An autoradiographic study of the cells accumulating $^3$H $\gamma$-aminobutyric acid in the isolated retinas of pigeons and chickens

John Marshall and Mary Voaden

Light and electron microscopic autoradiography have been used to identify the cells accumulating $^3$H-GABA into the chicken retina and both the "red spot" and peripheral areas of the pigeon retina. The results are essentially the same in both species. $^3$H-GABA is incorporated into horizontal, amacrine, and ganglion cells and there is also a localized uptake into a synaptic stratum in the proximal regions of the inner plexiform layer. The findings are discussed in terms of retinal organization.

Key words: retina, autoradiography, neurotransmitters, amino acids.

Species differences exist in the cells taking up $\gamma$-aminobutyric acid (GABA) into retinal tissue. In the rat retina it is the Müllér's fibers that are active and in the rabbit retina, while these cells show incorporation, uptake also occurs into amacrine cells. In contrast, in the frog and goldfish, the uptake is solely neuronal and occurs into ganglion, amacrine, and horizontal cells.

The species variation in the patterns of incorporation may, in part, relate to functional differences in the retinal ganglion cells but before a comprehensive analysis can be made more species must be studied. For the present investigations we have chosen retinas from the pigeon and chicken. These are of interest, not only because of the anatomic and functional characteristics of the retinas (see Discussion), but also because our species survey has, so far, lacked studies on the avian retina. Results on the pigeon retina have been reported in a preliminary form.

Materials and methods

The pigeons (Columba livia) and chickens (Gallus domesticus) were maintained under normal room light conditions, 12-hour diurnal cycle, before use. Pigeons were killed by an overdose of nembutal injected into the air sac, and chickens by decapitation.

General experimental procedure. The eyes were enucleated, hemisected circumferentially at the equator, the vitreous body removed, and the retinas gently teased from the back of the eye with...
Figs. 1 and 2. Light microscopic autoradiographs of the red spot area of pigeon retina, showing the distribution of cells incorporating $^3$H-GABA, both with (Fig. 1) and without (Fig. 2) an intact pigment epithelium. N-nerve fiber layer, G-ganglion cells, S-innermost synaptic region in the inner plexiform layer, A-amacrine cells, and H-horizontal cells. (×901.)

an iris repositor. These manipulations were performed under normal room light conditions. Portions of retinas from the red spot and the periphery were isolated and incubated both with and without pigment epithelium.

A Kreb's bicarbonate medium (pH 7.4) was used for the incubations. The bicarbonate and then the whole medium were gassed with 5 per cent carbon dioxide in oxygen. Each portion of retina was placed in 2.0 ml. of medium containing 100μCi per milliliter of $^3$H-GABA (1.0 × 10$^{-3}$ M; NEN Chemicals Ltd., GmbH., Frankfurt, West Germany). Incubations were performed for 45 minutes at 37° C. in 15.0 ml. specimen bottles covered in tinfoil. The bottles were agitation slowly and the medium was bubbled, throughout the incubation, with a 95 per cent O$_2$/5 per cent CO$_2$ gas mixture. Care was taken not to disrupt the tissue structure. At the end of the incubation period the retinas were rinsed, fixed in glutaraldehyde followed by osmium tetroxide, and processed for light microscopic autoradiography as described by Marshall and Voaden.

For electron microscopic autoradiography, thin sections were picked up on carbon-coated stainless-steel grids. The emulsion coating procedure is a modification of that developed by Dr. S. A. Hodson (personal communication).

Collodion membranes are prepared by allowing single drops of 1 per cent collodion in amyl acetate to fall onto the surface of clean distilled water in a crystallizing dish. The membranes so formed are picked up by lowering 60 mm. squares
Figs. 3 and 4. Light microscopic autoradiographs of the peripheral area of pigeon retina incubated in $^3$H-GABA, both with (Fig. 3) and without (Fig 4) intact pigment epithelium. Note the reduction in both ganglion cell and amacrine uptake. Labels are as in Figs. 1 and 2.

of 5 mm. thick perspex, in the center of which 40 mm. diameter holes have been machined, down onto them. The membrane covering the hole is carefully lifted from the water surface and dried. This procedure is then repeated with the other side of the perspex square, so that the system then has a double-membrane coat over the central hole. When dry, this membrane system is dipped in a solution of Ilford L-4 emulsion diluted one to three with distilled water and held at 40°C in a water bath. The procedure is carried out under an Ilford 902 safe light. Care must be taken to keep the double-membrane system vertical when moist, as the two membranes will collapse together and adhere. After coating, and when partially dried, one of the membranes is destroyed by piercing with forceps.

The experimental grids are arranged on the polished end of a short perspex rod, 30 mm. in diameter. The single remaining emulsion—collodion membrane is lowered emulsion side down onto the grids. The weight of the perspex square causes the membrane to break at the periphery and the square drops down the perspex rod. After a short drying period the grids are pricked out of the collodion emulsion sheet and are dropped into repeated changes of amyl acetate to dissolve away the collodion. The grids are then dried and loaded into a stainless-steel grid holder, before being immersed in a toluene-based scintillator in the dark, for one week.

