

THE BIOSYNTHESIS AND CONTENT OF GAMMA-AMINOBUTYRIC ACID IN THE GOLDFISH RETINA

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ABSTRACT

Goldfish retinas incubated with L-glutamate-¹⁴C (UL) were found to synthesize γ -aminobutyric acid-¹⁴C (GABA-¹⁴C). The accumulation of newly synthesized GABA was enhanced by physiological stimulation of the retina with flashing light; and this increase was directly proportional to the logarithm of the light intensity. The total GABA content was also higher in light-stimulated than in dark-adapted retinas, although the glutamate content remained unchanged. No differences were found in the cell-free activities of glutamate decarboxylase (EC 4.1.1.15) and GABA-glutamate transaminase (EC 2.6.1.19) extracted from light-stimulated and dark-adapted retinas. These findings, together with other physiological and morphological evidence, suggest that GABA plays a functional role in synaptic transmission in the goldfish retina.

INTRODUCTION

The vertebrate retina has for many years been used as a model system to study the organization and synaptic interactions in the central nervous system. Although most retinal studies have emphasized morphology and electrophysiology (1-6), in recent years there have been a number of investigations on retinal synaptic chemistry. The presence of several possible neurotransmitters, for instance acetylcholine, γ -aminobutyric acid (GABA), and dopamine, have been directly demonstrated or inferred (7-12). In particular, several groups of workers have shown that GABA and glutamate decarboxylase (EC 4.1.1.15), the enzyme responsible for the biosynthesis of GABA, were present in the rabbit and frog retinas (7, 8).

In an attempt to localize GABA-containing cells in the retina, Ehinger found by radioautography that in the rabbit retina some cells in the inner nuclear layer and ganglion cell layer incorporated GABA-³H (9). Neal and Iversen (9 a) reported that GABA-³H was taken up by Müller (glial) cells

in the rat retina. In the goldfish retina, Lam and Steinman (10) showed that GABA-³H was also taken up by external and internal horizontal cells. Furthermore, they found that light stimulation enhanced the uptake of label into horizontal cells (10).

In the present investigation the effect of light stimulation on GABA content, biosynthesis, and metabolic enzymes in the retina is examined. It is shown that physiological stimulation of the goldfish retina by flashing light increases the amount of newly synthesized GABA in the retina. In addition, the total concentration of GABA increases when a dark-adapted retina is light stimulated and decreases when a light-stimulated retina is subsequently dark adapted. Although the mechanisms underlying the regulation of GABA accumulation by light stimulation are not known, these findings suggest that GABA plays a functional role in the goldfish retina.

METHODS

Media

Sterile L-15 medium and L-15 medium deficient of L-glutamate (Grand Island Biological Co., Grand Island, N. Y.) were diluted to make them isotonic for fresh-water fish (260 mosmols) and supplemented with 1000 units/ml Penicillin G and 0.5 mg/ml streptomycin sulphate. L-glutamate-¹⁴C (UL, 255 Ci/m from Schwarz Bioresearch Inc., Orangeburg, N. Y.) was dried under a stream of nitrogen, purified by high voltage paper electrophoresis, and added to L-15 medium deficient of L-glutamate. The radioactive media were sterilized by passing them through millipore filters (Swinnex-13 Filter Unit, 0.22 μ pore diameter, Millipore Corporation, Bedford, Mass.) that had been washed with boiling water.

Injections and Incubations

The goldfish (*Carissus auratus*, 15–18 cm long) and the general procedures used in this investigation were similar to those reported in an earlier article (10). The retinas received light stimulation from a 60 W tungsten filament lamp that was about 30 cm away; the lamp flashed at a rate of 15 flashes/min (2 sec on and 2 sec off) while the environment was kept at subscotopic conditions. In all experiments, the temperature was kept at $21 \pm 2^\circ\text{C}$. 10 μl of glutamate-free medium containing 2 μCi of L-glutamate-¹⁴C (UL) was injected into the vitreous humor through the corneal-scleral junction of a goldfish with a Hamilton microsyringe. After injection of both eyes, the eyes were covered with contact lenses made of gelatin filters (Eastman Kodak Co., Rochester, N. Y.) of different neutral densities (0–12 log units). The fish were kept in a water tank and exposed to light stimulation for various periods of time. The retinas were then isolated, washed in excess medium under total darkness for 1–10 min, and processed for paper electrophoresis. The release of L-glutamate-¹⁴C from the retina into the medium followed a biphasic decay, the first phase having a half-time of 2.5 ± 0.7 min and the second 14 ± 2.7 min, irrespective of light stimulation. The time required for removal of half of the sucrose-¹⁴C from the extracellular space of the retina was about 2.5 ± 0.7 min, also irrespective of light stimulation (10). Less than 5% of the newly synthesized GABA-¹⁴C was released into the medium after 10 min of washing under subscotopic conditions.

