Proc. Natl. Acad. Sci. USA
Vol. 76, No. 7, pp. 3557–3561, July 1979
Neurobiology

The γ-aminobutyric acid system in rabbit retina: Localization by immunocytochemistry and autoradiography

(γ-aminobutyric acid uptake/protein A-peroxidase-antiperoxidase method/amacrine cells

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Communicated by P. D. Boyer, April 26, 1979

ABSTRACT **The localization of γ-aminobutyric acid (GABA) neurons in the rabbit retina has been studied by immunocytochemical localization of the GABA-synthesizing enzyme L-glutamate decarboxylase (L-glutamate 1-carboxy-lyase, EC 4.1.1.15) and by [^{3}H]GABA uptake autoradiography. When Triton X-100 was included in immunocytochemical incubations with a modified protein A-peroxidase-antiperoxidase method, reaction product was found in four broad, evenly spaced laminae within the inner plexiform layer. In the absence of the detergent, these laminae were seen to be composed of small, punctate deposits. When colchicine was injected intravitreally before glutamate decarboxylase staining, cell bodies with the characteristic shape and location of amacrine cells were found to be immunochemically labeled. Intravitreally administered [^{3}H]GABA produced a diffuse labeling of the inner plexiform layer and a dense labeling of certain amacrine cell bodies in the inner nuclear layer. Both immunocytochemical and autoradiographic results support the notion that certain, if not all, amacrine cells use GABA as their neurotransmitter.**

Evidence has accumulated to support the idea that γ-aminobutyric acid (GABA) is a neurotransmitter in the vertebrate retina (for review, see ref. 1). Both GABA and its rate-limiting synthetic enzyme L-glutamate decarboxylase (GADase; L-glutamate 1-carboxy-lyase, EC 4.1.1.15) have been measured after microdissection of the retina and shown to be concentrated in the inner plexiform layer (2, 3). Use of immunocytochemical techniques for the visualization of GADase has permitted an even more accurate localization of GABA neurons. By using this approach, GADase has been localized to H_1 horizontal cells and certain amacrine cells of the goldfish retina (4). In mammals, Barber and Saito (5) demonstrated a wide band of GADase-positive immunochemical reaction product throughout the inner plexiform layer of the rat, which appeared to be subdivided into an indeterminate number of narrower bands. On the basis of preliminary electron microscopic examination, Wood *et al.* (6) suggested that the stained terminals were those of certain amacrine cells.

Among mammalian retinas, the rabbit retina has been the most extensively studied (7, 8). In addition, the development of a satisfactory isolated retinal preparation has made possible quantitative biochemical, physiological, pharmacological, and biochemical studies of this retina *in vitro* (9–12). More recently, Caldwell and Daw (13–15) have also developed a system for pharmacological studies of the rabbit retina *in vivo*. In view of these developments, we have begun immunocytochemical studies of neurotransmitter-synthesizing enzymes in the rabbit retina, and we describe here the localization of GADase in this retina. In addition, the results of autoradiographic studies on the uptake of GABA are presented and compared to those reported for various species (16–23).

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MATERIALS AND METHODS

Immunocytochemistry. *Tissue fixation.* To make a normal tissue preparation, eyes were removed from sodium pentobarbital-anesthestized rabbits. In some experiments, colchicine (0.50 mg in 0.50 ml of sterile isotonic saline) was injected intravitreally (rabbit under ether anesthesia) 36–48 hr before the eye was removed. After removal of the cornea, the lens and vitreous humor were gently removed with a cotton-tipped swab, and the entire eye cup was immersed in the periodate/lysine/paraformaldehyde fixative of McLean and Nakane (24). After 4 hr at room temperature, the retina was gently separated from the underlying pigment epithelium and transferred to a fresh solution of 2% formaldehyde/0.12 M phosphate buffer, pH 7.4, and stored overnight at 4°C.

Tissue sectioning. Small pieces of fixed retina (about 3 × 5 mm) were embedded in molten low-gelling-temperature agarose [Miles; 4% in phosphate-buffered saline (25 mM sodium phosphate/0.85% NaCl, pH 7.4)] at about 35°C, chilled for 30 min, and then cross sectioned at 50 μm with a Vibratome; the bathing solution was phosphate-buffered saline..