After exposure, the grids are rehydrated through an alcohol/water series before being developed and fixed in Kodafix. The grids are then removed from the holder and stained with uranyl acetate and lead hydroxide. All grids were examined in an AEI 801 microscope operating at 60 kv. and images were recorded on Ilford EM 6 photographic plates.

Results
The results are shown in Figs. 1 through 7.

In both the pigeon and chicken retinas, $^3$H-GABA is concentrated by horizontal,
Fig. 5. Electron microscopic autoradiograph of the innermost retinal layers in the red spot area of pigeon retina $^3$H-GABA can be seen to have been incorporated into neural components of the inner plexiform layer (P) and over both ganglion cell bodies (G) and their nerve fibers (F). No grains can be seen over the Müller's fibers (M). (x3,275.)

Fig. 6. Electron microscopic autoradiograph of an amacrine cell in the red spot area of pigeon retina showing the incorporation of $^3$H-GABA. Note the presence of mitochondria (E) within this cell. No grains are present over the Müller's fiber (M). (x10,275.)

Fig. 7. Light microscopic autoradiograph of chicken retina showing the distribution of cells incorporating $^3$H-GABA. Note the presence of high incorporation over the innermost boundary of the inner plexiform layer (S). Labels are as designated in Figs. 1 and 2. (x781.5.)

Discussion

The pattern of $^3$H-GABA uptake in chicken and pigeon retinas is essentially the same as those seen previously in the goldfish and frog: amacrine, horizontal, and ganglion cells are labeled but there is no evidence for GABA accumulation by the retinal glia. The similarities are of interest as it is recognized that all four are complex retinas and have a number of functional and morphologic similarities. In contrast, the species in which GABA en-

amacrine, and ganglion cells. In addition, there is a localized uptake in the inner plexiform layer; this is particularly pronounced in the region adjacent to the ganglion cells. There was no evidence for GABA uptake by Müller's fibers—either at the LM or the EM (Figs. 5 and 6) level.

Less $^3$H-GABA was taken up into retinas that had the intact pigment epithelium still attached (Figs. 1 and 3). The most obvious decrease occurred in the amount of GABA that entered the horizontal cells.
ters only into the glial cells, the rat, cat, and baboon are markedly different.

In the "complex" retinas a large number of ganglion cells respond to stimuli such as size, shape, and direction of motion of an object, rather than the apparently more simple, antagonistic, center-surround responses found, for example, in the rat, cat, and primates. Associated with the "complex" responses is a large increase in the proportion of amacrine cells in the retina and a consequent greater development of the inner plexiform layer. Several synaptic sublayers may be recognized, both morphologically and chemically, and catecholamine fluorescence and acetylcholinesterase activity have been shown to be associated with specific regions. The present results show that, in the avian retina, differences exist also in the ability of the sublayers to accumulate GABA—the most intense activity occurring in the region bordering on the ganglion cells. The predominant synaptic terminals in this region have not been identified. However, reports do suggest an increase in the amacrine terminal population in the proximal regions of the inner plexiform layer. The area is of interest as it could prove a suitable one in which to study potential transmitter actions of the amino acid.

No fundamental differences were seen between the incorporation of GABA in the "red spot" and peripheral regions of the pigeon retina, except that there was a decrease in the uptake into ganglion cells in the latter. The anatomy of the peripheral retina does suggest a more simple functional organization of the ganglion cells than in the red spot area, but both are "complex" when compared with the retinas of primates and other mammals.

It is recognized that "rod" cells predominate in the peripheral retina of the pigeon and chicken. It is of interest, therefore, that GABA was accumulated into horizontal cells in these areas. In previous studies, horizontal cells have not shown activity in species with predominantly rod photoreceptor populations (rat and rabbit). The photoreceptors in the avian retina differ from those in mammals in that they are less clearly differentiated at the synaptic level and both rods and cones have synaptic pedicles with multiple postsynaptic contacts. In mammals, rod photoreceptors usually make single contacts only. Mammalian cone photoreceptors do make multiple postsynaptic contacts and studies are now needed on a retinal area rich in these, e.g., the primate macula.

The decrease in the uptake of the amino acids observed when the pigment epithelium remained attached to the neural retina (Figs. 1 and 3) is consistent with the presence of the zonulae occludens at the inner margin of these cells—the probable location of part of the blood-retinal barrier. Entry of the amino acids into the retina will then occur only from the vitreal surface. The uptake would appear to be limited solely by the rate of free diffusion as there is no evidence for a barrier at the ganglion cell level; peroxidase diffuses freely through retinas from this surface.

In the course of this study, many electron micrographs of the inner layers of the pigeon retina have been prepared. It has been a consistent finding that mitochondria were present in all the cell types observed (Fig. 6). This is contrary to the observations of Hughes, Jerrome, and Krebs who concluded that mitochondria did not exist in the inner retinal layers of this species.

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