

Dopaminergic and Indoleamine-Accumulating Amacrine Cells Express GABA-Like Immunoreactivity in the Cat Retina

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Colocalization of indoleamine uptake and GABA-like immunoreactivity was studied in the cat retina. Consecutive, semithin sections were incubated in antisera to either 5-HT (5-hydroxytryptamine) or GABA. More than 90% of all 5-HT-accumulating amacrine cells expressed GABA-like antigens. With the same approach, the colocalization of 5-HT uptake and GABA-like immunoreactivity was studied in rabbit and 75–80% of the 5-HT-accumulating amacrine cells expressed GABA-like immunoreactivity, thus confirming a previous study (Osborne and Beaton, 1986). Since, in both cat and rabbit, endogenous 5-HT could not be found by immunocytochemistry, one must consider the possibility that some GABAergic amacrine cells take up indoleamines.

In the cat retina, antibodies against tyrosine hydroxylase (TH) label dopaminergic amacrine cells (Oyster et al., 1985). By incubating consecutive, semithin sections in antisera to either TH or GABA, it was found that 84% of the dopaminergic amacrine cells also expressed GABA-like immunoreactivity. GABA-like immunoreactivity and ³H-muscimol uptake were found to be colocalized in more than 90% of the amacrine cells labeled. However, dopaminergic amacrine cells did not accumulate ³H-muscimol.

Evidence is presented from colocalization studies for 2 types of interplexiform cell in the cat retina. One is stained by GABA-like immunocytochemistry and by ³H-muscimol uptake. The other is the dopaminergic amacrine cell, which also expresses GABA-like immunoreactivity, but does not accumulate ³H-muscimol.

Dopamine, serotonin (5-HT), and GABA have all been reported to be putative neurotransmitters in the mammalian retina (see Massey and Redburn, 1987, for review). However, evidence that they are actually used in retinal function is not equally compelling for the 3 substances.

Dopamine seems to be the predominant catecholamine transmitter in the retina (reviewed by Kamp, 1985), and was localized with a variety of techniques. Dopaminergic neurons in the mammalian retina were identified by Falck-Hillarp formaldehyde-induced fluorescence (Malmfors, 1963); by autoradiography of ³H-dopamine accumulation (Ehinger and Falck, 1971; Kramer

et al., 1971); and by immunocytochemical methods using antibodies against tyrosine hydroxylase (TH) (Hendrickson et al., 1981; Brecha, 1983) or antibodies against dopamine (Geffard et al., 1984). In the cat retina, all these methods have labeled an amacrine cell type that has an unusually large cell body, occurs at low density (0.1% of all amacrines), and has a predominant stratification level close to the amacrine layer in stratum 1 of the inner plexiform layer (IPL) (Törk and Stone, 1979; Pourcho, 1982; Oyster et al., 1985; Voigt and Wässle, 1987).

Indoleamine-accumulating neurons in the cat retina were first reported by Ehinger and Florén (1976). A set of neurons that preferentially took up the 5-HT analog 5,6-dihydroxytryptamine (5,6-DHT) was identified by fluorescence microscopy with the Falck-Hillarp technique. The specific uptake of indoleamines was later confirmed, using either uptake of tritiated 5-HT (Ehinger and Florén, 1980) or immunocytochemical staining of 5-HT-accumulating neurons (Osborne et al., 1982; Tornqvist et al., 1983; Osborne and Patel, 1984). The morphology of 5-HT-accumulating neurons in the cat retina has recently been investigated by intracellular dye injection (Wässle et al., 1987a).

Although minute amounts of 5-HT have been detected in mammalian retinal tissue (Osborne et al., 1982; Tornqvist et al., 1983), endogenous 5-HT has not yet been localized. Thus it remains unknown whether 5-HT is used as a transmitter in the mammalian retina.

By contrast, nonmammalian retinas contain much higher concentrations of endogenous 5-HT, which has been localized by immunocytochemistry in a variety of neurons (Osborne et al., 1982; Tornqvist et al., 1983; Witkovsky et al., 1984; Weiler and Schütte, 1985; Negishi et al., 1986). This, together with physiological evidence, makes 5-HT a likely transmitter in nonmammalian retinas.

The role of GABA as a transmitter in the retina is well established (reviewed by Morgan, 1985). In the cat retina, uptake of tritiated GABA or muscimol and immunocytochemistry with antibodies against glutamic acid decarboxylase (GAD) labeled approximately 30% of all amacrine cells in the inner nuclear layer (Pourcho, 1981; Freed et al., 1983; Pourcho and Goebel, 1983; Bolz et al., 1985) and the majority of displaced amacrine cells in the ganglion cell layer (Wässle et al., 1987b). Recently, immunocytochemistry with antibodies directed against GABA conjugated to bovine serum albumin labeled as many as 40% of all amacrine cells in cat retina (Mosinger et al., 1986). Next to glycine, which is found in 45% of all amacrine cells (Pourcho, 1980; Wässle et al., 1986; Pourcho and Goebel, 1987), GABA is the predominant transmitter of amacrine cells in the cat retina.

There are several lines of evidence suggesting that 5-HT-accumulating neurons of the cat retina might also express GA-

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BAergic markers. In the rabbit retina, it has been shown that some amacrine cells that accumulate the indoleamine 5,7-DHT also express GAD or GABA-like immunoreactivity (Osborne and Beaton, 1986), and since GABA staining in all mammalian retinas is very similar (Mosinger et al., 1986), this makes colocalization also likely for the cat retina. Staining of 5-HT-accumulating amacrine cells with intracellular dye injection revealed 3 types of amacrine cells in the cat (Wässle et al., 1987a). Two of these had a striking similarity in their dendritic branching pattern to that of GABA-accumulating amacrine cells (Pourcho and Goebel, 1983). Immunocytochemical staining of vertical sections for GABA or serotonin accumulation showed a very similar laminar distribution. Amacrine cells and displaced amacrine cells were labeled at a ratio of 3:1 and the whole IPL showed strong immunoreactivity with both markers (Wässle et al., 1986, 1987a, b). Finally, there is ultrastructural evidence for possible colocalization. Nearly all reciprocal synapses with rod bipolar cells in the IPL of the cat retina are labeled by uptake of ^3H -GABA (Freed and Sterling, 1982) and the same synapses may accumulate indoleamines (Holmgren-Taylor, 1982).

Concerning the coexistence of dopamine and GABA, there is a recent report on various regions of the rat brain and the retina showing immunoreactivity for TH and GABA/GAD to be colocalized (Kosaka et al., 1987).

The first part of the present paper describes 5-HT-accumulating amacrine cells of the cat retina stained in semithin sections (1 μm thick) with antibodies against 5-HT. Every other section was stained for GABA-like immunoreactivity to test for the coexistence of both markers within the same neuron. This technique was also applied in the rabbit retina to confirm and quantify the colocalization of 5-HT uptake and GABA immunoreactivity described in this species by Osborne and Beaton (1986).

The second part of the paper addresses the coexistence of TH and GABA-like immunoreactivity in consecutive, semithin sections. Finally, labeling of the retina by GABA-like immunocytochemistry will be compared with uptake of tritiated muscimol, and the coexistence of all 3 markers (GABA, muscimol, TH) will be tested for the dopaminergic amacrine cells.

Materials and Methods

Uptake of indoleamines. Following neurophysiological experiments done for other projects, 3 cats with otherwise normal eyes were used in uptake studies. The animals were deeply anesthetized with 3% halothane in a mixture of 70% nitrous oxide and 30% carbogen (95% O_2 , 5% CO_2). In addition, one normal cat, which had not been in a neurophysiological experiment, was deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (Nembutal; 40 mg/kg). One rabbit was used and was anesthetized by intravenous injection of Nembutal into the ear vein. In all animals, a small incision was made in the sclera, and, under ophthalmic control, 10 μl of a 5-HT solution was injected with a Hamilton syringe at several locations within the vitreous. The 5-HT solution was made up by dissolving 20 mg of 5-hydroxytryptamine creatine sulfate (Sigma) in 10 ml distilled water containing 1% ascorbic acid. In the cat eye, the injected amount (20 μg) would produce a concentration of 2.5×10^{-5} M when homogeneously distributed within the vitreous. Four hours later, the eyes were enucleated under deep anesthesia and the animals were killed by an overdose of Nembutal. A small, encircling cut was made around the ora serrata and the eyecup was placed in the fixative (see below).

