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The Uptake of [γ-^{3}H] Aminobutyric Acid in the Goldfish Retina

(*Carissius auratus*/horizontal cells/amacrine cells/light-stimulated retinas)

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ABSTRACT **After goldfish retinas had been incubated for 1 hr with [γ-^{3}H]aminobutyric acid, we found by autoradiography that the label was localized to a few restricted types of retinal cells. In particular, external and internal horizontal cells from light-stimulated retinas were more heavily labeled than corresponding cells from retinas kept in darkness. Some other cells and tissues in the retina also incorporated the labeled acid. Light stimulation, however, did not cause a pronounced change in the amount of label associated with these cells. Among these were some heavily labeled cells on the vitreal side of the inner nuclear layer, and scattered grains associated with the ganglion cell and optic nerve layers. Electrophoresis of retinal extracts after incubation with the labeled acid also showed that light-stimulated retinas contained about 40–100% more radioactivity than retinas kept in darkness, and that 90% of this activity remained as [γ-^{3}H]aminobutyric acid. The role of the acid in the retina is not known; it is not clear if horizontal cells normally synthesize or store it. The stimulation-dependent accumulation of the labeled acid into horizontal cells suggests that it plays a functional role in these cells.**

Electrophysiological studies have shown both excitatory and inhibitory interactions in the retina (1–3). Very little, however, is known about the synaptic chemistry and neurotransmitters involved in these interactions. There is evidence that γ-aminobutyric acid (GABA) acts as an inhibitory neurotransmitter in the crustacean neuromuscular junction (4, 5), and possibly in some vertebrate central nervous systems (6–8). Recently, several groups of workers have reported that GABA and glutamate decarboxylase (EC 4.1.1.15) were present in vertebrate retinas (9–12). The GABA-containing cells and the possible functional role played by GABA in the retina are, however, unknown.

The vertebrate retina offers unique advantages for the study of neuronal organizations and synaptic chemistry in the central nervous system. We have chosen to study the chemical mechanisms of synaptic transmission in the goldfish retina, because it has been used extensively in electrophysiological (13) and morphological (14) studies. Results to be reported separately show that GABA is present and synthesized in the goldfish retina*. In this article, we present autoradiographic and radiochemical evidence indicating that [^{3}H]-GABA is taken up by a few restricted cell-types in the goldfish retina, and that this uptake is influenced by light stimulation.

METHODS

[^{3}H]GABA (specific activity 2 Ci/mmol, New England Nuclear Corp., Boston, Mass.) was dried by flash evaporation and redissolved in Leibovitz medium (L-15, Grand Island Biological Co., Grand Island, N.Y.), which had been diluted to make it isotonic for fresh-water fish (260 mOsm). This medium was used throughout the investigation. In all our experiments, the environment was kept in total darkness (at subscotopic conditions). The eyes were stimulated with a 60W tungsten filament lamp that was placed 30 cm above the water tank or incubation dish; the lamp flashed at a rate of 15 flashes/min (2-sec on and 2-sec off). The temperature was kept constant at 19 ± 2°C throughout the experiments. The goldfish (*Carissius auratus*, 15–18 cm long) used in these experiments were kept in well-aerated water, between 19 and 20°C, under very dim illumination.

In Vitro Incubations. Goldfish eyes were enucleated in very dim light and the cornea, lens, and any muscle attached to the eye cup were removed by dissection. The eye cup was washed with 3 ml of medium for 1 min, and placed in a small Petri dish containing 200 μCi of [^{3}H]GABA in 2 ml of medium, which completely covered the eye cup, for 1 hr under various light conditions. After incubation, the eye cup was cut radially into two equal halves. The retina from each half was detached from the pigment epithelium with fine-tip forceps and washed with shaking under subscotopic conditions with 3 ml of medium for 2–3 min for the removal of the extracellular [^{3}H]-GABA. This washing procedure was repeated three times. The approximate time required for removal of half of the [^{14}C]sucrose (specific activity 346 Ci/mol, New England Nuclear Corp., Boston, Mass.) from the extracellular space of goldfish retinas was found to be 2.5 ± 0.7 min, irrespective of light stimulation.