Extraction and Paper Electrophoresis

Retinas were weighed (60–110 mg per retina) and homogenized vigorously with a ground glass homogenizer in two times the retinal wet weight of 0.47 M

formic acid–1.4 M acetic acid, containing 5 mg/ml of unlabeled GABA (grade A, Calbiochem, Los Angeles, Calif.) as a marker. 5–15 μl of the homogenate was used for high voltage paper electrophoresis (6 kv, 1.5 hr) according to the method of Hildebrand et al. (13). After electrophoresis, the paper was dried, cut into 3 cm strips, and dipped into saturated iodine vapor or 2% ninhydrine dissolved in acetone to reveal the position of GABA. The locations of glutamate-¹⁴C and GABA-¹⁴C on the paper were identified by radioactive scanning using a chromatogram scanner (Model 880, Vanguard Instrument Corp., Melville, N. Y.). The regions occupied by GABA-¹⁴C and glutamate-¹⁴C in unstained strips were then cut out and each was placed in 10 ml of scintillation fluid (4g/liter Omnifluor, from New England Nuclear Corp., Boston, Mass., in toluene). The radioactivity was measured with a scintillation counter. Occasionally, the identity of GABA-¹⁴C in unstained strips was checked by eluting the label associated with the GABA region with 2 ml of 0.01 M HCl for 4 hr. The eluate was divided into two equal portions and each was dried by flash evaporation. One portion was then incubated with a solution containing 5 μl Tris (1 M, pH 7.9), 15 μl Na₂SO₄ (1 M), 50 μl β -mercaptoethanol (0.1 M), 5 μl nicotinamide adenine dinucleotide phosphate (50 mM), 10 μl α -ketoglutarate (20 mM), and 10 μl GABAse (5 mg/ml, GABACF, Worthington Biochemical Corp., Freehold, N. J.) for 2 hr at 25°C (14), this incubation converted GABA in the solution to succinate. The other portion was incubated in the same solution in the absence of GABAse. After the incubation, 10 μl from each portion was used for high voltage paper electrophoresis, and the label associated with the GABA region was measured by scintillation. The portion treated with GABAse had less than 5% of GABA-¹⁴C activity found in the untreated portion.

Amino Acid Analysis

Retinas were isolated under dim illumination, washed in isotonic Ringer's solution (125 mM NaCl, 3 mM KCl, 1.8 mM CaCl₂, 1.1 mM MgCl₂, 6.2 mM NaHCO₃, 0.6 mM NaH₂PO₄, pH 7.2) for 10 sec and weighed. Six to eight retinas were homogenized vigorously in 1 ml of cold 10% trichloroacetic acid (TCA) with a ground glass homogenizer. The homogenate was centrifuged at 10,000 g for 10 min. Cold 5% TCA was added to the supernatant until a final volume of 2 ml was obtained. The amino acid contents, in particular the glutamate and GABA concentrations, in the 2 ml sample were measured with a Jeol Amino-Acid Analyser (Tokyo Electronic Co., Tokyo, Japan). During each analysis, GABA solutions of known concentrations were run as a marker and internal standard.