Uptake of ^3H -muscimol. *In vitro* uptake of ^3H -muscimol was performed in one cat and one rabbit retina. The eyes were enucleated from deeply anesthetized animals, quickly opened by an encircling cut, and the eyecup was transferred to the perfusion chamber. Immediately after enucleation the animals were killed by an overdose of Nembutal. The eyecup was incubated for 30 min in Krebs bicarbonate solution saturated with carbogen (Lee and Iversen, 1981) at 38°C. It contained ^3H -mus-

cimol (sp act, 17.5 Ci/mmol; New England Nuclear). Subsequently the eyecups were fixed.

Tissue preparation. The eyecups were fixed in 4% paraformaldehyde (PA), 0.05% glutaraldehyde (GA), and 0.2% picric acid in 0.1 M phosphate buffer (PB), pH 7.4. After 30 min fixation, the retinae were dissected from the sclera, cut into small pieces, and fixed for another 2 hr in the same fixative at 4°C. After washing in 0.1 M PB containing 4.5% sucrose, the tissue was dehydrated in a graded series of ethanol, infiltrated in propylene oxide, and embedded in Epon 812. The retinal pieces were oriented before embedding according to whether horizontal or vertical sections were to be taken. After curing for 2 d at 50°C, serial semithin sections were cut at a thickness of 1 μm either horizontally or vertically. The sections were collected on gelatinized slides (0.5% gelatin) and stained for immunocytochemistry.

Immunocytochemical staining procedures. The sections were etched with alcoholic sodium hydroxide for 15 min at room temperature, washed in distilled water for 5 min and in 0.1 M PBS, pH 7.4, for another 5 min. The sections were then treated with 0.5% hydrogen peroxide in absolute methanol for 30 min at room temperature to destroy endogenous peroxidase activity. After three 5 min washes in PBS, the sections were preincubated for 30 min at room temperature in PBS containing 10% normal goat serum (NGS), 1% BSA, and 0.5% Triton X-100 to reduce nonspecific staining. They were then incubated for 2 hr at room temperature or 18–24 hr at 4°C with primary antibodies against GABA or 5-HT, respectively. Anti-GABA IgG was produced from rabbits injected with GABA conjugated to BSA using GA (Storm-Mathisen et al., 1983). It was diluted (1/1000–1/2000) in PBS containing 3% NGS and 1% BSA. Anti-5-HT IgG (Immunonuclear) was diluted (1/200) in PBS containing 5% BSA and 1% NGS. Following three 5 min washes in PBS, the sections were incubated for 1 hr at room temperature with the secondary antibody goat anti-rabbit IgG (Miles) diluted (1/20) in PBS. The sections were then washed in PBS and incubated for 30 min at room temperature with rabbit peroxidase-antiperoxidase (PAP; Miles) diluted (1/50) in 0.1 M PBS containing 1% NGS. Finally, the sections were washed in 0.05 M Tris-HCl buffer (pH 7.6) and incubated with a freshly prepared solution of 0.05% diaminobenzidine tetrahydrochloride (DAB) and 0.05% hydrogen peroxide in the same Tris buffer. The progress of the reaction was monitored on a staining microscope and terminated at the appearance of nonspecific staining. After a final wash in distilled water, the sections were dehydrated in a graded series of ethanol, cleared in xylene, and mounted with Canada balsam.

For TH immunocytochemistry, the rabbit primary antibody (Eugen Tech) was diluted (1/200) in PBS containing 3% NGS, 1% BSA, and 0.5% Triton X-100. The sections were incubated for 2 hr at room temperature or 12–18 hr at 4°C. After three 5 min washes in PBS, the sections were incubated for 1 hr at room temperature with biotin-conjugated goat anti-rabbit IgG (Sigma) diluted (1/50) in PBS. After 3 washes in PBS, the sections were incubated for 30 min at room temperature with the avidin-HRP complex (Sigma) diluted (1/1000) in PBS. The DAB reaction and mounting were performed as described above.

Autoradiography. Following the immunocytochemical staining procedures, sections from retinae preincubated with ^3H -muscimol were washed and dried. They were coated with photoemulsion (Ilford L4, diluted 1/1) and exposed in the dark for 10–30 d at 4°C. They were developed (Kodak D-19), fixed, and mounted.

Specificity tests. The GABA antibody was raised in rabbits against GABA conjugated to BSA with GA. The method of purification and testing was as described in the literature (Storm-Mathisen et al., 1983; Ottersen and Storm-Mathisen, 1984a, b; Ottersen et al., 1986). However, additional tests were performed to show that no cross-reactivity occurred with indoleamines (5-HT) and dopamine (DA) linked to BSA with PA or GA. In an ELISA test, the reaction of the GABA antibody was tested with respect to the GABA-GA-BSA complex, the 5-HT-PA-BSA complex, the BSA-GA conjugate, the DA-PA-BSA complex, and the DA-GA-BSA complex. The antibody only showed a reaction with the GABA-GA-BSA complex; the other antigens were not recognized. Details of this specificity test will be given elsewhere (I. Wulle and J. Schnitzer, unpublished observations).

Specificity was further tested by preabsorbing the anti-GABA antibody with BSA, 5-HT-GA-BSA, or GABA-GA-BSA. Only preabsorption with the GABA-GA-BSA complex abolished the immunostaining obtained in retinal sections with anti-GABA serum.

The specificity of the immunostaining with all 3 antibodies (GABA, 5-HT, TH) was also tested by omitting the incubation with the primary antiserum.

