

Birth and Fate of Proliferative Cells in the Inner Nuclear Layer of the Mature Fish Retina

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ABSTRACT

In teleost fish, unlike other vertebrates, the retina continues to grow throughout the animal's life both by stretching of the mature tissue and by the addition of new cells. Following larval development, new retinal cell birth is known to occur in a rim at the periphery of the mature retina and in the outer nuclear layer (ONL). We have now found that cell birth and proliferation also occurs in the inner nuclear layer (INL) of the mature fish retina. In rainbow trout (*Onchoryncus mykiss*), proliferative cells exist in the INL of fish of all ages, at least up to 2 years posthatching. The proliferative cells form clusters in the INL that align in radial columns, reaching from the inner to the outer plexiform layers. The density of proliferative cell clusters changes along the equatorial plane of the retina and is highest near both the nasal and temporal poles. Our data suggest that, after birth, the proliferative cells migrate away from the INL and into the ONL, with a half-time of about 3 days, and their cell bodies can be seen in the outer plexiform layer. Once they are in the ONL, the proliferative cells continue to divide and likely give rise to the precursor cells that differentiate into new rod photoreceptors. *J. Comp. Neurol.* 394:271-282, 1998. © 1998 Wiley-Liss, Inc.

Indexing terms: teleost fish; development; rod photoreceptors; neurogenesis; cell migration

Retinal growth in teleost fish has served as a model system to study processes that regulate and modulate neuronal proliferation, specification, and repair. Whereas eye and retinal development is completed during embryogenesis in most vertebrates, it continues in teleost fish throughout much of the animal's life (for review, see Fernald, 1991). Furthermore, in teleost fish, unlike most other vertebrates, the retina regenerates when it is damaged by chemical or mechanical injury (for review, see Raymond and Hitchcock, 1997). In young fish, most retinal growth arises from the continuous development of new retinal tissue in a narrow ring at the periphery of the mature tissue and in a fissure that extends along the ventral pole from the center to the edge of the retina. The ring of developing tissue is known as the peripheral growth zone (PGZ), and the fissure is known as the embryonic or choroid fissure. In older fish, retinal development continues at the PGZ and choroid fissure, but a greater proportion of retinal growth reflects stretching of the mature tissue (trout, *Salmo* spp.: Lyall, 1957; Kunz and Callaghan, 1989; Schmitt and Kunz, 1989; goldfish, *Carassius auratus*: Johns, 1977; Johns and Easter, 1977; Meyer, 1978; carp, *Cyprinus* spp.: Kock, 1982; Negishi et al., 1985).

Stretching of the mature retina as the eye grows causes a continuous decrease in the packing density of all retinal neurons, except for the rod photoreceptors (Lyall, 1957; Johns, 1977; Kock, 1982). Nearly constant rod density is maintained by the continuous birth of new rods in the outer nuclear layer (ONL; for reviews, see Raymond, 1985; Fernald, 1991). These new rods arise from the differentiation of precursor germinal cells embedded in the ONL (Sandy and Blaxter, 1980; Johns and Fernald, 1981; Johns, 1982). The proliferation of these precursor cells changes with a diurnal rhythm (Chiu et al., 1995), and it can be altered by specific growth factors (Mack and Fernald, 1993). These precursors have also been hypothesized to be the cellular source from which the entire retinal tissue regenerates following injury (Raymond et al., 1988).

The developmental origin of the rod precursor cells is not fully understood. During embryogenesis, retinal develop-

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ment is characterized by a sequence of cellular changes that culminate with the formation of the typical retinal layers in an area that later becomes the centralmost part of the growing eye (Johns, 1982; Schmitt and Kunz, 1989; Hagedorn and Fernald, 1992). Retinal development thereafter progresses by addition of new cells at the PGZ and embryonic fissures. Johns (1982), by using pulse injection of [³H]thymidine to label proliferating cells, observed in larval goldfish (those within 1 week of hatching) the expected mitotic activity in the PGZ. Surprisingly, she also observed clusters of labeled cells in the ostensibly mature inner nuclear layer (INL), immediately adjacent to the PGZ. Hagedorn and Fernald (1992) have also observed [³H]thymidine-labeled cells in the INL of larval retina in African cichlids. Johns did not find proliferating cells in the INL of fish at postlarval stages. Therefore, she proposed that the clusters of proliferating cells in the INL at the larval stage, which she called 'neurogenic clusters,' migrate across the outer plexiform layer and into the ONL. There, they become the precursors of rod photoreceptors. The rod precursors 'seeded' during larval development were presumed to sustain themselves thereafter through a continuing process of symmetric cell division.

We report here on a study of growth in the mature retina of trout reared in the laboratory under conditions optimized to favor rapid development. We have discovered that mitotically active cells exist in the INL of the mature retina throughout the animal's life and not, as might have been expected, just during larval development. We describe some of anatomical features of the clusters of proliferating cells, and, through a kinetic analysis, we demonstrate that the cells migrate from the INL to the ONL soon after their birth. The cells, once they are in the ONL, continue to divide and likely give rise to the rod precursors.

MATERIALS AND METHODS

Rearing trout in the laboratory

To control the developmental progress of the animals used in this study, we reared rainbow trout (*Onchorynchus mykiss*) in a small aquaculture facility in the laboratory. Eyed eggs (approximately 4 weeks postfertilization) were obtained year round from Mount Lassen Trout Farms (Red Bluff, CA). Fish were held in a linear, insulated fiberglass trough (3.3 / 0.4 / 0.3 m; length / width / depth). Water flowed vigorously (about 80 liters/minute) along the full length of the trough in a closed system. Water quality was maintained by biological filtration (aerobic and anaerobic bioreactors) and active filtration (activated charcoal, protein skimmer, and mechanical filter). Water quality was monitored for amines, nitrates, and nitrites. About one-quarter of the tank water was exchanged biweekly. A chiller maintained the water at 15°C, and a Servo system maintained pH at 7.0 ± 0.1. The trough was kept indoors under artificial illumination that matched the light spectrum of normal daylight. Light cycled with 14-hour/10-hour light/dark periods.

Proliferating cell nuclear antigen immunohistochemistry

Proliferating cells were identified by immunostaining with a monoclonal antibody (mAb) against proliferating cell nuclear antigen (PCNA). Fish were anesthetized by

immersion in MS-222 and then decapitated and pithed. Intact eyes were removed and fixed overnight at 4°C by continuous agitation in alcoholic formaldehyde (9:1 95% ethanol:40% formaldehyde; Bancroft and Stevens, 1990). Each eye was rinsed with water, the cornea was removed, and the lens was extracted. The eyecup was immersed in 30% sucrose for several hours. The sucrose was replaced overnight at 4°C with 1:1 30% sucrose:Tissue-Tek OCT compound (Miles Inc., Elkhart, IN). The tissue was freeze-embedded in the OCT compound, and 16-µm sections were cut with a freezing microtome. Sections were then transferred to gelatin-subbed slides and dried.

