Specific Cell Types in Cat Retina Express Different Forms of Glutamic Acid Decarboxylase

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ABSTRACT

We studied the expression of glutamate decarboxylase (GAD), GAD_{65} and GAD_{67}, in cat retina by immunocytochemistry. About 10% of GABAergic amacrine cells express only GAD_{65} and 30% express only GAD_{67}. Roughly 60% contain both forms of the enzyme, but GAD_{67} is present only at low levels in the majority of these double-labeled amacrine cells. The staining pattern in the inner plexiform layer (IPL) for the two GAD forms was also different. GAD_{65} was restricted to strata 1-4, and GAD_{67} was apparent throughout the IPL but was strongest in strata 1 and 5. This indicates that somas, as well as their processes, are differentially stained for the two forms of GAD. Cell types expressing only GAD_{65} include interplexiform cells, one type of cone bipolar cell, and at least one type of serotonin-accumulating amacrine cell. Cell types expressing only GAD_{67} include amacrine cells synthesizing dopamine, amacrine cells synthesizing nitric oxide (NO), and amacrine cells accumulating serotonin. Cholinergic amacrine cells express a low level of both GAD forms. Our findings in the retina are consistent with previous observations in the brain that GAD_{65} expression is greater in terminals than in somas. In addition, in retina most neurons expressing GAD_{67} also contain a second neurotransmitter as well as GABA, and they tend to be larger than neurons expressing GAD_{65}. We propose that large cells have a greater demand for GABA than small cells, and thus require the constant, relatively unmodulated level of GABA that is provided by GAD_{67}.

Indexed terms: GABA, cholinergic amacrine, dopaminergic amacrine, interplexiform cell, bipolar cell

Two molecular forms of glutamate decarboxylase (GAD), which are the product of two different genes, have recently been identified (Erlander et al., 1991; one has a molecular weight of 65 kD (GAD_{65}) and the other has a molecular weight of 67 kD (GAD_{67}). These two forms differ in subcellular localization (Erlander et al., 1991; Kaufman et al., 1991; Escalada et al., 1994), expression in different brain regions (e.g. Gonzales et al., 1991; Feldblum et al., 1993), expression during development (Greif et al., 1992; Hendrickson and Erickson, 1993), and interaction with the cofactor pyridoxal phosphate (Kaufman et al., 1986; Martin et al., 1991a,b). These differences suggest that the two GAD forms provide flexibility for regulating gamma-aminobutyric acid (GABA) synthesis, but how the expression of each form is related to neuronal function is unknown. The retina offers a good model system to study these questions because many retinal cell types can be identified, and much is known about their morphology, transmitter content, and physiology.

In mammalian retina both forms of GAD are expressed, but with a different distribution (Sarthy and Egal, 1992; Hendrickson and Erickson, 1993; Vardi et al., 1994). Most notably, cat horizontal cells stain exclusively for GAD_{67}, whereas Macaque (mulatta and fascicularis) horizontal cells stain for GAD_{65} (Vardi et al., 1994). In the amacrine cell layer, numerous somas express each GAD form, but whether both forms are present in the same cells and which cell types express which GAD form is unknown. Immediate identification of the stained amacrine cell types was not possible by morphology because the cells that stain for GAD constitute a heterogeneous population with dense staining throughout the inner plexiform layer. We took advantage of the fact that many cells expressing GABA also express another neurotransmitter (Chun et al., 1988; Muller et al., 1988; Wissle and Chun, 1988), permitting us to identify specific subtypes within the GABAergic amacrine cell population. Here we show that retinal neurons exclusively expressing GAD_{65} tend to be small and do not contain any other neurotransmitter, whereas amacrine cells exclusively expressing GAD_{67} tend to be larger and contain another neurotransmitter.