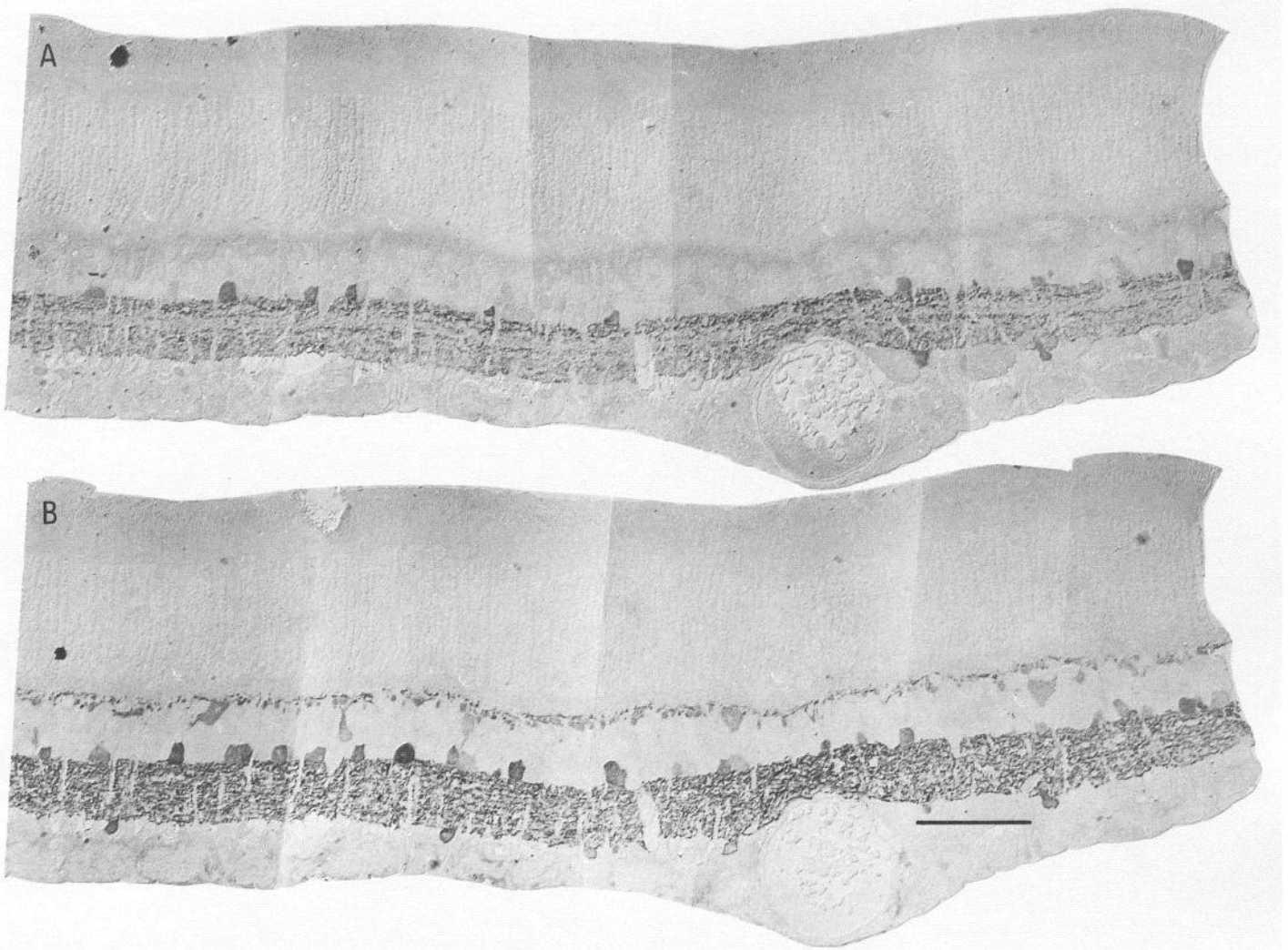


Figure 1. Low-power micrographs of 2 consecutive 1- μ m-thick vertical sections through a cat retina. *A*, Stained for 5-HT-like immunoreactivity; *B*, for GABA-like immunoreactivity. This and all following micrographs were taken with interference-contrast illuminations; hence, not only labeled cells, but also unlabeled cells, became visible, making the comparison between adjacent sections easier. Scale, 50 μ m.

Results

Colocalization of 5-HT uptake and GABA-like immunoreactivity in cat retina

Immunocytochemical staining of normal cat retina with antibodies against 5-HT never revealed any labeled neurons in vertical sections or whole-mount preparations. However, when the retina was preloaded by injecting 20 μ g of 5-HT into the vitreous, consistent labeling was observed (Fig. 1*A*). Amacrine cells in the inner nuclear layer (INL) and displaced amacrine cells in the ganglion cell layer (GCL) were labeled, and the total inner plexiform layer (IPL) was filled with immunoreactive processes. The percentage of labeled amacrine cells varied from 10–20%, apparently the result of a nonuniform 5-HT concentration within the vitreous (Wässle et al., 1987a).

Immunostaining of cat retina with antibodies against GABA labeled more cells than did 5-HT immunocytochemistry. The sections in Figure 1 are consecutive, each 1 μ m thick, and are therefore directly comparable. In the section stained for GABA-like immunoreactivity (Fig. 1*B*) amacrine cells, displaced amacrine cells, and the dense plexus of their processes in the IPL

are labeled. Strong labeling is also present in the outer plexiform layer (OPL), and the cell bodies of horizontal cells showed GABA-like immunoreactivity. Very rarely, bipolar cells were also labeled, as well as some interplexiform cells. Approximately 30% of all amacrine cells in the INL were found to express GABA-like immunoreactivity. This percentage and the cell types labeled were independent of whether 5-HT uptake was performed or whether a normal retina was stained. This would indicate that the GABA antibody recognizes endogenous GABA, which, in contrast to 5-HT, seems to be present in detectable amounts.

A closer look at the consecutive sections of Figure 1, *A*, *B*, shows that all amacrine cells and displaced amacrine cells labeled by 5-HT uptake in Figure 1*A* also express GABA-like immunoreactivity in Figure 1*B*. A small part of Figure 1 is shown at higher magnification in Figure 2, *A–C*. Three consecutive sections were alternately stained for 5-HT or GABA-like immunoreactivity. The 3 amacrine cells strongly labeled in Figure 2*A* by 5-HT uptake can also be detected in Figure 2*B* (arrows); hence, they express GABA-like immunoreactivity. In addition to these 3 cells, another amacrine cell is labeled by 5-HT uptake in Figure 2*C*; this cell also shows GABA-like im-

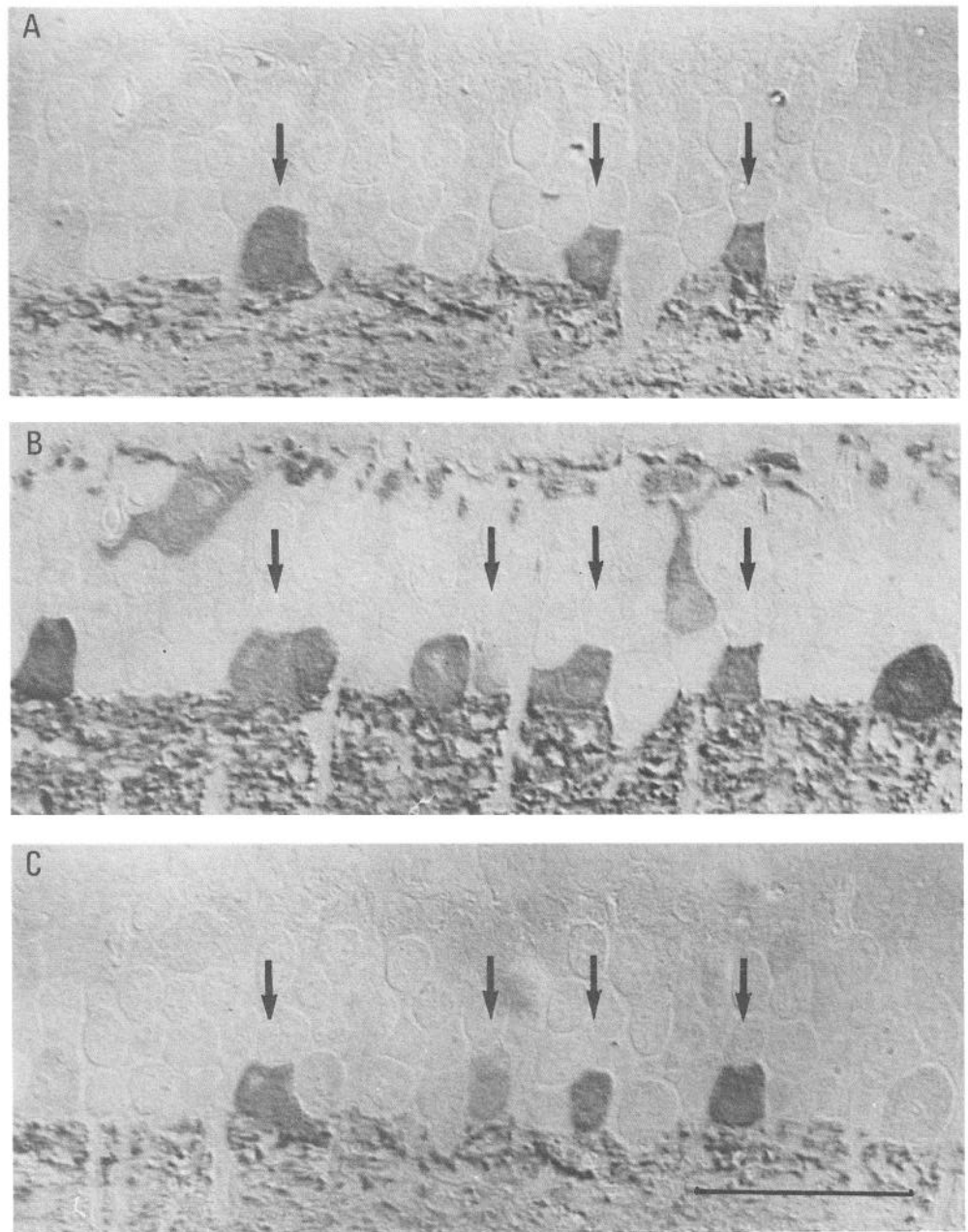


Figure 2. High-power micrographs of 3 consecutive vertical sections through the cat retina. The micrographs show part of the left half of Figure 1. *A, C*, Stained for 5-HT-like immunoreactivity; *B*, stained for GABA-like immunoreactivity. *Arrows*, amacrine cells, which express both GABA- and 5-HT-like immunoreactivity. Scale, 25 μ m.

munoreactivity. Two more amacrine cells, only very weakly labeled by 5-HT uptake in Figure 2, *A, C*, show strong GABA staining in Figure 2*B*. It is clear from Figure 2 that individual amacrine cells can be recognized in consecutive sections of 1 μ m thickness.