Half-Light and Half-Dark Retina. An eye cup was partitioned radially into two equal halves by a piece of 4-log unit of a neutral-density gelatin filter (Eastman-Kodak Co., Rochester, N.Y.), that had been cut to fit exactly the circular shape of the cup (Fig. 1). The right half was covered completely with 8-log unit filters (subscotopic). The whole eye cup was incubated *in vitro* with [^{3}H]GABA under flash

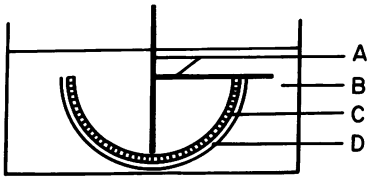

FIG. 1. Schematic diagram of a half-light and half-dark retina. (*A*) neutral density filters; (*B*) [^{3}H]GABA medium; (*C*) retina; (*D*) eyecup.

Abbreviation: γ-aminobutyric acid, GABA.

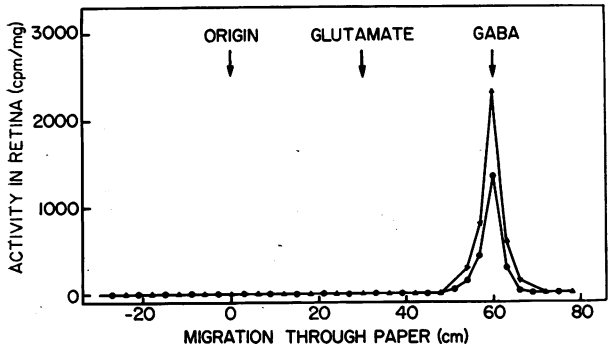

FIG. 2. Paper electrophoresis of homogenates from light-stimulated (▲——▲) and unstimulated (kept in the dark) (●—●) retinas. Activity in retina is expressed as cpm/mg of wet weight. Wet weight per retina averaged from 60 to 110 mg.

stimulation for 1 hr, washed with medium, and then processed for autoradiography. In some experiments, retinas were first isolated from the eye cups and then incubated with [^{3}H]GABA.

In Vivo Injections. 10 μl of medium containing 100 μCi of [^{3}H]GABA was injected with a Hamilton microsyringe into the vitreous humor through the corneal–scleral junction of a goldfish. The amount injected corresponded to about 2% of the volume of the vitreous humor. After injection of both eyes, the right eye was covered with a contact lens made of an 0-log unit of a neutral-density gelatin filter, while the left eye was covered with 8-log unit filters. The fish was kept in a water tank and exposed to flash stimulation for 1 hr. The retinas were then isolated, washed in medium, and processed for autoradiography or electrophoresis.

Autoradiography. Retinas were fixed for 2 hr at 5°C with the following fixative (15): 20 ml of 25% glutaraldehyde, 25 ml of medium, and 5 ml of distilled water. The tissue was post-fixed with 1% OsO_4, dissolved in 0.2 M Sorenson phosphate for 1 hr at 5°C, dehydrated in ethanol, embedded in Epon 812, and sectioned at 3–4 μm thickness with a LKB Ultratome. Slides of the retinal sections were dipped in Kodak NTB2 emulsion that had been diluted 1:1 with distilled water, exposed for various periods of time (*in vitro* experiments: 2–10 days; *in vivo* experiments: 30–60 days) in a light-tight box, developed for 3 min in Kodak Dektol (diluted 1:1 with water), followed by Kodak Rapid Fix for 3 min, and finally washed for 10 min with distilled water. Some slides were subsequently stained with 1% toluidine blue in 18 mM sodium benzoate (pH 4.3).

Extraction and Paper Electrophoresis. Retinas were weighed and homogenized with a ground-glass homogenizer in 50 μl of 0.47 M formic acid–1.4 M acetic acid (pH 1.9, EP buffer), containing 10 mg/ml of unlabeled GABA (grade A, Calbiochem, Los Angeles, Calif.) as a marker. 10 μl of the homogenate was used for high-voltage paper electrophoresis (6 kV, 1.5 hr), as described* (16). After electrophoresis, the paper was dried, then dipped in 2% ninhydrin dissolved in acetone. This test revealed the position of the marker GABA, which was stained purple (17). The paper was then cut into 3-cm strips, and the radioactivity on each strip was eluted with 5 ml of formic acid–acetic acid buffer. Each eluate was dried in a scintillation vial with a stream of air; 10 ml of scintillation fluid (4 g/liter of Omnifluor, New England Nuclear Corp., in toluene) was added to each vial; the radioactivity was measured with a liquid scintillation counter.