Cell-Free Enzyme Activities

Glutamate decarboxylase (GAD) activity was assayed by a modification of the method described by Molnoff and Kravitz (15). Four retinas were weighed and homogenized in four times the retinal wet weight of GAD buffer (100 mM β -mercaptoethanol, 1 mM pyridoxal phosphate, 200 mM KH_2PO_4 , pH 7.3). 15 μl of the retinal extract (3 mg wet weight), and 25 μl of glutamate- ^{14}C (UL) (SA 50 Ci/M, concentration 10 $\mu\text{Ci/ml}$) were added to 150 μl of GAD buffer. The incubation was carried out at $19 \pm 2^\circ\text{C}$ for 0.5–3 hr, and was stopped by injecting 10 μl of 5 N HCl into the reaction mixture. $^{14}\text{CO}_2$ formed during the incubation period was collected in 0.1 ml of 1 M hyamine hydroxide in methanol. At least 10 hr after the end of the incubation, the hyamine was dissolved in 10 ml of toluene-Omnifluor scintillation fluid and the radioactivity associated with $^{14}\text{CO}_2$ was measured with a scintillation counter. In some cases, 10 μl of the incubation mixture was used for paper electrophoresis to determine the amount of GABA- ^{14}C that was synthesized. Theoretically, the total GABA- ^{14}C activity in the mixture should be four times the total activity of $^{14}\text{CO}_2$ generated during the incubation. It was found that results obtained from measurements of both GABA- ^{14}C and $^{14}\text{CO}_2$ formation agreed to within 15% of each other.

GABA-glutamate transaminase (EC 2.6.1.19) activity was measured by a radiochemical procedure, as described by Hall and Kravitz (16). Four retinas were weighed and homogenized vigorously with a ground glass homogenizer in four times the retinal wet weight of 100 mM Tris buffer (pH 8.5). The reaction was started by adding 20 μl of the retinal homogenate (4 mg wet weight) to a solution containing 10 μl α -ketoglutarate (20 mM), 20 μl pyridoxal phosphate (15 mM), 10 μl succinate (10 mM), 10 μl Tris (1 M, pH 8.5), 10 μl nicotinamide adenine dinucleotide (30 mM), 10 μl β -mercaptoethanol (100 mM), 10 μl GABA- ^{14}C (SA 3.7 Ci/M, concentration 30 mM, from Schwarz Bio Research.). The reaction proceeded at $19 \pm 2^\circ\text{C}$ for 0.5–3 hr and was ended by injecting 10 μl of 40% TCA into the reaction mixture, which was then applied directly to a column (0.5 cm \times 5 cm) of Dowex-50- H^+ , (100–200 mesh, X2). Since GABA was retained by the column, the radioactive products succinate and succinic semialdehyde were collected in the eluate by washing the column with 6 ml of water. 2 ml of the eluate was added to 8 ml of Aquafuor (New England Nuclear Corp.) and the radioactivity in the eluate was measured by scintillation counting. The data obtained were corrected for quenching and background.

Aminooxyacetic acid is known to inhibit the activity of GABA-glutamate transaminase. It was found that in the presence of 10 $\mu\text{l/ml}$ aminooxyacetic acid, a concentration which did not ap-

preciably affect glutamate decarboxylase activity, the cell-free activity of GABA glutamate transaminase measured by this assay was reduced by more than 90 per cent.

The cell-free activities of both glutamate decarboxylase and GABA-glutamate transaminase were found to be linear with reaction time from 0 to 4 hr and with enzyme (homogenate) concentrations.

RESULTS

Effect of Light on GABA Biosynthesis

Preliminary investigations on neurotransmitter synthesis, together with the findings concerning GABA uptake in the retina (9, 10), led to studies on the regulation of GABA biosynthesis and storage in the goldfish retina. GABA biosynthesis was demonstrated *in vivo* by the isolation of GABA- ^{14}C from the goldfish retina after an intracocular injection of L-glutamate- ^{14}C (UL). 4 hr after the injection, the radioactivity in the retina was extracted and analyzed by paper electrophoresis. Typical results from such an experiment showed that the goldfish retina synthesized GABA from the precursor glutamate (Fig. 1).

