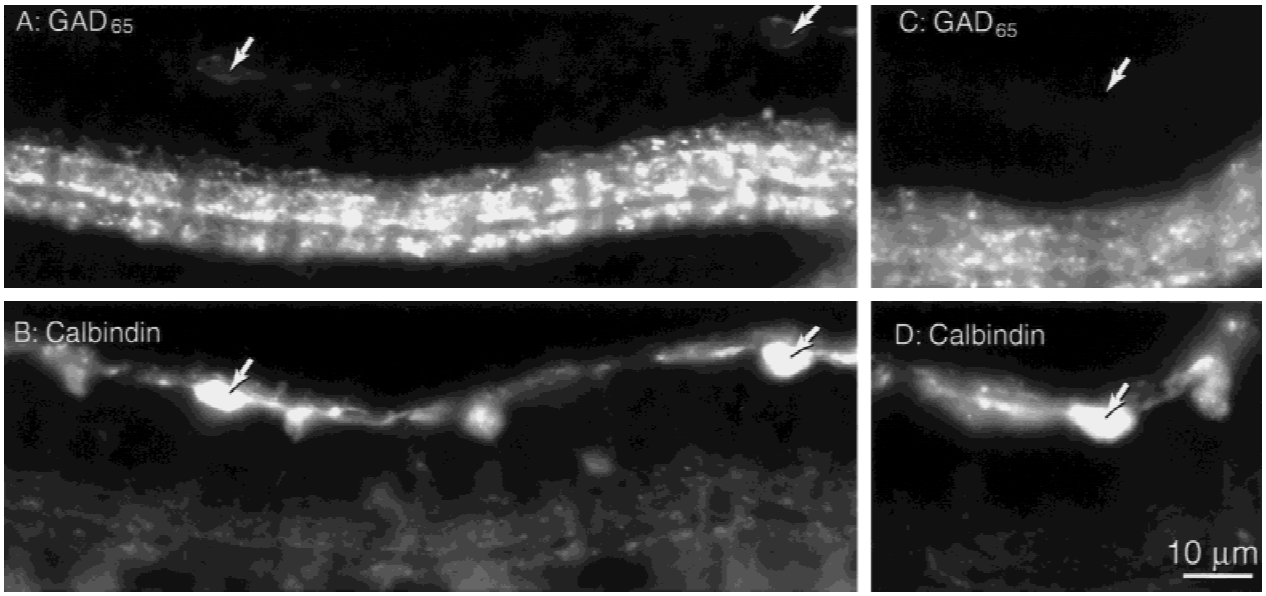

Fig. 7. Double labeling for GAD_{65} [visualized with F(ab) rhodamine] and calbindin (visualized with FITC) (cryosections). A, B: Visual streak. Type A horizontal cells stained brightly for calbindin also stained for GAD_{65}. Type B horizontal cells must be present but are apparently unstained by either antibody. C, D: Type A horizontal cell stained for calbindin is unstained for GAD_{65}. This shows that the bright stain under the FITC filter is not detectable under the rhodamine filter.

If the horizontal cell terminals do synthesize GABA, why has GABA remained undetected? Several possibilities exist: (1) GAD_{67} is restricted to the small volume of a terminal, so it would probably produce a limited amount of GABA. (2) Horizontal cell terminals in rabbit have not been shown to contain synaptic vesicles, so GABA must be present diffusely in the cytosol, probably at a low concentration. Concentrations of antigen below 40 μM are probably undetectable by conventional immunocytochemical methods (Pow & Crook, 1994). (3) Horizontal cell terminals probably release GABA by a transporter mechanism (Ayoub & Lam, 1984; Schwartz, 1987; Haugh-Sheïdt et al., 1995). This mechanism is very sensitive to external and internal ionic concentrations of sodium, chloride, and probably other molecules, so GABA may be transported out of the cell during anesthesia and/or fixation. (4) The antibody for GABA scarcely penetrates the tissue. It is therefore possible that the terminals on the surface do not contain GABA because they have been cut during sectioning, and the deeper, intact terminals are not reached by the antibody.

Our conjecture that GABA is the neurotransmitter of rabbit horizontal cells does not conflict with a developmental function for GABA. It appears that horizontal cells in young rabbits use GAD_{65} to synthesize GABA, which is packaged into synaptic vesicles. Indeed, at these stages the horizontal cells express synaptic proteins not expressed in adulthood (Mandell et al., 1990; Haas et al., 1990). As the horizontal cells mature, they down-regulate GAD_{65} and probably up-regulate GAD_{67} (Rowe-Rendleman & Redburn, 1994). The function of GABA could change at different developmental stages, as could the method of synthesis and release (see discussion below). Hence, both hypotheses may be true.