Sections were rehydrated in PBST (0.06 M phosphate buffer, 0.15 M NaCl with 0.1% Tween-20, pH 7.4) for 10 minutes, incubated for 1 hour in blocking serum (20 µl/ml horse serum; Vector Laboratories, Burlingame, CA), and then incubated overnight at 4°C in a mixture consisting of 1:50 horse serum and 1:100 of mAb against PCNA (PC10; Santa Cruz Biotechnology, Santa Cruz, CA) in phosphate-buffered saline (PBS; 0.06 M phosphate buffer in 0.15 M NaCl, pH 7.4) with 0.3% Triton X-100 and 1% dimethylsulfoxide. Following this incubation, sections were rinsed with PBST and incubated for 2 hours in 1:50 horse serum and 1:100 biotinylated horse anti-mouse mAb (Vector Laboratories). To reduce nonspecific background staining, the sections were again rinsed in PBST and incubated for 30 minutes in casein blocking solution (0.5% casein in Dulbecco's PBS with 0.05% Tween-20 and 0.5 g/liter Na azide). After rinsing with PBST, the sections were incubated for 1 hour with reagents of the Vectastain ABC-Elite kit (Vector Laboratories). After a thorough rinsing with PBST, peroxidase activity was detected with a substrate kit (SK-4600; Vector Laboratories). Sections were rinsed with water, dehydrated through graded alcohol, followed by xylene, and mounted in Cytoseal 60 (Stephens Scientific, Camden, NJ).

Prevalence of PCNA⁺ cells in adult trout

Retinas from trout of various ages were immunostained with PCNA to determine the effect of age and size on the prevalence of proliferative activity in the mature INL. Young fish (up to 2 months posthatch or about 2 g body weight) were reared in the laboratory. Older animals were collected at the trout farm that provided us with the fertilized eggs. The oldest fish we studied was 24 months posthatch (about 3 kg body weight). The eyes were fixed and processed for PCNA immunohistochemistry, as described above. In this and all subsequent experiments, cells were counted following the methods and recommendations of Saper (1996). We counted every PCNA⁺ cell profile in the INL and ONL of sections that extended from nasal to temporal PGZ along the equatorial plane. In each section, we also measured the distance between the nasal and temporal PGZs along the photoreceptor margin by using software tools on digital image files (NIH-Image video acquisition and analysis software; NIH, Bethesda, MD). Each value represents the average cell count in four to six sections from an individual fish, with 26 fish sampled. To compare data among various animals and stages of growth, we expressed our results in units of cell density, not as absolute numbers. This method is valid, because, as the eye grew, PCNA⁺ cells did not change in size but changed only in prevalence (50 cells measured in each of six different fish, ranging in eye diameter from 3.5 mm to 15 mm; $P > 0.05$ by analysis of variance; ANOVA).

Diurnal rhythm in the rate of cell proliferation in the INL and the ONL

To determine whether ONL and INL proliferative activity exhibits a diurnal rhythm, PCNA-immunostained retinas were collected at various times throughout a single 24-hour period. These measurements were conducted in midsummer with fish that were raised indoors at the Mount Lassen Trout Farm but that were at the same developmental stage normally studied in the laboratory (about 40 days posthatch). Fish were maintained under normal seasonal light supplemented with timed-fluorescent lighting. Sunrise was at 6:00 a.m. and sunset was at 8:30 p.m. Over a 24-hour period, we collected and fixed eyes at 3-hour intervals, commencing and ending at 9:30 a.m. In darkness, animals were collected under infrared illumination with a viewing device equipped with an image intensifier. Tissues were processed for PCNA immunohistochemistry as described above. To assay the rate of proliferation, the number of PCNA⁺ cells along the equatorial plane of the retina from nasal to temporal poles was determined. Cells were counted in four equatorial sections from each of three to four fish at each time point (30 fish total).

Continuous provision of labeled deoxyuridine

Either brominated deoxyuridine (BrdU) or iodinated deoxyuridine (IdU) was delivered by either of two different methods to sustain elevated systemic levels of labeled deoxynucleotide in 1–2 g trout. In the first method, the solution was infused continuously through an indwelling, subdermal catheter. The catheter consisted of about 15 cm of vinyl tubing 0.25/0.75 mm (inner diameter/outer diameter) encased by a second, snug-fitting vinyl tube. The small tube extended about 1 cm past the end of the larger tube. Prior to insertion, the catheter tips were dipped in ethanol and Betadine (Purdue Frederick Co., Norwalk, CT), rinsed, and then filled with the solution containing the labeled deoxyuridine. To insert the catheter, fish were first anesthetized in ice-cold MS-222 solution (7.5 mg/100 ml) and then placed on an aluminum block kept on ice. Under a dissection microscope, the catheter was inserted at the base of the tail and tunneled forward under the skin, alongside the dorsal ridge, until its tip was approximately at the thorax. The outside vinyl tubing was used to secure the catheter by suturing it to the dorsal aspect of the tail. The insertion wound and the suture points were dabbed with Betadine, and the fish was returned to the aquaculture trough. Catheter insertion typically took about 1 minute. In the trough, individual fish were maintained within a narrow cage that allowed them to swim but did not allow them to 'flip,' which would otherwise tangle the catheter line. The catheter was connected to a syringe pump that continuously infused nucleotide-containing solution at a rate of about 10 μ l/hour. The infusion solution was 5% dextrose in water with 8 mg/ml BrdU and 3 μ l/ml Tetracycline stock (50 mg/ml). To prevent infection of the wounds, individual fish were dipped every 3 days for 10–15 seconds in a solution of malachite green (66 mg/liter). We infused fish for periods ranging from 1 day to 8 days.

A second method was simpler but yielded less intensely labeled cells, probably because the concentration of labeled nucleotide in the metabolic pool was smaller than that achieved by infusion. Up to 25 fish were kept at 15°C in

0.5-liter volume of water containing either 2 g/liter IdU or 4 g/liter BrdU. Water was treated with Neutral Regulator (0.08 g/liter; SeaChem Laboratories, Stone Mountain, GA) and continuously aerated. About half of the water volume was exchanged at least every other day. Fish were maintained in the labeled water for periods ranging from 1 day to 10 days.

BrdU and IdU immunohistochemistry

In some of the experiments reported here, proliferating cells were identified following incubation with BrdU alone. In other experiments, the fate of cells was followed by using two different markers of proliferation, BrdU and IdU, which can be identified selectively in the same section with specific antibodies and appropriate enzymatic amplification systems. The double-label methods described below were modified from Miller et al. (1991). Antibodies Bu1/75, a rat mAb that identifies BrdU specifically (SeraLab, Crawley Down, Sussex; supernatant obtained through Accurate Chemical Corp, Westbury, NY), and IU-4, a mouse mAb that recognizes both BrdU and IdU (Caltag, South San Francisco, CA), were used in this technique. We confirmed that, in the trout retina, Bu1/75 does specifically recognize BrdU and does not recognize IdU, because, with the procedures described below, the antibody failed to label any cells in the retina of fish maintained for several days in water containing IdU.

