Local Regeneration in the Retina of the Goldfish

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SUMMARY

We have studied regeneration of the retina in the goldfish as a model of regenerative neurogenesis in the central nervous system. Using a transscleral surgical approach, we excised small patches of retina that were replaced over several weeks by regeneration. Lesioned retinas from three groups of animals were studied to characterize, respectively, the qualitative changes of the retina and surrounding tissues during regeneration, the concomitant cellular proliferation, and the quantitative relationship between regenerated and intact retina. The qualitative and quantitative analyses were done on retinas prepared using standard methods for light microscopy. The planimetric density of regenerated and intact retinal neurons was computed in a group of animals in which the normal planimetric density ranged from high to low. Cell proliferation was investigated by making intraocular injections of 5-bromo-2'-deoxyuridine (BUdr) at various survival times to label proliferating cells and processing retinal sections for BUdr immunocytochemistry. The qualitative analysis showed that the surgery created a gap in the existing retina that was replaced with new retina over the subsequent weeks. The BUdr-labeling experiments demonstrated that the excised retina was replaced by regeneration of new neurons. Neuroepithial-like cells clustered on the wound margin and migrated centripetally, appositionally adding new retina to the old. The quantitative analysis showed that the planimetric density of the regenerated neurons approximated that of the intact ones.

Keywords: bromodeoxyuridine, BUdr, rod precursors, differentiation, blastema.

INTRODUCTION

Matthey (1927) was the first to demonstrate convincingly that adult central nervous tissue regenerates. He showed that in urodele amphibians, the destruction of the retina was followed by regeneration of the entire tissue. This work initiated the study of optic nerve regeneration that continues to this day. In contrast, the study of retinal regeneration—the restitution of the retina after it has disappeared in whole or in part—has proceeded more slowly, drifting in and out of fashion, and generally not receiving the sustained attention that has been accorded axonal regeneration. Since this original study, several labs have continued investigating the process in urodeles (see reviews by Reyer, 1977; Stroeva and Mitashov, 1983), while others have shown that it also occurs in anurans (Levine, 1981; Reh and Nagy, 1987), teleost fish (Lombardo, 1968, 1972; Maier and Wolburg, 1979; Raymond, Reifler, and Rivlin, 1988b), and chicken embryos (Coulombre and Coulombre; 1965, 1970; Park and Hollenberg, 1989).

Two fundamentally different mechanisms are triggered following damage or removal (retinectomy) of the existing retina. In amphibians and chicken embryos, a subset of cells of the retinal pigment epithelium (RPE) transdifferentiates to form a neuroepithelium from which a new retina differentiates. In contrast, in teleost fish, the missing neurons are replaced by the rod precursors

(Raymond, et al, 1988b) specialized neuroepithelial cells that reside within the outer nuclear layer (ONL) and normally divide to produce rod photoreceptors only (Johns and Fernald, 1981; Raymond, 1985).

In our investigation of retinal regeneration in the goldfish we have three aims: (1) to describe the cellular proliferation giving rise to regenerated retina, using modern techniques, (2) to attempt to identify general regulatory principles of retinal regeneration, and (3) to begin constructing the cascade of cellular and molecular events involved.

We used the thymidine analog given in summary BUdr and immunocytochemistry to identify proliferating cells during regeneration. Short survival periods revealed the proliferating cells; long survivals showed what the proliferating cells became. Planimetric density (number of neurons per mm^2 of retinal surface) was used as a quantitative descriptor of the regenerated retina. We used fish that ranged in size and age from small and young to large and old in order to vary the retinal "environment" during regeneration. As fish grow, the planimetric density of all retinal neurons, except the rods, changes continuously (Johns and Easter, 1977; Johns, 1982), from high in small, young goldfish to low in large, old ones. These growth-related changes allowed us systematically to vary the age and planimetric density of the retinal neurons surrounding regenerating ones simply by making lesions in fish that ranged in size from small to large.

Portions of the data described here have been reported in preliminary form (Easter, Mangione, and Malinoski, 1986; Hitchcock, 1990; Lindsey and Hitchcock, 1991).

METHODS

Goldfish (1.6- to 16-cm standard body length; 1.6- to 4.5-mm lens diameter) were purchased (Grassyfork Fisheries, Martinsville, IN and local pet stores) and maintained in areated aquaria at 18°–26°C. All surgical procedures and other procedures requiring physical restraint of the fish were performed after they were deeply anesthetized by immersion in a 0.1% aqueous solution of tricaine methane-sulfonate.

Surgery

A small rectangular patch of retina, typically 0.5–2.0 mm on a side, was removed from either the ventral or dorsal region of the nasal retina. These locations and this size were chosen for reasons of convenience. We did not evaluate different sizes or locations. A slit, parallel to the retinal margin and about 0.5 mm central to it, and extending into the vitreal chamber, was made with a microknife. Then, two perpendicular cuts extending centrally from both ends of the slit produced a tongue-shaped flap, that included all ocular and retinal layers, that was then reflected outward. The blade of a forceps or small scissors was introduced at the base of the tongue, between the neural retina and retinal pigmented epithelium, and gently drawn toward the tip of the tongue to separate neural and pigmented layers. The retina was cut at the base of the tongue and removed as one piece. One ophthalmic suture, which generally fell out after several days, tacked the sclera at the tip of the tongue to the marginal sclera, and the fish was revived and returned to its home tank. The scleral incisions sealed within 24 h, and the globe had a normal external appearance within 2 weeks.

Ordinarily, this was a nearly bloodless operation because both the systemic blood pressure is so low and the major annular vessel at the retinal margin was spared. Also, care was taken to avoid the large blood-filled choroidal organ that straddles the subscleral optic nerve.

Histology and Immunocytochemistry

For both qualitative and quantitative transmitted-light microscopy, the eyes were enucleated, the cornea and lens removed, and the eyecups fixed by immersion for 1 h in 2.5% glutaraldehyde/2.0% paraformaldehyde in 0.8 M Sorensen's buffer, pH 7.4. After several buffer rinses, the vitreous was aspirated. the eyecups were dehydrated in a graded series of ethanol, and infiltrated overnight in catalyzed JB-4 glycomethacrylate at 4°C. The eyecups were then hemisected along a line adjacent to the regenerated patch and placed into molds where the plastic was allowed to polymerize. Radial. 5-μm thick sections were cut on a rotary microtome, mounted onto gelatinized slides, stained with 0.25% toluidine blue, and coverslipped with Histoclad.

Proliferating cells were marked by injecting BUdr intraocularly at various time points after the surgery (see Results). BUdr-labeled cells were detected in frozen sections using indirect immunofluorescence and in whole mounts using peroxidase-antiperoxidase (PAP) immunocytochemistry. In all instances, the globe was punctured at the limbus with a micropipette, and 2 μl of 1 mM BUdr was injected into the vitreal chamber through a 30-gauge needle attached to a 10-μl Hamilton syringe. Eyecups were fixed in fresh, room temperature 4% paraformaldehyde in 0.1 M phosphate buffer for 1 h, rinsed in buffer, prepared for cryosectioning (Knight and Raymond, 1990), and frozen in a 2:1 mixture of 20% sucrose/phosphate buffer: OCT (Tissue Tek). Radial, 10-μm thick sections were collected through the regenerated patch, and mounted on poly-L-lysine- (Sigma) coated slides. For immunostaining, the sections were thor-

oughly air dried—so that sections would adhere to the slides—and rinsed with PBS to remove any residual sucrose/OCT. The slides were then soaked in 2 N HCl/ phosphate-buffered saline (PBS) solution for 30 min— to fragment the DNA and expose the incorporated BUdr —and rinsed for 30 min in PBS plus 0.5% triton X-100. Then, with the slides horizontally oriented in a moist chamber, nonspecific binding by the primary antibody was blocked by incubating the sections in PBS/0.5% triton X-100 plus 20% normal goat serum (NGS) for 30 min. The slides were drained, and the sections were incubated for 1-2 h at room temperature with anti-BUdr (Beckton-Dickenson) diluted 1:20 in PBS/triton X-100 plus 1% NGS. Sections were rinsed in the antibody diluent for 30 min, incubated in a goat anti-mouse secondary antibody conjugated to tetrarhodamine isothiocyanate (TRITC; Sigma), rinsed in PBS, and coverslipped with Gel/Mount (Biomeda Corp.). Fluorescence photomicrographs were taken with Kodak Tri-X, at ASA 1000, which was developed with Kodak Accufine.

