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Localization of ^{3}H-GABA, -Muscimol, and -Glycine in Goldfish Retinas Stained for Glutamate Decarboxylase

Alexander K. Ball* and Christopher Brandon†

*Department of Anatomy, McMaster University, Hamilton, Ontario L8N 3Z5, Canada, and †Department of Biological Chemistry and Structure, The Chicago Medical School, North Chicago, Illinois 60064

Glutamic acid decarboxylase (GAD), the synthesizing enzyme for the neurotransmitter GABA, has been localized in goldfish retina using a new antiserum. We observed at least six types of GAD-immunoreactive amacrine cells, one of which was large and pyriform (Ab type). In addition, immunoreactive synaptic terminals were located throughout the inner plexiform layer (IPL). Amacrine cells that were GAD-immunoreactive also had high-affinity uptake mechanisms for both ^{3}H-GABA and ^{3}H-muscimol that were detectable autoradiographically. Type Ab pyriform amacrine cells were heavily labeled because of ^{3}H-GABA uptake and were GAD-immunoreactive. Other types of GAD-immunoreactive amacrine cells, including a subpopulation of Ab amacrines, were lightly labeled because of ^{3}H-GABA uptake. Because the same neurons that were GAD-immunoreactive also accumulated ^{3}H-GABA and ^{3}H-muscimol, these three are appropriate markers for GABAergic cells in the goldfish retina. However, the uptake of ^{3}H-muscimol by many non-GAD-immunoreactive cells, detectable at longer autoradiographic exposure times, indicates that this label must be used with caution. Thirty percent of goldfish retinal amacrine cells are GABAergic, and their processes are distributed throughout all levels of the IPL. Few GAD-immunoreactive amacrine cells accumulated ^{3}H-glycine, so the goldfish retina contains distinct populations of glycinergic and GABAergic amacrine cells.

High-affinity uptake of GABA has been shown to be a reliable marker for GABAergic neurons (Bloom and Iversen, 1971), and autoradiographic methods are frequently used for the detection of these neurons. A high-affinity uptake mechanism for GABA has been identified autoradiographically in H1 horizontal cells, and in amacrine cells of the Ab type, in the goldfish retina (Marc et al., 1978). This serial section analysis showed these Ab amacrine cells to have large pyriform cell bodies with single primary processes that ramify in the proximal half of the inner plexiform layer (IPL) sublamina b (Famiglietti et al., 1977). Furthermore, the cells accumulated ^{3}H-GABA maximally in the dark and minimally in red light. These findings, and the observation that the cells were postsynaptic to B1 (red-depolarizing) bipolar cells (Stell et al., 1977), suggested that these cells were depolarized by red light and hyperpolarized in the dark. Although many other amacrine cells, and the distal IPL, also showed some accumulation of ^{3}H-GABA, this labeling was viewed with caution because it could not be modulated by changes in illumination. As a result, only H1 horizontal cells and the Ab pyriform amacrines were considered to be GABAergic.

These conclusions were reinforced by the immunocytochemical identification of L-glutamic acid decarboxylase (GAD), the synthesizing enzyme for GABA, in H1 horizontal cells and Ab-like amacrine cells (Lam et al., 1979). However, when the binding and uptake of the GABA analog ^{3}H-muscimol were compared to ^{3}H-GABA binding and uptake in the goldfish retina, the ^{3}H-muscimol uptake pattern bore little resemblance to the ^{3}H-GABA uptake pattern (Yazulla and Brecha, 1980). In contrast to the 2% of cell bodies (Ab amacrines) in the amacrine cell layer that were heavily labeled by ^{3}H-GABA uptake (Marc, 1982), a large number of amacrine cell bodies were labeled lightly by ^{3}H-muscimol uptake, and muscimol also evenly labeled the entire IPL (Agardh and Ehinger, 1982; Yazulla and Brecha, 1980). Similar results have been obtained with the GABA analog ^{3}H-isoguvacine (Agardh and Ehinger, 1983). Furthermore, ^{3}H-muscimol and ^{3}H-GABA binding sites have been localized to both sublamina a and sublamina b of the IPL (Yazulla, 1981). The effects of exogenous GABA on ganglion cell activity of the superfused carp retina also suggest that GABAergic neurons are involved in transmission in both sublaminae (Glickman et al., 1982; Negishi et al., 1978).

Improvements in antibody isolation and immunocytochemical technique have recently resulted in the improved localization of GAD-immunoreactivity (GAD-IR) in goldfish retina (Brandon, 1985a, b; Zucker et al., 1984). These localizations show that GAD-IR resembles the labeling due to ^{3}H-muscimol or ^{3}H-isoguvacine uptake and ^{3}H-muscimol or ^{3}H-GABA binding more than it resembles the labeling due to ^{3}H-GABA uptake. Together, these results have called into question the suitability of ^{3}H-GABA uptake as a marker for GABAergic transmission in the retina (Yazulla, 1981; Yazulla and Brecha, 1980; Yazulla et al. 1984).

Recent studies have combined EM autoradiography (of ^{3}H-GABA uptake) and immunocytochemistry (of GAD) to examine the incidence of colocalization of the two markers in IPL synaptic terminals (Zucker and Yazulla, 1983; Zucker et al., 1984). Correspondence of ^{3}H-GABA uptake and GAD-IR occurred in only 18% of IPL synaptic terminals, suggesting that ^{3}H-GABA uptake and GAD-IR occurred in different amacrine cells (Zucker et al., 1984). However, this analysis was carried out at only one exposure time, positive identification of Ab amacrine terminals was not possible, and the techniques were working near the limits of their resolution.