Intact eyes were fixed overnight at 4°C in Bouin's fixative (Polysciences, Warrington, PA) and were then rinsed with water, and the cornea and lens were removed. The eyecup was dehydrated through a series of alcohols and infiltrated overnight at 4°C with JB-4 (Polysciences). The JB-4 was exchanged once again, polymerization was initiated, and embedding was completed overnight at 4°C. Three-micron-thick sections were cut on glass knives, transferred to water droplets on aminosilanized glass slides (Henderson, 1989), and dried overnight at 37°C.

To identify cells labeled with either of the two deoxyuridines, retinal sections were rehydrated in distilled water for 10 minutes, incubated in 4 M HCl for 30 minutes, and again rinsed with distilled water. Following 10 minutes of immersion in a solution consisting of 0.15 M NaCl, 0.05 M Tris, and 0.1% Tween-20, pH 7.4 (0.15 TBST), slides were incubated in pronase E (1 mg/ml) in 0.15 M TBST for 30 minutes at room temperature. The enzyme was thoroughly rinsed with 0.15 M TBST, and sections were then incubated for 30 minutes in blocking solution. For Bu1/75, the blocking solution consisted of 1:50 rabbit serum (Vector Laboratories) in 0.15 M TBST. This was followed by incubation in the primary antibody solution containing 1:13 Bu1/75 and 1:50 rabbit serum for 3 hours. After rinsing with 0.15 M TBST, sections were incubated in the biotinylated secondary antibody (rabbit anti-rat immunoglobulin [IgG]; 1:100; Vector Laboratories) with 1:50 rabbit serum in 0.15 M TBST for 2 hours at room temperature or overnight at 4°C. The slides were rinsed, incubated in casein blocking solution for 30 minutes, and treated with the reagents of the ABC-AP kit (Vector Laboratories). After thorough rinsing with 0.15 M TBST, alkaline-phosphatase activity was detected with red AP substrate (SK-5100; Vector Laboratories). This substrate rendered the cells red under transmitted light and fluorescent under epiillumination. Sections were rinsed with water, air dried, and mounted with Gel/Mount (Biomed, Foster City, CA).

In double-labeled sections, BrdU was always immunolabeled first, as described above. After reacting with the red AP substrate, sections were washed with distilled water and placed in 0.5 M TBST for 30 minutes. Next, they were incubated for 30 minutes in blocking serum consisting of 1:50 horse serum and two drops/ml Avidin D blocking solution (Vector Laboratories) in 0.5 M TBST. This was followed by a 3-hour reaction with IU4-mAb (1:100) in a solution of 1:50 horse serum and two drops/ml biotin blocking solution (Vector Laboratories) in 0.5 M TBST. Sections were rinsed in 0.5 M TBST and then incubated for either 2 hours at room temperature or overnight at 4°C in biotinylated secondary antibody (horse anti-mouse antibody; 1:100) with 1:50 horse serum in 0.5 M TBST. The slides were rinsed, incubated in casein blocking solution for 30 minutes, and treated with the reagents of the ABC-AP kit. IdU was recognized selectively, because the alkaline-phosphatase activity in this reaction was detected with blue AP substrate (SK-5300; Vector Laboratories). Slides were rinsed in water, air dried, and mounted with Gel/Mount.

Distribution of proliferating cells across the retina

The distribution of the density of INL and ONL proliferating cells was determined along the equatorial plane from nasal to temporal poles in juvenile fish. In these experiments, six fish, all with approximately the same weight (range 3.5–4.5 g, about 2 months posthatch), were exposed to continuous BrdU for 24–36 hours through an indwelling catheter, as described above. The eyes were fixed and processed for BrdU immunohistochemistry in 3- μ m-thick sections, as described above. To compare data among the various experimental animals, nine equal-sized fields along the retina from nasal to temporal poles were defined, each 720 μ m in length, excluding the PGZ. The optic nerve was defined as the origin. Because of the asymmetry in optic nerve location, there were five counting fields on the nasal side of the sections and four on the temporal side. Retina-to-retina variation in size was a relatively small problem, because the fish studied were reared together and were all of the same age and about the same size. The area nearest the optic nerve was not counted in any of the retinas. The small retina-to-retina size variability was reflected in a varying distance between the end bin and the PGZ. Cells in this area were not counted. BrdU⁺ cell profiles in the INL and ONL were counted, and the results were expressed simply as the density of cells in each counting field. Cells were counted in four to six equatorial sections from one eye of each of the six fish.

Migration of proliferating INL cells

To determine the fate of proliferating cells in the INL, double labeling of BrdU and IdU was utilized to investigate whether these cells were migrating out of the INL or were continuing to divide in the INL. Nineteen trout raised from the same batch of eggs and all of nearly the same size and weight (1.06 ± 0.08 g; average \pm S.D.; range 0.99–1.23 g) were transferred to 0.5 liter of water with IdU (2 g/liter), fed as usual, and kept at 15°C for a total of 43 hours. Water was exchanged after 24 hours with fresh IdU solution. After 43 hours, three fish were removed, and their eyes were processed for BrdU and IdU double labeling, as described above. These animals defined the conditions at time zero. The remaining animals were

rinsed in water and then transferred to 0.5 liter of water with BrdU (4 g/liter) maintained at 15°C. The water was exchanged for fresh BrdU solution every 24 hours. Two or three animals were removed from the BrdU-containing water at selected time points up to 196 hours after transfer to BrdU. Immediately upon removal, fish were killed, and their eyes were processed as described above.

To analyze the number of BrdU⁺ and IdU⁺ cells in the INL and ONL of animals exposed to both labels, equatorial sections running from nasal to temporal PGZs were photographed on color-reversal film under both brightfield and epifluorescence illumination (Texas red filter set; Chroma Technology, Brattleboro, VT). All analyses were conducted on these photographic images. Because all fish in these experiments were of similar weight, their eye diameters and the size of each histological section were also similar. In each image, a field was defined that was 1.80 mm in length, starting just inside the PGZ in the nasal pole at the position where the outer plexiform layer is just discernible. Labeled cell profiles were counted within that field in the INL and ONL of every section. BrdU⁺ or IdU⁺ cells were easily identified by their distinct color under transmitted light. In addition, the BrdU-labeled cells were also fluorescent. To minimize the ambiguity of distinguishing overlapping cell bodies in the fluorescent images, we scored the cells by identifying individual profiles in the transmitted light photomicrograph and subsequently established whether each was also fluorescent.

The total number of cell profiles in the INL within a field 180 μ m in length was also measured. The number of labeled cell profiles in each section could then be normalized by an unvarying feature, because the density, size, shape, and orientation of the INL cells in the mature retina of fish of nearly the same size do not change over a period of 8 days, which was the maximum length of our experiments. Therefore, although we cannot provide an absolute number of BrdU⁺ or IdU⁺ cells, we can compare normalized values measured in the many sections investigated, because the values are a ratio in which the denominator is constant among the sections. Cells were counted in four equatorial sections from one eye each of two to three fish collected at each selected time point after transfer to BrdU (19 fish total).