For PAP immunocytochemistry on whole mounts, fish were dark adapted for at least 1 h, the retinas were isolated, then fixed free floating in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (see Hitchcock and Easter, 1986, for a detailed description of the dissection procedures). The retinas were rinsed in buffer, slowly frozen, and thawed to aid antibody penetration, soaked in 2 N HCl/PBS for 30 min, rinsed again, and incubated in 20% NGS in PBS/0.5% triton X-100 for 2 h. Next, the retinas were incubated in anti-BUdr diluted 1:80 in PBS/0.5% triton X-100/1% NGS for 48-72 h with gentle agitation at 4°C. Following the incubation in the primary antibody, the retinas were rinsed for 1 h in the antibody diluent and incubated with goat anti-mouse IgG (Sigma) diluted 1:20 for 2 h. After 30 min of rinsing, the retinas were incubated with mouse PAP (Sigma) diluted 1:200 for 1 h, rinsed in PBS alone, and reacted with 3,3'-diaminobenzidine (Adams, 1977). Retinas were rinsed a final time, mounted onto gelatin-coated slides, air dried, dehydrated in alcohols, and coverslipped with Histoclad. Whole mounts stained with toluidine blue were processed similarly, with the exception that the immunocytochemistry was omitted.

Quantitative Methods

The planimetric density of neurons was determined in the following manner. For each eye, evenly spaced sections within the regenerated patch were selected for cell counts. Control densities were determined from counts taken from retina adjacent to the regenerated patch at the same centroperipheral position. Regenerated patches were identified by one or several of three features: the laminar fusions marking its boundary, the thick bundle of axons around its periphery, and the scar in the overlying scleral cartilage (see Results). Planimetric densities were computed separately for four groups of neurons: rods and cones in the outer nuclear layer, all cells with

somata in the inner nuclear layer [INL (this group also included Müller cells, the principal glial cell of the retina)], and neurons in the ganglion cell layer (GCL). Nuclei were traced at a final magnification of 1100×. The nuclei of rods, cones, and ganglion cells were identified according to previously published criteria (Johns and Easter, 1977; Raymond, Hitchcock, and Palopoli, 1988a). Nuclear diameters and retinal lengths were measured from the tracings using a Zeiss MOP-3 digitizing system, and the raw counts were corrected for split nuclei (Konigsmark, 1970). Planimetric densities were computed by dividing the corrected number of neurons by the product of the retinal length sampled (along the inner limiting membrane) and thickness of the section (0.005 mm). No corrections were made for tissue shrinkage.

Within-animal comparisons were made using a nonparametric, pairwise rank statistic (Wilcoxon matched-pairs signed-ranks test; Daniel, 1978). This test utilizes both the sign and the magnitude of the difference between paired observations, and allows one to determine if the median of the differences between each pair is significantly different from zero. Between-animal comparisons were made with a least-squares regression analysis to determine if neuronal density varied as a function of eye size. Lens diameter was selected as the independent variable (Easter, Johns, and Baumann, 1977; Johns, 1982). A T statistic was used to test whether or not the slopes of the regression lines through scatter plots of density versus lens diameter were significantly different from zero.

RESULTS

Regeneration and Cell Proliferation

The photomicrographs in Figures 1-3 illustrate the progression from lesion to complete regeneration. Twenty animals were used in this analysis. A photomicrograph of an undamaged retina, at approximately the same centroperipheral position as the lesions, is shown in Figure 1(A). [What appear as slight differences in the magnification of the panels in this and subsequent photomicrographs are due to slight differences in the size of the fish used; larger fish have larger eyes and retinas (Johns and Easter, 1977)].