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METHODOLOGY

Tissue preparation

Retinas were obtained from adult cats by enucleation under deep sodium pentobarbital anesthesia (40 mg/kg). Most eyes were taken at the end of acute physiological experiments, but these experiments had no effect on the immunostaining reported here. For cholince pre-treatment, three cats were injected intraocularly with 0.35–0.625 mg cholinc in 10 μL D,H2O under a combination of ketamine (100 mg, i.m.) and metofane anesthesia. In each case, one eye was left intact as a control. Enucleation was performed 48 hours after injection, under pentobarbital anesthesia as above. Eyes were hemisectioned at the equator, and the posterior half was fixed by immersion in buffered 4% paraformaldehyde containing 0.01% glutaraldehyde for 1 hour at room temperature. After rinsing and overnight cryoprotection in 0.1 M phosphate buffer containing 30% sucrose, tissue was taken from the central area (up to 2 mm eccentricity) and frozen in a 2:1 mixture of 0.1 M phosphate buffer containing 20% sucrose and O.C.T. embedding medium (Barthel and Raymond, 1990).

Immunostaining

Immunocytochemistry was performed on 6–10-μm frozen sections according to the following protocol: preincubate in diluent containing 10% normal goat or donkey serum, 5% sucrose, and 0.15–0.3% Triton X-100 in 0.1 M phosphate buffer for 1/2 hour at room temperature; incubate in primary antibodies overnight at 4°C; wash and incubate in secondary antibodies conjugated to either horseradish peroxidase (HRP) or a fluorescent marker for 3 hours at room temperature; incubate (HRP-conjugated antibodies) in 0.05% diaminobenzidine (DAB) + 0.01% hydrogen peroxide in phosphate buffer for 15 minutes; wash and mount in Vectashield mounting medium for fluorescence (Vector Laboratories, Inc., Burlingame, CA). For 5-hydroxytryptamine (5-HT) staining, retinas were incubated in oxygenated Ames medium containing 0.05 mM 5-HT for 1 hour at room temperature, followed by a brief rinse, prior to fixation.

For most double-labeling experiments, both primary antibodies were applied simultaneously. However, double labeling for tyrosine hydroxylase (TH) and GAD67 (both made in rabbit) was done sequentially as follows: incubate in antibodies for GAD67; incubate in anti-rabbit conjugated to HRP; process for DAB reaction; elute antibodies with glycine-HCl buffer (pH 2.2) for 5 minutes; incubate in antibodies for TH; incubate in anti-rabbit conjugated to Cy3. To reduce possible crossreaction between the antibodies for GAD67 and GAD65 in the tissue, in some experiments the antibodies for GAD67 were preabsorbed with Escherichia coli lysate containing GAD65 protein (kindly donated by Dr. Daniel Kaufman) in a ratio of 9:1 (antibody [2×] concentrated lysate). To eliminate the possibility of crossreaction between secondary antibodies and primary antibodies from the wrong species, we incubated each of our secondary antibodies with every incompatible primary antibody with which it was used in combination for multiple labeling. For example, for the triple-labeling experiment shown in Figure 1, we first incubated sections in primary antibodies for GAD65 (mouse) and GAD67 (rabbit), followed by incubation in anti-guinea-pig secondary antibodies conjugated to 7-amin-4-methylcoumarin-3-acetic acid (AMCA). No staining was revealed in any of these combinations.

Nicotinamide adenine dinucleotide phosphate (NADPH)

The protocol of Sagar (1986) was followed. Retinas were fixed in 4% paraformaldehyde for 1/2 hour at room temperature and then placed in 0.1 M Tris buffer, pH 8.2, containing 15 mM malic acid, 1.0 mM NADP, 0.2 mM nitro blue tetrazolium (NBT), 1.0 mM CoCl₂, and 0.4% Triton X-100 for 2 hours at 37°C. The reaction was stopped by rinsing in phosphate buffer, and the retina was then cryoprotected and embedded as above.

