Growth of the Adult Goldfish Eye

II. INCREASE IN RETINAL CELL NUMBER

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ABSTRACT The retinas of adult goldfish, one to four years of age, 4-23 cm in length, were examined with standard paraffin histology to determine if new cells were being added with growth. Retinal cell nuclei were counted and the area of the retina was measured. An analysis of cell densities in various regions throughout the retina showed that the cells are distributed nearly homogeneously. The density (No./mm^2 of retinal surface) of ganglion cells, inner nuclear layer cells and cones decreases with growth, but the density of rods remains constant. Thus the rods account for a larger proportion of the cells in larger retinas. The total number of cells per retina increases: the ganglion cells from 60,000 to 350,000; the inner nuclear layer cells from 1,500,000 to 4,000,000; the cones from 250,000 to 1,400,000; the rods from 1,500,000 to 15,000,000. This increase in the number of retinal neurons implies the formation of even more new synapses, and suggests the adult goldfish retina as a model for both neuro- and synaptogenesis.

Although it is generally acknowledged that vertebrate eyes continue to grow postembryonically (Walls, '47; Mann, '69; Easter et al., '77), the mechanism of this growth has not been studied in detail. Neurogenesis is normally thought of as an embryonic phenomenon, and later increases in the area of the neural retina are usually attributed to vaguely defined forces tending to stretch the eyeball. Some workers have suggested that these stretching forces might result from vitreal secretion and the accompanying increases in intraocular pressure (Weiss, '49; Coulombre, '55; Ali, '64; Mann, '69). The possibility that postembryonic retinal growth might also involve neuronal addition has been considered in only a few species of teleost fish and in one amphibian. These studies showed that the number of retinal cells does increase with growth even in the adult (Müller, '52; Lyall, '57a; Blaxter and Jones, '67; Wilson, '71).

The simultaneous requirements of visual function make postnatal neurogenesis in the retina particularly intriguing. Goldfish have been studied extensively by both physiologists and anatomists (for a list of references see Rodieck, '73), so this species is an appropriate choice for a quantitative study of cell numbers in which the goal is to understand the integration of retinal development with visual function.

In this study, we report on the numbers of retinal cells in goldfish of different sizes. We believe that differences in size are related to age and reflect growth. The possibility that such differences could be due to polymorphism in body size was tested using a standard ichthyological technique for relating size and age. This analysis showed that our fish varied in size according to their age. In the following study (Johns, '77) goldfish were maintained over long periods of time so that retinal growth and cell addition could be directly assessed using the technique of ^{3}H-thymidine radioautography.

METHODS

Common goldfish (*Carassius auratus*), 4-23 cm long tip-to-tip, were obtained from Ozark Fisheries, Stoutland, Missouri. They were kept in aerated aquaria at room temperature (18-22°C), and fed dry commercial fish food (TetraMin).

Age analysis. The ages of 101 fish of various sizes were determined using a standard ichthyological technique (Lagler, '56). Fish scales grow at a non-uniform rate, adding a new increment each year, so that the age of the fish can be determined simply by counting the number of annual growth rings.

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Two scales were removed from each fish from a region dorsal to the lateral line and just caudal to the opercular flap. They were viewed at × 40 with a rear-projection device (Lagler, '56).

Histological methods. Smaller fish were fixed *in toto;* larger animals were decapitated and their heads fixed in Bouin's for at least 48 hours. The cornea was punctured to expedite the penetration of fixative. Following fixation, the eyes were enucleated, a notch cut in the iris at the dorsal pole for purposes of orientation, and the lens removed. The eyes were embedded in paraffin (Paraplast, m.p. 61°C) and sectioned at 5 μm. In some cases, the sections were treated prior to staining with a 1-5% solution of potassium permanganate followed by 2% oxalic acid to bleach the pigment in the pigmented retinal epithelium (A. Lockett, personal communication).

Computation of retinal area. In frozen sections, and presumably in vivo, the goldfish retina is approximately spherically symmetric, and subtends an angle, ρ, of about 185° with respect to its center (Easter et al., '77). Therefore, the retinal area, A, is given by:

$$A = \int_0^{92.5} 2\pi r^2 \sin\rho \, d\rho,$$

where r = radius of the sphere,
ρ = angle of the retinal locus with respect to the center of the sphere, $\rho = 0$ corresponds to the pole.