Histochemical detection of cells in apoptosis

The presence of short stretches of DNA in cell nuclei, which is characteristic of apoptosis, was examined by utilizing the TUNEL method (Gavrieli et al., 1992). Fish were anesthetized in ice-cold MS-222, decapitated, and pithed. Eyes were removed, the cornea was cut, and the lens was extracted. These eyecups were fixed overnight at room temperature by gentle agitation in 4% paraformaldehyde in PBS. They were then embedded in paraffin, and 7- μ m sections were cut and transferred onto gelatin-subbed slides (Bancroft and Stevens, 1990). The sections were then TUNEL stained by using a commercial kit (Boehringer-Mannheim, Indianapolis, IN) with alkaline-phosphatase converter. To confirm that our method could detect apoptotic cells in retinal sections (positive control), the following controls were used: 1) DNAase I-treated sections prepared from normal animals and 2) sections prepared from animals exposed to 5-fluorouracil (5-FU). This cytotoxic agent is known to cause apoptosis in dividing cells (Sakaguchi et al., 1994). It was determined independently that single intraperitoneal injections of

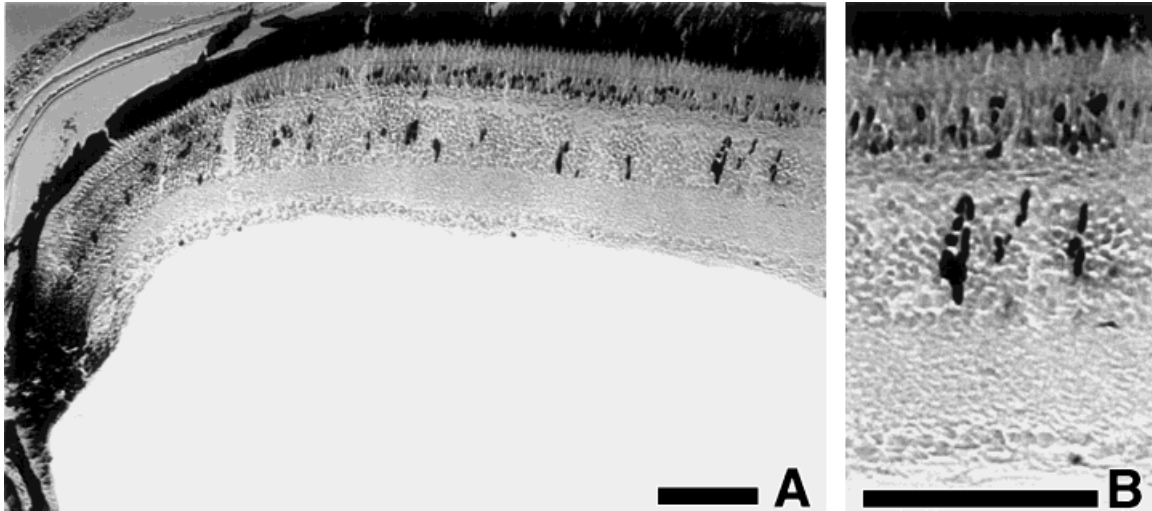


Fig. 1. Photomicrograph of a radial section of trout retina immunolabeled with an antibody against proliferating cell nuclear antigen (PCNA). The 16- μ m cryosection was cut from nasal-to-temporal pole along the equator of the eyeball of a 2 g trout. PCNA-expressing cells appear as darkly stained cell bodies in the section. **A**: Section that includes the nasal peripheral growth zone (PGZ). PCNA⁺ cells are

found in the PGZ and in the outer nuclear layer (ONL) and the inner nuclear layer (INL). **B**: Higher magnification of a portion of A illustrating that PCNA⁺ cells in the mature INL form clusters that align vertically and reach from the inner to the outer plexiform layers. Scale bars = 100 μ m.

5-FU (0.02–0.2 mg 5-FU/g fish weight in 5% dextrose) caused apoptosis in the proliferative zone of the trout retina within 2 days of injection.

Data presentation

Statistical analyses were performed with SPSS for Windows (ver. 6.1.3; SPSS Inc., Chicago, IL). Photomicrographs were recorded by standard 35-mm photography onto Kodak Ektachrome 160T professional color-reversal film (Eastman Kodak, Rochester, NY) and commercially digitized (Photo CD; Eastman Kodak Co.). Digitized images were processed with Adobe Photoshop (ver. 3.0; Adobe Systems Inc., Mountain View, CA) and printed by dye-sublimation at an 8-bit color depth (Phaser II; Tektronix Inc., Beaverton, OR). Image processing was limited to cropping and adjustment of contrast and brightness. All experiments in this study were approved by the UCSF Committee on Animal Research.

RESULTS

Proliferative cells exist in the retinal INL of trout up to 24 months posthatch

To investigate cell division in the growing retina of trout, we measured the cellular expression of the PCNA, which specifically identifies proliferating cells within which DNA is replicating (Negishi et al., 1990; Yu et al., 1992). A typical photomicrograph of trout retina immunoreacted with an antibody against PCNA is shown in Figure 1. We found, as expected, that there were PCNA⁺ cells in the PGZ at the margin of the retina and in the ONL. The former are cells dividing in the course of retinal development, whereas the latter are progenitors of rod photoreceptors. We were surprised, however, to discover large numbers of PCNA⁺ cells in the mature INL. These cells, which we will refer to as proliferating inner nuclear cells (PINC), frequently existed as clusters that aligned in vertical

columns spanning the nuclear layer from inner to outer plexiform layers (Fig. 1).

Johns (1982) and Hagedorn and Fernald (1992), as discussed above, have observed proliferating cells in the mature INL of embryonic and larval fish but not in older animals. We explored whether PINCs exist in mature trout and not only in larval stages of development. We determined the density of PINCs in fish, which ranged from 1 day to 2 years posthatch, representing a range in size from less than 0.1 g to 3 Kg. Cell counts as a function of eye diameter are illustrated in Figure 2. To compare among individual fish, cell counts were normalized with respect to the length of the retinal section, as detailed above in Materials and Methods. Although we detected PCNA⁺ cells in the INL of every one of the fish we studied, the density of PINCs declined exponentially as eye diameter increased. Thus, PINCs exist in the retina of even the oldest animals we tested, but their prevalence declines with age.

Diurnal rhythm in the rate of cell proliferation in the INL and the ONL

The rate of cell division in the ONL exhibits a diurnal rhythm in African cichlid fish that is higher during the night phase of a 12-hour dark/light cycle (Chiu et al., 1995). We tested whether comparable rhythms exist for the ONL in trout and whether PINCs also exhibit diurnal rhythms of proliferation. To compare data among the many fish investigated, we defined the average cell density measured at 6:30 a.m. as unity.