Antibodies employed

Monoclonal antibody specific for GAD67 (GAD-6; 1:20; a high concentration was used to maximize the number of stained somas, but the background remained low). GAD-6 was prepared by Chang and Gottlieb (1988) and obtained from the Developmental Studies Hybridoma Bank (Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and Department of Biology, University of Iowa, Iowa City, IA, contract No.1-HD-6–2915). Polyclonal antiserum for GAD67 (K2) was obtained from two sources: that donated directly by Dr. Kaufman was used at a dilution of 1:200; that obtained from Chemicon International Inc. (Temecula, CA) was used at a dilution of 1:1,000. In both cases, the concentration was chosen to optimize the signal-to-noise ratio. This antibody was produced in rabbit against purified recombinant GAD polypeptide (Kaufman et al., 1991). The polypeptide was synthesized in E. coli transfected with DNA encoding feline GAD77. Polyclonal antibody to GABA (1:30; Insectar Corp., Stillwater, MN) was prepared in rabbit. Polyclonal antibody for GABA was raised in guinea pig (1:500–1,000; Chemicon). Monoclonal antibody for choline acetyltransferase (ChAT; 1:2.5; Boehringer Mannheim, Indianapolis, IN) was raised from rat–mouse hybridoma. Polyclonal antibody for tyrosine hydroxylase (1:200; Eugene Tech International, Inc., Baxley, NJ) was raised in rabbit. Rat monoclonal antibody for 5-HT (1:100; Chemicon). Secondary antibodies: peroxidase-conjugated Fab’ fragment goat anti-mouse, rabbit, and rat antibodies (Protos Immunoresearch, San Francisco, CA); peroxidase-conjugated goat anti-guinea pig (Sigma Chemical Co., St. Louis, MO); Cy3, fluorescein isothiocyanate (FITC), Texas Red, and AMCA-conjugated goat or donkey anti-mouse, rabbit, rat, and guinea pig antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove).

Cell counting

To determine the percentage of GABAergic amacrine cells immunoreactive for each GAD type, immunoreactive cells were visualized with DAB-reaction product, and unstained cells were visualized with Nomarski optics. All counting and analysis was done either directly from the tissue or from unmodified, conventional photomicrographs. Threshold was defined as the level of staining observed in (non-GABAergic) bipolar cells in the inner nuclear layer (INL), and cells that appeared darker than the bipolars were judged to be positively stained. Only somas in the amacrine cell layer were considered for this purpose because it is difficult to distinguish between amacrine cells and small ganglion cells in the ganglion cell layer. Counts performed independently by the two authors were generally in good agreement. When the same section was counted, the average between the two was taken, and when different sections were analyzed, the numbers were pooled.
Fig. 1. Triple labeling for GAD$_{65}$ (A, visualized with Cy3), GAD$_{67}$ (B, fluorescein isothiocyanate (FITC)), and GABA (C, AMCA). All somas stained for GAD also stained for GABA. Arrowheads point to somas stained for GAD$_{65}$ and GABA (A, C), arrows point to somas stained for GAD$_{67}$ and GABA (B, C). GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer.
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Image processing

To produce figures for publication, photomicrographs of stained sections were scanned with an Optronics Photocan P-1000 microdensitometer at a 25-μm resolution and imported to Adobe Photoshop. Images were then cropped, contrast enhanced (analogous to printing on paper of a chosen contrast in the darkroom), and imported to Adobe Illustrator for mounting and labeling. All manipulations on the digitized images were applied equally to the entire image.

RESULTS

Colocalization of GABA and GAD

The first set of experiments was designed to confirm the specificity of our antibodies for GAD. This was done by double labeling for each of the GAD forms and GABA. In both cases, all GAD-immunoreactive amacrine cells also stained for GABA, indicating that GABA is synthesized and GAD is present in these cells. Figure 1 depicts this colocalization in a triple-labeling experiment for GAD_65, GAD_67, and GABA.

Of the GABA-immunoreactive amacrine cells, 56% (157/280) also stained for GAD_65. Lower antibody concentration (1:100) resulted in a very low signal in somas. Higher concentration (1:5) did not increase the stained fraction. The percentage of immunoreactive amacrine cells staining for GAD_67 varied with the antibody source. When the antibody from Dr. Kaufman was used, 57% (152/268) of the GABA-immunoreactive cells also stained for GAD_66, but when the antibody from Chemicon was used (preabsorbed with GAD_66) this percentage was 92% (373/403). The percentages for each of the two GAD forms taken separately sum to more than 100%, indicating that some cells express both GAD forms.