The value of r could not be obtained directly from radial sections of histological material, since they were usually distorted from the situation in life. However, r can be estimated from the "retinal length," l, the distance from one retinal margin to the other, along a path through the retina. Since l corresponds to ρ = 185°, then:

$$\frac{l}{2\pi r} = \frac{185}{360}$$

$$r = 0.31l.$$

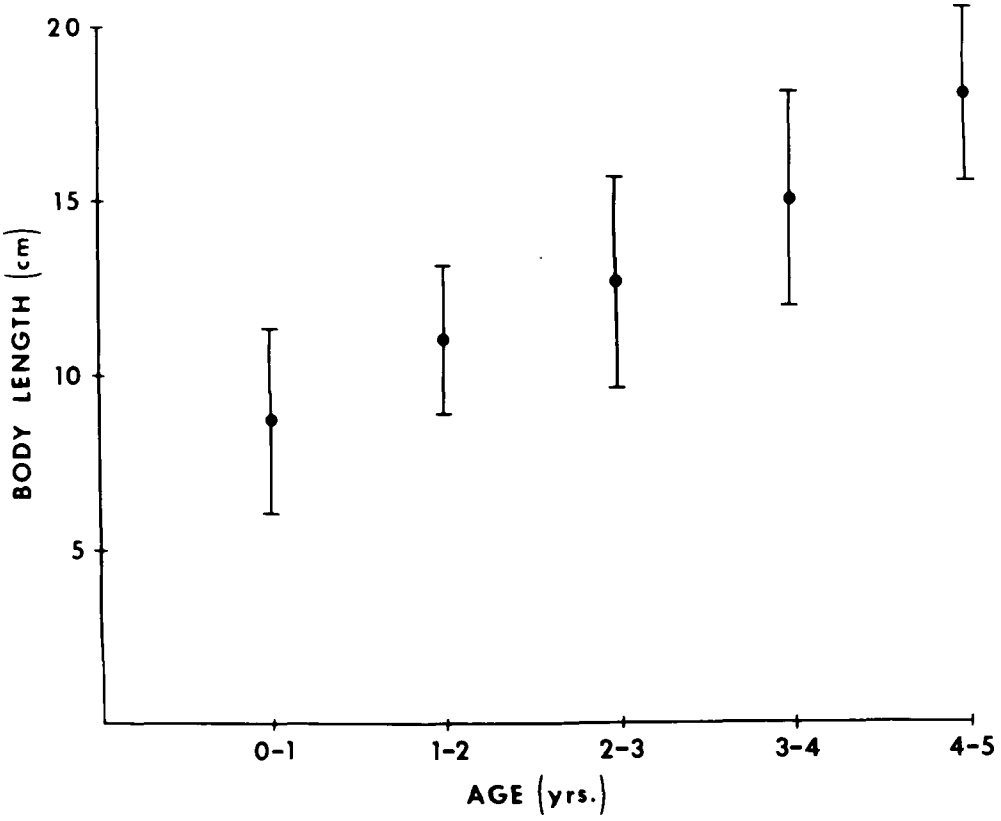

Fig. 1 The relation between size and age in goldfish. The mean body length ± 1 S.D. of fish of five different ages is ploted. The age of the fish was determined from the number of growth rings on the scales (METHODS).

Eyes were sectioned perpendicularly to the pupil, and, in the largest section of each eye, *l* was measured with an ocular micrometer (estimated error < 0.15 mm) at both the inner and outer limiting membranes. All retinal dimensions were corrected for an histological shrinkage of 30% (linear), determined by comparing paraffin-embedded and frozen sections through the eyes of fish of comparable sizes (Johns, '76).

Retinal cell counts—radial sections. Cells were counted in one to three sections viewed at 450 or 900 × cut horizontally (nasotemporal axis) or vertically (dorsoventral axis) through the center of each eye. The number of each type of cell counted ranged from 100 to 1,200 per section. Two counting methods were used. One involved determining the number of nuclei in a given length of retina and correcting for split nuclei using the Abercrombie method (Abercrombie, '46) as modified by Konigsmark ('70). The other method, which proved most useful for counting multilaminate nuclei, was the line sampling technique described by Trowell and Westgarth ('59). This involved counting the number of nuclei which touched a line oriented perpendicularly to the retinal layers. In both cases, the cell density per mm^2 of retinal surface was calculated.

Retinal cell counts — tangential sections. Eyes were cut into nine approximately equal pieces representing the dorsal, dorsonasal, nasal, nasotemporal, ventral, ventrotemporal, temporal, temporodorsal, and central regions of the retina. Serial, 5 μm sections were cut tangential to the convex surface of each piece, and *camera lucida* drawings were made of selected sections through the ganglion cell layer and the layer of cone nuclei at 900 × using an oil immersion objective. The density of ganglion cells was determined from the drawings in areas ranging from 1,200-2,750 μm^2 in each of the nine retinal regions; the cone density was determined likewise in areas ranging from 700-39,000 μm^2. The number of nuclei counted in individual sections ranged from 20 to 140.