In order to more accurately determine whether GAD-IR and GABA uptake occur in the same populations of amacrine cells, we have used LM autoradiography to identify the amacrine cells that have high-affinity uptake mechanisms for ^{3}H-GABA, ^{3}H-muscimol, and ^{3}H-glycine in goldfish retinas stained immunocytochemically for glutamate decarboxylase.

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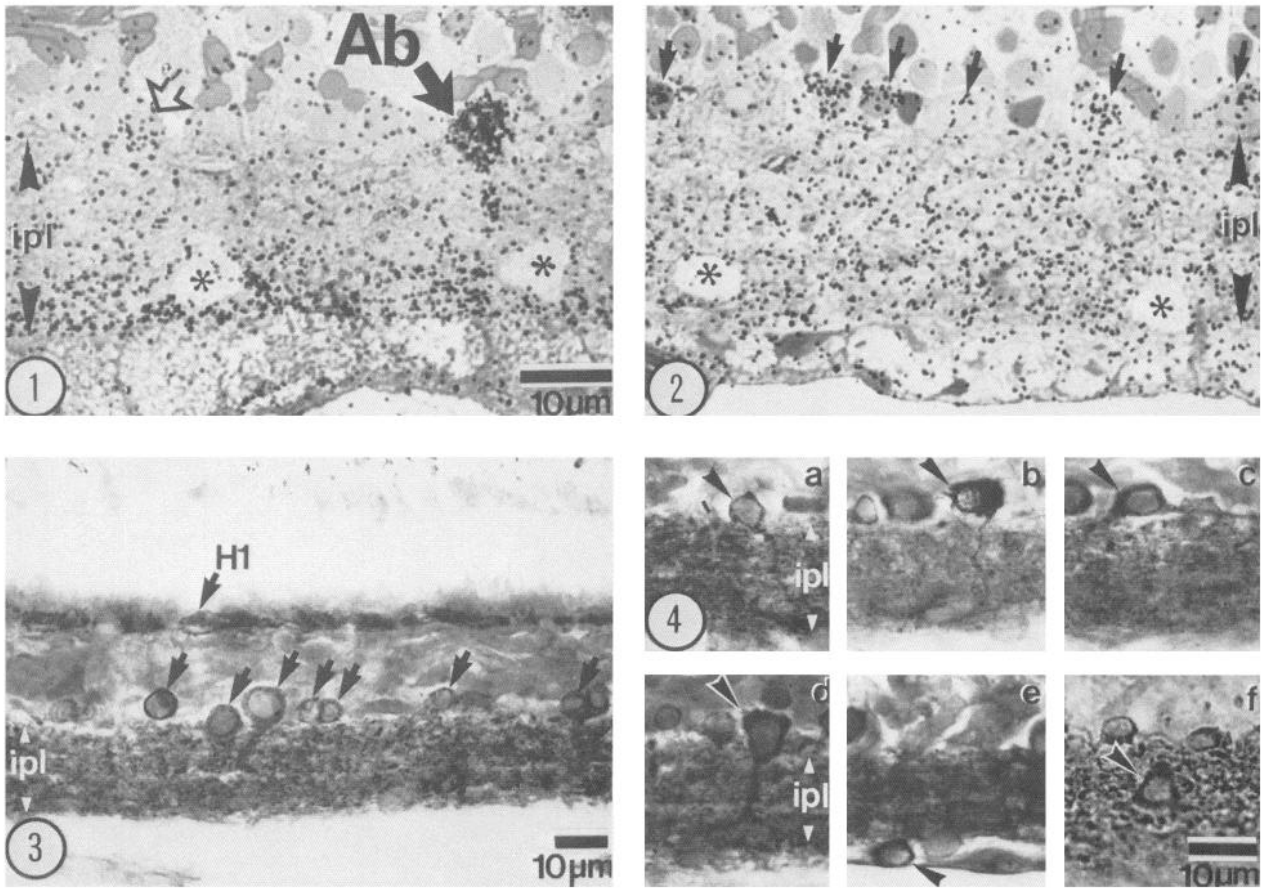
Correspondence should be addressed to Dr. Ball at the above address.



Figures 1, 2, 3, and 4. 1, Autoradiograph showing uptake of ^{3}H-GABA in a dark-adapted goldfish retina. Labeling of H1 horizontal cell axon terminals is minimal, while an Ab pyriform amacrine cell (*Ab, solid arrow*) is heavily labeled, as are its processes in the proximal third of the IPL. Labeled processes are clustered around the synaptic terminals of B1 bipolar cells (*asterisk*). Other amacrine cells (*open arrow*) and the distal IPL are lightly labeled. Section, 0.5 µm, lightly stained with toluidine blue, 1 week exposure. *ipl* = Inner plexiform layer. ×1200. *2,* Autoradiograph showing uptake of ^{3}H-muscimol in a dark-adapted goldfish retina. A large number of amacrine cells are lightly labeled because of ^{3}H-muscimol uptake (*arrows*). The entire IPL is evenly labeled, with labeled processes clustered around the synaptic terminals of B1 bipolar cells. Section, 0.5 µm, lightly stained with toluidine blue, 2 week exposure. *ipl* = Inner plexiform layer. ×1200. *3,* Frozen section of goldfish retina stained for GAD-IR using the F(ab)-PAP method. Horizontal cell perikarya (*H1, arrow*) and a large number of amacrine cells (*arrows*) are GAD-immunoreactive. GAD-containing processes are located throughout the IPL. Section, 20 µm. *ipl* = Inner plexiform layer. ×700. *4,* Micrographs showing six types of GAD-immunoreactive amacrine cells observed in goldfish retina. Small cells with a thin rim of cytoplasm around the nucleus, and with processes distributed to all levels of the IPL, were in the majority (*a*). Other cells were densely stained, with an eccentrically placed nucleus (*b*); large and fusiform, sending primary processes along the IPL–INL border (*c*); large and pyriform or round, sending a single primary process to IPL substrata 3 or 4 (*d*); displaced to the ganglion cell layer (*e*); or large interstitial cells, with primary processes extending laterally in substratum 3 (*f*). Frozen sections, 20 µm, F(ab)-PAP method. *ipl* = Inner plexiform layer. ×1000.