Most GABAergic amacrine cells express GAD_67 and half express both forms

Simultaneous staining for the two GAD forms confirmed the above prediction by revealing three subsets of amacrine somas: somas stained for GAD_65 only, somas stained for GAD_66 only, and somas stained for both (Fig. 2). The existence of these subsets was confirmed by the staining pattern in the inner plexiform layer (IPL): GAD_65 was restricted to strata 1–4 and GAD_66 was expressed at all levels (Fig. 3), with the highest concentration in strata 1 and 5 (Vardi et al., 1994). In addition, confocal optical sections of strata 1–4 show regions expressing GAD_65 only (especially in the border between INL and IPL) and regions expressing GAD_66 only, as well as regions expressing both forms.

In these experiments (staining simultaneously for both GAD forms), 62% of all somas stained for GAD stained for GAD_66 and either 50 or 90% stained for GAD_67, depending on the source of antibodies (see below). These percentages of GAD_65/GAD_total and GAD_66/GAD_total are consistent with the percentages of GAD_65/GABA_total and GAD_66/GABA_total reported above. The change in the number of GAD_67 immunoreactive cells from about 50% to about 90% resulted from a change in the source of the antibodies employed. Although the antiserum to GAD_67 obtained from Chemicon should have been identical to that obtained directly from Dr. Kaufman, this did not turn out to be the case. When used side by side in the same experiment, the antiserum from Chemicon labeled a much larger number of cells than did that from Dr. Kaufman. The increase in GAD_67-stained somas (using antibodies from Chemicon) was accompanied by an increase in the percentage of cells stained for both GAD_65 and GAD_67 and a decrease in the percentage of cells stained exclusively for GAD_65 (Table 1). From this we conclude that cells expressing relatively low levels of GAD_67 also express GAD_65.

Controls. The relatively high level of colocalization of the two GAD forms found with the Chemicon antiserum for GAD_67 raised the possibility that the concentration of the Chemicon antibodies was higher in our experiments than the Kaufman antibodies (even though it was used at a 5-times higher dilution) and crossreacted with GAD_66. We therefore preabsorbed the antibody for GAD_67 with GAD_66 protein prior to incubation, but the percentages of cells staining for GAD_67 did not change significantly (Table 2). We also excluded crossreactivity by observing that certain cells stained brightly for GAD_66 but not for GAD_67. Specifically, neither interplexiform cells nor bipolar cells stained for GAD_67, although they stained strongly for GAD_66. In addition, colchicine treatment, which elevated the percentage of somas stained for GAD_66 to 67% (Tables 1, 2), did not change the percentage of GAD_67-stained cells.

We conclude that (1) most GABAergic amacrine cells express GAD_67; (2) about half express a relatively lower level of GAD_67, and these also express GAD_66; (3) interplexiform cells, a subtype of cone bipolar cells, and a few amacrine cell types express only GAD_66; and (4) GAD_66 in a cell's processes is expressed at higher concentrations than in its soma (inferred from weaker soma staining with low antibody concentration and the elevation of this staining by colchicine treatment).

Identification of GAD form in specific amacrine cell types

Many amacrine cells immunoreactive for GABA also contain an additional neurotransmitter. We used this feature to identify several types of GABAergic amacrine cell and the GAD form they express.

Amacrine synthesize NO express GAD_65. We used an NADPH-diaphorase assay (as detected with nitro blue

<table>
<thead>
<tr>
<th>Source</th>
<th>% 65/GAD</th>
<th>% 67/GAD</th>
<th>% Colocalization</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaufman</td>
<td>10</td>
<td>37</td>
<td>14</td>
<td>30</td>
</tr>
<tr>
<td>Chemicon</td>
<td>10</td>
<td>33</td>
<td>57</td>
<td>455</td>
</tr>
</tbody>
</table>

1Numbers are percent amacrine somas stained for GAD_65 only, GAD_66 only, and both (% colocalized) as a fraction of total number of amacrine somas stained for GAD.
2Data combined from three experiments.
3Data is from a colchicine pretreated retina.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%GAD_65/GAD</th>
<th>%GAD_67/GAD</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>61</td>
<td>95</td>
<td>96</td>
</tr>
<tr>
<td>Colchicine</td>
<td>47</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>Colchicine + preabsorbed</td>
<td>43</td>
<td>343</td>
<td></td>
</tr>
</tbody>
</table>