Staining. Immunohistochemical staining was carried out by using a modification of the peroxidase-antiperoxidase method of Sternberger and coworkers (25) in which the second antibody (goat antiserum to rabbit IgG) was replaced with protein A. This method is based on the ability of the protein A component of the cell wall of *Staphylococcus aureus* to bind to the Fc portion of most mammalian IgG (26). Although somewhat less sensitive then the original, this method has the advantage of decreased "nonspecific" background staining. In separate experiments, staining was carried out both with and without Triton X-100 in the bathing medium. In the former case, the medium was phosphate-buffered saline containing 0.25% Triton X-100; in the latter, it was phosphate-buffered saline containing 0.1% ovalbumin (Sigma, type V). All staining reagents and sera were diluted with these media.

For staining, sections were first agitated for 30 min in either phosphate-buffered saline in a petri dish and then transferred to individual wells of a disposable multiwell titer-plate containing 250 μl of the diluted primary antiserum (one or two sections per well). The anti-GADase serum used here was raised against purified mouse brain GADase; both antigen and antiserum have been extensively characterized (27–29). Optimal dilutions were 1:250 and 1:500. The plate was covered and agitated for 4 hr at room temperature on an orbital rotator and then for an additional 12 hr at 4°C. All subsequent operations were carried out at room temperature. At the end of this time, sections were transferred to individual 15 × 60 mm plastic petri dishes containing about 7 ml of washing medium, agitated on the orbital rotator for 45 min, and then transferred to dishes with fresh medium and washed for an additional 45 min. Sec-

Abbreviations: GABA, γ-aminobutyric acid; GADase, glutamate decarboxylase.