RESULTS

Size and age. Figure 1 shows the relation between body size and age. Although the stan-

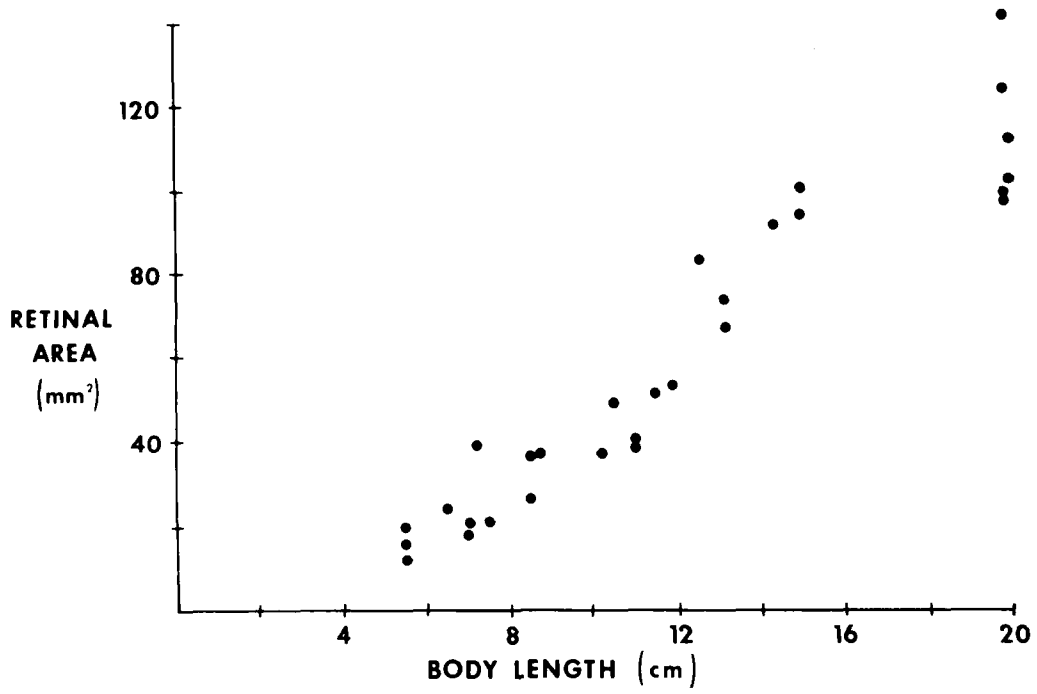

Fig. 2 The increase in retinal area with growth. The retinal area at the internal limiting membrane is given as a function of body length. Each point is one retina. The measurements were corrected for an histological shrinkage of 30% (linear).