* Lam, D. M. K., in preparation.

RESULTS

Uptake of GABA *in vitro*

Two eye cups were incubated *in vitro* with [^{3}H]GABA. One eye cup was covered completely with an 0-log unit density filter (light), the other with 8-log unit filters (dark); both retinas were exposed to light stimulation for 1 hr. Half of each retina was fixed for autoradiography for the identification of the cells that incorporate [^{3}H]GABA; the other half was processed for electrophoresis for the determination of the amount of label and the percentage of radioactivity that was still present as [^{3}H]GABA after the incubation. The results reported in this communication are from experiments that have each been performed four to eight times, to ensure consistent interpretation of our observations.

In all our analyses of retinal extracts by electrophoresis, we find that light-stimulated retinas contain 40–100% more [^{3}H]GABA than retinas kept in the darkness. One such typical finding is shown in Fig. 2. In addition, more than 90% of the label retained by the retinal cells after 1 hr of incubation in either light or darkness remains as [^{3}H]GABA. The demonstration that a large percentage of the radioactivity present in the retina after the incubation was indeed [^{3}H]GABA is a prerequisite for the interpretation of the autoradiographic results shown in Figs. 3 and 4

An autoradiographic comparison between the uptake of [^{3}H]GABA by retinas incubated in the light and in the dark is shown in Figs. 3*A* and *B*. To ensure comparable results, all retinal slides were processed in parallel. Two types of cells in the inner nuclear layer are much more heavily labeled in the light-stimulated retina (Fig. 3). These cells are identified as external and internal horizontal cells by their locations, sizes, and shapes. Both types of horizontal cells are in fact so heavily labeled in the light-stimulated retina that it was possible to compare their geometries with those of Golgi-impregnated (14, 18) or dye-injected (13) horizontal cells (Fig. 4). This comparison strongly supports our conclusions about the identity of these cells.

Although the amount of label associated with the other [^{3}H]GABA-containing cells and tissues in some experiments appears to be higher in the light-stimulated retina, this difference cannot be clearly established due to the insensitivity of our method. A few cells on the vitreal side of the inner nuclear layer are heavily labeled; their location and geometry suggest that they are amacrine cells. Scattered grains are associated with the ganglion cell and optic nerve layers. There is, however, no clear incorporation of [^{3}H]GABA into ganglion cell bodies. The identity of the GABA-containing tissues in the optic nerve layer is not known.

We have also incubated isolated retinas with [^{3}H]GABA under various conditions of light stimulation. We found that the types of cells and tissues that took up the label were identical to those obtained from eye-cup preparations. In addition, the amount of radioactivity associated with horizontal cells again increased with light stimulation. However, under identical processing conditions for autoradiography,