In order to test this colocalization on a larger number of amacrine cells, horizontal sections were stained for 5-HT uptake and GABA-like immunoreactivity. Figure 3, *A, B*, shows high-power drawings made from such consecutive horizontal sections through the INL, stained for 5-HT and GABA, respectively. Superimposition of the 2 drawings shows that 85% of all amacrine cells labeled by 5-HT uptake in Figure 3*A* also express GABA-like immunoreactivity in Figure 3*B*.

From 2 cats and 3 large horizontal sections, 879 amacrine cells were labeled by 5-HT uptake. Of these, 92% also expressed GABA-like immunoreactivity. Thus, practically all 5-HT-ac-

cumulating amacrine cells express GABA-like immunoreactivity. When all GABA-positive amacrine cells were counted in the same material and the results compared with the number of 5-HT-accumulating amacrine cells, only 70% of all amacrine cells that expressed GABA-like immunoreactivity took up 5-HT. Double-labeling of horizontal or bipolar cells was never observed.

Colocalization of 5-HT uptake and GABA-like immunoreactivity in rabbit retina.

It has been shown in rabbit retina by Osborne and Beaton (1986) that some amacrine cells that accumulate the indoleamine 5,7-DHT also express GABA-like immunoreactivity. There are 2 morphologically distinct types of serotonin accumulating amacrine cells in rabbit (Sandell and Masland, 1986; Vaney, 1986), and it is not known whether both express GABA-like immu-

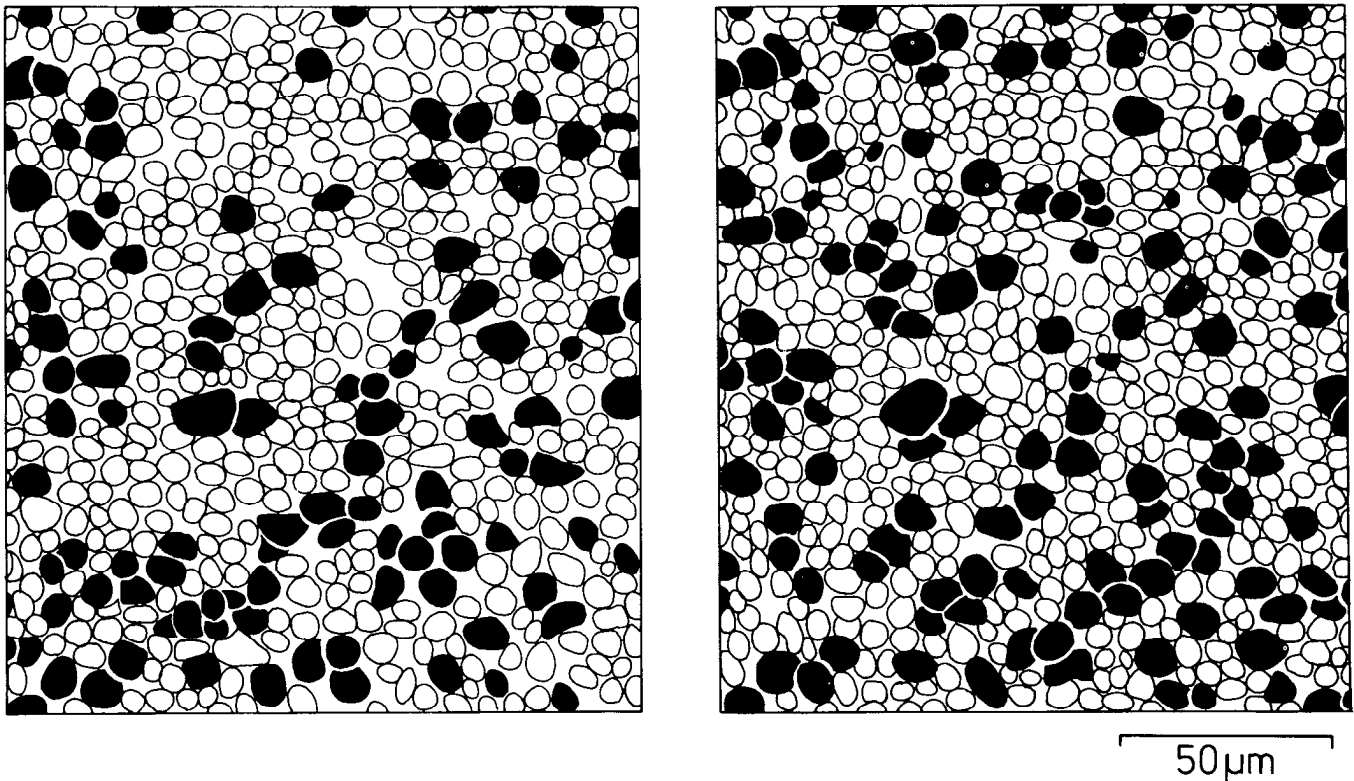


Figure 3. Drawings of all cell bodies found in 2 consecutive horizontal sections through the inner border of the inner nuclear layer. *Filled profiles* represent immunolabeled cell bodies; *open profiles* are unlabeled cells. *Left*, Cells were labeled by uptake of 5-HT. *Right*, Labeled by GABA-like immunoreactivity.

noreactivity. Therefore, a quantitative evaluation of the colocalization of 5-HT uptake and endogenous GABA was performed. Figure 4*A* shows a vertical section of rabbit retina, stained with antibodies against 5-HT following uptake of 5-HT. Five amacrine cells are labeled, and a dense band of labeled dendrites close to the GCL can be observed. The consecutive section was stained for GABA-like immunoreactivity (Fig. 4*B*). Many more amacrine cells and displaced amacrine cells express GABA-like immunoreactivity and their processes fill the whole IPL. Three out of the 5 amacrine cells labeled in Figure 4*A* by 5-HT uptake also contain endogenous GABA (black arrows, Fig. 4*B*). Two of the 5-HT-accumulating cells show hardly any GABA label (open arrows, Fig. 4*B*). To increase the cell sample, consecutive horizontal sections were compared. A total of 273 amacrine cells accumulated 5-HT and 205 of them (75%) also expressed GABA-like immunoreactivity.

Approximately 50–60% of 5-HT-accumulating amacrine cells have been defined morphologically as S1 cells, and the remaining 40–50% as S2 cells (Vaney, 1986). The present result of 75% double-labeling means that both S1 and S2 cells can express GABA-like immunoreactivity. Quite frequently, 2 immediately adjacent 5-HT-labeled cell bodies both expressed GABA-like immunoreactivity as well. Since both S1 and S2 cells are distributed in a regular mosaic, one member of such adjacent pairs is probably an S1 cell and the other an S2 cell.

Colocalization of TH and GABA-like immunoreactivity in the cat retina

Staining the retina with antibodies to TH revealed a population of amacrine cells with rather large cell bodies (15 μm) and prom-

inent dendritic stratification levels close to the INL in stratum 1 of the IPL (Fig. 5*A*). When the consecutive section was stained for GABA-like immunoreactivity (Fig. 5*B*), TH cells were also found to be labeled. This makes it likely that GABA and dopamine are colocalized in these cells. The series of sections and alternating staining is continued through Figure 5, *C* and *D*. There is a consistent difference between TH and GABA-like immunoreactivity: while TH is found only in the cytoplasm and not in the cell nucleus, GABA-like immunostaining is uniform throughout the cell. It is possible that the GABA labeling of the nucleus is an artefact—probably the nuclear membranes were opened by the fixation (de Jong et al., 1987); however, it indicates that the antigens recognized by the 2 antibodies are distributed within the cell in different spatial patterns. Since the density of TH cells is rather low, colocalization was tested with only a limited number of cells from both serial vertical and horizontal sections. Of the 19 TH-immunoreactive cells encountered, 16 also expressed GABA-like immunoreactivity; hence 84% seem to be double-labeled.