1All data are from the same experiment. In this particular experiment % GAD_65 stained somas was unusually low; however, the experiment demonstrates the enhancement of GAD_65 staining due to colchicine and the lack of effect of preabsorption on GAD_65 staining.
tetrazolium) to identify neurons containing NO synthase (Dawson et al., 1991) and then stained for the GAD forms. All of these cells (comprising at least three cell types; Wässle et al., 1987a; Vaccaro et al., 1991; personal observation) expressed GAD<sub>67</sub> but not GAD<sub>56</sub> (Fig. 4).

**Cholinergic amacrine cells express both GAD<sub>56</sub> and GAD<sub>67</sub> but at a low level.** In retinas not treated with colchicine, amacrine somas immunoreactive for choline acetyltransferase stained for neither GAD<sub>56</sub> nor GAD<sub>67</sub> (not shown), but most of them did stain lightly for GABA (Fig. 5A,B). When the retina was pretreated with colchicine to enhance accumulation of newly synthesized protein in the soma, however, a small percentage of cholinergic amacrine somas did stain for GAD<sub>56</sub> (Fig. 5C,D) and GAD<sub>67</sub> (Fig. 5E,F). This includes both the on and off (displaced and nondisplaced) types of cholinergic amacrine cell. Apparently, levels of both GABA and GAD in cholinergic amacrine cells are lower than in most GABA-immunoreactive amacrine cells.

**Dopaminergic amacrine express GAD<sub>67</sub>**. Figure 6 shows that amacrine cell somas immunoreactive to tyrosine hydroxylase stain for GAD<sub>67</sub> and not for GAD<sub>56</sub>. This is consistent with the observation mentioned above that stratum 1, especially its outer substratum where the dopaminergic A18 cell stratifies (Türk and Stone 1979; Oyster et al., 1985; Voigt and Wässle, 1987), stains primarily for GAD<sub>67</sub> (Fig. 3).

**Most serotonin-accumulating amacrine express GAD<sub>67</sub> and others express GAD<sub>56</sub>.** Amacrine cells stained for preloaded 5-HT consist of more than two cell types (Wässle et al., 1987b), but these have not been differentiated. Thus, when the retina was double labeled for 5-HT and GAD<sub>56</sub> (Fig. 7A,B), about 10% of the 5-HT-accumulating cells also stained for GAD<sub>56</sub>. When the retina was double labeled for 5-HT and GAD<sub>67</sub> (Fig. 7C-F), most of the cells were double labeled but a few (about 10%) did not stain for GAD<sub>67</sub>. Because all somas that stained for 5-HT also stained for
GABA (Wässle and Chun, 1988; personal observation), cells not stained for GAD_67 must be those that stain for GAD_68.

**Approximately 45% of amacrine cells are GABAergic**

The percentage of amacrine cells estimated to be GABAergic has been reported to be about 30% when using methods such as GABA accumulation (Freed et al., 1983; Pourcho and Goebel, 1983) and immunoreactivity for GAD (Bolz et al., 1985) and GABA (Pourcho and Owczarzak, 1989; Wässle and Chun, 1989). These estimates appear low because, to date, every amacrine cell type shown to contain a transmitter other than glycine also contains GABA. This includes cells containing acetylcholine (Chun et al., 1988), NO (Müller et al., 1988), substance P (Pourcho and Goebel, 1988; Vaney et al., 1989), vasoactive intestinal peptide (VIP) (in rabbit: Casini and Brecha, 1992), dopaminergic (Wässle and Chun, 1988), and cells that accumulate serotonin (Wässle and Chun, 1988; in rabbit: Massey et al., 1992). Glycnergic amacrine cells have been estimated to account...
DISCUSSION