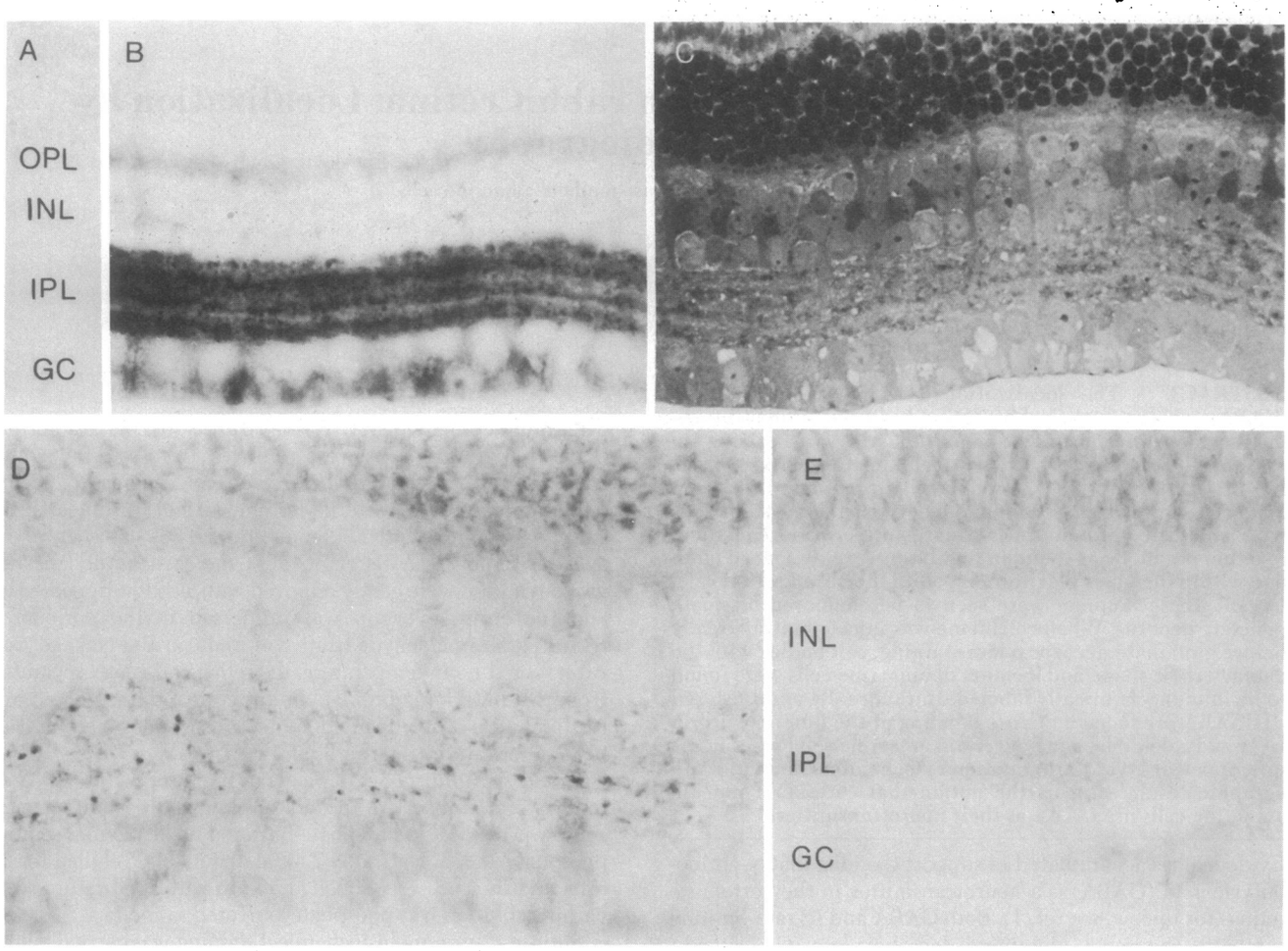

FIG. 1. Lamination of GADase-containing structures in the rabbit retina. OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GC, ganglion cell layer. (*A*) Control section, incubated with preimmune serum in the presence of Triton X-100. (*B*) Fifty-micrometer epoxy-embedded section of Triton-treated, GADase-stained retina. Four dense bands of reaction product fill the IPL; other areas are unstained. (*C*) One-micrometer epoxy embedded section of stained tissue, counterstained with toluidine blue. Laminated reaction product is restricted to the IPL. (*D*) Fifty-micrometer section of retinas stained in the absence of Triton X-100. Lamination of punctate deposits is still discernible. (*E*) Control section, no Triton. (*A–C*, ×400; *D* and *E*, ×750.)

tions were then placed in individual wells of a black porcelain spot dish with 100 μl of protein A solution (Pharmacia; 50 μg/ml) per well and rotated for 1.5 hr in a humid atmosphere. After two more 45-min washes, sections were treated in a spot dish with a 1:50 dilution of peroxidase-antiperoxidase (Sternberger-Meyer Immunocytochemicals, Inc., Jarrettsville, MD) for 1.5 hr, given two more 45-min washes, and then stained for peroxidase. The staining solution contained 30 mg of diaminobenzidine·4HCl and 20 μl of 30% H_2O_2 in 50 ml of phosphate-buffered saline; sections were generally incubated in this medium for 5–8 min.

The stained sections were briefly washed in phosphate-buffered saline, postfixed for 1 hr in 1% glutaraldehyde in phosphate-buffered saline, treated with 1% OsO_4 in phosphate-buffered saline for 2–3 hr, stained *en bloc* with 1% uranyl acetate in 0.1 M sodium acetate, dehydrated with graded ethanols, and infiltrated with Spurr's resin. For embedding, sections were placed in small drops of resin at intervals on a microscope slide that had been treated with dimethyldichlorosilane to prevent epoxy from adhering to it. Another treated slide was placed over the first and, after the resin had spread completely, the sandwich was incubated overnight at 70°C to cure the plastic. After cooling, the slides could easily be pried apart with a razor blade; the result was a 50-μm-thick sheet of epoxy containing the stained sections mounted with the stained face upward, suitable for cover-slipping with oil for light microscopy.

In the absence of detergents, immunocytochemically stained tissue generally showed reaction product only in the few microns near the surface. As a result, semi- or ultrathin sectioning could yield false negative results unless the tissue could be cut absolutely *en face*. Tissue flat-embedded between two treated microscope slides, as described above, could be satisfactorily sectioned *en face* by removing one slide and gluing a smooth-faced epoxy block over the embedded section with cyanoacrylate glue (Super-Glue), curing the glue for 1 hr at 60–70°C, and then breaking the section off from the remaining slide (30). It was generally possible to section such blocks serially through both stained surfaces.