The rate of mitosis in the ONL in trout, as in African cichlids, has a diurnal rhythm that is higher during the dark phase of the daily cycle (Fig. 3). The number of PCNA⁺ cells in the ONL was significantly higher at 12:30 a.m. and 3:30 a.m., than at 6:30 a.m. ($P < 0.05$ with Tukey's multiple comparison test). All other time points were not significantly different. In contrast, in the INL, only the cell

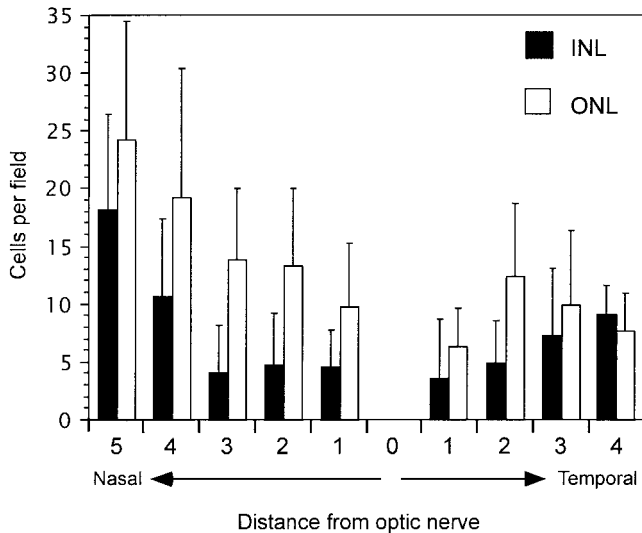


Fig. 4. Density distribution of bromodeoxyuridine-positive (BrdU⁺) cells along the equatorial plane from nasal to temporal poles in the retina of rainbow trout. The number of cells in each of nine fields (720 μm wide) was counted in both the outer nuclear layer (ONL) and the inner nuclear layer (INL). Each bar represents the mean ± S.D. of counts from six fish, with four equatorial sections counted in each fish.

Figure 5 shows typical photomicrographs of retinal sections immunoreacted to selectively identify cells labeled with IdU alone (blue), cells labeled with BrdU alone (red), and those labeled with both nucleotides (red and blue). It was critically important to distinguish cells labeled with IdU alone from those labeled with BrdU only or with a combination of labels. This task was made easier by the fact that the red label (BrdU) also fluoresces, whereas the blue one (IdU) does not.

Figure 6 illustrates cell counts in the INL and ONL. Counts of cells labeled with IdU alone are presented on the left, and the right illustrates counts of cells labeled with BrdU or with a mixture of BrdU and IdU. Cells labeled with IdU alone are cells that were in S phase sometime in the 43-hour period when only IdU was available and that did not then divide again; that is, they were in their terminal mitosis sometime in the 43-hour period. Cells that were labeled with both labels were in S phase when IdU was available and again when BrdU was available. BrdU-only-labeled cells were in S phase perhaps more than once, exclusively when BrdU was available.

The results indicate that IdU-only-labeled cells continuously disappeared from the INL. The loss of the cells had a roughly exponential time course, with a half-time of about 3 days following the switch to BrdU. At the same time that these cells were disappearing, BrdU-only-labeled and double-labeled cells appeared in the INL and reached a steady-state level about 4 days after the switch to BrdU. These results indicate that the PINCs disappear from the INL because they migrate or they die, but not because they divide continuously. If PINCs were simply dividing repeatedly, then the number of double-labeled and BrdU-only-labeled cells should have increased linearly in time. The fact that a steady-state is reached indicates that, in the INL, there exists a balance between the birth of new cells and either their migration away from the INL or their death.

The experimental results also demonstrate that IdU-only-labeled cells actually migrate out of the INL and appear in the ONL. The data shown in Figure 6 indicate that, at the time fish were switched from IdU to BrdU, there already existed a steady number of IdU-only-labeled cells in the ONL. These cells were either born in the ONL or migrated from the INL during the incubation in IdU. About 1 day after switching to BrdU, there occurred a large and transient increase in the number of IdU-only-labeled cells in the ONL. These added cells can only have come from the INL, because they cannot arise from cell division in the ONL. Any cell that divides in the ONL after switching to BrdU would be labeled with BrdU only or with a mixture of the nucleotides. Also, the added IdU-only-labeled cells that migrated from the INL likely divided again in the ONL, thus explaining why the rise in cell count was transient. The fact that continuous cell division occurs in the ONL far in excess of any cell loss is apparent, because the numbers of BrdU-only-labeled or double-labeled cells increased linearly with time. Contrast this linear rise with the saturation in labeled cell numbers observed in the INL.

Cells newly born in the INL can be seen to cross the outer plexiform layer

Our kinetic analysis of the movement of double-labeled cells indicates that newly born cells in the INL migrate into the ONL and likely replenish the pool of rod progenitor cells. Direct observation of cells in these clusters crossing from the INL to the ONL would make the evidence presented above even more compelling. In Figure 7, we show selected examples of clusters of PCNA⁺ cells crossing the outer plexiform layer. The frequency of these events was small, and we could not use an unbiased method to calculate this frequency, because we specifically searched sections to find examples of this event. Nonetheless, Figure 7 demonstrates that newly born cells can be seen to cross the outer plexiform layer. Because these cells are elements in a cluster of cells and clusters exist in the INL and not in the ONL, these cells are likely migrating from the INL to the ONL and not the other way around.

There are no detectable apoptotic cells in the mature INL

If, in addition to migration, PINCs were to die a programmed death through apoptosis, then this event could be diagnosed through specific histochemical reactions. We used the TUNEL method to identify cells undergoing apoptosis (Gavrieli et al., 1992). Few cells were positively identified as apoptotic by using this method. In 32 histologic sections from five fish, a total of 15 apoptotic cells were identified in the neural retina, and almost all of these were in or near the PGZ. We applied the TUNEL method to retinal sections of 5-FU-treated fish as a positive control to establish that our failure to detect apoptotic cells in the mature INL was not due to the inadequacy of our histochemical method. In these animals, TUNEL detected apoptotic cells in the PGZ, as expected, because 5-FU causes apoptosis of proliferating cells (data not shown).

DISCUSSION

Rainbow trout is a useful species in which to study retinal growth and development. Fertilized eggs are available on demand year round, small-scale aquaculture facili-