Figure 1(B) shows the retina and supporting tissue immediately after the surgery. The surgery produced a sharply defined zone of missing retina that is in quite good register with the cuts in the scleral cartilage. The underlying RPE, although not intentionally removed, was extensively damaged. The outer segments of photoreceptors adjacent to the margin of the wound [Fig. 1(B)] are sheared off,
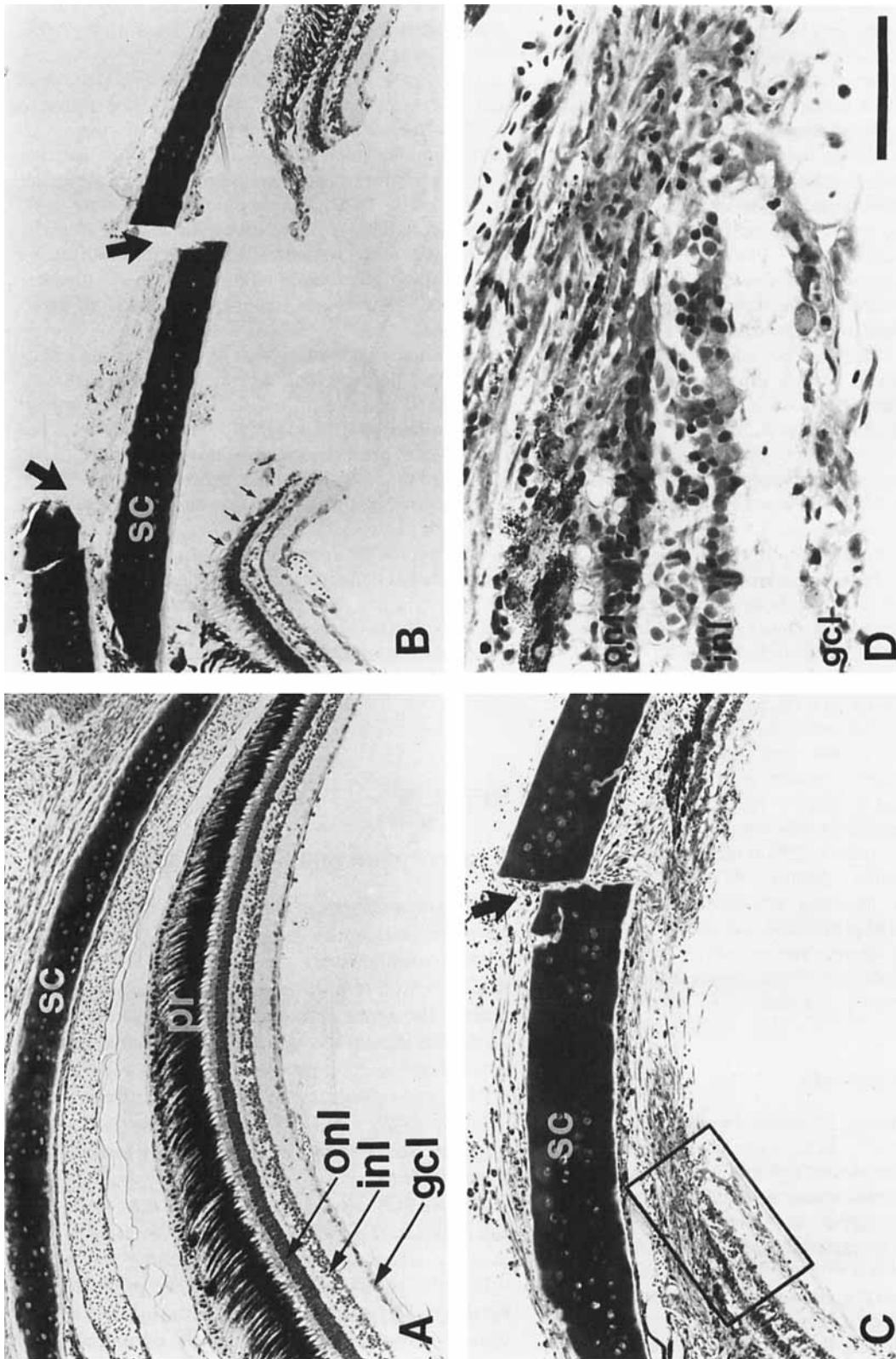
Figure 1 Light photomicrographs of regenerating retina. Panel A is a normal retina at the same location as the lesions illustrated in all the subsequent Figures. Panels B and C represent survival times of 0 and 1 week, respectively. The large arrows identify the cuts made in the scleral cartilage during surgery. The small arrows in panel B mark the location of photoreceptors sheared from the intact retina surrounding the excised patch. The retina enclosed in the rectangle in panel C is shown at higher magnification in panel D. In each photomicrograph, the retinal margin is toward the right. gcl = ganglion cell layer; inl = inner nuclear layer; onl = outer nuclear layer; pr = photoreceptor layer; sc = scleral cartilage. Scale bar = 200 μm for A–C and 50 μm for D.
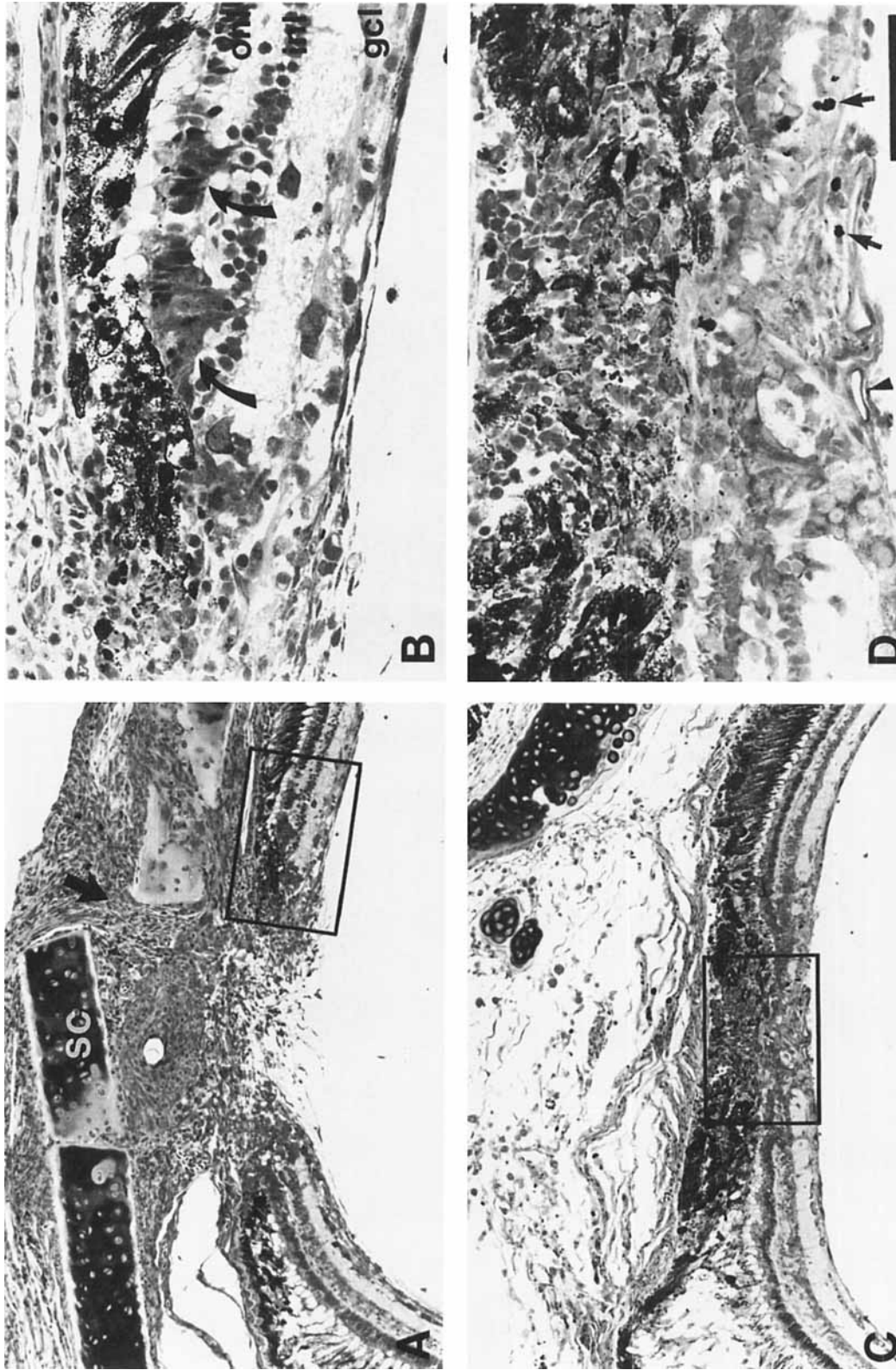
Figure 2   Light photomicrographs of regenerating retina. Panels A and C represent survival times of 2 and 4 weeks postlesion, respectively. The retinas enclosed in the rectangles in panels A and C are shown at higher magnification in panels B and D, respectively. In panel A, the large arrow identifies the cut made in the scleral cartilage during surgery. The arrows in panel B identify intraretinal clusters of proliferating cells. In panel D, the arrows identify clumps of pigment that were frequently enclosed within the regenerating retina. The arrowhead identifies a retinal vessel cut in cross section. In each photomicrograph the retinal margin is toward the right. Abbreviations as per Figure 1. Scale bar = 200 μm for panels A and C and 50 μm for panels B and D.
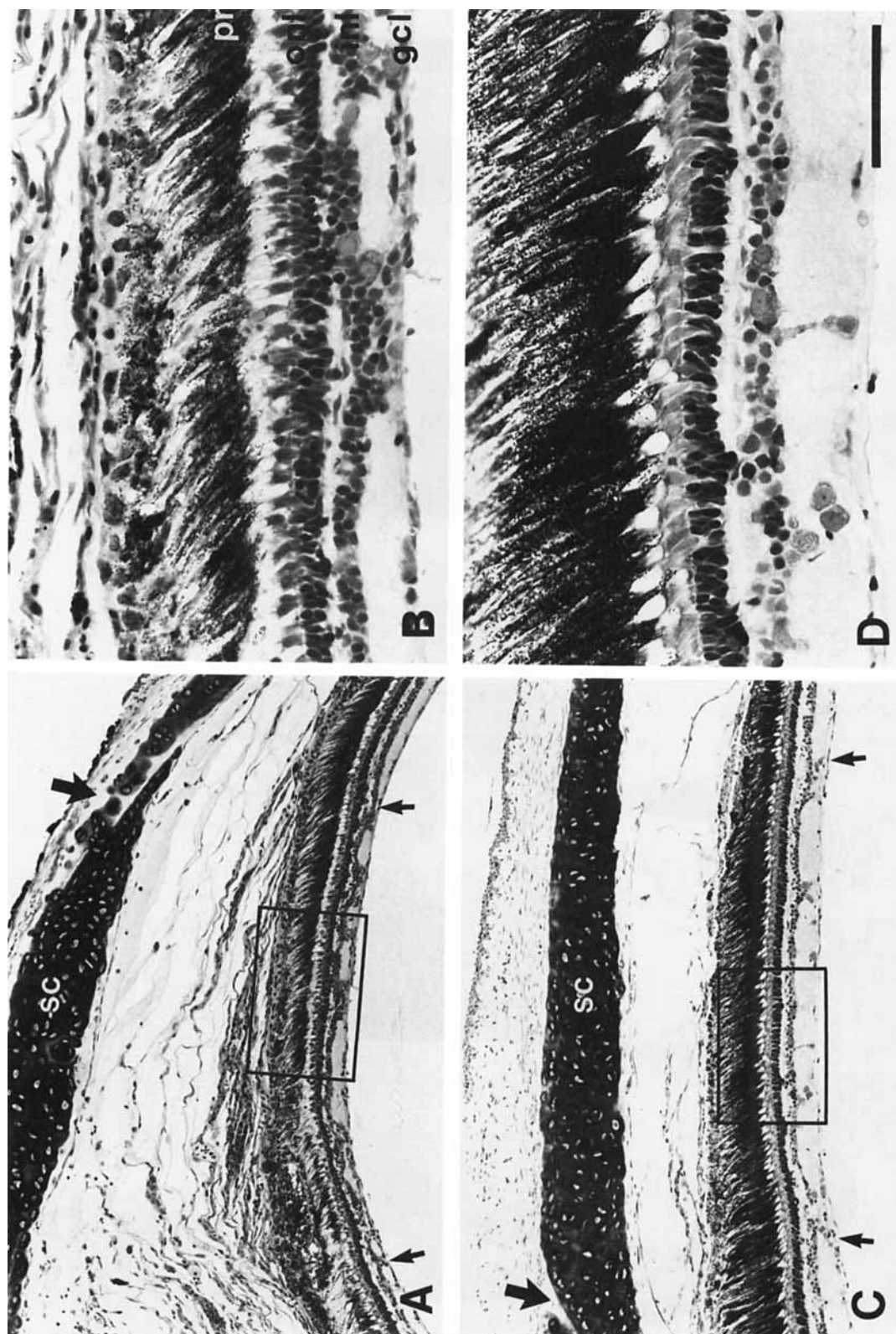
Figure 3 Photomicrographs of regenerating retina. Panels A and C represent survival times of 8 and 20 weeks postlesion, respectively. The retinas enclosed in the rectangles in panels A and C are shown at higher magnification in panels B and D, respectively. In panels A and C, the large arrows identify scars in the scleral cartilage overlying the regenerated retina. The small arrows identify laminar fusions seen frequently within the regenerating retina and at the interface between the regenerated and intact retinas. Panels B and D illustrate the continuing maturation of the regenerated retina between 8 and 20 weeks. In each photomicrograph the retinal margin is toward the right. Abbreviations as per Figure 1. Scale bar = 200 µm for panels A and C and 50 µm for panels B and D, respectively.