Autoradiography. [^{3}H]GABA [New England Nuclear; specific activity 45.3 Ci/mmol (1 Ci = 3.7 × 10^{10} becquerels)], 100 μCi in 50 μl of isotonic saline solution, was injected through the sclera into the vitreous body of a light-adapted rabbit eye. After 2 hr, the animal was anesthetized with pentobarbital, and the eye was enucleated. Isolated retinas and eyecups were fixed in 3% glutaraldehyde/0.10 M sodium phosphate buffer, pH 7.4, for 1 hr at room temperature and overnight at 4°C, osmicated, dehydrated in graded ethanols, and embedded in Epon. Three-micrometer sections were coated with Kodak NTB-2 emulsion and developed in Dektol after 1 week of exposure (21). The sections were counterstained with toluidine blue.

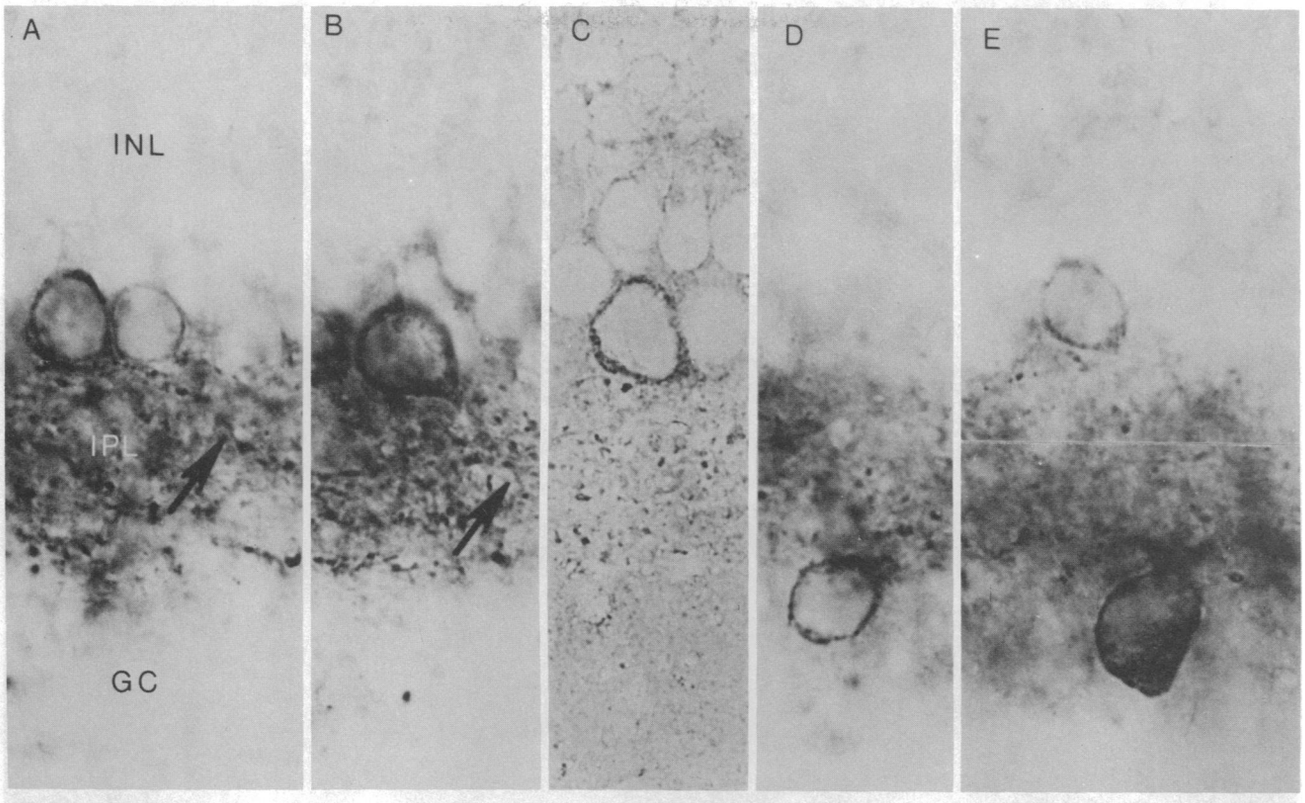

FIG. 2. Montage showing several cell bodies in a colchicine-treated rabbit retina stained immunohistochemically for GADase. Triton X-100 was omitted from staining reactions in order to get a more discrete staining. (*A* and *B*) Fifty-micrometer epoxy-embedded sections, showing stained amacrine cell processes descending into the IPL (arrows). (*C*) One-micrometer epoxy-embedded section through a labeled cell body. (*D*) A probable misplaced amacrine cell body, positively stained, in a 50-μm section. (*E*) Upper cell is an amacrine cell. The lower one is probably a misplaced amacrine; its processes ramify in the innermost layer of the IPL. (×1000.)