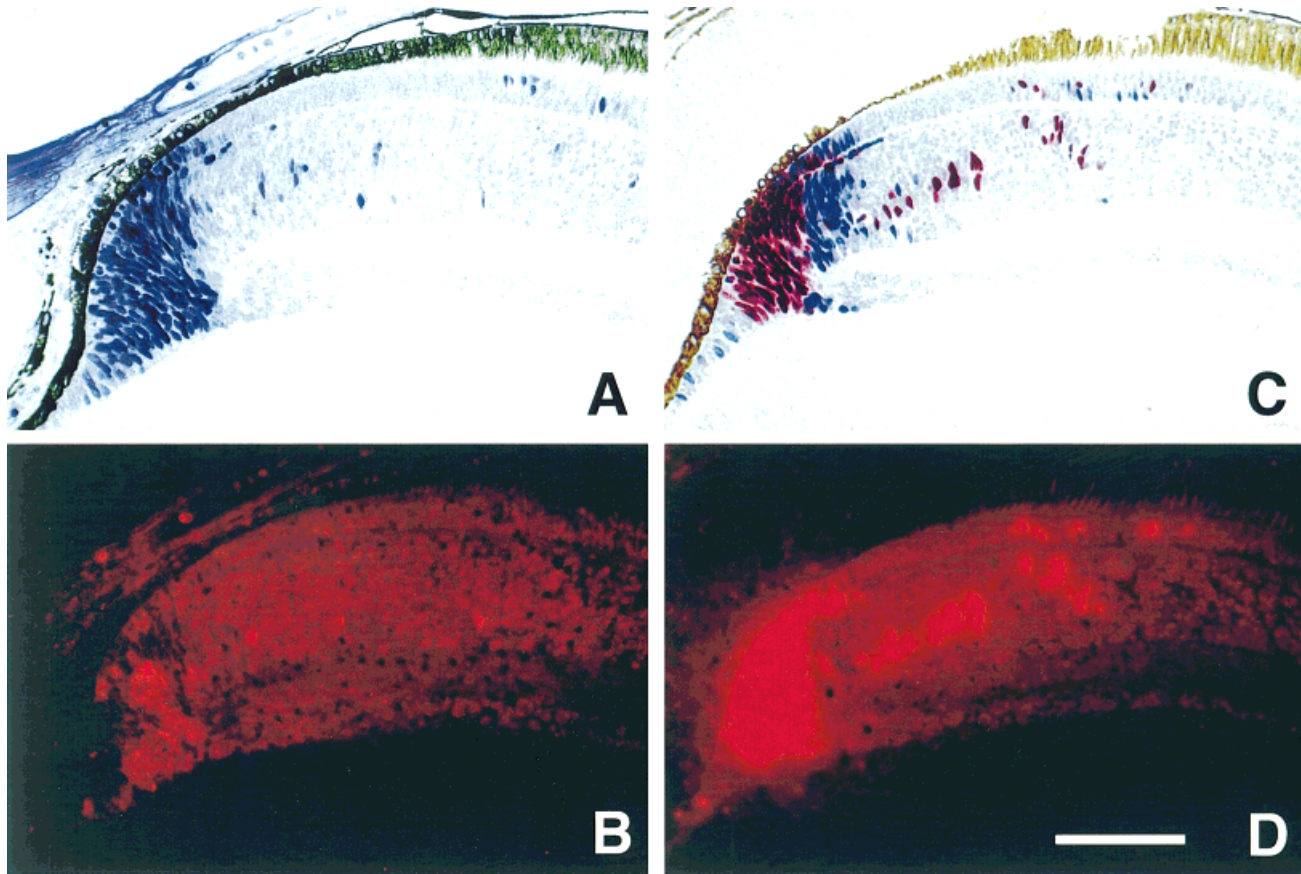


Fig. 5. Photomicrograph of radial sections of trout retina immunolabeled with antibodies specific for bromodeoxyuridine (BrdU) and iododeoxyuridine (IdU). The 3- μ m sections were cut from temporal to nasal poles along the equator. Shown are the nasal poles, including the PGZ. Under transmitted light, BrdU⁺ cells are red, and IdU⁺ cells are blue. Under fluorescent epiillumination, cells positive for BrdU fluoresce bright red, and cells positive only for IdU do not fluoresce at all. Fish were first incubated in IdU-containing water for 43 hours and

were then transferred to BrdU-containing water. **A** and **B** are the same section, which was obtained from a fish killed 6 hours after its transfer to BrdU. **C** and **D** are the same section, which was obtained from a fish killed 196 hours after its transfer to BrdU. **A** and **C** were photographed under transmitted light, and **B** and **D** were photographed under epiillumination. The limited fluorescent labeling in **B**, compared with **D**, confirms that the BrdU antibody does not recognize IdU. Scale bar = 100 μ m.

ties can be constructed in the research laboratory, and the fish are sturdy and are relatively easy to raise. 'Age' in fish is a poorly determined characteristic, because growth and development reflects environmental factors, such as availability and quality of food, water temperature, light cycles, etc. (Ali, 1975). Trout studied in this report were raised under laboratory conditions, such that, over the first 3 months posthatching, eye diameter increased linearly with time. If we assume that the back of the eye is hemispherical and that the retina covers all of its surface, then the observed change in eye diameter indicates that the retina grows at a rate of about 10% increase in surface area per week (Julian and Korenbrot, unpublished observations).

Fate of the proliferating INL cells

We have found cells that divide and proliferate in the INL of the retina in the growing eyes of mature trout. The cells, which are referred to here as PINCs, form clusters that align vertically, reaching from the inner to the outer plexiform layers. The INL is composed primarily of the nuclei of fully differentiated neurons (horizontal, bipolar, and amacrine cells) that are not expected to undergo cell

division. PINCs could be dividing Müller (glial) cells, but they are almost certainly not, because we have evidence that the nuclei of the progeny of the dividing cells do not accumulate in the INL. Indeed, double-label experiments have allowed us to demonstrate that PINCs migrate from the INL to the ONL, with a time constant of about 3 days after their birth. The PINCs, once they are in the ONL, continue to divide, and they most likely then differentiate into rod precursors. The precursors give rise to rod photoreceptors that are continuously added to the ONL of the growing fish retina (for review, see Fernald, 1991).

PINC in the mature trout retina are almost certainly the same cells identified by Johns (1982) in the retina of the larval goldfish and referred to as 'neurogenic clusters.' Those cells also form vertically aligned clusters in the INL that can be seen to extend into the outer plexiform layer (Rivlin and Raymond, 1987). The normal function of PINCs, to replenish the pool of rod precursors in the ONL, must be regulated to achieve a balance in which the rate of replacement of precursors is matched to the rate of rod differentiation. The rate of rod production, on the other hand, must be controlled to match the rate of retinal