Materials and Methods

Uptake of ^{3}H-GABA or ^{3}H-muscimol

^{3}H-GABA or ^{3}H-muscimol, 5 µCi (New England Nuclear, Lachine, Quebec; nos. NET-191X, NET-574), was evaporated with dry nitrogen and reconstituted with 5µl saline (Marc et al., 1978) containing the GABA-transaminase inhibitor amino-oxyacetic acid (AOAA; Sigma, St. Louis) (Iadarola and Gale, 1981). The solution was injected intraocularly into 10–15 cm goldfish (*Carassius auratus,* Grassyforks) that had been dark-adapted 2 hr prior to injection. After 30 min in the dark, the fish were decapitated, pithed, and enucleated, and the retinas were dissected from the pigment epithelium in saline under dim green light. The retinas were then placed in fresh, cold fixative. The estimated concentrations of ^{3}H-GABA, ^{3}H-muscimol, and AOAA at the retinal surface were 0.5, 2.0, and 100 µM, respectively.

Uptake of ^{3}H-glycine

^{3}H-glycine, 5 µCi (New England Nuclear, no. NET-004), was evaporated with dry nitrogen and reconstituted with 5 µl saline. The solution was injected intraocularly into light-adapted goldfish, and the fish were placed in an opaque container illuminated by red light for 30 min. Retinas were then dissected and fixed under red light as described above. The estimated concentration of ^{3}H-glycine at the retinal surface was 1.0 µM.

Fixation

Retinas were fixed at 4°C for 20 min in a solution containing 2% formaldehyde, 0.1% glutaraldehyde, and 0.1% acrolein in 0.1 M Sorensen's phosphate buffer with 0.2 mM $CaCl_2$ and 3% sucrose, pH 7.3, then titrated over 30 min to a fixative consisting of 2% formaldehyde in 0.05 M sodium bicarbonate buffer with 3% sucrose, pH 10.4, and fixed overnight. Retinas were then titrated to K-PBS (0.25 M NaCl, 0.25 M KCl, 0.1 M sodium phosphate, pH 7.4) over 1 hr.

Immunocytochemistry

Retinas were sliced to 1 mm thickness and the retinal strips placed in K-PBS containing 2% normal goat serum (NGS; Sigma). After 2–8 hr, the slices were transferred to a solution of F(ab) fragments (40 µg/ml) prepared from a monospecific antiserum directed against rabbit brain GAD (Brandon, 1985a). Sections were incubated at room temperature for 20 hr on an orbital rotator. The following steps were then carried

out: (1) titration to PBS over 1 hr; (2) goat anti-rabbit serum [F(ab)'2-specific; Cappel Laboratories, Cochranville, PA], diluted 1:50 in PBS with 2% NGS, 4 hr; (3) PBS wash, 2 × 1 hr; (4) F(ab)-PAP (Jackson ImmunoResearch; Avondale, PA) diluted 1:100 in PBS with 2% NGS, 4 hr; (5) PBS wash, 2 × 1 hr; (6) titration to 0.05 M Tris-HCl buffer with 1% NaCl, pH 7.6, over 30 min; (7) incubation in 0.2% diaminobenzidine-HCl (Sigma), 0.7% imidazole (Sigma), and 0.01% (H_2O_2 (Sigma) in Tris-HCl buffer for 15 min; (8) wash in 0.15 M sodium cacodylate buffer, pH 7.3, 2 × 15 min; (9) postfixation in cacodylate-buffered 2% osmium tetroxide, 2 hr; (10) dehydration in an acetone series, infiltration, and flat-embedding in TAAB low-viscosity resin (Reading, England).

Autoradiography

Serial sections, 2–5-μm-thick, were cut *en face* from embedded slices and sequentially mounted on four clean glass slides (to be exposed for four different times). The slides were then dipped in undiluted Kodak NTB-2 nuclear track emulsion and stored at 4°C in light-tight boxes containing silica gel. After exposure times of 2 d, 4 d, 1 week, or 2 weeks, the slides were developed in undiluted Kodak D-19 for 2 min, fixed, washed, and dried.

Cells were considered to have accumulated radiochemical when they were labeled significantly above background levels. At 1 week exposure, cells that were lightly labeled due to ^{3}H-GABA uptake typically had 4–8 times more silver grains over their perikarya than background (as measured over the distal IPL). Cells that were heavily labeled due to ^{3}H-GABA uptake had greater than a 25× background silver grain density over their perikarya.