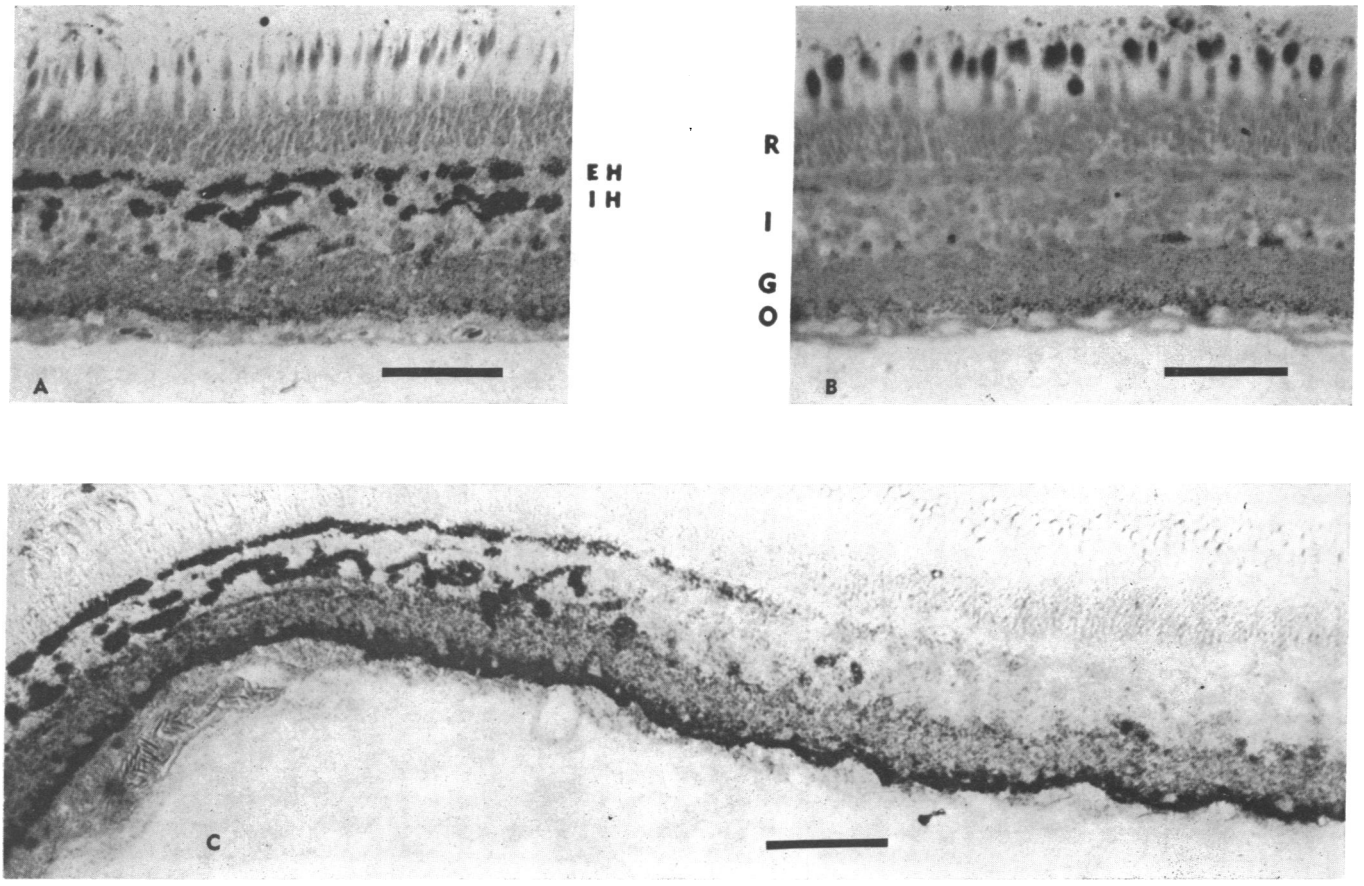

FIG. 3. Autoradiography of goldfish retinas that have been incubated for 1 hr in the light (*A*) and in the dark (*B*) with [³H]GABA. *C*, shows autoradiography of a retina, *left* half was stimulated by light; the *right* half was kept in the dark. *R*, receptor cell layer; *I*, inner nuclear layer; *G*, ganglion cell layer; *O*, optic nerve layer; *EH*, external horizontal layer; *IH*, internal horizontal cells. Scales (Horizontal Bars): 60 μm.

cells from isolated retinas that had been incubated with [³H]GABA were in general more heavily labeled than corresponding cells from eye-cup incubations. The reasons underlying this observation are not clear. To some extent, the difference in the amount of label between isolated retina and eye-cup preparations may be due to the possibility that [³H]-GABA is more readily accessible to the extracellular space from the medium when retinas are isolated, than when they are attached to eye cups.

Half-light-stimulated and half-unstimulated (kept in the dark) retina

A more direct demonstration of the difference in GABA uptake between stimulated and unstimulated retinas was obtained by the use of a single eye cup, half or which was exposed to light stimulation, while the other half was kept at subscoptopic conditions (covered with 8-log unit filters), during the incubation period (Fig. 1). Autoradiography of a radial section of the retina, cut perpendicular to the "light–dark" partition (Fig. 3*C*), again shows that the external and internal horizontal cells from the light-stimulated half of the retina are much more heavily labeled than the corresponding cells from the half-retina that was kept in the dark. Light stimulation, however, seems to have a less pronounced influence on the amount of label taken up by the other [³H]GABA-containing cells and tissues.