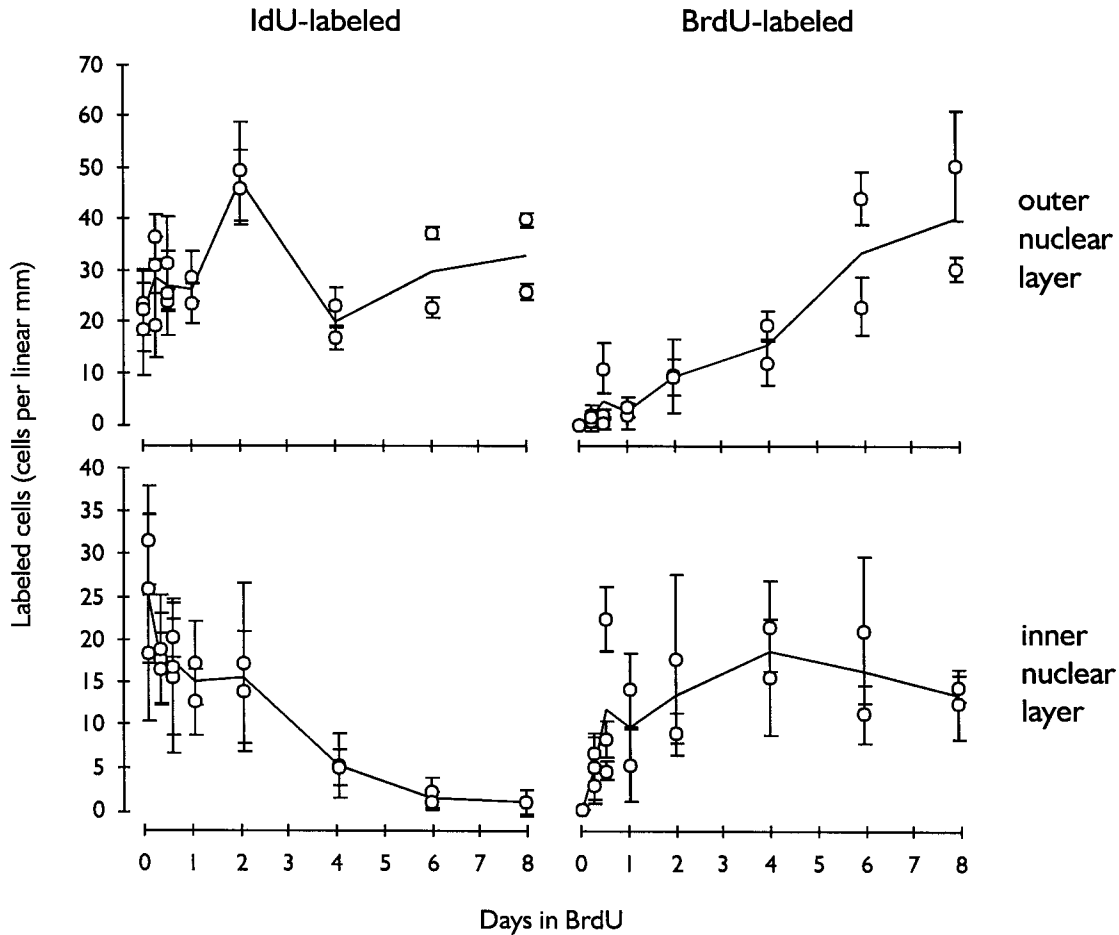


Fig. 6. Counts of labeled cells in the outer nuclear layer (ONL) and inner nuclear layer (INL) of rainbow trout maintained in iododeoxyuridine (IdU)-containing water for 43 hours and then transferred to bromodeoxyuridine (BrdU)-containing water at a time indicated as zero in the abscissa. The abscissa is the duration of continuous exposure to BrdU. The density of IdU- and BrdU-labeled cells in the ONL (**top**) and in the INL (**bottom**) was determined in the equatorial

plane in a 1.8-mm length of retina adjacent to the PGZ at the nasal pole. Each data point is the average \pm S.D. of counts in four to six sections of a single fish, with two to three fish per time point (19 fish total). The IdU-labeled cells can be seen to disappear from the INL with a half-time of between 2 and 3 days and then appear in the ONL. The total number of labeled cells increases linearly in the ONL.

growth. Trout grow much more rapidly than goldfish. For example, goldfish take about 2 years to reach a body length of about 8 cm (Johns, 1982), but it only takes about 8 weeks posthatch for trout to reach this size. Because eye size and body size are approximately proportional, it follows that the eye in the trout grows more rapidly than in the goldfish; therefore, the rate of rod production is higher in trout than in goldfish. It is not surprising, therefore, that PINCs are readily observed in trout at least over their first 2 years of life, but they have been observed previously only in larval goldfish, at which stage retinal growth must be substantially faster than in adults.

There are also technical aspects to consider in explaining the experimental detection of PINCs in the retina of adult trout but not in adult goldfish or cichlids. Previous studies used single intraperitoneal or intraocular injections of [3 H]thymidine to discover mitotic cells in the retina (Sandy and Blaxter, 1980; Johns, 1982; Hagedorn and Fernald, 1992). This method only detects cells that divide within a time window defined by the metabolic half-life of the injected nucleotide (about 2 hours following

intraperitoneal injection; Hagedorn and Fernald, 1992). The methods we used (continuous subdermal delivery or continuous immersion) created longer lasting labeling windows. Indeed, we have observed PINCs in both adult goldfish and zebrafish after immersion in BrdU-containing water for 24–36 hours. Even under these experimental conditions, however, clusters of PINCs are infrequent in these species. Thus, extended exposure to labeled deoxy-nucleotides favors detection of infrequent events of cell division and indicates that PINCs exist in the retina of adult fish other than trout. In adult trout, however, PINCs are more abundant than in goldfish or zebrafish, probably because the rate of retinal growth is faster in trout than in the other species tested.

Although PINCs may be difficult to detect in slow-growing retinas, because they divide infrequently, the cells are likely to exist in all teleost retina. Their prevalence and, thus, the ability to detect them are simply a reflection of the physiological state of the retina. They reproduce at a rate that is just sufficient to meet the demands of tissue growth. This possibility is made strikingly apparent by the