Sections exhibiting good immunocytochemical staining (complete Ab penetration, low background) were used for analysis. With the aid of a camera lucida, proximal inner nuclear layer (INL) perikarya (presumed amacrine cell bodies) were scored as (1) immunoreactive, (2) exhibiting uptake, (3) double-labeled, or (4) unlabeled, at all four exposure times. The total numbers of cells categorized in this manner were 3297, 4144, and 3123 for experiments with ^{3}H-GABA, ^{3}H-muscimol, and ^{3}H-glycine, respectively.

Results

Autoradiography: ^{3}H-GABA and ^{3}H-muscimol

Intraocular injection of radiochemical was chosen to avoid uptake of ^{3}H-GABA or ^{3}H-muscimol by H1 horizontal cells. When goldfish were given intraocular injections in the light, or when isolated retinas were incubated *in vitro,* the labeled H1 axon terminals in the proximal INL could be confused with amacrine perikarya. This finding is consistent with the observation that the uptake of ^{3}H-GABA *in vitro* is modulated by light (Marc et al., 1978). The inclusion of AOAA in the injection solution also prevented the degradation of GABA and the appearance of labeled metabolic products in other cells during the postinjection period. Retinas exposed to ^{3}H-GABA showed large, heavily labeled pyriform cells with a single primary process extending through the IPL (Fig. 1, 0.5 μm section, 1 week autoradiographic exposure). These cells comprised about 2.5% of proximal INL perikarya and were Ab amacrine cells. The proximal half of the IPL was heavily labeled, and silver grains were clustered around the axon terminals of presumed B1 bipolar cells (Fig. 1, asterisk). The distal IPL was lightly labeled, as were some small amacrine perikarya (Fig. 1, open arrow). A similar labeling pattern has been obtained with *in vitro* incubations (Marc et al., 1978) and with freeze-dried retinas (Agardh and Ehinger, 1982).

Muscimol uptake yields a labeling pattern that is qualitatively different from the GABA labeling pattern. In retinas exposed to ^{3}H-muscimol, a large number (about 30%) of proximal INL perikarya were labeled (Fig. 2, 0.5 μm section, 2 week exposure). All cells showed an equal density of labeling. The entire IPL was evenly labeled, and silver grains were clustered around presumed B1 bipolar cell terminals (Fig. 2, asterisk). Similar ^{3}H-muscimol uptake labeling patterns have been reported before (Agardh and Ehinger, 1982; Yazulla and Brecha, 1980).

Immunocytochemistry

Frozen 20 μm retinal sections stained for GAD-IR contained some labeled horizontal cell perikarya (Fig. 3, H1) and amacrine cell perikarya (Fig. 3, arrows). H1 axons, passing through this layer to their terminals in the proximal INL, were lightly labeled. The entire IPL was labeled with dense deposits of immunochemical reaction product that represent synaptic varicosities and terminals of amacrine cell processes (Brandon, 1985b). The GAD-IR labeling pattern was more similar to the ^{3}H-muscimol uptake pattern (Fig. 2) than to the ^{3}H-GABA uptake pattern (Fig. 1). A large number of morphologically diverse amacrine cell perikarya were GAD-immunoreactive (Fig. 4). In addition to Ab-like amacrine cells (Fig. 4*d*), we observed small, round cells with diffuse processes (Fig. 4*a*), densely stained cells with eccentrically placed nuclei and diffuse processes (Fig. 4*b*), fusiform cells with primary processes extending along the INL–IPL border (Fig. 4*c*), a variety of presumed displaced amacrine cells (Fig. 4*e*), and interstitial cells (Fig. 4*f*). All cells could be placed into one of six categories. The most commonly observed cells were the small round cells (Fig. 4*a*), while interstitial cells (Fig. 4*f*) were the least common. GAD-immunoreactive amacrine cells resembled cells heavily labeled by ^{3}H-GABA uptake (Ab amacrines); they differed in the shape of their perikarya (round to pyriform) and in the length and thickness of their primary process (Figs. 3 and 4*d*).

With thicker, unfrozen sections, limited antibody penetration permitted uniform labeling only within 20 μm of the surface of the retinal slices, while high background staining obscured specific perikaryal labeling within the most superficial 5 μm. Analysis was therefore restricted to the 5–20 μm region in which perikaryal staining was obvious (Fig. 5, arrows). GAD-immunoreactive processes were observed throughout the IPL, clustered around the synaptic terminals of presumed bipolar cells in both sublamina a and sublamina b (Fig. 5*a, b*).