Different localization and possible function of the two forms of GAD

The major aim of this study was to identify the GAD forms expressed in specific retinal cell types to relate this information to the known morphological and anatomical features of these cells. We found two features that tend to be associated with the expression of GAD$_{65}$ and not GAD$_{67}$. One is the copresence of GABA and another neurotransmitter and the second is a relatively large dendritic field size (Fig. 8). GAD$_{65}$ was expressed primarily in small neurons not known to contain another neurotransmitter (a subtype of cone bipolar cell), the GABAergic interplexiform cell, and monkey horizontal cells. In contrast, GAD$_{67}$ was expressed primarily in larger neurons, many of which colocalize GABA with another neurotransmitter (dopamine, acetylcholine, and NO). In addition, the compartmentalization reported for brain, that GAD$_{65}$ concentrates in synaptic terminals and GAD$_{67}$ concentrates in soma and dendrites (Erlander et al., 1991; Kaufman et al., 1989; Mercugliano et al., 1992), appears to hold true for retina as well.

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*This type of cone bipolar may also contain glutamate. Because glutamate is a precursor for GABA, it may not function for neurotransmission in this cell.*

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**Fig. 5.** Cholinergic cells express low levels of GABA, GAD$_{65}$, and GAD$_{67}$. Choline acetyltransferase (ChAT) immunoreactivity is visualized with DAB and GABA/GAD immunoreactivity with fluorescent markers. A,B: Double staining for ChAT and GABA. Only a low level of GABA was revealed in the ChAT-immunoreactive soma (arrows) compared with other GABA-immunoreactive cells, which appear brighter. C,D: Double staining for ChAT and GAD$_{65}$. Colocalization is apparent in one cell (arrow, lower right), whereas two more somas were only lightly labeled (arrows, center and lower left). E,F: Double labeling for ChAT and GAD$_{67}$. Approximately half of the amacrine cells staining for ChAT also stained for GAD$_{67}$ (arrows). bv, blood vessel. Scale bars = 20 μm.
Several hypotheses regarding the differential function of the two GAD forms have been offered based on the difference in compartmentalization of the two GAD forms and the different dependence of their activity on cofactor pyridoxal-P (reviewed by Erlander and Tobin, 1991; Martin and Rimvall, 1993). Two of these are particularly attractive because they can be related to our findings. The first suggests that GAD_{65} is used to produce GABA in synaptic terminals for conventional vesicular release, and GAD_{67} is used to produce GABA throughout the cell by employing a transporter mechanism (Kaufman et al., 1991). The second hypothesis suggests that GAD_{65} may be specialized to respond to short-term changes in demand for GABA release in neuronal activity when short-term regulation is required. GAD_{67} may provide a constitutive level of GABA required to support a tonic or high level of synaptic release (Martin and Rimvall, 1993), and thus constant activity of the enzyme is required. Regulation could be achieved on a long-term basis, possibly through regulation of its mRNA level.

Vesicular vs. transporter release. This hypothesis is consistent with our finding that the interplexiform cell and the cone bipolar cell, which use vesicular release, express GAD_{65}, whereas cat horizontal cells, which presumably release GABA through a transporter (Ayoub and Lam, 1984; Schwartz, 1987), express GAD_{67}. In addition, because most amacrine cells that colocalize GABA with another transmitter express GAD_{67}, it is possible that vesicular release is reserved for the other transmitter and that GABA in these cells is released through a transporter. One exception to this hypothesis is the differential expression of GAD by cat and monkey horizontal cells (Vardi et al., 1994). At present, we have no reason to believe that cat and monkey horizontal cells employ a different release mechanism.

Short- vs. long-term regulation. The correlation between dendritic field size and GAD form reported here could support this hypothesis if we assume that small neurons that perform local operations in space are subjected to faster changes (e.g., because of eye movements). In these neurons, short-term regulation of GAD_{65} would be adequate. Larger neurons may have a greater metabolic demand and greater requirement for GABA release. Such demand could possibly be met by GAD_{67}, which is present largely as active holoenzyme. It is worth noting that horizontal cells are more depolarized in the dark than in the light, and therefore release more GABA in the dark. Because cats are nocturnal animals, their horizontal cells probably have a greater demand for GABA release than
**Fig. 7.** A small percentage of 5-HT accumulating amacrine cells express GAD_{65}, and a large percentage of these cells express GAD_{67}. A, B: Double staining for 5-HT and GAD_{65}; arrows point to a soma stained for both. C–F: Double staining for 5-HT and GAD_{67}. In C and D, all somas stained for 5-HT also stained for GAD_{67} (arrows). In E and F, one soma is stained for 5-HT but not for GAD_{67} (arrowhead), whereas another soma is stained for both (arrow).