Close inspection of Figure 5*D* reveals a small process (arrows) leaving the cell body of a dopaminergic amacrine cell and running towards the OPL. Figure 6*B* shows another example of an ascending dendrite originating from the cell body of a dopaminergic amacrine cell. In addition to these cells, sometimes smaller cells, which also have processes ascending to the OPL (see Fig. 6*B*), were labeled exclusively by GABA immunoreactivity. Such putative GABAergic interplexiform cells have been described previously in ^3H -GABA and ^3H -muscimol uptake studies (Nakamura et al., 1980; Pourcho and Goebel, 1983). Figure 8*B* presents evidence that these small interplexiform cells

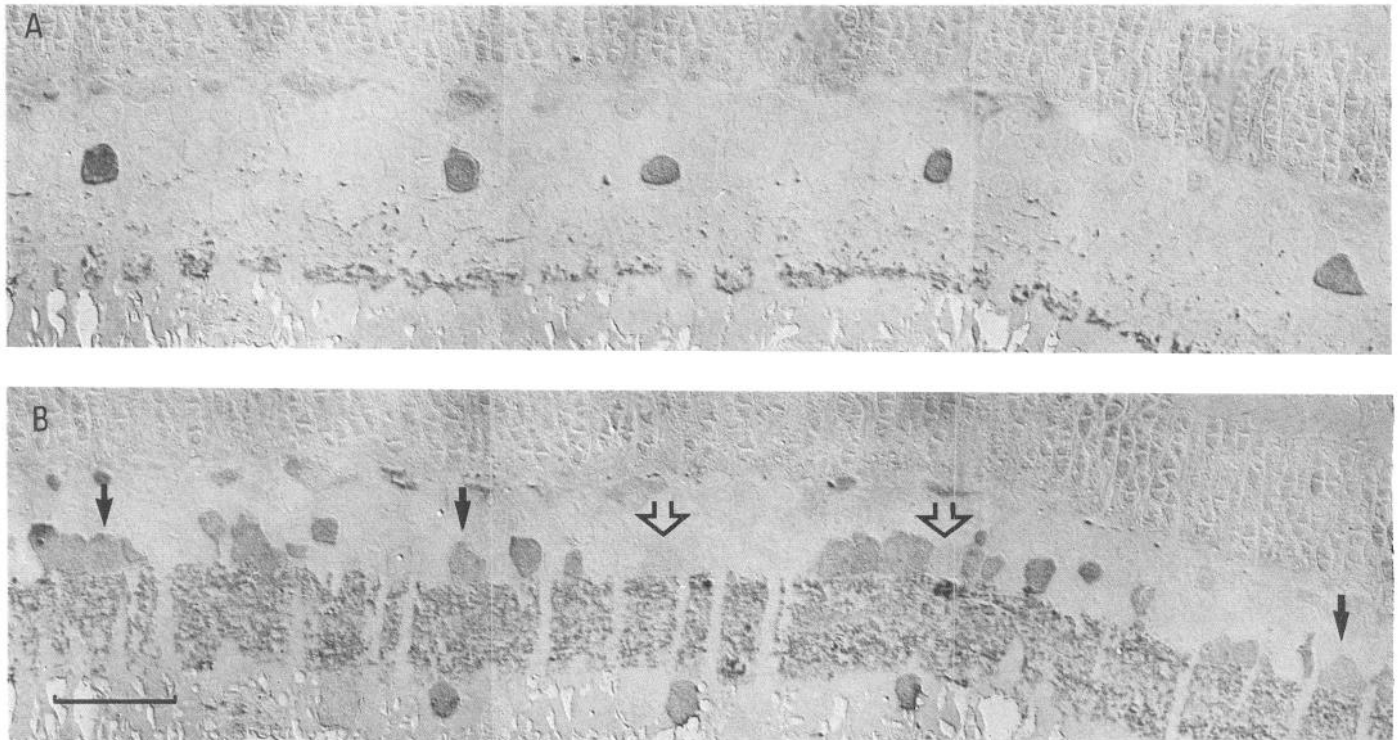


Figure 4. Micrographs from 2 consecutive vertical sections through a rabbit retina. Following uptake of 5-HT, *A* is stained for 5-HT-like immunoreactivity. *B* is labeled by GABA-like immunoreactivity. Arrows in *B*, the 5 cells that were labeled in *A*. Only 3 show a clear GABA-like immunoreactivity. Scale, 25 μ m.

exhibit both GABA-like immunoreactivity and ^3H -muscimol uptake. The ascending processes stained in Figures 5 and 6 present further evidence for 2 types of interplexiform connections in the cat retina (Oyster et al., 1985), one of which seems to be based on the dopaminergic amacrine cell (Boycott et al., 1975), with the second seemingly purely GABAergic (Nakamura et al., 1980) (see also Fig. 8).

^3H -Muscimol uptake and GABA-like immunoreactivity

Uptake of ^3H -muscimol both in monkey (Hendrickson et al., 1985) and in goldfish (Ball and Brandon, 1986; Ball, 1987) retina is colocalized to a large extent with GABA- or GAD-like immunoreactivity in the same amacrine cells. It was used in the present study for 2 reasons. First, it is an independent test for the specificity of the GABA antibody. Second, since muscimol is a GABA analog, its high-affinity uptake would fulfill a second criterion for the acceptance of GABA as a transmitter of cells showing GABA-like immunoreactivity. Uptake of GABA itself was not used, since heavy uptake by Müller cells makes localization difficult (Bolz et al., 1985).

Sections labeled for GABA-like immunoreactivity were also exposed for ^3H -muscimol autoradiography. As can be seen from Figure 7, ^3H -muscimol was taken up into amacrine cells, displaced amacrine cells, and a dense labeling of the IPL was observed. No uptake was found in horizontal cells or bipolar cells. A direct comparison could be made between both labels in Figure 7, and, with a few exceptions, all amacrine cells showing GABA-like immunoreactivity had also taken up ^3H -muscimol. The IPL is densely labeled by both markers. However, no colocalization is observed in the OPL, and horizontal cells only

express GABA-like immunoreactivity. The few bipolar cells that showed GABA-like immunoreactivity did not take up ^3H -muscimol (not shown).

Colocalization in amacrine cells was investigated on a more quantitative basis. Of 199 amacrine cells, which accumulated ^3H -muscimol, 190 showed GABA-like immunoreactivity. Hence, practically all (95%) amacrine cells that show a high-affinity uptake of muscimol also express GABA-like immunoreactivity. Of 372 cells that were GABA-positive, 336 (90%) also accumulated muscimol.

One might question the significance of the result, whereby 10% of the amacrine cells show GABA-like immunoreactivity but no accumulation of ^3H -muscimol. However a close inspection of the TH cells shows this difference to be important (Fig. 7). The retina illustrated in Figure 7 was stained for GABA-like immunoreactivity, and alternate sections were also stained for TH-like immunoreactivity. In addition, they were exposed for ^3H -muscimol autoradiography. A dopaminergic amacrine cell shown in Figure 8*A* has not taken up ^3H -muscimol. In the consecutive section (Fig. 8*B*), this cell expresses GABA-like immunoreactivity, while the other 3, smaller cells have also accumulated ^3H -muscimol. One of them has an ascending process and is an interplexiform cell. The third section of this series was again stained for TH-like immunoreactivity. No ^3H -muscimol uptake by the dopaminergic amacrine cell could be detected, in contrast to the 3 other cells, which were again labeled. This result was tested in 5 more TH-immunoreactive cells, of which all showed GABA-like immunoreactivity, but no accumulation of ^3H -muscimol.