which, as can be inferred by the subsequent thinning of the ONL [Fig. 1(D)], kills the cells.

One week after the surgery [Fig. 1(B)], nonneuronal cells, presumably phagocytes and fibroblasts, began accumulating in the area of the missing retina. By 2 weeks [Fig. 2(A)], a large bolus of nonneuronal cells lay in and around the retinal lesions. Within the retina adjacent to the margin of the retinal wound [Fig. 2(B)], clusters of columnar, neuroepithelial-like cells are present. They clearly lie within the neural retina, and are most numerous within the ONL. Such cells are not ordinarily seen in the central retina; their resemblance to the proliferative cells of the retinal margin suggests that they are proliferative.

By 4 weeks [Fig. 2(C,D)], the initial influx of nonneuronal cells has subsided, and loose connective tissue is forming at the site of the scleral wound. The retinal wound is closed by presumptive neural tissue, which is overlain by loosely organized pigment-containing cells, presumably from the RPE. The inner boundary of the retina is reestablished by this time and underlain by blood vessels (arrowhead). In addition, clumps of pigment appear to be enclosed within the regenerating retina. These were commonly seen, both in radial sections and whole mounts (see below). By 8 weeks [Fig. 3(A)], the original gap created by the lesion now contains retina with clear, albeit uneven, and relatively thin plexiform and cellular layers [Fig. 3(B)], which are fused at some places. This is completely overlain by a layer of RPE nuclei, although the photoreceptor outersegments, which they envelope, appear somewhat disorganized. By 20 weeks (panel F), the neural retina is essentially normal, except for the several INL–GCL fusions. The RPE and photoreceptor layers also appear normal.

We have assumed that the laminar fusions marked by the arrows in Figure 3(A,C) mark the boundary of the original lesion. First, they are never seen in the undamaged retina. Second, they are in good register with the overlying scars in the scleral cartilage and disruptions in the photoreceptor/RPE layers. Third [Fig. 6(B)], the axons of ganglion cells form large fascicles around the edge of the regenerate, and the laminar fusions lie just inside these fascicles. Finally, after retinal regeneration following a neurotoxic lesion, similar laminar fusions were seen at the interface between the regenerated retina and the normal one produced by the marginal germinal zone (Raymond et al., 1988b).

Figure 4 again illustrates the progression from lesion to complete regeneration, except that in this figure each retina was exposed to BUdr 24 h prior to sacrifice. Twelve animals were used in this analysis. In this figure, the labeled cells, which appear as bright spots, identify the momentary sites of cell proliferation. Panel A shows that after 1 week of survival proliferating cells are diffusely distributed throughout the wound from retina to sclera. In contrast, within the retina, BUdr-labeled cells are found in clusters at the wound margin and within the adjacent ONL [Fig. 4(B)]. Examination of other sections revealed that the two clusters of labeled cells seen here are part of a continuous band along the entire wound margin. We have chosen to call this band the *blastema,* analogous to the cap of proliferating cells seen in amphibians on the proximal stump of a regenerating limb (Brockes, 1991). After 2 and 4 weeks [Fig. 4(C,D)], the blastema still lies at the wound margin, but a comparison with panel A suggests that the two edges have narrowed the original gap in the retina. In addition, over time the number of BUdr-labeled, nonneuronal cells decreases as well. By 8 weeks [Fig. 4(E)], only a few, scattered BUdr-labeled cells are present, comparable with intact retina.

We also observed labeled cells outside the blastema, in the ONL immediately surrounding the wound [Fig. 4(A,C,D)]. At 1 week after the surgery [Fig. 4(A,B)], the ones close to the blastema are likely to be replacements for the photoreceptors that were damaged during the initial surgery [Fig. 1(B)]. At 8 weeks [Fig. 4(E)], the labeled cells are probably rod photoreceptors that continue to be added, as in normal retina, to the regenerated retina. But at 2 and 4 weeks [Fig. 4(C,D)], labeled cells within the ONL were much more numerous than normal, particularly between the lesion and the retinal margin. Presumably, the lesion stimulated the proliferation of these cells, probably the rod precursors, as they are the only proliferative cells with their nuclei in this layer.