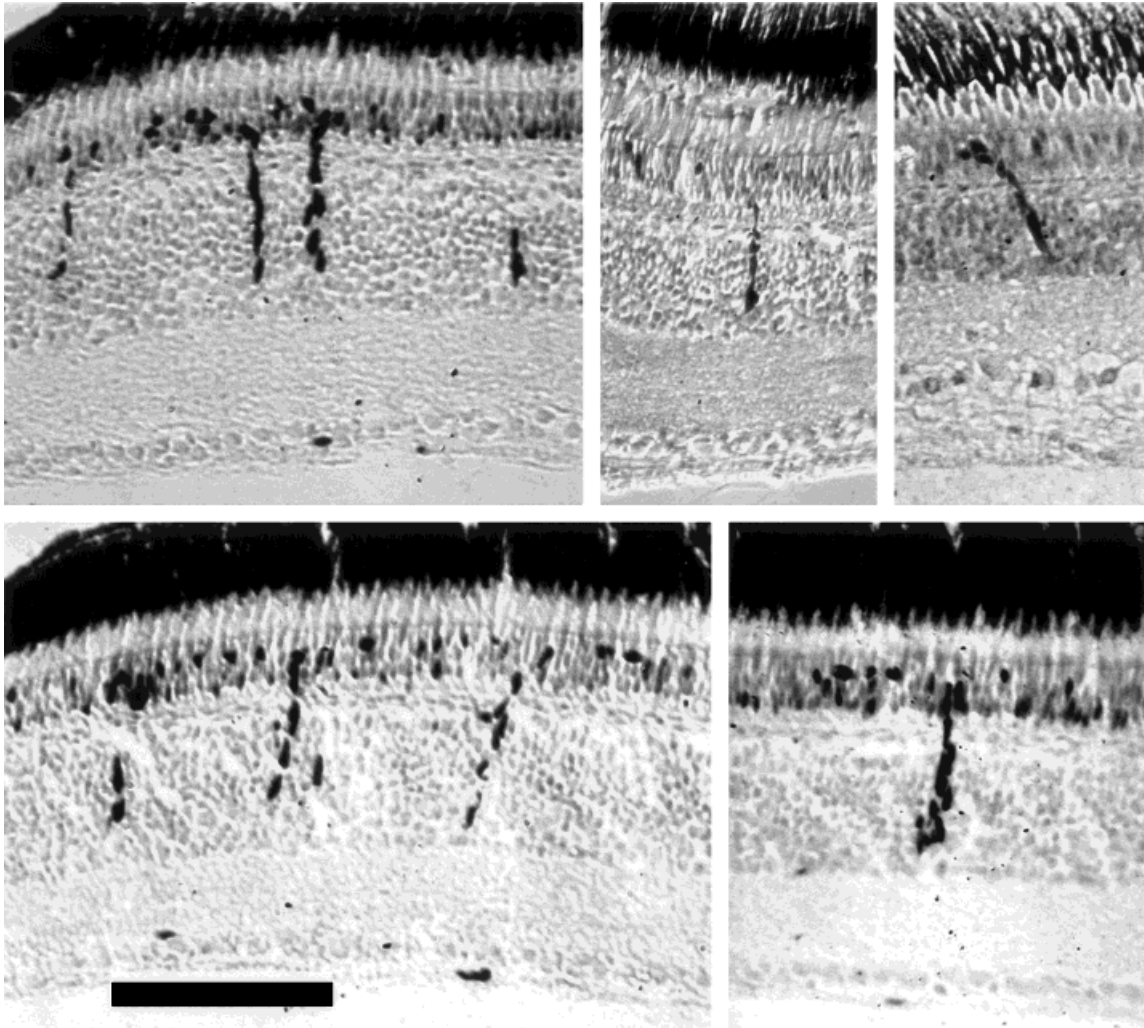


Fig. 7. Photomicrographs of radial retinal sections immunoreacted to identify proliferating cell nuclear antigen-positive (PCNA⁺) cells. Sections from different fish were selected to demonstrate PCNA⁺ cells that form parts of clusters and are traversing the outer plexiform layer. Scale bar = 100 μ m.

experimental results of Negishi and Shinagawa (1993) and Hitchcock et al. (1996). Negishi and Shinagawa found that, in 5–6 cm long goldfish, intraocular injection of several growth factors, control proteins, and even saline alone resulted in the appearance of clusters of PCNA⁺ cells in the INL. These clusters align radially from the inner to the outer plexiform layers and appear identical to the PINCs we have described here. Hitchcock et al. found that, in goldfish, excision of small pieces of retina leads to the appearance of proliferative cells in the mature INL of the retina near the area of damage. These proliferative cells were arranged in clusters that spanned the INL from the inner to the outer plexiform layers and that appeared, again, identical to the PINC clusters we have described here. It is possible, then, that the experimental data of both Negishi and Shinagawa and Hitchcock et al. reveal that the retinal tissue response to damage includes signals that up-regulate the proliferation of germinal cells, which are present in the INL but which divide infrequently, preceding injury.

In the trout, the density of PINCs is not uniform across the nasotemporal axis of the retina. The density along this axis is highest nearer either PGZ than the center of the eye, and it is higher on the nasal than on the temporal side. The nonuniform distribution of PINCs is mirrored in the density pattern of proliferating ONL cells, as might be expected if ONL proliferation is dependent on migration of cells from the INL. A similar density pattern of ONL proliferation is seen in a cichlid fish (Kwan et al., 1996). Furthermore, the nasal/temporal asymmetry is consistent with the findings of Easter (1992), who demonstrated that retinal growth is asymmetric, with the nasal side growing more rapidly than the temporal side, in foveated teleost eyes. The apparent interdependence between proliferating cells in the INL and the ONL, however, is not reflected in a daily rhythm of proliferation; whereas the proliferation in the ONL has a strong diurnal rhythm, that in the INL does not.

In addition to migration, the loss of labeled cells in the INL could arise from dilution of the label, because of

continuing cell division, or because of programmed cell death. Dilution of the label is an unlikely mechanism. If PINCs divided continuously and their progeny resided in the INL, then labeled cells would disappear, but the density of INL cells would also increase with time. However, it is well known that, in all teleosts, including trout, the density of cells in the INL decreases continuously as the retina ages (Johns, 1977; Johns and Easter, 1977; Kock, 1982). We explored the possibility that cell death occurs in the INL, but we found no evidence that PINCs undergo apoptosis. However, it is possible that some of them may still undergo a program of cell death that is not characterized by the production of small fragments of nuclear DNA, the feature tested by our method of assay.

Teleost retina regenerates when it is damaged mechanically (Hitchcock et al., 1992) or chemically (Maier and Wolburg, 1979; Raymond et al., 1988). The tissue regenerates from stem cells located within the retina itself. The hypothesis has been advanced that these stem cells are located in the ONL and are the same cells that generate rods under normal conditions (Raymond et al., 1988; Hitchcock et al., 1992). This hypothesis is supported by the findings that retinal damage causes an increase in the rate of cell division in the ONL and that regeneration occurs only if the ONL is damaged (Raymond et al., 1988). Damage limited only to the INL does not activate tissue regeneration (Raymond et al., 1988; Hitchcock et al., 1992). In light of this hypothesis, it is surprising that an antibody against *pax6*, an early developmental regulatory gene, does not label rod precursors in the normal retina but does label the neural progenitor cells that give rise to the regenerated tissue (Hitchcock et al. 1996). It is surprising, because *pax6* antibody labels all cells in the PGZ and in the earliest embryonic retina; therefore, it appears to be transiently expressed in retinal cells at a developmental stage that precedes rod specification. Thus, for the hypothesis to be correct, it would be necessary for rod precursors, which do not express *pax6*, to dedifferentiate to an earlier, *pax6*-expressing stage. The identification of PINCs as the cells that generate the rod precursor cells may explain this experimental finding. It may simply be that the mature retina has two types of undifferentiated cells: PINCs, which are totipotential cells able to regenerate every one of the retinal neurons, and the rod precursor cells, which are descendants of the PINCs and have a developmental fate that has become restricted to photoreceptors.

In fish killed immediately at the end of BrdU delivery, we observed numerous clusters of PINCs as well as labeled nuclei in the ONL. In contrast, in fish killed 30 days after the end of BrdU delivery, there were few if any BrdU-labeled cells in the INL (data not shown). The ONL, of course, had numerous labeled nuclei, presumably all in rod photoreceptors (Johns and Fernald, 1981). Therefore, most PINCs do not undergo terminal mitosis and differentiation in the INL of normal retina. In *Xenopus* larvae, retinal development occurs in a ciliary margin that is structurally analogous to the PGZ of fish (Beach and Jacobson, 1979). Recently, Dorsky et al. (1997) observed radially aligned clusters of cells embedded in the otherwise mature INL of the retina in larval *Xenopus*. Anatomically, these clusters resemble the PINC clusters we have described here. However, PINCs are not likely to be analogous to the INL clusters seen in *Xenopus*, because, in *Xenopus*, the clusters identify postmitotic cells, which are recognized by their expression of delta and notch gene products (Dorsky et al.,

1997). It is a possibility, however, that, in injured teleost retinas, PINCs might undergo their last mitosis in the INL as they differentiate and contribute to retinal repair. If this is the case, then the progeny of PINCs might be found to express notch or delta in regenerating teleost retina.

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