To determine whether the accumulation of newly synthesized GABA was dependent upon physiological stimulation, the amounts of GABA- ^{14}C present in light-stimulated and nonstimulated

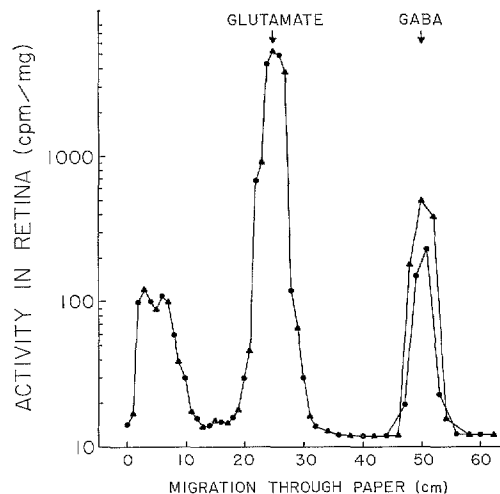


FIGURE 1. Paper electrophoresis of homogenates from light-stimulated (\triangle - \triangle - \triangle) and nonstimulated (kept in the dark) (\bullet - \bullet - \bullet) retinas incubated *in vivo* with L-glutamate- ^{14}C (UL). Activity in retina is expressed as cpm per mg wet weight. Wet weight per retina averaged from 60 to 100 mg.

(kept in total darkness) retinas 4 hr after intraocular injections of L-glutamate- ^{14}C (UL) were compared. It was found in four in vivo (Fig. 1) and four in vitro (eye cup or isolated retina preparations) experiments that, although the amount of L-glutamate- ^{14}C in the retina remained unchanged, the level of GABA- ^{14}C in light-stimulated retinas was 70–100% higher than that in dark-adapted retinas.

Neural activities in the retina are dependent on the intensity, frequency, and other parameters of the light stimulation received by the retina. To study in more detail the influence of light on the accumulation of newly synthesized GABA, the effect of varying light intensity was then examined by injecting L-glutamate- ^{14}C (UL) into both eyes of seven goldfish that had been kept in darkness for 12 hr (dark adapted). The left eyes were covered with neutral density filters (1–12 log units), the right eyes were covered with 0 log unit filters, and the fish were exposed to light stimulation for 5 hr. Analyses of retinal extracts by paper electrophoresis showed that the amount of GABA- ^{14}C present in goldfish retinas was directly proportional to the logarithm of the light intensity over a range of 8 log units (Fig. 2).

Endogenous Levels of GABA

The finding that light stimulation increased the accumulation of newly synthesized GABA suggested that this increase might also be reflected in the total content of GABA in the retina. The in-

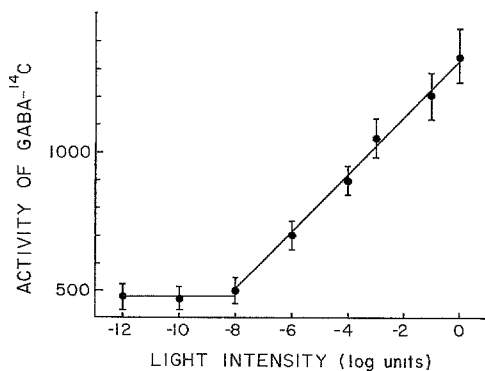


FIGURE 2 Accumulation of GABA- ^{14}C in retinas that received different intensities of light stimulation. Activity is expressed as cpm of GABA- ^{14}C accumulated per 0.5 hr (averaged over 4 hr of incubation) per 10 mg wet weight of retina. The bars represent standard deviations from at least four observations per points.

fluence of light stimulation on total GABA level in the goldfish retina was therefore studied. Dark-adapted goldfish were light stimulated for various periods of time (0–300 min) and the amount of retinal GABA was measured by means of amino acid analysis. As shown in Fig. 3 (A), GABA concentration in dark-adapted retinas increased during the first 90 min of light stimulation and then gradually reached an equilibrium level. It is not known whether the time taken to reach this level and the magnitude of the equilibrium level itself vary with the intensity and rate of light stimulation received by the retinas. No measurement was made of these variables or of the equilibrium level at adaptation to continuous illumination.

The change in GABA content during dark adaptation was also studied. Goldfish that had been light stimulated for at least 12 hr were kept in darkness for various periods of time (0–300 min) and the GABA concentration on these retinas was determined. During the first 2 hr in the dark, the GABA content in the retina decreased until it reached a steady level equal to that of the dark-adapted retina (Fig. 3 [B], Table I). These findings suggested that the total GABA content in the goldfish retina was regulated by light stimulation.