The presence of the blastema at the wound margin and the sequence of events represented in Figures 1–4 suggest the following: (1) The repair of the lesion is due to regeneration, and (2) the wound is closed by the migration of the blastema into the wound and the appositional addition of new retina to the old. The presence of the blastema, however, does not exclude the possibility that the wound might be closed by the old retina pinching shut, a simple "purse stringing," without the addition of new retina. To investigate this possibility, a third group of animals ($n = 9$) was labeled cumula-
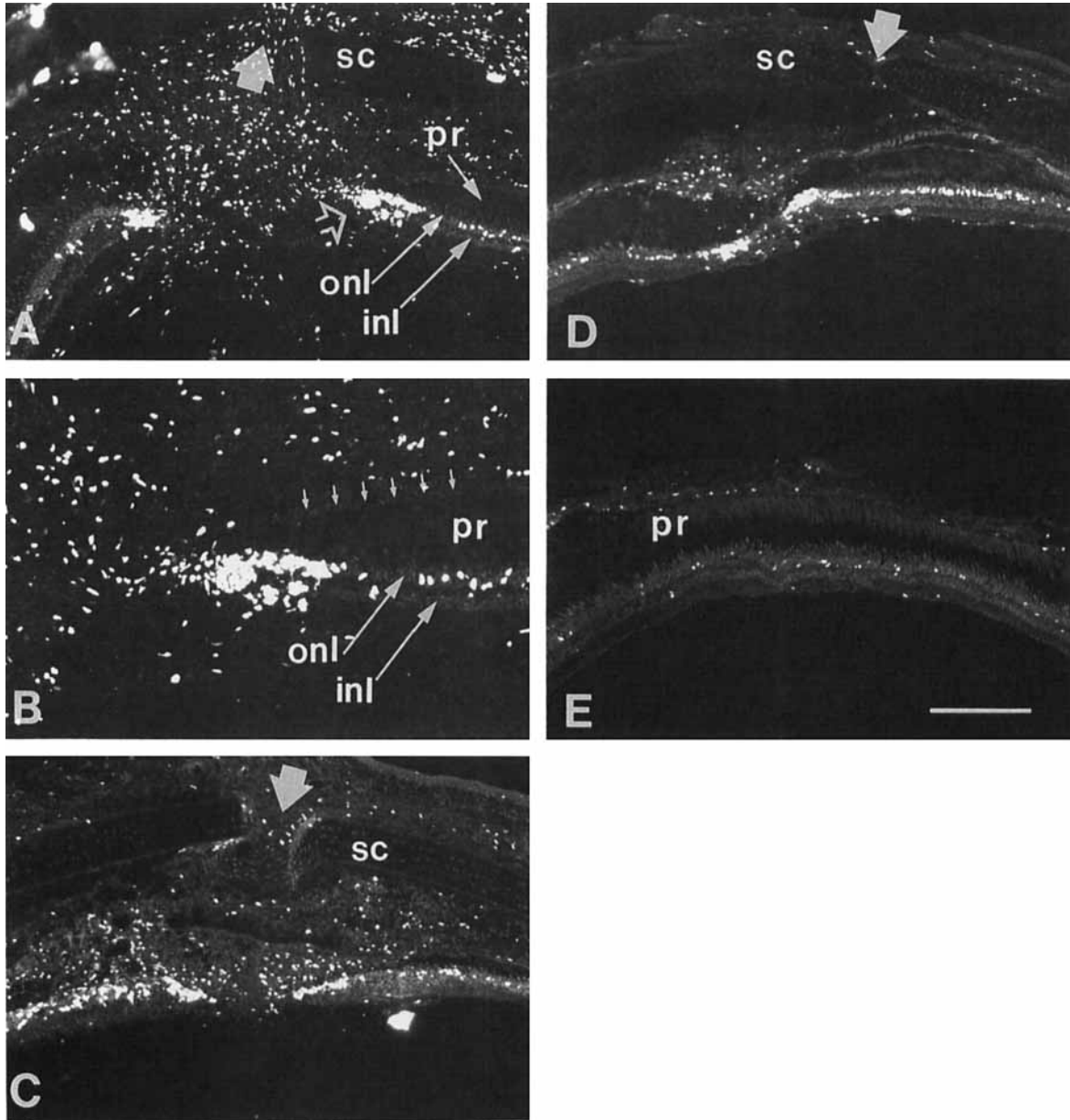
Figure 4   Light photomicrographs of BUdr-labeled, regenerating retinas. The animals were sacrificed 24 h after BUdr injection. The bright spots are fluorescent, BUdr/TRITC-labeled cells. (These photomicrographs were slightly underexposed so that the background cellular staining could be seen. As a result, the BUdr cells at each wound margin appear fused into a single cluster.) Panels A, C, D, and E represent survival times of 1, 2, 4, and 8 weeks postlesion [cf. Figs. 1(A), 2(C), and 3(A), respectively]. The large arrows indicate the cuts and scars in the scleral cartilage overlying the regenerating retina. The sclera was lost during processing of the retina illustrated in panel E. The open arrow in panel A indicates that part of the regenerating retina illustrated at higher magnification in panel B. In panel B, the small arrows indicate nuclei of RPE cells, which remain unlabeled with BUdr. In each photomicrograph, the retinal margin is toward the right. Abbreviations as per Figure 1. Scale bar = 200 μm for panels A and C–E and 50 μm for panel B.

tively with BUdr. All received lesions and were labeled according to one of three different schedules. BUdr was administered at survival times of 1, 2, and 3 weeks (group 1), 2 and 3 weeks (group 2), or 3 weeks (group 3), respectively. Each group survived 8 weeks postlesion. Two predictions were made. First, if the retinal wounds were repaired by regeneration of new retina, then each retina should contain a patch in which cells in all three nuclear layers are labeled. Second, if the new retina is laid down appositionally, adjacent to the blastema, then there should be a progressive restriction in the extent of the labeled cells. The retina that had BUdr available from 1 week should contain labeled cells over most of the regenerated patch, whereas those that received BUdr later should have smaller, labeled patches, lacking the retina produced earlier.

The predictions were confirmed as Figure 5 illustrates. Multiple exposures to BUdr begun at 1 week resulted in a patch of retina that contained labeled cells in all layers from one boundary to the other [Fig. 5(A)]. Successive delays in BUdr administration [Figs. 5(B,C)] produced patches of labeled retina that were progressively smaller and further inside the boundary of the original lesion. We conclude that the retina that was surgically removed was replaced by the appositional regeneration of new tissue.

We also viewed regenerated patches in the retinal whole mount. Figure 6(A) illustrates a regenerated patch in a Nissl-stained whole mount. The regenerated patch was identified by the subtle differences in the pattern of stained cells, and, as was frequently seen, an inclusion of pigment, presumably from the RPE, trapped within its middle [Fig. 2(D)]. Further, large fascicles of axons outlined the presumptive boundaries of the regenerate. In a normal, Nissl-stained retina, the overlying optic fiber layer appears as a spoke-like arrangement of unstained fascicles of axons coursing from the margin (rim) toward the optic disc (hub). We suggest that the fascicles coursing around the periphery of the patch are made up of both the regenerated axons of ganglion cells that lay distal to the lesion and the new ganglion cells added at the margin since the lesion was made. Figure 6(B) illustrates a retina that received three successive injections of BUdr (1, 2, and 3 weeks after the lesion) and was processed for BUdr and PAP immunocytochemistry. The roughly rectangular, dark patch of labeled cells shows the original lesion.

In Figure 6(B), the broad, lighter band of labeled cells bridging the space from the lesion to the margin includes mostly cells in the ONL. This suggests that those cells outside the regenerating retina that were labeled following short survival times [Fig. 4(C,D)] persisted and differentiated into photoreceptors. Evidence for this is shown in Figure 7—a photomicrograph taken between the lesion and the margin in a retina that was exposed to BUdr at 2 and 3 weeks postlesion and sacrificed at 8 weeks postlesion. Although occasional BUdr-labeled cells were seen in other layers, as shown here, the vast majority were nuclei of rod photoreceptors.

Quantitative Analysis

For purposes of computing the planimetric density of regenerated neurons, cell counts were made in a total of 17 eyes in 15 fish that survived 20 wks. From each eye, an average of 6304 and 6166 μm^2 of intact and regenerated retina were sampled, respectively. An average of 2748 rods, 323 cones, 1110 INL neurons, and 55 ganglion cells were counted in each retina.

Figure 8 illustrates the ratio of the planimetric densities in intact and regenerated retina (intact density/regenerated density) plotted on a logarithmic scale as a function of lens diameter. A ratio of 1 indicates that the planimetric density of the regenerated cells matched that of the intact retina, that is, the number of neurons produced during regeneration matched that of the surrounding intact retina. Ratios greater than 1 indicate that regenerated neurons were underproduced, whereas ratios less than 1 indicate that regenerated neurons were overproduced.