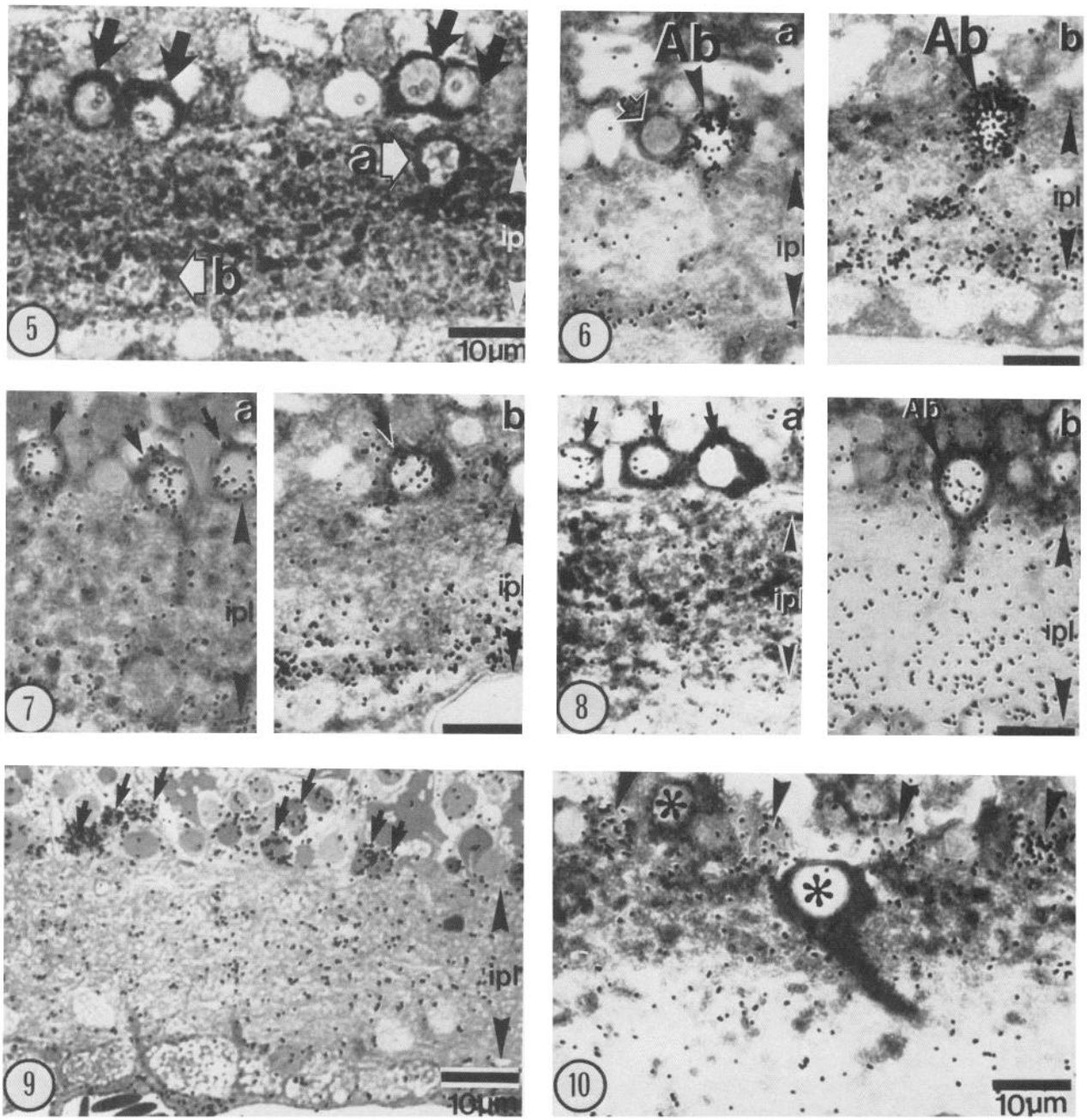
Double-labeling (GAD and GABA)

At short exposure times (2–4 d), the only amacrine cells labeled due to the uptake of ^{3}H-GABA were Ab amacrines (Fig. 6*a, b*). Silver-grain density over these cells was sufficient to allow their identification as Ab amacrines without obscuring their immunocytochemical labeling (Fig. 6, Ab). Smaller GAD-immunoreactive cells (Fig. 6*a,* arrow) did not show measurable ^{3}H-GABA uptake at this short exposure time. However, longer exposure times (1–2 weeks) revealed ^{3}H-GABA uptake by other GAD-immunoreactive amacrine cells (Fig. 7*a, b*). The silver-grain density over Ab amacrines obscured their GAD-IR at these longer exposure times, but other types of GAD-immunoreactive amacrines (like those in Fig. 4) were lightly labeled due to ^{3}H-GABA uptake, including some Ab-like amacrines (Fig. 7*a*), interstitial, and presumed displaced amacrine cells. Few nonimmunoreactive cells accumulated ^{3}H-GABA.