RESULTS

Immunocytochemistry. When GADase immunocytochemical localization was carried out in the presence of 0.25% Triton X-100 to enhance antibody penetration, the pattern shown in Fig. 1 *B* and *C* was obtained. In 50-μm epoxy whole mounts dense GADase-positive deposits formed four broad laminae within the inner plexiform layer (Fig. 1*B*). The laminae were of approximately equal intensity and were evenly spaced throughout the layer. Control sections, incubated with preimmune sera, showed no staining (Fig. 1*A*).

In 1-μm plastic sections (Fig. 1*C*) these dense laminae were seen to be formed by the superposition of many small, punctate deposits of cytochemical reaction product in structures which, even at this thickness, showed some lamination. The toluidine blue counterstain confirmed that reaction product was confined to the inner plexiform layer.

When Triton X-100 was omitted from the staining solutions, GADase-positive reaction product was observed in much smaller, more discrete deposits and was restricted to the surface and a few micrometers into each section. In these sections, some lamination was still discernible but was less distinct than in Triton-treated tissue (Fig. 1*D*). No staining was present in control sections incubated in preimmune rabbit serum (Fig. 1*E*).

The high concentration of GADase within axon terminals permitted its ready visualization there by antibody staining, even at 1:1000 dilutions of the primary antiserum. However, in cell bodies and axons, normal GADase levels were apparently too low for reliable localization. Ribak *et al.* (31) first showed that *in vivo* adminstration of colchicine before staining caused GADase to accumulate within cell bodies and axons due to a blockage of axon transport; the accumulated enzyme could then be visualized immunocytochemically. In order to visualize the cell bodies corresponding to the stained GADase-containing inner plexiform layer terminals, localization experiments were carried out using retinas from rabbits that had been injected intravitreally with colchicine 36 hr before enucleation. Under these conditions, stained cell bodies were routinely observed in the inner nuclear layer (Fig. 2). Except as noted below, these cell bodies were always located in the vitreal third of the inner nuclear layer and had the general unipolar morphology of amacrine cells. Often, partially stained processes were visible emerging from the cell body; by through-focusing in the light microscope, these could always be traced descending into the inner plexiform layer, but never in the opposite direction. On rare occasions, small stained cells with the shape of amacrine neurons were found within the ganglion cell layer (Fig. 2 *D* and *E*); their shape, location, and the absence of stained axons suggest that these neurons were displaced amacrine cells, although the cell in Fig. 2*E* may be a ganglion cell. The ratio of stained soma in the inner nuclear layer and in the ganglion cell layer was roughly 10:1. A 1-μm plastic section (Fig. 2*C*) containing such stained cells in the inner nuclear layer showed that reaction product was present within the perinuclear and apical cytoplasm, as expected for a protein accumulating at its site of synthesis.

Autoradiography. After uptake of [^{3}H]GABA *in vivo* by rabbit retina, silver grains were observed in a broad band over the full depth of the inner plexiform layer (Fig. 3); there was little if any apparent lamination within this band. In addition, numerous cell bodies within the inner half of the inner nuclear layer showed heavy labeling. Occasional cell bodies in the