Kinetics of GABA- ^{14}C Accumulation during Stimulation

In an attempt to understand how retinal GABA concentration changed with visual stimulation, the time course of the accumulation of newly synthesized GABA was examined. A group of dark-adapted goldfish were injected intraocularly with L-glutamate- ^{14}C (UL) and kept in darkness for 30

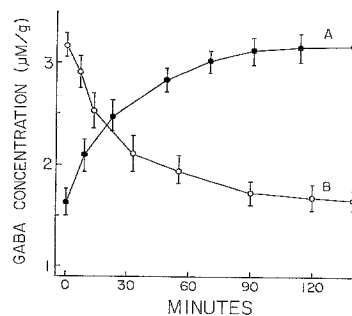


FIGURE 3 Effect of dark adaptation (—●—●—) and light stimulation (—○—○—) on GABA content ($\mu\text{moles per g wet weight}$) in the retina. The bars represent standard deviations from at least four observations per point.

TABLE I
Effect of Light Stimulation on Cell-Free Enzyme Activities, Glutamate and GABA Concentrations in the Retina

	Light	Dark	Change
			%
Glutamate decarboxylase	1.08 ± 0.12	0.99 ± 0.10	9
GABA-glutamate transaminase	4.05 ± 0.25	4.02 ± 0.21	0.7
Glutamate	3.87 ± 0.45	3.80 ± 0.40	1.9
GABA	3.15 ± 0.35	1.65 ± 0.21	93*

Substrate: μ moles/g wet weight of retina.

Enzyme activity: μ moles/hr per g wet weight of retina.

* Difference highly significant by student's *t* test, $p < 0.0005$.

min, the amount of GABA-¹⁴C in the retinas was then measured. Other dark-adapted goldfish were light stimulated for various periods of time (0-300 min) and then injected with L-glutamate-¹⁴C (UL). After the injection, these fish were light stimulated for a further 30-min and the amount of GABA-¹⁴C accumulated in the retinas during this period of time was measured by paper electrophoresis and scintillation counting. As shown in Fig. 4, during the first 30 min when dark-adapted retinas were light stimulated, the retinas contained about eight times more GABA-¹⁴C than did dark-adapted retinas kept in darkness for a similar period. The rate of GABA-¹⁴C accumulation then decreased with further light stimulation until after about 2 hr of light stimulation, when it reached a steady level equal to about twice the accumulation rate of GABA-¹⁴C in dark-adapted retinas.

Cell-Free Enzyme Activity

The preceding findings indicated that visual stimulation affects the accumulation of newly synthesized GABA and its total content in the goldfish retina. To determine whether light stimulation influences the biosynthesis or breakdown of GABA, the substrate (glutamic acid) concentrations in light-stimulated and nonstimulated (dark-adapted) retinas were measured. As shown by amino acid analysis, the level of glutamic acid in the goldfish retinas was not significantly changed with visual stimulation (Table I). Unfortunately, the intracellular concentrations of glutamic acid in specific retinal cells, especially in GABA-containing cells, could not be determined by the measurement of glutamate content in the whole tissue. The enzyme activities of glutamate decarboxylase and GABA-glutamate transaminase

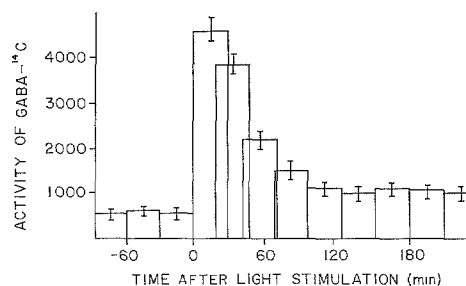


FIGURE 4 Accumulation of GABA-¹⁴C in the retina at various time intervals after light stimulation. Activity is expressed as cpm of GABA-¹⁴C accumulated in the retina per 0.5 hr per 10 mg wet weight of retina. The bars represent standard deviations from at least four observations per point.

(the enzyme responsible for the degradation of GABA in tissue) in homogenates of dark-adapted and light-stimulated retinas were next measured. Results from such analyses showed that the cell-free activities of both enzymes prepared from light-stimulated and dark-adapted retinas remained unchanged (Table I). This observation suggested that light stimulation did not induce the specific biosynthesis or breakdown of the enzymes glutamate decarboxylase and GABA-glutamate decarboxylase in the goldfish retina.