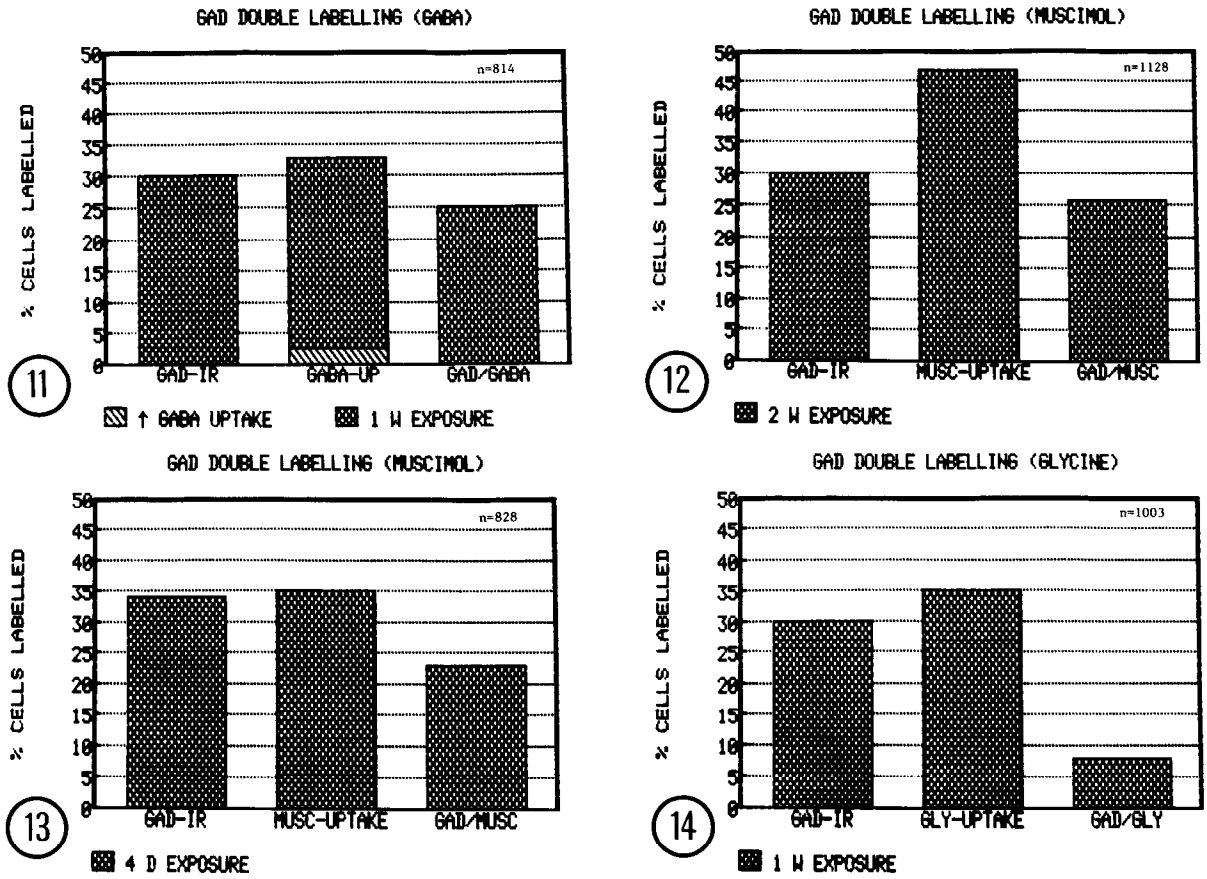
Retinas in which GAD-IR and ^{3}H-muscimol uptake were simultaneously localized required longer exposure times (1–2 weeks) to reveal ^{3}H-muscimol-labeled cells. No cell type was heavily labeled due to uptake, so that GAD-IR cells were never obscured. All types of GAD-IR amacrines (Fig. 4) were labeled due to ^{3}H-muscimol uptake (Fig. 8*a, b*). Almost all GAD-IR cells accumulated ^{3}H-muscimol, but many non-GAD-IR cells did so as well.

Autoradiography (glycine)

About 35% of INL perikarya (Fig. 9, arrows) and the entire IPL were evenly labeled owing to ^{3}H-glycine uptake after a 1-week autoradiographic exposure. A similar result has been reported using *in vitro* incubations (Marc, 1982; Marc and Lam, 1981) or freeze-dried retinas (Ehinger, 1981). These labeled cells there-



Figures 5–10. *5,* Goldfish retina, lightly fixed with formaldehyde/acrolein/glutaraldehyde and processed for GAD-IR using the pre-embedding F(ab)–PAP method. Numerous amacrine perikarya (*solid arrows*) and synaptic terminals throughout the entire IPL are GAD-immunoreactive. Immunoreactive GAD terminals are clustered around presumed bipolar cell synaptic terminals in sublamina a (*a* →) and b (*b* ←). Section, 2 µm; cut *en face* from the surface of a retinal slice. *ipl* = Inner plexiform layer. ×1200. *6,* Double-labeled goldfish retina. Autoradiograph of a goldfish retina that had been exposed to ^{3}H-GABA and was also stained immunocytochemically for GAD. At short exposure times (4 d), only Ab amacrines have detectable levels of ^{3}H-GABA. Silver-grain density over Ab amacrines is not so dense as to obscure GAD-IR in these cells (*Ab, arrows* in *a* and *b*). Other GAD-immunoreactive cells (*open arrow* in *a*) are not labeled at this short exposure time. *ipl* = Inner plexiform layer. ×1200. *7,* Double-labeled goldfish retina. Autoradiograph of a goldfish retina that had been exposed to ^{3}H-GABA and was also stained immunocytochemically for GAD. At longer exposure times (1–2 weeks), all other types of GAD-immunoreactive cells are lightly labeled because of ^{3}H-GABA uptake (*solid arrows* in *a* and *b*), including a subpopulation of Ab-like pyriform cells (*a*). *ipl* = Inner plexiform layer. ×1200. *8,* Autoradiograph of a goldfish retina exposed to ^{3}H-muscimol and also stained immunocytochemically for GAD. All GAD-immunoreactive cell types showed evidence of ^{3}H-muscimol accumulation (*arrows* in *a*), including Ab amacrines (*Ab* → in *b*). Heavy labeling due to ^{3}H-muscimol uptake was not evident in any GAD-immunoreactive amacrine cell type. One week exposure, 2 µm section. *ipl* = Inner plexiform layer. ×1200. *9,* Autoradiograph showing the uptake of ^{3}H-glycine by a light-adapted goldfish retina. A large number of amacrine cells are labeled (*arrows*), as is the entire IPL. Section, 0.5 µm, lightly stained with toluidine blue, 1 week exposure. *ipl* = Inner plexiform layer. ×1200. *10,* Autoradiograph of a goldfish retina exposed to ^{3}H-glycine and also stained immunocytochemically for GAD. GAD-immunoreactive large Ab amacrines and small cells (*asterisks*) show no evidence of ^{3}H-glycine accumulation. Small amacrine cells labeled because of ^{3}H-glycine accumulation (*arrowheads*) are not GAD-immunoreactive. One week exposure, 2 µm section. *ipl* = Inner plexiform layer. ×1200.