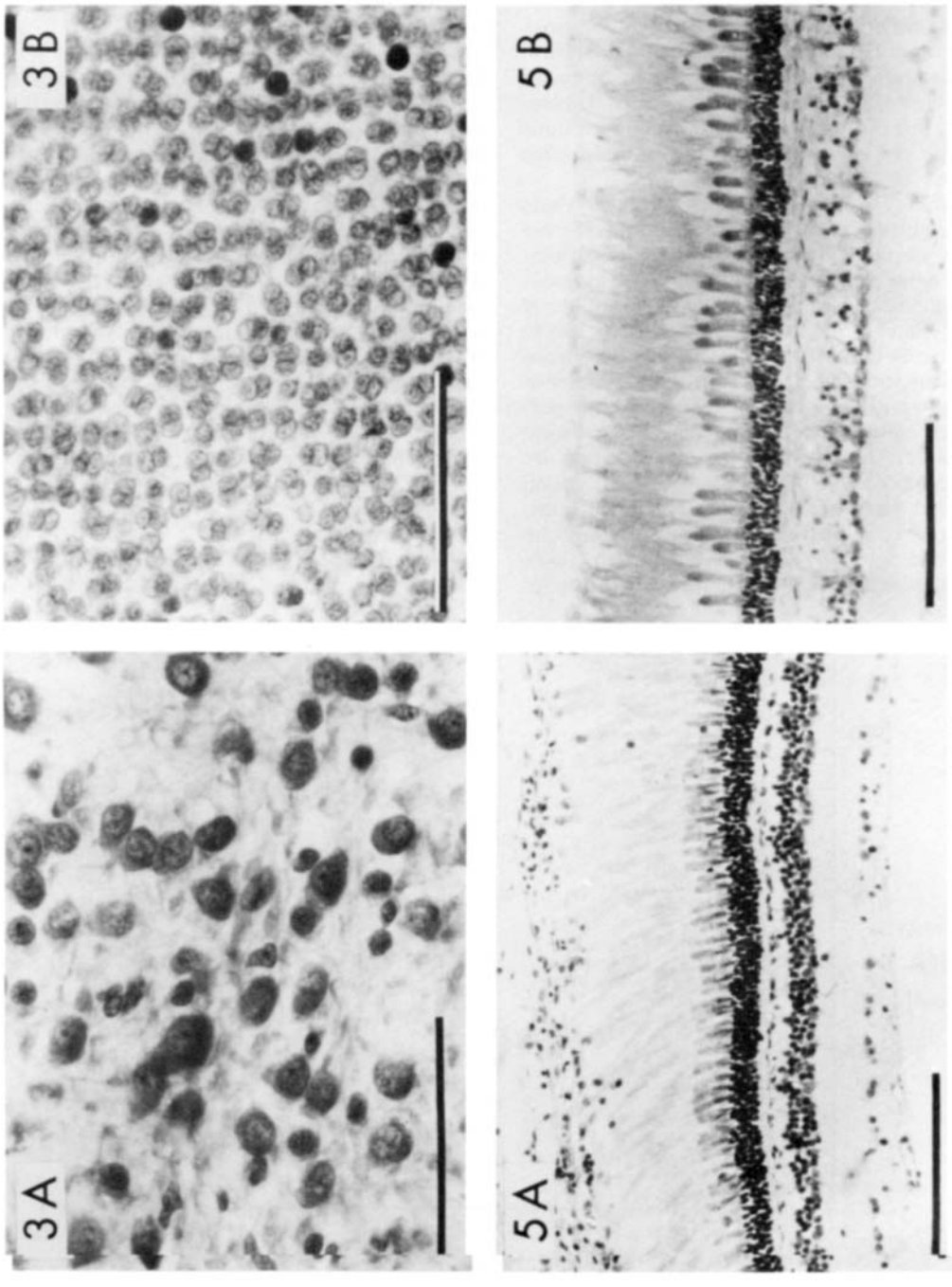

Fig. 3A Ganglion cells in a tangential section from the center of the retina. Foot-Masson, 5 μm. Calibration bar: 50 μm.
B Cone nuclei in a tangential section from the temporal region of the retina. Foot-Masson, 5 μm. Calibration bar: 50 μm.
Fig. 5 Comparison of the retinas of small and large fish. Gallocyanin and eosin, 5 μm, bleached sections. Calibration bar: 100 μm.
A The retina from a 7-cm fish.
B From a 20-cm fish.