Although uptake of ^3H -muscimol and 5-HT were not per-

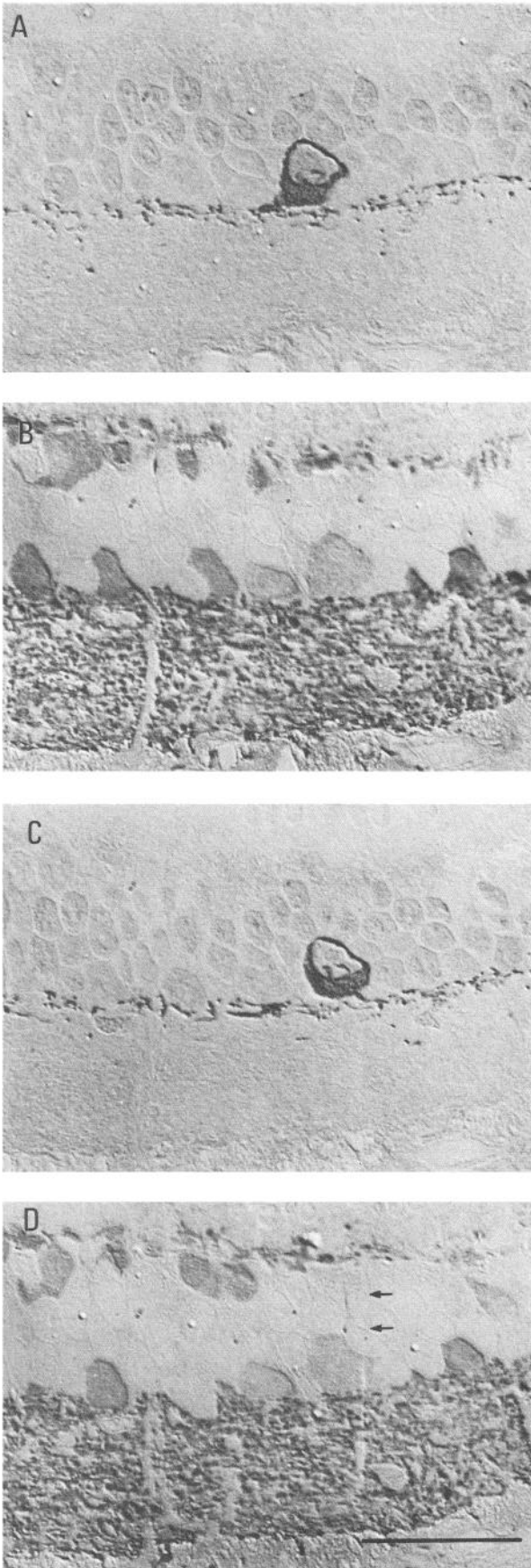


Figure 5. High-power micrographs from 4 consecutive vertical sections through a cat retina. From top to bottom, each micrograph shows

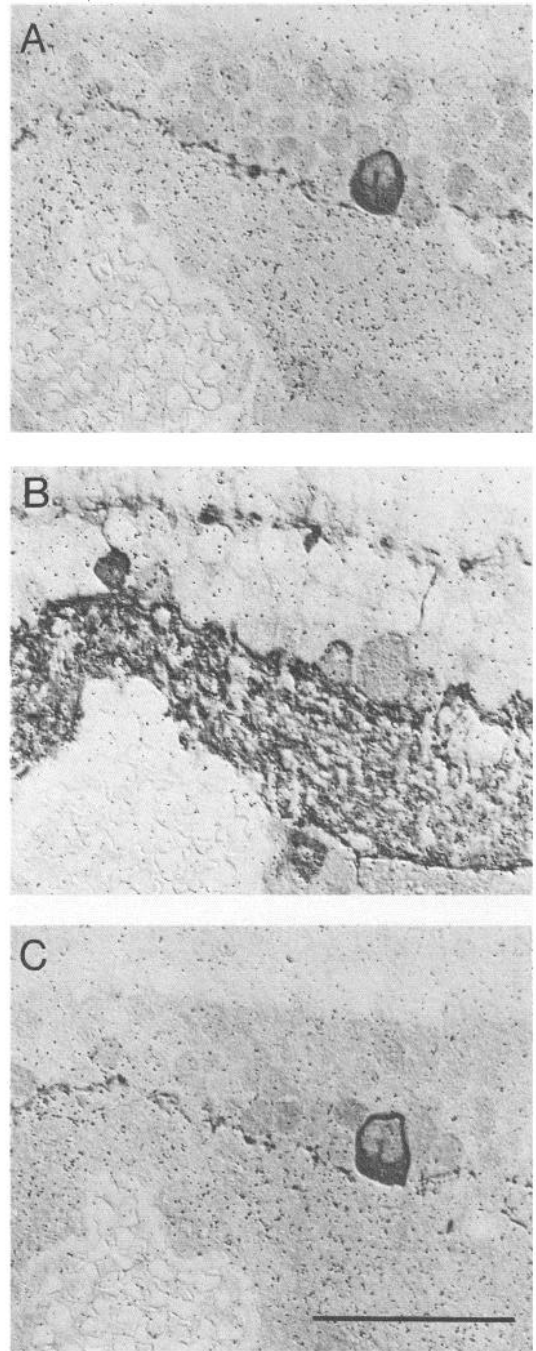


Figure 6. High-power micrographs from 3 consecutive vertical sections through a cat retina. From top to bottom, each micrograph shows the ONL, OPL, INL, and IPL. To the left, a thick blood vessel is transected. The little black dots are silver grains from autoradiography and ^3H -muscimol uptake and, because of underexposure, were not further analyzed. A, C, Stained for TH-like immunoreactivity. B, D, For GABA-like immunoreactivity. B, Processes can be detected ascending to the OPL from both the large dopaminergic amacrine cell (right) and the small putative GABAergic interplexiform cell (left). Scale, 25 μm .

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the outer plexiform layer (OPL), the inner nuclear layer (INL), and the inner plexiform layer (IPL). A, C, Stained for TH-like immunoreactivity. B, D, for GABA-like immunoreactivity. Arrows (D) indicate a small process from a dopaminergic amacrine cell oriented towards the OPL. Scale, 25 μm .

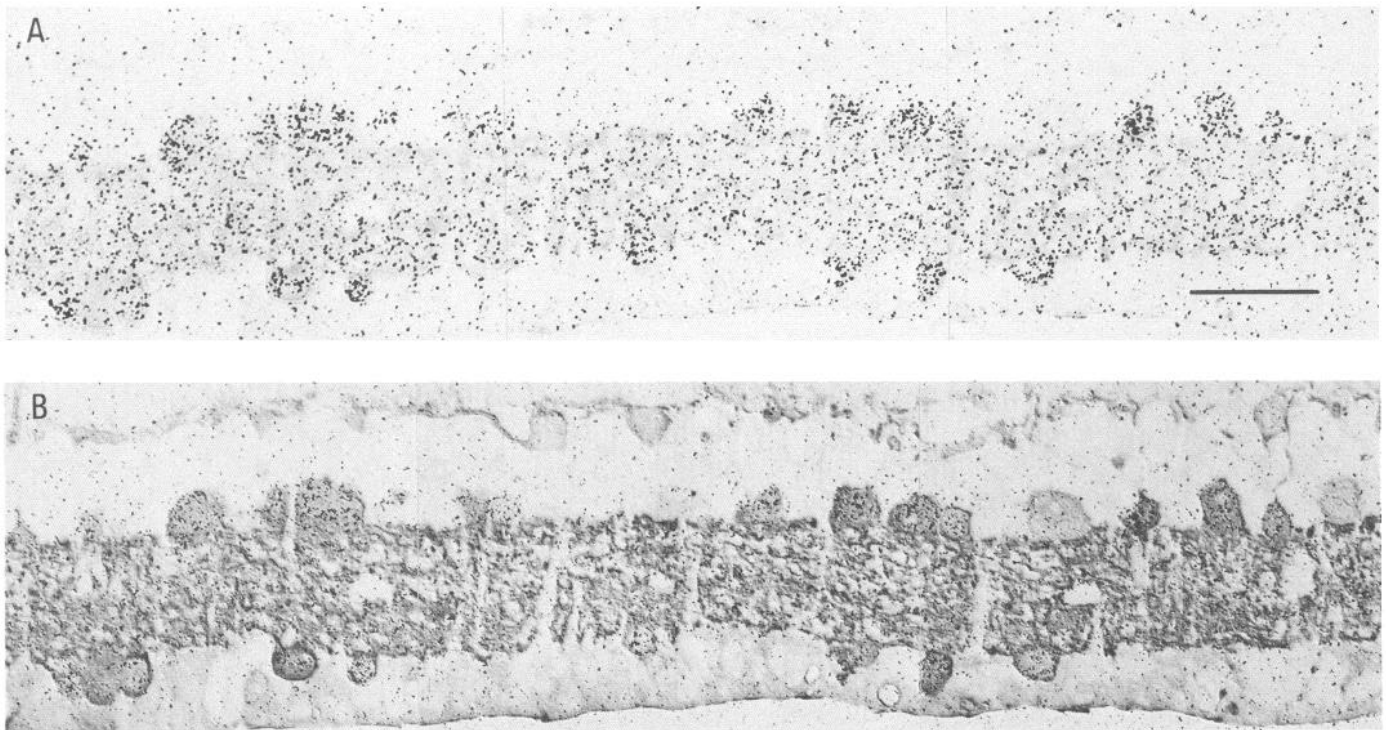


Figure 7. Low-power micrographs from the same vertical section through a cat retina. The section was labeled by ^3H -muscimol uptake, followed by autoradiography. *A*, Focus is on the silver grains. *A*, Also stained for GABA-like immunoreactivity, shown in *B*. With 2 exceptions, all amacrine cells expressed both markers. Scale, 25 μm .

formed together, it is clear from the percentage numbers that both markers must be colocalized in the majority of amacrine cells that show GABA-like immunoreactivity.