Uptake of [³H]GABA *in vivo*

To confirm the observations obtained from the *in vitro* incubations, similar *in vivo* experiments were performed, as described in *Methods*. In order to obtain grain densities comparable to those found in eye-cup incubations, slides with retinas from these experiments were exposed for a much longer time (30–60 days). These experiments also showed that the external and internal horizontal cells were more heavily labeled in the light-stimulated retinas. A similar amount of radioactivity was associated with a few amacrine cells, and some tissues in the ganglion cell and optic nerve layers, irrespective of light stimulation.

DISCUSSION

Since the demonstration that GABA and glutamate decarboxylase are present in rabbit and frog retinas (9, 10), various approaches have been used to localize GABA-containing cells. Kuriyama and coworkers (9) sectioned rabbit retinas into different tangential layers and reported that although GABA and glutamate decarboxylase were present in all retinal layers, they were most concentrated in the ganglion cell layer. Using the same technique, Graham and coworkers (10), who worked with frog retinas, reached the same conclusions, and, in addition, showed that GABA concentration decreases with the adaptation to darkness. Localization of GABA-containing cells was also studied by autoradiography of [³H]GABA uptake into rabbit retina, and

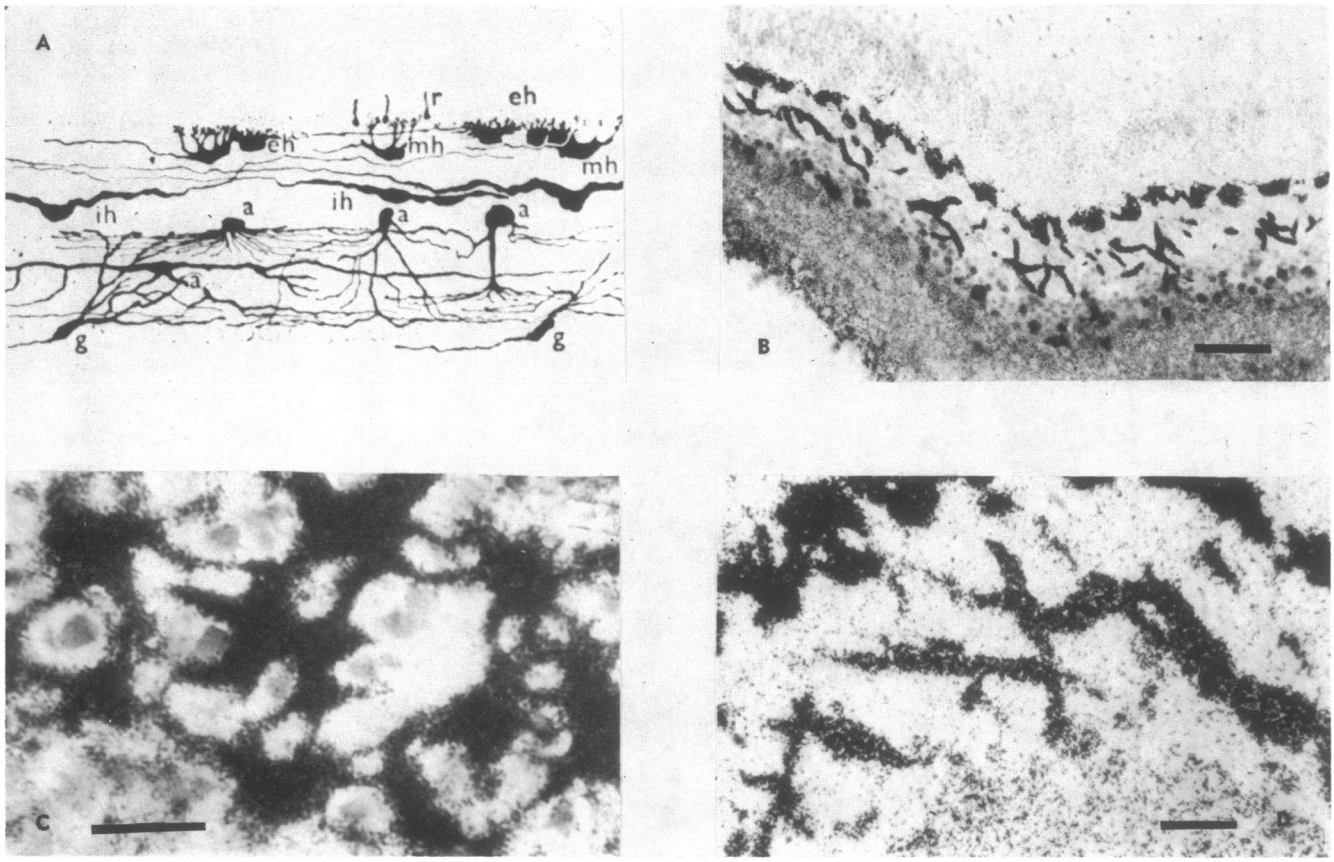

FIG. 4. (*A*) A vertical section of the teleost retina, stained by the Golgi method from Cajal (19). The section shows the horizontal connections in the retina. *r*, rods; *eh*, external horizontal cells; *mh*, intermediate horizontal cells; *ih*, internal horizontal cells; *a*, amacrine cells; *g*, ganglion cells.

B, *C*, and *D* are sections through retinas that have been incubated in [^{3}H]GABA under light stimulation for 1 hr. Scale 40 μm. (*B*) Oblique section showing external horizontal cells and their processes, which extend vertically toward receptors and laterally toward neighboring horizontal cells. The stellate geometry of the internal horizontal cells is also revealed. On the vitreous side of the inner nuclear layer, there is heavy labeling in what may be amacrine cells. Scale: 40 μm. (*C*) *En face* section through external horizontal cells, revealing extensive coupling between neighboring cells. Scale: 20 μm. (*D*) Oblique section through the internal horizontal cells, showing their stellate shape. Scale: 20 μm.

Ehinger (11), reported that the radioactivity was associated with ganglion cells and cells on the vitreal-side of the inner nuclear layer. The extent of stimulation received by the retina during [^{3}H]GABA incubation was, however, not specified.