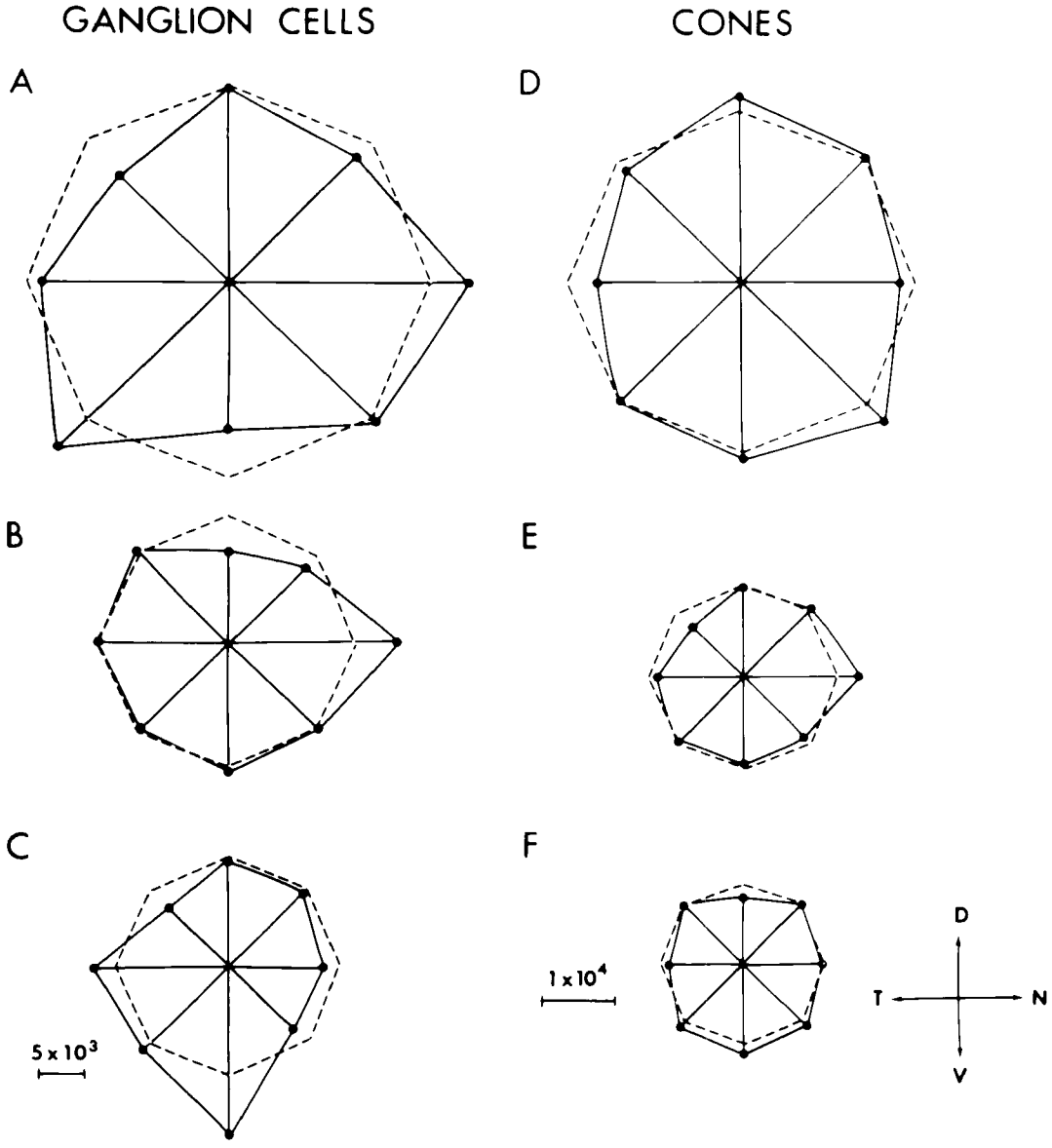

Fig. 4 Ganglion cell and cone densities in tangential sections. The length of each ray indicates the mean cell density (No./mm^2 of retinal surface) in corresponding retinal regions in the two eyes from one fish. The dashed lines represent the overall mean density; a scale for each cell type is given at the bottom. The direction of the ray indicates the location of the retinal sample as shown in the lower right: nasal, N; temporal, T; dorsal, D; ventral, V. The upper two graphs (A and D) are from a 7.1-cm fish, B and E are from a 13.0-cm and C and F from a 23.3-cm fish.

dard deviations are large, in general older fish are larger.

Growth of the eye and retina. The diameter of the goldfish eye increased 3-fold over a fivefold increase in body length [eye diameter (mm) = 0.45 body length (cm) −1.39]. The growth of the eye is thus negatively allometric with respect to the growth of the body; that is, the eyes of large fish are relatively smaller compared with the overall body size than are the eyes of small fish.

Figure 2 shows the increase in retinal area at the vitreal surface (inner limiting membrane) as a function of body length. Some of the scatter is probably due to differences in the ages of the fish, since the eyes of older fish tend to be slightly larger than those of younger fish of the same body length (Müller, '52; Johns, '76). An additional source of scatter is an asymmetry which has been noted previously in the goldfish retina (Stell and Hárosi, '76) and which we have also seen (Johns, '76). The extent of the retina is greater in the horizontal than in the vertical meridian, so the areas calculated from sections through the former axis will be greater than those from the latter.

Identification of cell types. For counting, retinal cells were separated into four categories: ganglion cells, inner nuclear layer cells, rods and cones.

Ganglion cells were identified by their position and distribution in the ganglion cell layer, by their cytological characteristics (a large, round, pale nucleus) and by their hyperchromic response to optic nerve section (Murray and Grafstein, '69; Johns, '76). Neuroglia and vascular elements present in the same layer were not counted.

The inner nuclear layer (INL) contained several cell types, but no attempt was made to differentiate among them when counting cells. The nuclei of the primary retinal glial cell, the Müller cell, is found in this layer (Rodieck, '73), and they were included in the cell counts.

The nuclei of the photoreceptor cells are segregated into two distinct laminae within the outer nuclear layer. The rod nuclei are located proximal and the cone nuclei at, or distal to, the external limiting membrane (ELM). This spatial separation, in addition to the marked cytological differences in their nuclei, allowed the photoreceptor cells to be tallied separately. No attempt was made to identify cone types.

The diameters of the retinal nuclei were on the average 20% greater in the largest fish than in the smallest ones.

Retinal cell density. The cell density distribution in the retina was determined from tangential sections. Photomicrographs of the ganglion cell layer and the layer of cone nuclei are shown in figures 3A and 3B, respectively. All of the ganglion cells in the entire depth of the ganglion cell layer were usually included in one tangential section, and this is the section which was drawn and counted. Similarly, the cone nuclei in a more distal section were drawn and counted. The density of cells in eight of the retinal regions in the three fish are shown in the polar plots in figure 4. The average density in comparable segments from the two eyes in each fish is given by the length of the ray; its direction indicates the retinal region in which the density was sampled. The endpoints of adjacent rays are connected. The cell density in the center of the retina is not shown in these plots. In some cases the proximity of the optic disk interfered with the measurements in this region; where made, they were similar in magnitude to the other regions. Homogeneity of cells would result in regular octagonal figures (dotted lines) in these polar plots; although the shapes in figure 4 are not regular octagons, they are not systematically misshapen, as would be expected if certain regions had higher cell densities. The cone plots are more nearly regular octagons than are the ganglion cell plots, reflecting their more regular spacing (compare fig. 3B with 3A). Figure 4 also demonstrates the decrease in cell density which occurs with growth: note that the size of the fish increases from top to bottom, while the cell density decreases. This point will be discussed more fully later.

We conclude that cell density is essentially homogeneous in the goldfish retina throughout growth. Although only the ganglion cells and cones were analysed so quantitatively, the other retinal cells, the rods and the INL cells, did not show any regions of increased density either.