Discussion

GABA-like immunoreactivity in the cat retina

In the present paper, antisera raised against GABA coupled to BSA with glutaraldehyde (Storm-Mathisen et al., 1983) were used to label putative GABAergic neurons in the cat retina. The specificity of the antibody binding was tested by the usual immunocytochemical controls, including ELISA tests, preabsorption with the antigens, and omission of the first antibody; however, the possibility of cross-reactivity with some unknown antigen can never be excluded. Hence, GABA labeling in the present study is compared with that in previous investigations and with results from staining putative GABAergic neurons using other methods.

In a recent comparison of GABA-like immunoreactivity among vertebrate retinas, Mosinger et al. (1986), using an antiserum produced according to the same prescription as ours (Storm-Mathisen et al., 1983), reported a staining pattern in the cat identical to our findings. They labeled horizontal cells, interplexiform cells, amacrine cells, and displaced amacrine cells. The same staining pattern, using antibodies against GAD, was also described in a brief report by Bolz and McGuire (1985). In the rabbit retina, Agardh et al. (1987) described GABA-like immunoreactivity similar to that shown in Figure 4*B*. In further experiments, not documented in detail here, we compared the GABA antibody used in the present study with a monoclonal GABA antibody produced and characterized by Matute and Streit (1986). In consecutive, semithin sections, both antibodies stained exactly the same neurons. Thus, 3 different anti-GABA

antibodies, as well as staining the retina with GAD immunocytochemistry, produced comparable results.

Uptake studies of ^3H -muscimol or ^3H -GABA in the cat retina labeled interplexiform cells (Nakamura et al., 1980), amacrine cells (Pourcho, 1980, 1981; Pourcho and Goebel, 1983; Freed et al., 1983; Bolz et al., 1985; Wässle et al., 1987*b*) and displaced amacrine cells. With all markers applied (GABA or GAD antibodies, ^3H -GABA or ^3H -muscimol uptake), the same proportion of amacrine cells (30%) was labeled. As shown in the present paper for amacrine cells of the cat retina, and previously for other species (monkey: Hendrickson et al., 1985; rabbit: Mosinger and Yazulla, 1985; goldfish: Ball and Brandon, 1986; Yazulla et al., 1987), the markers for GAD immunoreactivity, GABA immunoreactivity, and uptake of tritiated GABA or muscimol are colocalized to a large extent in the same amacrine cells. Thus, there remains little doubt that the antibodies used in the present study label amacrine cells because they have detectable amounts of endogenous GABA.

In most mature mammalian retinas, neither GAD immunocytochemistry nor GABA-like immunoreactivity labels horizontal cells (Brandon et al., 1979; Schnitzer and Rusoff, 1984; Hendrickson et al., 1985; Mosinger and Yazulla, 1985; Mosinger et al., 1986), although there is evidence for GABAergic staining in early postnatal mouse (Schnitzer and Rusoff, 1984) and rabbit (Osborne et al., 1986) retinas. In the mature cat retina, both GABA- and GAD-like immunoreactivity are found in horizontal cells (Bolz and McGuire, 1985; Mosinger et al., 1986). However, cat horizontal cells were not labeled by uptake of tritiated GABA or muscimol (Pourcho, 1980, 1982; Pourcho and Goebel, 1983; Bolz et al., 1985; Wässle et al., 1987*b*). Hence, in contrast to the nonmammalian retina, where GABA is well established as a horizontal cell transmitter (Yazulla, 1986), the

role of GABA with respect to mammalian horizontal cells remains uncertain.

Colocalization of GABA- and TH-like immunoreactivity

TH-like-immunoreactive amacrine cells of the cat retina (Oyster et al., 1985) are identical to presumed dopaminergic amacrine cells identified by the Falck-Hillarp histofluorescence method (Törk and Stone, 1979) or by high-affinity uptake of ^3H -dopamine (Pourcho, 1982). The agreement of the results of these independent histochemical methods, and additional biochemical (Kramer, 1971) and physiological (Thier and Alder, 1984) evidence, suggests that these cells are dopaminergic amacrine cells. The present paper has shown, for the cat, and a previous study for the rat (Kosaka et al., 1987), that these dopaminergic amacrines also express GABA-like immunoreactivity. In the rat they also expressed GAD-like immunoreactivity; hence it is possible that the 2 classical transmitters coexist in these amacrine cells. There is, however, a difference with respect to other putative GABAergic amacrine cells: no muscimol uptake is found in the dopaminergic cells. It cannot be determined whether the cells lack an uptake system for GABA in general, or whether higher concentrations than the 10^{-6} M used in the present experiments are required. Since it was found in a previous study that indoleamine accumulation is concentration-dependent (Wässle et al., 1987a), uptake of ^3H -muscimol by dopaminergic amacrine cells is not ruled out by the present experiment.

Serial sectioning of these dopaminergic amacrine cells sometimes revealed little processes running towards the OPL (Figs. 5D, 6B). These processes originated from the cell bodies of 3 out of 19 dopaminergic amacrine cells. In whole-mount preparations of cat retinas stained for TH-like immunoreactivity, Oyster et al. (1985) observed interplexiform processes originating from dendrites of dopaminergic amacrine cells. They considered these cells to be of a different class from normal dopaminergic amacrine cells. However, we propose that interplexiform processes, which can originate from cell bodies or from the dendritic plexus, are a normal feature of dopaminergic amacrine cells, and that all cells are of one kind or the other. Since they are very fine, the cell body processes were probably not detected in whole-mount preparations (Voigt and Wässle, 1987).

5-HT-Like immunoreactivity in the cat retina

The antibody against 5-HT was raised in rabbits against 5-HT coupled to BSA with paraformaldehyde (Steinbusch et al., 1978). As with the GABA antibody, one must determine the specificity of this antibody (Tandler et al., 1986). However, there was a very powerful test inherent in our study. When the cat retina was not preloaded with 5-HT, no staining of the retina was achieved with the 5-HT antibodies. Thus, there is no endogenous antigen cross-reacting with the antibody. When the retina was preloaded by injecting 20 μg 5-HT into the vitreous, a reliable staining pattern emerged. That the antigen causing this labeling was actually 5-HT was tested in a previous study by comparing the immunocytochemical staining with the formaldehyde-induced fluorescence following 5,6-DHT uptake (Wässle et al., 1987a). All cells labeled by immunocytochemical staining also showed the Falck-Hillarp type of fluorescence.

5-HT Uptake by GABAergic amacrine cells?