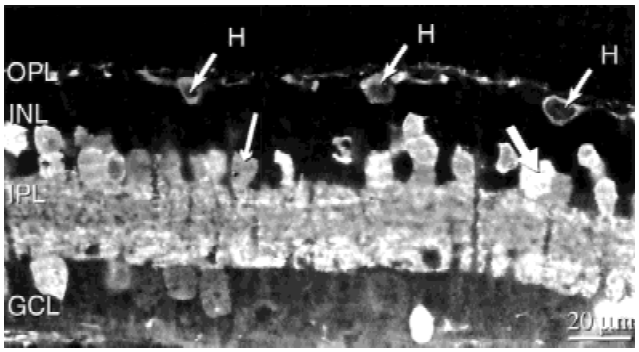

Fig. 8. Horizontal cells (H) in the visual streak stain for GABA (70-μm vibratome section). The stain in the horizontal cell somas is weaker than the most intensely stained amacrine cells (thick arrow), but stronger than the weakly stained amacrine cell somas (thin arrow). The brighter and darker horizontal stripes in amacrine cells and in the IPL result from lack of penetration of this antibody combined with chatter due to vibratome sectioning.

Differential functions of GAD_{65} and GAD_{67}

Where GAD_{65} and GAD_{67} were localized to the same cell (type A cells in visual streak), they were segregated into different compartments. This is consistent with previous studies showing these isoforms to be segregated in both central nervous system and retina (Erlander et al., 1991; Kaufman et al., 1991; Esclapez et al., 1994; Vardi & Auerbach, 1995; Brandon & Criswell, 1995). However, the restriction of GAD_{67} to synaptic endings and GAD_{65} to somatic regions is opposite to the segregation pattern previously reported in the literature. Nonetheless, this differential expression is consistent with hypotheses concerning the differential function of these two isoforms. One hypothesis is that GAD_{65} is correlated with synaptic release of GABA, whereas GAD_{67} is correlated with a transporter-mediated release (Erlander et al., 1991; Vardi & Auerbach, 1995). This is supported by the observation that GAD_{65} is

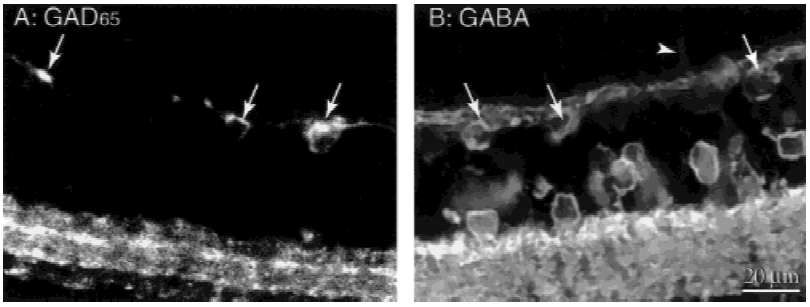

Fig. 9. Double labeling for GAD_{65} (Cy3) and GABA (FITC). Horizontal cells stained for GAD_{65} also stained for GABA (cryosection). B is a mirror image of A. Three somas are labeled for both antibodies (arrows). Müller cell processes (arrowhead) also stain for GABA in this section.

expressed in primary dendrites of type A horizontal cells which appear to form conventional synapses (Fisher and Boycott, 1974; Brandstätter et al., 1996). Synaptic vesicles in conventional synapses from horizontal cell primary dendrites were also shown in goldfish (Marshak & Dowling, 1987). GAD_{67} is expressed in horizontal cell terminals, which probably release GABA *via* a transporter mechanism (Ayoub & Lam, 1984; Schwartz, 1987; Haugh-Sheïdt et al., 1995). An interesting finding which might be related to the function of GAD_{67} in horizontal cells is that GABA down-regulates the activity of GAD_{67} but not GAD_{65} (Rimvall & Martin, 1994). It is also known that GABA affects the activity of the GABA transporter. Therefore, it appears that GABA content in GAD_{67} expressing cells is highly regulated by GABA itself, as it affects both synthesis by GAD_{67} and release by the transporter.

Another hypothesis, which has been suggested for cat retina, is that GAD_{65} is associated with cone vision, whereas GAD_{67} is associated with rod vision (Vardi & Auerbach, 1995). Stated in other words, GAD_{65} is associated with faster GABA regulation, whereas GAD_{67} is associated with slower regulation. This is consistent with the observation that GAD_{65} is expressed in the visual streak, which, with an approximately two-fold higher cone density (Juliusson et al., 1994), may transmit on average a faster signal than other retinal regions. Finally, we also note that type Ae horizontal cells, the putative orientation selective horizontal cell type, are also regionally specific to the visual streak (Kolb & Normann, 1982; Bloomfield, 1992). There may be a correlation between the GAD_{65}-immunoreactive type A cell and the Ae cell.

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