**Fig. 8.** Relationship between dendritic field diameter and GAD form. Dendritic field diameter for glycineric amacrine cells (Gly; Pourcho and Goebel, 1983) is also plotted. A (+) or (−) sign under a cell type indicates colocalization or lack of colocalization with another neurotransmitter. The lines indicate the range of dendritic field diameters for each cell type. Sizes were taken from the following references: MHC, monkey horizontal cell (Wässle et al., 1989); CBP, cone bipolar (largest bipolar from Kolb et al., 1981); IPC, interplexiform cell (Boycott et al., 1987); CHC, cat horizontal cell (Boycott et al., 1978); ChAT, cholinergic cells (rabbit: Tauchi and Masland, 1985; Pourcho and Osman, 1986; Schmidt et al., 1987); NO, cells expressing NADPH-diaphorase activity (Vaccaro et al., 1991; personal observation); TH, dopaminergic amacrine A18 (Kolb et al., 1981; Voigt and Wässle, 1987).
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Macaque horizontal cells, which operate primarily in the daytime. The colocalization of GAD67 with other neurotransmitters may also support this hypothesis because it is very likely that GABA in these cells is not mediating a simple, fast, inhibitory process (e.g., object detection) but rather a more complicated and slower process that might be associated with the state of retinal adaptation. For example, the dopaminergic cell, which could modulate gap junction conductance in transitions between different light levels (Hampson et al., 1992), contains high levels of GAD67. In addition, the strong immunoreactivity for GAD67 in the layer of the rod bipolar axon terminals and in dopaminergic and NADPH-labeled amacrine cells suggests that this form of the enzyme is primarily involved with modulations of the rod pathway and this pathway is indeed slower than the cone pathway.

Interestingly, there is another correlation between dendritic field size in amacrine cells and their neurotransmitter content. Glycinergic amacrine cells are usually narrower than GABAergic amacrine cells (Pourcho and Goebel, 1985). Thus, it appears that size and transmitter type, as well as transmitter regulation, are highly coordinated.

Other implications for amacrine cells

We estimated that 45% of amacrine cells are probably GABAergic because they express GAD and contain GABA. This is higher than the 25-30% previously reported for cat (Pourcho and Owczarzak, 1989; Wässle and Chun, 1989) and close to that reported for peripheral retinas in monkey (Koontz et al., 1993). We attribute this higher estimate to our use of two antibodies for two different GAD forms and possibly to the use of a high concentration of antibody for GABA (as could be inferred from the bright staining of horizontal cells). The finding that each GAD form alone did not account for all GABA-immunoreactive amacrine cells but both together did explains why in other studies (Hendrickson et al., 1985; Yazulla, 1986; Moseing and Yazulla, 1987; Agardh et al., 1987) the amacrine cell population immunoreactive for GAD was generally smaller than the population immunoreactive for GABA. This result also supports the third form of GAD (see Wu et al., 1973) is unlikely to be present in retina (unless it colocalizes with GAD65 and GAD67).

With the fraction of GABAergic amacrine cells estimated to be 45% and the glycinergic amacrine cells 50% (Jäger and Wässle, 1987; Pourcho and Goebel, 1987), virtually all amacrine cells in cat are now accounted for. This was verified here by double staining for GABA and glycine: only about 5% of the amacrine cells did not stain for either neurotransmitter (data not shown; see also Pourcho and Owczarzak, 1991). We suggest that most of these are the cholinergic neurons, which expressed only a low level of GABA and therefore can go undetected. That GABA and glycine account for almost all amacrine cells is also true in monkey (70-95% for GABA and glycine combined: Koontz et al., 1993) and human (50% glycine: Marc and Liu, 1985; 60% glycine and 40% GABA: Crooks and Kolb, 1992; but also 42% glycine and 26% GABA: Davanger et al., 1991) and thus seems to be a general feature of the vertebrate retina.

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LITERATURE CITED