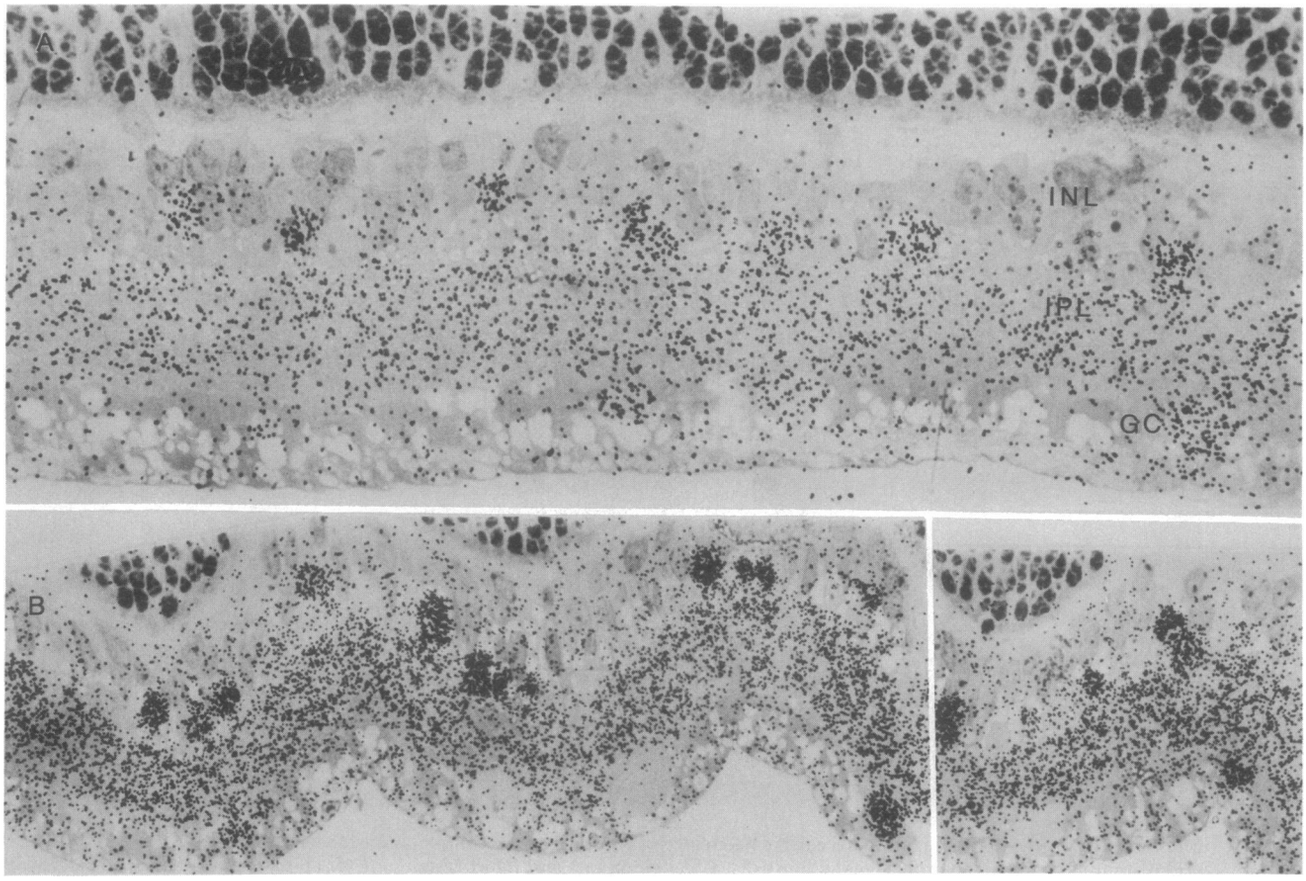

FIG. 3. Bright-field micrographs of autoradiograms of rabbit retina incubated *in vivo* with [^{3}H]GABA. Silver grains appear diffusely over the IPL, over amacrine cell bodies in the INL, and over some cell bodies in the GC. The outer plexiform layer is unlabeled. (*Upper*, ×725; *Lower*, ×650.)

ganglion cell layer were also labeled, although many were clearly devoid of grains (Fig. 3 *lower*). The outer plexiform layer and horizontal cell layer were relatively free of label. The different grain densities of the areas shown in Fig. 3 probably reflect the different distances of these areas from the site of injection of [^{3}H]GABA.