The ratios of the computed planimetric densities varied depending on the particular cell type and the size of the animal. The planimetric densities of rods [Fig. 8(A)] were generally lower in the regenerated retina, indicating that they were underproduced during regeneration. This was especially so in three of the largest retinas, where there were 1.9–2.7 times more rods/mm^2 in the intact region. The pairwise comparisons showed that the differences in the densities between regenerated and intact rods were statistically significant (p = 0.003). The cones, in contrast, were overproduced [Fig. 8(B; p = 0.012]. (The apparent underproduction of rods and overproduction of cones is addressed further in the Discussion) Like rods, the density of INL neurons [Fig. 8(C)] was slightly lower in the regenerated retinas (p = 0.0495), with the exception of three of the largest eyes. The data
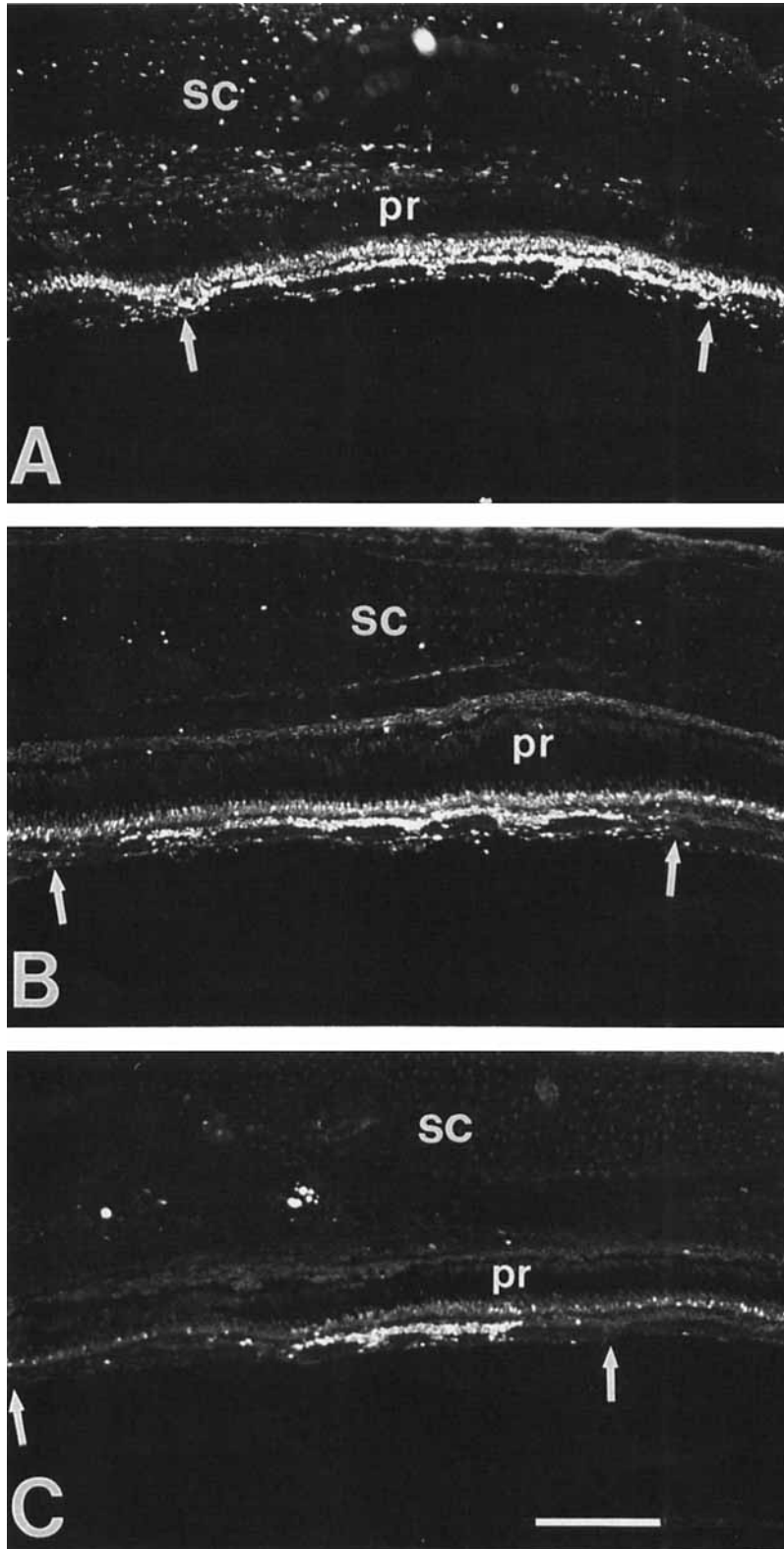
Figure 5 Light photomicrographs of retinas that were exposed to BUdr at 1, 2, and 3 weeks postlesion (A), 2 and 3 weeks postlesion (B), or 3 weeks postlesion (C), and survived 8 weeks postlesion. In each, the arrows indicate the boundaries of the original lesion. Abbreviations as per Figure 1. Scale bar = 300 μm.
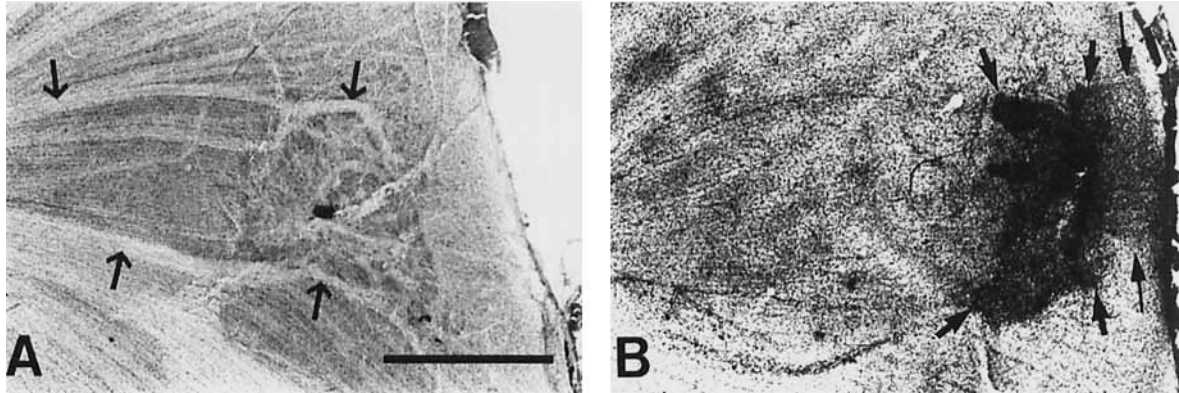
Figure 6 Photomicrographs of patches of regenerated retina in whole mounts. Panel A is a low-power photomicrograph of a Nissl-stained whole mount 45 weeks postlesion. The regenerate can be recognized by the subtle differences in staining and the boundary formed by the thick fascicles of ganglion-cell axons (arrows). The retina in panel B was exposed to BUdr at 1, 2, and 3 weeks and survived 8 weeks postlesion. Note the roughly rectangular patch of densely labeled cells (short arrows) and the band of labeled cells (long arrows) bridging the gap between the regenerated patch and the margin. Scale bar = 1 mm.

for ganglion cells were extremely variable [Fig. 8(D)]; the ratios were equally distributed about 1.0, and their planimetric densities did not differ significantly ($p = 0.98$).

Figure 9 shows the planimetric densities plotted as a function of lens diameter of regenerated cones, INL neurons, and ganglion cells pooled together [Fig. 9(A)] and rods [Fig. 9(B)]. Rods were ana-

Figure 7 Photomicrograph of BUdr-labeled cells between the lesion and the retinal margin in an animal that was exposed to BUdr at 2 and 3 weeks postlesion and survived 8 weeks postlesion. The BUdr-labeled cells are rod nuclei lying within the ONL. The arrowhead indicates the outer limiting membrane. c = layer of cone nuclei within the ONL; r = layer of rod nuclei within the outer nuclear layer; other abbreviations as per Figure 1. Scale bar = 50 μm.