There are several implicit indications in the literature that 5-HT uptake and GABA markers might label the same amacrine cells

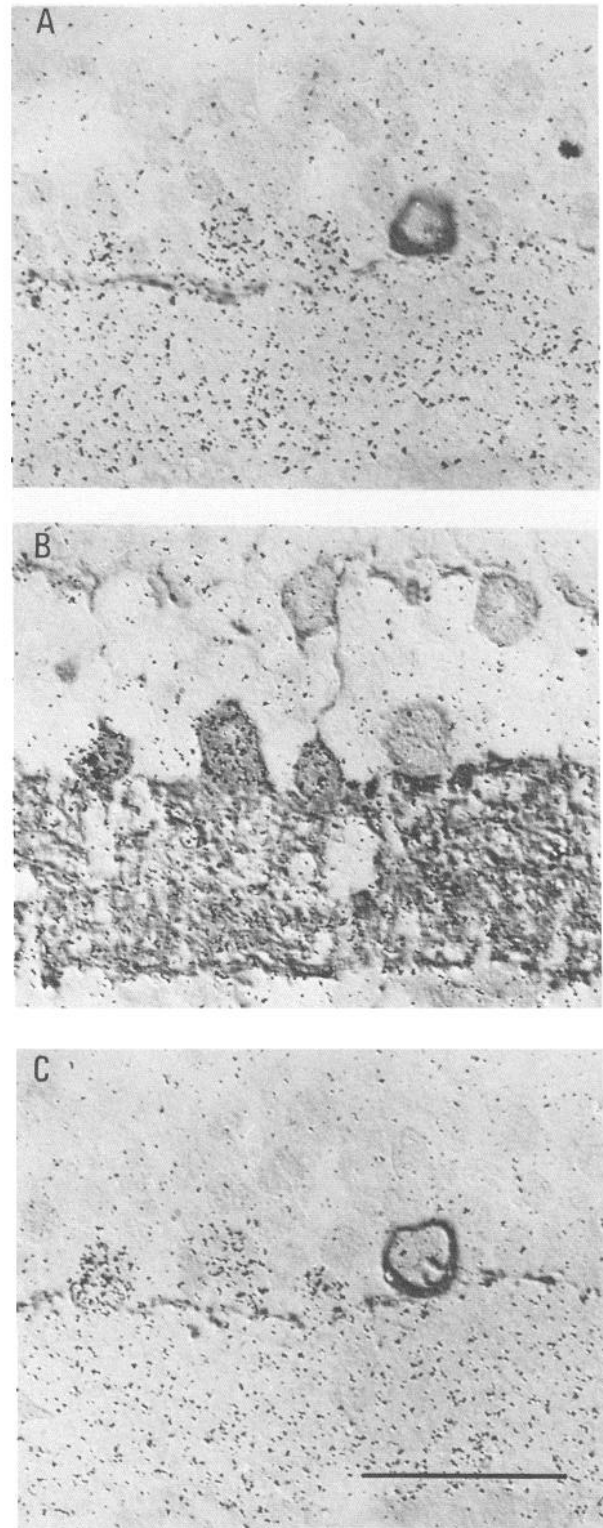


Figure 8. High-power micrographs from 3 consecutive vertical sections through a cat retina following ^3H -muscimol uptake and autoradiography. *A*, Stained for TH-like immunoreactivity. Accumulation of silver grains was only detected in 3 unstained small cell bodies to the left. *B*, Stained for GABA-like immunoreactivity; section was taken from the right border of Figure 7*B*. The dopaminergic amacrine cell expressed GABA-like immunoreactivity, but in contrast to the 3 other cells stained, no accumulation of silver grains could be detected over its cell body. *C*, Stained for TH-like immunoreactivity; the section confirms the lack of ^3H -muscimol uptake. Scale, 25 μm .

in the cat retina. Indoleamine-accumulating neurons were pre-labeled by uptake of 5,6-DHT, which could then be visualized using formaldehyde-induced fluorescence. Under direct microscopic control, intracellular injection of Lucifer yellow into labeled cells revealed 3 distinct morphological types of amacrine cell (Wässle et al., 1987a). They correspond to the A17, the A20, and the A19/22 amacrine cells defined from Golgi staining (Kolb et al., 1981). In a combined Golgi/³H-muscimol uptake study, Pourcho and Goebel (1983) have shown that A17 and A19/22 accumulate muscimol. This makes it likely that the same amacrine cells have the capacity to accumulate both 5-HT and ³H-muscimol.

Uptake of indoleamines into cat amacrine cells and the synaptic organization of the labeled neurons have been studied extensively, and it has been found that their terminals synapse with bipolar terminals and provide synapses with other amacrine cells (Ehinger and Holmgren, 1979; Dowling et al., 1980; Holmgren-Taylor, 1982). Their main synaptic contacts are reciprocal synapses with rod bipolar terminals, and 75% of these synapses involve indoleamine-accumulating processes (Holmgren-Taylor, 1982). Freed and Sterling (1982) found that virtually all reciprocal synapses with rod bipolar cells are labeled by ³H-GABA uptake. This apparent controversy would be solved if the same amacrine cells accumulated both indoleamines and GABA.

In the rabbit retina, 2 morphologically distinct types of amacrine cell accumulate indoleamines (Sandell and Masland, 1986; Vaney, 1986). One of these cells has a striking similarity to the A17 cell of the cat, which, as mentioned above, seems to accumulate both GABA and indoleamines. Osborne and Beaton (1986) were able to show by uptake of 5,7-DHT and GABA/GAD immunocytochemistry that some putative GABAergic amacrine cells accumulate indoleamines.

All these findings suggest that, in cat retina, indoleamine uptake and endogenous GABA might be colocalized within the same amacrine cells. In the present study, the technique of labeling consecutive sections with different antibodies was used. This procedure is more difficult to evaluate, since the same neurons have to be detected in the 2 sections. However, with respect to immunocytochemical staining, it causes fewer problems. Staining the same section with 2 different primary and secondary antibodies is difficult because of cross-reactivities and overlapping fluorescence spectra. Two consecutive sections can both be labeled permanently (PAP method; biotin-Avidin method) and therefore a careful quantitative analysis can be performed. It was found that 92% of all 5-HT-accumulating amacrine cells also expressed GABA-like immunoreactivity. The possibility cannot be excluded that the remaining 8% is a separate population of cells that expresses an endogenous transmitter other than GABA. However we are inclined to consider this more of a technical problem, since double-labeling mostly failed in regions where GABA staining was weak.

Up to 70% of amacrine cells that expressed GABA-like immunoreactivity also accumulated 5-HT. This raises the question of why all of these cells are not double-labeled. As studied previously (Wässle et al., 1987a), uptake of indoleamines depends critically on the concentration available. In addition, injection into the vitreous inevitably causes concentration differences along the retinal surface, and individual cell types require differing amounts to become labeled. The lowest 5-HT dose (10^{-7} M) was taken up by A20 amacrine cells, followed by A19/22 cells. A17 amacrine cells were only labeled when twice the amount of 5-HT

(40 μ g) was injected into the vitreous. Even higher amounts of 5-HT injection labeled ganglion cells and Müller cells. Thus, it is possible that higher concentrations of 5-HT would be taken up by all putative GABAergic amacrine cells; however, it is likely that other cells would then also be labeled.

The uptake of 5-HT shows a pattern comparable to that of ³H-muscimol uptake: while amacrine cells seem to accumulate 5-HT and ³H-muscimol, neither substance was taken up into horizontal cells, despite the fact that they express GABA-like immunoreactivity.

The interpretation of 5-HT uptake is flanked by 2 extreme positions. One assumption is that GABA and 5-HT are indeed transmitter substances of the same amacrine cells and are colocalized. Such a colocalization has been found in other parts of the brain (Belin et al., 1983; Millhorn et al., 1987). If so, it would not be surprising to find a high-affinity uptake system for both substances. However there is still the problem that endogenous 5-HT has not been detected in these cells. There are also conflicting reports concerning whether, following uptake, 5-HT is released by high concentrations of potassium ions (Thomas and Redburn, 1979; Osborne, 1980; Nowak et al., 1985; Redburn, 1985). Thus, the available data on mammalian retinal 5-HT are not sufficient to postulate a neurotransmitter role for 5-HT in this tissue. Perhaps, as suggested by Florén (1979), serotonin is taken up and might be a precursor for other active substances.

There is substantial evidence that mammalian retinas contain GABAergic amacrine cells. These amacrine cells contain the GABA-synthetizing enzyme GAD; GABA is present in detectable amounts; the cells have a high-affinity uptake system for GABA and a Ca-dependent release has been demonstrated (for reviews, see Morgan, 1985; Yazulla, 1986). On this basis, the following interpretation of 5-HT uptake is an alternative position. GABAergic amacrine cells have a high-affinity uptake system for GABA; however, the specificity of the transport carrier is such that 5-HT is also taken up along with GABA, muscimol, and isoguvacine (Agardh and Ehinger, 1983). Thus, 5-HT uptake, like that of muscimol, would label only GABAergic amacrine cells. Clearly, more experiments are necessary to determine whether the role of 5-HT uptake is functional, or whether it merely labels GABAergic amacrine cells.

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