Age related changes in cell density and number. For the analysis of retinal cell numbers as a function of size, median radial sections from 25 goldfish retinas were used. Counts were made at regular intervals across one entire section through the center of the eye. Because of the variability in the nuclear diameters of the cells in the INL these counts

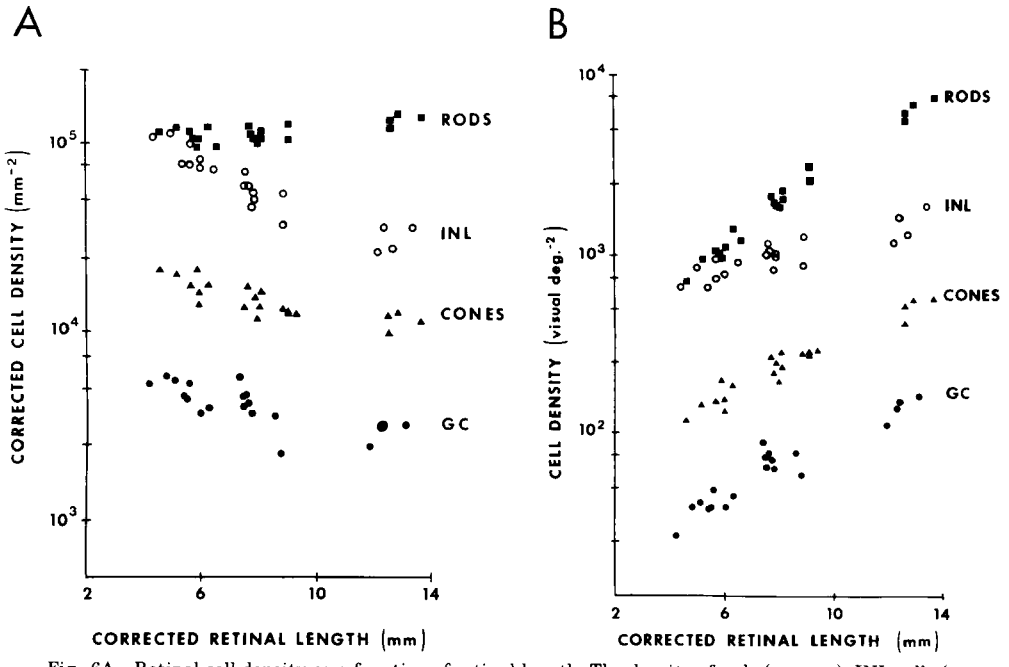

Fig. 6A Retinal cell density as a function of retinal length. The density of rods (squares), INL cells (open circles), cones (triangles), and ganglion cells (filled circles) is given in No./mm^2. Each point is one retina. The retinal length was measured along the ILM for the ganglion cells and along the ELM for the rods and cones; the mean retinal length was used for the inner nuclear layer cells.

B Retinal cell density as a function of visual angle. These are the same data as in A, plotted in terms of No./visual degree^2.

were not as accurate as were those for the more homogeneous cell populations, the rods, cones and ganglion cells. The magnitude of this error in the INL counts may have been up to 20-30%.

The retinas from a small (7 cm) and a large (20 cm) fish are compared in figure 5. The retina between the external and internal limiting membranes increased in thickness from about 135 to 175 μm during growth, primarily due to increases in the thickness of the inner plexiform and nuclear layers. The layer of rod nuclei remained relatively constant in thickness and density, while the cell density in the inner nuclear layer decreased dramatically. Figure 6A is a semi-logarithmic plot of cell density (No./mm^2 of retinal surface) versus retinal length. Retinal length was used as the independent variable since it covaried more tightly with the retinal cell counts than did body length. This graph indicates that the density of INL cells, cones, and ganglion cells decreased with growth, but the rod density remained constant or even increased.

The cell density can also be expressed in terms of visual angle in degrees^2, calculated using the retinal magnification factor (μm retinal surface/degree visual angle). As the goldfish eye grows, the extent of the retinal field remains constant at 185°, and the retinal magnification factor (RMF) increases linearly according to the formula:

RMF (μm/°) = 20.5 × lens diameter (mm)
(Easter et al., '77).

The number of cells per visual degree^2, given in figure 6B, increased with growth. This means that despite the spreading apart of retinal cells with growth, an image of constant angular subtense is projected upon an ever increasing number of cells as the fish gets larger.

The total cell numbers as a function of retinal length are given in figure 7. Despite the decrease in density of all but the rods, the total number of cells increased with growth: the ganglion cells increased from 60,000 to 350,000; the INL cells from 1,500,000 to 4,000,000; the cones from 250,000 to 1,400,000; and the rods from 1,500,000 to 15,000,000.