lyzed separately because they outnumber all other retinal neurons combined, and their inclusion would obscure the data for the other neurons. Moreover, during the normal retinal development, rods are the last cell type to be produced, and they are added continually with growth (Raymond, 1985), so it might be expected that with respect to other retinal neurons, they would accumulate differently during regeneration. For this analysis, we anticipated one of three possible outcomes. First, if regeneration serves *only* as a mechanism for repairing damage, then the planimetric density of the regenerated neurons might assume a constant, perhaps intermediate value, regardless of the size or age of the retina. Second, if regeneration recapitulates embryonic retinal development, regardless of retinal size, then the planimetric density of the regenerated neurons should be very high as in a very young animal. Third, if regeneration is regulated in some fashion, perhaps by the local environment (Negishi, Teranishi, and Kato, 1982; Reh and Tully, 1986; Reh, 1988), then the planimetric density of the regenerated neurons might be expected to approximate that of the surrounding intact retina. The data illustrated in Figure 9 show that the planimetric density of regenerated, nonrods decreased with increasing lens diameter, consistent with the proposal that the regenerative neurogenesis is under some form of regulatory control. The slope of the least-squares linear regression line was significantly different from zero ($p = 0.006$). This analysis also showed that the regenerated retina
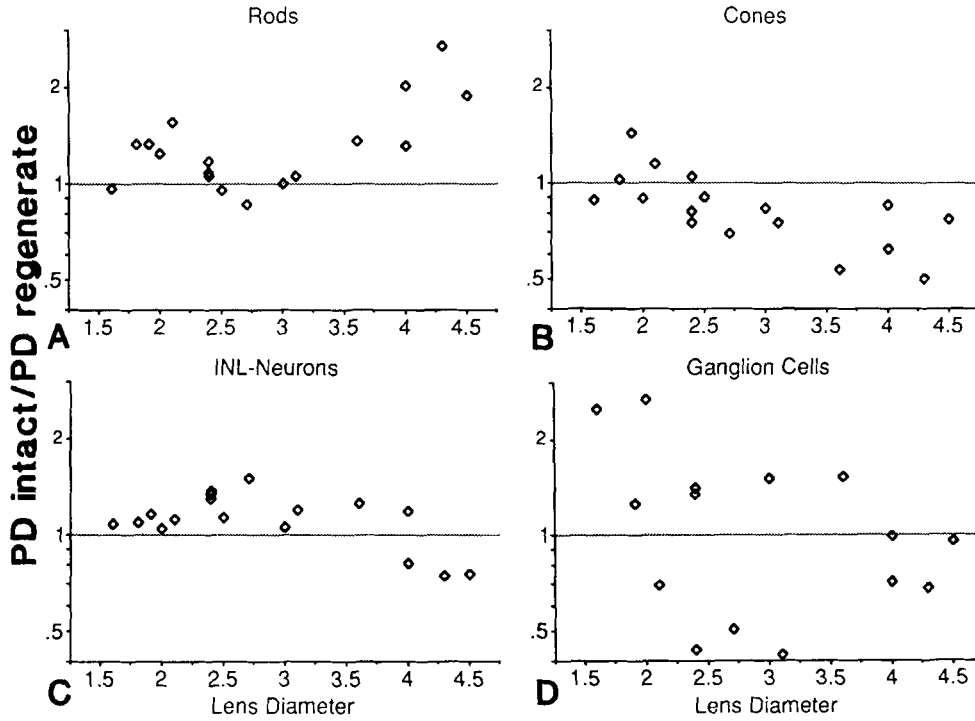
Figure 8 Ratios of the planimetric density of neurons in the intact and regenerated retina plotted as a function of lens diameter. Log scale on the ordinate. (A) Rods. (B) Cones. (C) INL neurons. (D) Ganglion cells. PD = planimetric density.

was not an exact match of the intact one. The slope of the least-squares regression line for the regenerated cells was more shallow than for cells in the surrounding normal retina ($-13{,}326$ versus $-23{,}869$ cells per millimeter of lens diameter), and the r^2 value was lower (0.79 for normal neurons versus 0.41 for regenerated neurons) indicating a greater variability in the values. In contrast to the nonrod cells, the densities of the regenerated rods

were invariant with lens diameter [Fig. 9(B)]; the slope of the regression line (-6320 cells per millimeter of lens diameter) was not statistically significantly different from zero ($p = 0.28$). This suggests that rod photoreceptor production during regeneration was not under the same regulatory control as were the nonrod cells.

The trends evident in the quantitative analysis are qualitatively illustrated in the photomicro-
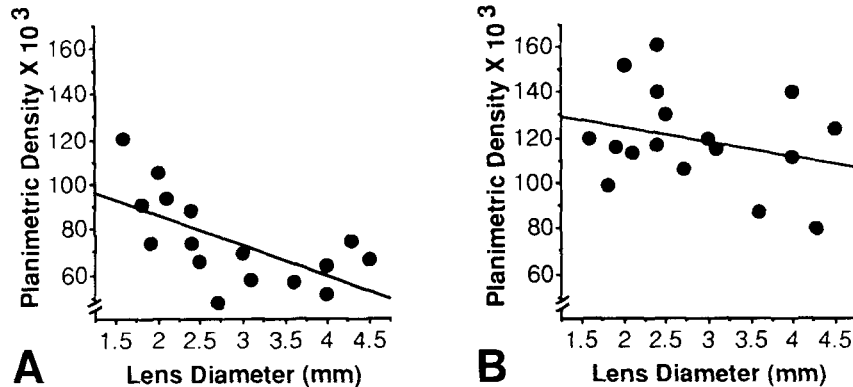

Figure 9 Scatter plots of the planimetric density of regenerated neurons versus lens diameter. Panel A shows the data of pooled cones, INL neurons, and ganglion cells. Panel B shows the data for rods. A least-squares linear regression line is drawn through each set of data.

graphs of normal [Fig. 10(A,B)] and regenerated [Fig. 10(C,D)] retinas from a small and large fish. As can be seen in Figure 10(A,B), with normal growth, the thickness of the retina increases, especially in the photoreceptor layer (PR), the number of rod photoreceptors (Johns and Easter, 1977) increases, and there are concomitant decreases in the densities of cones, ganglion cells, and cells within the INL (Johns and Easter, 1977; Johns, 1982; Raymond, 1990). Figure 10(C,D) show that the regenerated retinas qualitatively resemble their normal counterparts, with the exceptions that were apparent in the quantitative analysis (see above). In the largest eyes, the overproduction of INL neurons is probably due to the absence of large, regenerated horizontal cell axons that appear as open spaces between the nuclei in the INL [Fig. 10(B)]. These processes hypertrophy with retinal growth (Raymond, 1990), and this feature apparently was not recreated during regeneration.

DISCUSSION

We have shown that in the goldfish, local damage to the retina is repaired by local regeneration. We used a procedure similar to that of Lombardo (1968, 1972), and our data confirmed and extended his original observations. Locally excising all layers in a small patch of retina resulted in the formation of a ring of proliferating cells at the margin of the wound that migrated centripetally to close the wound by adding new retina to the old. We excluded the possibility that the retinal lesion was repaired simply by the old retina stretching to fill in the gap; cumulative labeling with BUdr after the lesion resulted in a rectangle of retina in which all three nuclear layers contained labeled cells.

The data from the BUdr experiments suggest that the retina was regenerated from an intrinsic population of cells. Although we cannot exclude the possibility that the blastema was created by cells that originated outside the retina, we think it unlikely. First, the blastema was always found within the bounds of the neural retina; second, there was no evidence of cells migrating from outside the retina into the blastema; RPE cells, the nearest candidates to invade retina, were never labeled with BUdr; and third, the blastema was generally contiguous with dividing cells within the ONL.

In the normal goldfish retina, there are at least four classes of proliferating cells: endothelial cells, glia (astrocytes, Müller cells, and microglia), neu-

roepithelial cells within the marginal germinal zone, and rod precursors. There is no evidence here or elsewhere to support the idea that endothelial cells or glia are competent to generate new retina, so they will not be discussed further.

The neuroepithelium at the retinal margin is known to produce all classes of retinal neurons under normal circumstances. It is possible that the cells at the margin migrated to the retinal wound. In animals that received BUdr injections after short survival times, there were always labeled cells within the ONL between the lesion to the marginal germinal zone, but they were not elongated in the direction of migration, as migrating cells tend to be. Instead, they were elongated in the radial direction, as rod precursors and neuroepithelial cells are, which suggests that they were not migratory. Moreover, labeled cells remained in this region long after regeneration was complete, indicating that some, and perhaps all, remained in place.

The size, shape, and restriction to the ONL suggest that these cells are the rod precursors, a widely scattered type of specialized neuroepithelial cell that lies in the ONL and normally gives rise to rods that are inserted interstitially into the existing layer of photoreceptors (Raymond, 1985). Several findings implicate these cells as the source of regenerated retina. First, damage to central retina, far removed from the proliferative margin, stimulates proliferation among an intrinsic population of cells followed by regeneration (Maier and Wolburg, 1979; Raymond et al., 1988b). Second, massive retinal damage that destroys the inner retina but spares the ONL, where the rod precursors lie, fails to stimulate regeneration (Raymond et al., 1988b). Third, our results (Fig. 7) show that the lesion stimulated rod precursors to proliferate and produce rods. We have not shown that the rod precursors give rise to the blastemal cells, but we consider them the most likely source.