In the lobster, it has been shown that the cell-free activity of glutamate decarboxylase extracted from inhibitory axons is inhibited by GABA (65% inhibition at 100 mM GABA, [17]), so that in such a system GABA biosynthesis might be regulated by product inhibition. In the nerve-ending particles of mouse brain, Kuriyama et al. (18) reported that significant inhibition (25%) of glutamate decarboxylase activity occurred in the presence of 100 mM GABA, but not at lower GABA concentrations

TABLE II
Effect of GABA on the Cell-Free Activity of Retinal
Glutamate Decarboxylase

Exogenous GABA Concentration (mM)	Decarboxylase activity μ moles/hr per g wet weight of retina
0	1.04 \pm 0.14*
0.1	1.05 \pm 0.13
1	1.06 \pm 0.14
10	1.00 \pm 0.17
100	0.90 \pm 0.15‡

* Each value represents the average and standard deviation of four observations.

‡ Difference not significant by student's *t* test.

In the goldfish retina, however, using the enzyme assay described in Methods, the cell-free activity of glutamate decarboxylase present in the retinal homogenate was not significantly inhibited (less than 15% inhibition) by exogenous GABA at a concentration of up to 100 mM (Table II). The reasons for this difference in product inhibition between the various vertebrate and invertebrate enzymes are not known.

DISCUSSION

In this paper, it is shown that the accumulation of newly synthesized GABA and total GABA contents are higher in light-stimulated than dark-adapted goldfish retinas. Graham et al., using frog retinas (8), also found a decrease in GABA content when light-adapted retinas were kept in the dark. In addition, they reported that the average cell-free activity of glutamate decarboxylase in light-adapted retinas was higher than in dark-adapted retinas. In this investigation, however, it was found that both glutamate decarboxylase and GABA-glutamate transaminase extracted from light-stimulated or nonstimulated (kept in darkness) retinas have the same cell-free enzymatic activities (Table I). Likewise, light stimulation did not significantly change the glutamate content in the goldfish retina.

The mechanisms whereby light stimulation influence GABA accumulation in the retina are not known. GABA concentration in the retina is determined by the biosynthesis, breakdown, uptake, and release of this compound, and changes in GABA accumulation might be explained by changes in one or more of these processes. Although the cell-free activities of glutamate decarboxylase and GABA-glutamate transaminase in

stimulated and nonstimulated retinas remain unchanged, these results do not exclude the possibility that the GABA biosynthetic or degrading activities are influenced by light stimulation in the intact retina. As to the uptake and release of GABA, it is similarly not clear how these variables are affected by light stimulation.

One of the major difficulties associated with the study of synaptic chemistry in the retina lies in our inability to localize the cells that synthesize and contain possible neurotransmitters. For instance, even after GABA concentration in the whole retina has been measured, the influence of light on the GABA contents of various types of retinal cells remains unknown unless each cell type can be analyzed independently. Although the vertebrate retina represents a relatively simple part of the central nervous system, it consists of one type of glial cells (Müller cells) and five major types of neurons (receptor, bipolar, horizontal, amacrine, and ganglion cells) each having numerous synaptic contacts. Although previous radioautographic findings (9, 10) show that horizontal and some amacrine cells probably contain and take up GABA, they fail to demonstrate that these cells normally synthesize GABA and use GABA as a neurotransmitter.

It is of interest to note that electrophysiological studies have suggested that horizontal and amacrine cells play a role in the organization of center-surround receptive fields of bipolar and ganglion cells (5, 6). In particular, Naka and Nye (19) have recently shown that electrical stimulation of horizontal cells influences the receptive-field organization of ganglion cells in the catfish retina. There is also evidence that the antagonistic surround is absent from ganglion cells of dark-adapted cat retinas (20). These physiological observations, together with our findings concerning GABA uptake (10) and accumulation, and the evidence that GABA acts as an inhibitory neurotransmitter in the lobster neuromuscular junction (21, 22), and probably in the vertebrate central nervous system (23, 24), suggest that, in the goldfish retina, GABA may play a functional role in synaptic transmission, particularly in the organization of center-surround receptive fields.

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