In the present article, we have attempted to identify GABA-containing cells in the goldfish retina by studying the uptake of [^{3}H]GABA, using autoradiographic and radiochemical techniques. We find that a few cells on the vitreal side of the inner nuclear layer (perhaps amacrine cells), and some of the tissues in the ganglion cell and optic nerve layers incorporated [^{3}H]GABA, in accordance with the results of GABA uptake into rabbit retinas (11). Although autoradiography is useful for the localization and identification of cells, the sensitivity of this technique does not enable us to observe an obvious difference in the amount of label associated with these GABA-containing cells and other tissues after incubation in light or darkness.

More surprisingly, however, our results show that during a 1-hr incubation with [^{3}H]GABA, light stimulation caused the external and internal horizontal cells to be much more heavily labeled than corresponding cells that were kept in the dark (Fig. 3). Chemical analysis also showed that light-stimulated retinas contained about 70% more [^{3}H]GABA than the retinas that were kept in the dark (Fig. 2).

The mechanisms responsible for these observations are not clear. The simplest explanation is that light stimulation directly regulates the rate or extent of GABA uptake (and perhaps release) by the horizontal cells. Alternatively, since in preliminary experiments we have found that the endogenous GABA concentration increases with retinal stimulation and light adaptation*, the effect of light on the accumulation of [^{3}H]GABA in horizontal cells may be a consequence of variations in endogenous GABA concentrations under different conditions of retinal stimulation. Experiments are in progress to distinguish between these possibilities.

Electrophysiological studies of retinal cells in the inner nuclear layer indicate that the horizontal cells may play an important role in lateral interactions, perhaps in the organization of on- and off-center receptive fields of bipolar cells (13, 19). It is not known if horizontal or amacrine cells in the goldfish retina normally contain and synthesize GABA, nor is it clear if GABA plays a functional role in lateral inhibitory mechanisms known to operate in the retina. The pronounced increase in [^{3}H]GABA accumulation in the horizontal cells

that are stimulated by light suggests that GABA plays a functional role in these cells. This role is currently under investigation.

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