Figures 11, 12, 13, and 14. *11,* Bar graph showing the results of categorizing 814 amacrine cells as (1) GAD-immunoreactive, (2) accumulating ^{3}H-GABA, or (3) double-labeled after 1 week of autoradiographic exposure. Thirty percent of INL perikarya were GAD-immunoreactive; 33% of INL perikarya showed some evidence of ^{3}H-GABA accumulation, and 2.5% of these cells were heavily labeled (*hatched bar*). Twenty-five percent of INL perikarya were GAD-immunoreactive and also accumulated ^{3}H-GABA, demonstrating good correlation between these two markers of GABAergic transmission. *12,* Bar graph showing the results of categorizing 1128 amacrine cells as (1) GAD-immunoreactive, (2) accumulating ^{3}H-muscimol, or (3) double-labeled after 2 weeks of autoradiographic exposure. Thirty percent of INL perikarya were GAD-immunoreactive, 46% of INL perikarya showed some evidence of ^{3}H-muscimol accumulation, and 26% of INL perikarya were GAD-immunoreactive and also accumulated ^{3}H-muscimol. Although most GAD-immunoreactive amacrine cells accumulated ^{3}H-muscimol, a large number of ^{3}H-muscimol accumulating INL perikarya were not GAD-immunoreactive. *13,* Bar graph showing the results of categorizing 828 amacrine cells as (1) GAD-immunoreactive, (2) accumulating ^{3}H-muscimol, or (3) double-labeled after 4 d of autoradiographic exposure. The percentage of double-labeled cells (*GAD/MUSC* = 23%) was not significantly different from the percentage of double-labeled cells obtained at longer exposure times (Fig. 12: *GAD/MUSC* = 26%). However, the percentage of ^{3}H-muscimol accumulating cells (*MUSC-UPTAKE* = 35%) was significantly lower than after a 2 week exposure (Fig. 12: *MUSC-UPTAKE* = 46%). These results suggest that, while ^{3}H-muscimol uptake may be an appropriate marker for GABAergic neurons at short exposure times, longer exposure times reveal the presence of ^{3}H-muscimol in many non-GABAergic cells. *14,* Bar graph showing the results of categorizing 1003 amacrine cells as (1) GAD-immunoreactive, (2) accumulating ^{3}H-glycine, or (3) double-labeled after 1 week of autoradiographic exposure. Thirty percent of INL perikarya were GAD-immunoreactive, 35% of INL perikarya contained amounts of ^{3}H-glycine that were detectable at this exposure time, and only 8% of INL perikarya were double-labeled owing to GAD-IR and ^{3}H-glycine uptake. These results demonstrate the existence of distinct GAD-immunoreactive (GABAergic) and ^{3}H-glycine-accumulating (glycinergic) amacrine cell populations.

fore probably correspond to I2 interplexiform cells and red-hyperpolarizing/green-depolarizing Aa amacrine cells (Marc, 1982; Marc and Lam, 1981).

Double-labeling (GAD and glycine)

Cells that were GAD-immunoreactive took up little or no ^{3}H-glycine (Fig. 10). In 1–5 μm sections (1 week exposure), numerous small INL perikarya were labeled (Fig. 10, arrows), while GAD-immunoreactive cells of all types were rarely labeled (Fig. 10, asterisk).

Quantitative analysis

Although observations were made 5–20 μm from the cut surface of retinal slices so as to avoid false-negative and -positive labeling, the thickness of this "window" varied among slices. As a result, we found numerous examples of GAD-immunoreactive perikarya that did not accumulate ^{3}H-GABA or ^{3}H-muscimol, as well as perikarya labeled, owing to uptake, that were not GAD-immunoreactive. We determined the extent of colocalization by categorizing cells as (1) demonstrating uptake, (2) GAD-immunoreactive, (3) double-labeled, or (4) unlabeled (Figs. 11–14).

After a 1 week exposure, 33% of proximal INL perikarya were labeled due to ^{3}H-GABA uptake (Fig. 11). Only 2.5% of these cells were very heavily labeled (Ab amacrines). Thirty percent of INL perikarya demonstrated GAD-IR; most of these cells also accumulated ^{3}H-GABA, since 25% of proximal INL perikarya were double-labeled.

After 2 weeks of exposure, when ^{3}H-muscimol labeling was qualitatively optimal, 46% of proximal INL perikarya were labeled because of ^{3}H-muscimol uptake (Fig. 12). Although 87% of GAD-immunoreactive perikarya accumulated ^{3}H-muscimol, a large number of cells that accumulated muscimol were not GAD-immunoreactive. At shorter exposure times (4 d, Fig. 13),

35% of proximal INL perikarya were labeled owing to ^{3}H-muscimol uptake. The number of cells that were double-labeled (23%) was not significantly different from that obtained at longer exposure times (26%; Fig. 12). Thus, while ^{3}H-muscimol was taken up by GAD-immunoreactive neurons, longer exposure times revealed uptake by other, non-GAD-immunoreactive neurons as well.