DISCUSSION

The observed pattern of lamination of GADase-positive terminals within the inner plexiform layer of the rabbit bears a striking resemblance to the lamination of amacrine cell processes in Golgi-impregnated retinas, in which one indistinct and four distinct laminae are clearly formed by the sharply laminated terminal processes of amacrine cells (32). On the basis of the shape of the observed GADase-positive cell bodies and their processes and on the striking lamination of their processes in the inner plexiform layer, we conclude that the stained terminals that we have described here are those of a population of amacrine cells that use GABA as their neurotransmitter. This interpretation is supported by the electron microscopic evidence of Wood *et al.* (6) and Vaughn *et al.* (33) in the GADase-stained rat retina. In a preliminary abstract (33), the latter workers described GADase-positive deposits within synaptic endings with the characteristic synaptic organization of amacrine cell terminals. Forming conventional synapses, they were presynaptic to bipolar, amacrine, and ganglion cell processes and postsynaptic to bipolar and amacrine cell processes. They were also occasionally postsynaptic to bipolar cell endings, in dyad configurations.

Lamination of amacrine cell processes has also been reported for putative dopaminergic neurons. Using a catecholamine fluorescence technique. Ehinger (34) observed brightly fluorescent amacrine cell bodies and at least three fluorescent laminae within the rabbit inner proximal layer. Nichols and Koelle (35) have also reported lamination of amacrine terminals and visualization of cell bodies after staining retinas of several species for acetylcholinesterase. In addition, autoradiographic studies have demonstrated the presence of high-affinity uptake sites for the putative neurotransmitters dopamine, glutamate, glycine, and taurine (36, 37). Finally, in the goldfish retina, autoradiographic studies have demonstrated that GABA, glycine, and dopamine are taken up by functionally and morphologically different populations of amacrine or interplexiform cells or both (36–38). Taken together, the above data suggest that several subpopulations of amacrine cells exist, each of which uses a different transmitter substance.

Recent pharmacological studies by Caldwell and Daw (13–15) have shed some light on this division of neurochemical labor. These workers infused strychnine, a glycine antagonist, or picrotroxin, a GABA antagonist, into the arterial blood supply of the eye of the intact rabbit, while recording extracellularly the responses of retinal ganglion cells to complex light stimuli. The effect of both drugs on cells with complex receptive fields was to simplify those fields. However, different aspects of the responses were affected by the two drugs. Strychnine diminished the time course of the transient portions of ganglion cell responses. Picrotoxin, on the other hand, abolished direction- and orientation-sensitivities and other responses attributed by these workers to lateral interactions within the inner plexiform layer. It is these lateral inhibitory interactions that seem to be

mediated through the system of laminated terminals visualized by GADase staining within the inner plexiform layer. Detailed electron microscopic analysis of these GADase-containing terminals may therefore lead to further understanding of the neuronal pathways mediating direction- and orientation-sensitivity in this retina.

The results from autoradiographic experiments reported above agreed at the light microscope level with the pattern of GADase immunocytochemical staining showing uptake into neuronal cell bodies and into structures within the inner plexiform layer. Similar labeling has been reported by Ehinger (17) in the same system. However, in the rat (18, 19), guinea pig, goat, and cat (20), uptake of [^{3}H]GABA was almost exclusively into Müller (glial) cells. In lower vertebrates, such as the goldfish (21), frog (22), pigeon, and chicken (23), [^{3}H]GABA uptake occurred exclusively into neuronal elements. It is of interest that most of the above experiments, except those of Ehinger (17), were carried out *in vitro*. Following our own *in vitro* studies of [^{3}H]GABA uptake in the rabbit retina (data not shown), autoradiographs showed accumulations of grains principally over Müller cells rather than neurons, in a pattern similar to that reported for the rat and other species. We interpret such a pattern as that of a lower-affinity uptake system for GABA which is operational, or at least detectable, only when the neuronal transport system has been impaired or has ceased to function due to neuronal death or damage. Such damage would be more likely to occur *in vitro* for mammalian retinas than those of the more resilient lower vertebrates and would be much less likely under the *in vivo* conditions used here for the rabbit retina.

It is difficult to discern reasons why subpopulations of amacrine cells subserving different information-processing functions should require different neurotransmitters, although this now appears to be the case. Studies involving simultaneous, separate visualization of two or more different neurotransmitter systems may shed light on this complex problem.

The technical assistance of Patricia Glazebrook is gratefully acknowledged. This work was supported by grants from the National Institutes of Health (NS-13224 and EY-02423) and the Huntington's Chorea Foundation. D.M.K.L. is recipient of a Research Career Development Award.

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