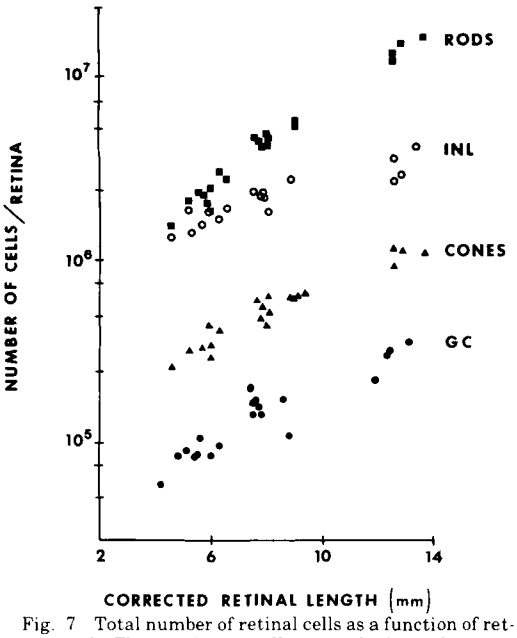

Fig. 7 Total number of retinal cells as a function of retinal length. The number of cells was calculated from the density and retinal area. Each point is one retina. The symbols are as in figure 6.

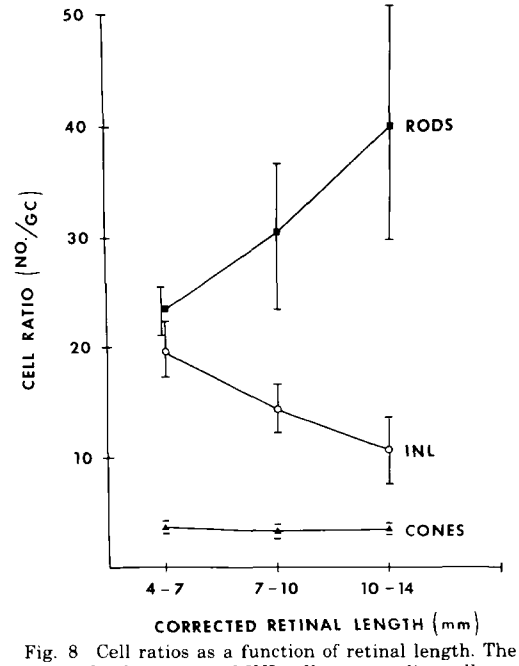

Fig. 8 Cell ratios as a function of retinal length. The number of rods, cones, and INL cells per ganglion cell was calculated from the cell densities. The data were grouped into three bins with respect to retinal length, and the mean ratios ± 2 S.E.M. are plotted. The symbols are as in figure 6.

Since the density of the rods was constant while that of all the other cells decreased, there were relatively more rods in a larger retina than in a smaller one. This is shown in figure 8, in which the mean ratio of rods, INL cells, and cones per ganglion cell is given for three ranges of retinal length. While the ratio of cones to ganglion cells remained constant at about 4:1, the number of INL cells per ganglion cell decreased from 20:1 to 10:1, but the number of rods per ganglion cell increased from 24:1 to 40:1.

DISCUSSION

The major conclusions of this study are four: (1) the adult goldfish retina grows, (2) one component of the retinal growth is cellular hypertrophy, (3) the hypertrophic component of retinal growth is insufficient to account for the entire increase in retinal area, so the total number of cells must increase, and (4) in contrast to the other cells, the rods do not decrease in density and so must be added in even greater numbers during growth.

Retinal cell density. Our estimations of cone density are in reasonable agreement with those reported previously for the goldfish (Hester, '68; Dawson et al., '71; Schellart, '73; Stell and Hárosi, '76). Each of these workers dealt with fish of a slightly different size, and since our values for cone density change with growth, only those for fish of corresponding lengths are comparable. In addition to the cones, Schellart ('73) also counted all of the other retinal cells, and his values for 18 to 21-cm fish agree with ours for fish of this size.

Concerning the homogeneity of cells in the goldfish retina, there is some disagreement. Both Hester ('68) and Schellart ('73) found a region of increased cone density in the dorsotemporal quadrant, and Schellart suggested that the rods were more dense in this region as well. Marc and Sperling ('76) found a higher cone density in the ventrotemporal region. Stell and Hárosi ('76) found, as we did, little evidence for an inhomogeneity in cell density. Our primary concern was whether the assumption of homogeneous density gave valid estimates of total cell numbers. Taking as a worst case Hester's maximum (22,000/mm^2) and minimum (14,000/mm^2) cone densities and assuming a density in one entire quadrant equal to the maximum and a minimum density in the remaining three quadrants, the total cell number is increased by only 8% over the value calculated assuming a homoge-