^{3}H-glycine was accumulated by 35% of proximal INL perikarya, presumably those of Aa amacrines (1 week exposure; Fig. 14). Only 8% of GAD-immunoreactive perikarya showed any accumulation of ^{3}H-glycine.

Discussion

Early studies on the autoradiographic localization of ^{3}H-GABA uptake in the goldfish retina revealed heavy uptake by a single class of large, pyriform amacrine cell ramifying in the proximal half of IPL sublamina b (Lam and Steinman, 1971; Marc et al., 1978). These cells made up 2–3% of the amacrine cells in the INL. Other small, lightly labeled cells were also observed but were not considered to be GABAergic because their accumulation of ^{3}H-GABA could not be modulated with colored lights. Our double-label analysis with three markers for GABA-mediated transmission demonstrates that these small cells, as well as the Ab amacrines, are probably GABAergic. These cells comprise approximately 30% of INL perikarya and ramify in both sublamina a and sublamina b of the IPL. That such a large population of GABAergic amacrine cells existed in the goldfish retina was suggested by uptake and binding studies using ^{3}H-muscimol (Yazulla, 1981; Yazulla and Brecha, 1980), uptake of ^{3}H-isoguvacine (Agardh and Ehinger, 1982), and the localization of GAD-IR (Zucker and Yazulla, 1983; Zucker et al., 1984).

The accumulation of ^{3}H-GABA by these small amacrine cells was probably not due to a low-affinity uptake mechanism, since we used radiolabel concentrations that were in the range of the K_m of the high-affinity uptake system (ca. 1 μM). In addition, AOAA was used to prevent the degradation of ^{3}H-GABA by GABA-transaminase, so the autoradiographic grains over labeled cells were due to ^{3}H-GABA rather than to a metabolic byproduct. Nonspecific labeling of these cells, by diffusion during tissue processing, was unlikely because uptake of ^{3}H-GABA has also been demonstrated using conditions (intravitreal injections and freeze-dry autoradiography) chosen to minimize diffusion (Agardh and Ehinger, 1982). Finally, as reported here, these cells contain the biosynthetic enzyme for GABA. In experiments using an antiserum to GABA itself, they have been shown to contain immunoreactive GABA as well (our unpublished observation).

Our results indicate that goldfish amacrine cells are morphologically diverse. At least six cell types contain GAD, accumulate ^{3}H-GABA, and accumulate the GABA analog ^{3}H-muscimol. These six categories were based solely on perikaryal morphology. Because of the density of GAD-immunoreactive neurites in the IPL, further characterization of these cells based on their ramification patterns was not possible. In view of such morphological diversity among the lightly labeled ^{3}H-GABA-accumulating cells, it is not surprising that the labeling pattern due to ^{3}H-GABA uptake could not be modulated by colored lights (Marc et al., 1978). This large population of GABAergic cells is undoubtedly involved in many complex retinal pathways in both IPL sublaminae (Negishi et al., 1978).

Amacrine cells of the Ab type were the only GAD-immunoreactive cells to be heavily labeled by ^{3}H-GABA uptake; other pyriform amacrines, and the smaller, nonpyriform types, were always more lightly labeled. The reason for this differential labeling is not known; it is possible that the heavily labeled Ab amacrines are completely hyperpolarized in the dark, while the other ^{3}H-GABA-accumulating amacrines (including the lightly labeled ones) are not completely hyperpolarized under any light condition. Large net accumulation of ^{3}H-GABA would therefore be observed only in Ab amacrines in the dark (Marc et al., 1978). Differential labeling of amacrine cells is not unique to the goldfish retina. It has also been observed in the cat, where four subpopulations of amacrine cells accumulate ^{3}H-glycine (Pourcho and Goebel, 1985).

Such differential uptake was not observed when retinas were exposed to ^{3}H-muscimol, and muscimol gave lower labeling densities than we obtained with ^{3}H-GABA. Longer autoradiographic exposure times revealed the presence of ^{3}H-muscimol in many cells that were not GAD-immunoreactive. This may be due to the binding of muscimol to sites that are inaccessible to GABA (DeFeudis, 1981) or to the interaction of muscimol with other neurotransmitter systems (Osborne, 1980), but in any event, caution is required in the use of this substance as a marker for GABAergic neurons.

We considered the possibility that the small GAD-immunoreactive cells that were lightly labeled due to ^{3}H-GABA or ^{3}H-muscimol uptake might have been the same cells that took up ^{3}H-glycine. Glycine is accumulated by many small amacrine cells and diffusely labeled processes in at least the distal two-thirds of the IPL (Marc et al., 1978), and these glycinergic Aa amacrines do resemble the small GAD-immunoreactive cells in number and size, and in the distribution of their processes. However, our colocalization experiments demonstrate that 92% of GAD-immunoreactive cells did not take up ^{3}H-glycine and, therefore, that the goldfish contains distinct populations of glycinergic and GABAergic amacrine cells.

In most species, ^{3}H-GABA uptake studies and GAD localization have demonstrated a large number of GABAergic cell types that send processes to all levels of the IPL (Brandon, 1985b; Brecha, 1983). Goldfish were thought to differ from other species in this regard, based on the labeling pattern due to ^{3}H-GABA uptake (Marc, 1982; Marc et al., 1978). Our results demonstrate that this difference is more apparent than real. ^{3}H-GABA uptake and GAD-IR label the same GABAergic neurons, which comprise approximately 30% of INL perikarya. Both markers of GABAergic transmission are useful for the identification of GABAergic amacrine cells in the goldfish retina.

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