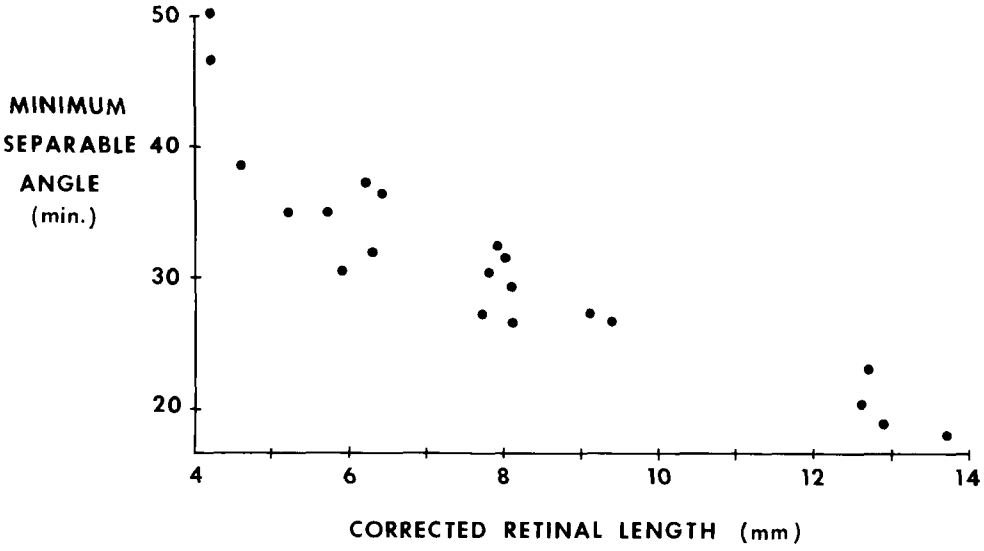

Fig. 9 Calculated visual acuity as a function of retinal length. The minimum separable angle was calculated as described in the text. Each point is one retina. The retinal length measured along the ELM is given by the abscissa.

neous, minimum density. We are therefore confident that our method of sampling cell density across a median radial section gave a reasonable estimate for calculating total cell numbers.

Changes in cell proportions with growth. The decrease in the relative number of cells in the INL could be explained either by cell death or by migration of cells out of this layer into another (e.g., the outer nuclear layer containing the rod nuclei). Cell death has been implicated in the embryonic development of the retina (Glücksmann, '40, '65; Silver and Hughes, '73) and may also play a role in postembryonic growth. Dying cells can be distinguished by several cytological criteria, including cytoplasmic edema and vacuolation, nuclear shrinkage and rounding-up (pyknosis), nuclear fragmentation and necrotaxis (Glücksmann, '30; Bessis, '64). We looked for pyknotic nuclei in the INL of the goldfish retina, but saw none. Whether instead some cells were migrating from inner to outer nuclear layers could not be determined from our material. We, therefore, remain uncertain as to the fate of the lost cells.

In contrast to the INL cells, the proportion of rods increased during retinal growth. A similar increase has been found in certain larval amphibians and in other teleosts (Bernard, 1900; Glücksmann, '40; Müller, '52; Lyall, '57a,b,; Blaxter and Jones, '67; Blaxter, '75). A change in the relative proportions of cells in the center of the retina is puzzling in view of the widely held belief that cell proliferation is restricted to the retinal margin in these animals. Several hypothesis have been suggested to account for this differential increase in the number of rods, and these will be discussed in the accompanying paper (Johns, '77) which deals with the source of the new retinal cells.

Physiological implications of retinal cell addition. The cone density is usually thought to define the resolving power, or acuity, of the eye. We calculated the minimum separable angle using Tamura's formulation ('57) and our previous estimation of the focal length of the lens, which was based on the assumption of emmetropy of the goldfish eye (Easter et al., '77). Based on the cone densities shown in figure 6A, the minimum separable angle decreased from 50 to 18′ as the retina grew (fig. 9). These calculated values agree quite well with both the physiological measurement of 17′ obtained by Schwassmann ('75) using single unit recordings in the optic tectum of goldfish about 14 cm in length and the behavioral measurements of Wilkinson ('72, cited by Schwassmann, '75). The visual acuity, calculated from cone densities, has also been shown to increase with growth in other teleosts (Müller, '52; Lyall, '57a; Tamura, '57; O'Connell, '67), and there is some behavioral

evidence for such an increase (Baerends et al., '60). Hester ('68) showed that the contrast threshold decreased with growth in goldfish, but this measure is difficult to relate directly to acuity.

Central connections. We have shown that millions of new retinal neurons are added over a few years, which implies that even greater numbers of new synapses must be formed concurrently. We suggest, therefore, that the goldfish retina might prove useful for studies of neuro- and synaptogenesis. The numbers are impressive; we estimate from figure 7 that in a goldfish between four and five years of age, only about one in six (60,000 of 350,000) of the retinal ganglion cells existed four years earlier; the others were added in the interim. If, as seems likely, all ganglion cell axons project and connect centrally, then we must suppose that synaptogenesis is occurring continuously even in the higher visual centers. Does this require new brain cells, or is adult neurogenesis restricted to the retina? Work by others (Rahmann, '68; Richter and Krantz, '70) has demonstrated cellular proliferation in adult teleost brains, but it is not clear whether the cells differentiate into neurons, so the question remains unanswered.

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