Regeneration of the retina by the rod precursors implies that retinal damage stimulates these cells to proliferate. Local injury to the brain of mammals is known to cause a local cascade of cellular changes, including the release of regulatory proteins that have both growth factor and mitogenic activities (e.g., Finklestein et al., 1988; Giulian and Lachman, 1985; Logan, 1990; Nieto-Sampedro, Saneto, de Vellis, and Cotman, 1985). For example, focal wounding of the neocortex in the rat causes a local accumulation of cells containing basic fibroblast growth factor (bFGF; Finklestein et al., 1988) as well as the release of related proteins into the extracellular space (Nieto-Sampedro, Lim, Hick-
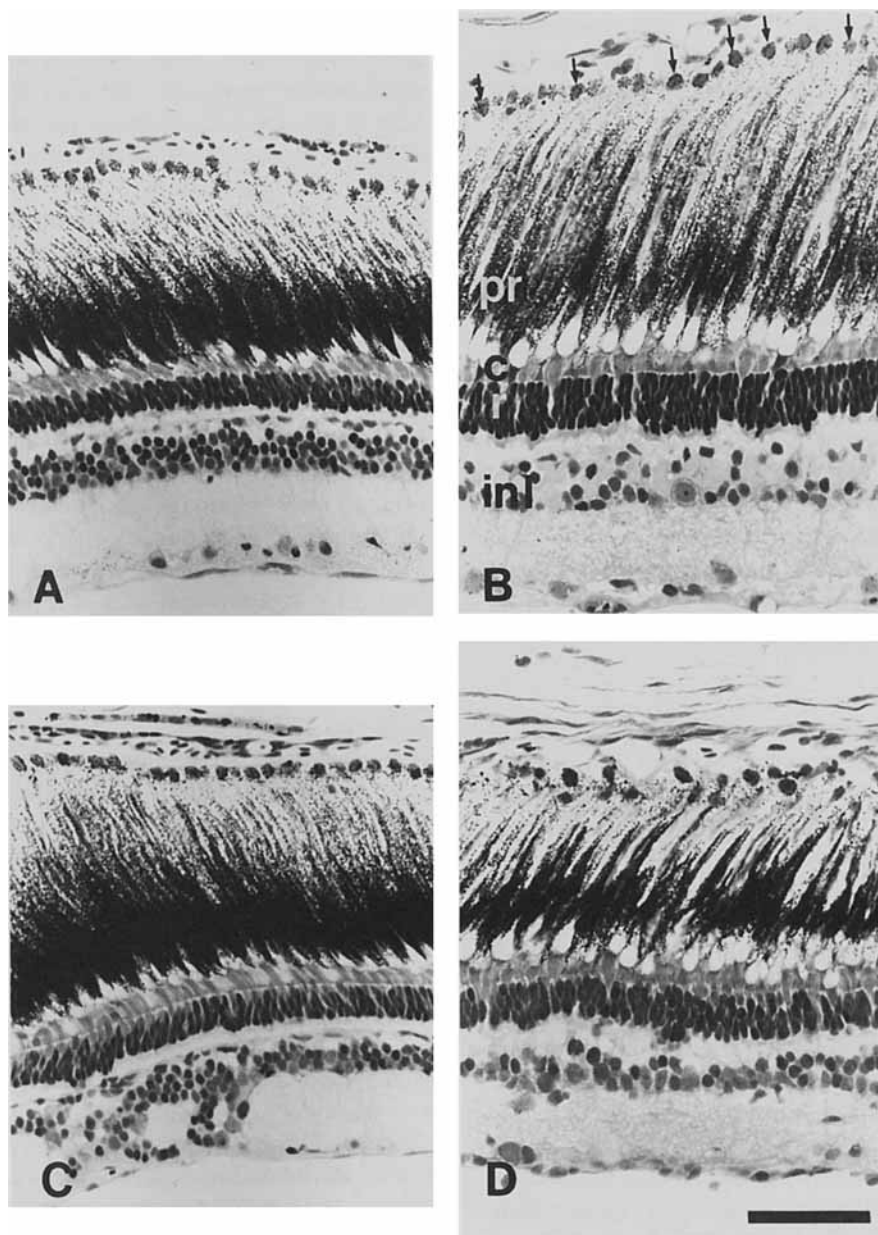
Figure 10 Photomicrographs of intact and regenerated retinas in small and large fish. Panels A and C are from a small fish; panels B and D are from a large fish. Upper panels are of intact retinas; lower panels are of regenerated retinas. The small arrows in panel B indicate the nuclei of RPE cells that can be seen in the other panels as well. Abbreviations as per Figures 1 and 7. Scale bar = 50 μm.

lin, and Cotman, 1988). The lesions we have made presumably cause the release of as of yet unidentified molecules that stimulate the rod precursors to divide and perhaps to migrate. Recent evidence indicates that rod precursors are responsive to injury-related changes in the retina. Crushing the intraorbital portion of the optic nerve induces a transient up-regulation in DNA synthesis in these cells (Henken and Yoon, 1989). Our results reinforce this; in the whole mount of Figure 6(A), the heightened proliferation outside the lesion was restricted to the part of the retina containing ganglion cells whose axons had been cut by the lesion.

If the rod precursors are the source of the regen-

erated retina, then the lesion has caused them to alter their fate, from rods to all retinal cells. The change in the fate of the rod precursors could be accounted for in two ways. One is to attribute it to the precursor, to suppose that it has been altered by the existence of retinal damage nearby, and that its normal program (produce rods) is changed (produce all retinal neurons). The other is to suppose that the postmitotic cell produced by the rod precursor is generally pluripotent, even under normal circumstances, and its fate is dictated by the presence, absence, or density of its neighbors. Under normal circumstances, all retinal cells are there in appropriate numbers, and only rods need to be added to maintain a constant density in the steadily stretching photoreceptor lawn (Raymond et al., 1988a). When the retina is damaged, this could be sensed by the postmitotic, pluripotent cell resulting in the missing cells being replaced.

Regeneration of the goldfish's retina from an intrinsic source is similar to that seen in *Xenopus laevis* (Levine, 1981). After removing small pieces of retina, proliferating cells were seen at the edge of the wound and in the surrounding retina. Unlike what we saw in the goldfish, in *Xenopus,* the retinal wound was initially filled with a sheet of neuroepithelial cells from which new retina subsequently differentiated. Retinal regeneration in goldfish and *Xenopus* contrasts with that in newts, tadpoles, and chicken embryos. In these animals, following either local or total destruction of the retina (Reyer, 1977), cells of the RPE transdifferentiate via a stereotyped sequence of events (Keefe, 1973; Reh and Nagy, 1987) to give rise to a neuroepithelium from which retina differentiates. In the present study, we saw no evidence of increased cell proliferation in the RPE [Fig. 4(B)], and we conclude that the RPE is not the source of regenerated retina in the goldfish. The RPE may play an indirect role in retinal regeneration, however. Schmidt, Cicerone, and Easter (1978) noted that when large areas of both neural and pigmented retina were removed from goldfish eyes, the neural retina did not regenerate. Our surgical technique generally destroyed the RPE within the lesion. As new retina was regenerated, it was always overlain by presumptive RPE, suggesting that the integrity of the former was contingent upon the presence of the latter.

The quantitative analysis showed that the planimetric density of newly regenerated, nonrod neurons generally approximated that in the surrounding, intact retina. The growth-related changes in the planimetric density of cones, INL neurons, and

ganglion cells was reproduced by the regenerated tissue. We interpret this finding as evidence that the regenerative neurogenesis is under regulatory controls that lead the regenerated retina to approximate the surrounding one. We cannot, however, identify the source of these regulatory signals. These factors could originate within the retina (Negishi et al., 1982; Reh and Tully, 1986; Reh, 1987), ocular tissues surrounding the wound, or even from more global factors, such as those that control the growth of the eye or animal.

The planimetric density of the regenerated rods was invariant with retinal size/age, in contrast to the other cells. In goldfish, rods have a unique developmental history and lineage (Raymond, 1985). They are added late in development, after all other cell types have differentiated, and they come from a separate pool of proliferating cells, the rod precursors. Further, new rods are continually added interstitially as the retina expands. During regeneration following neurotoxic destruction of the existing retina, rods recapitulate their *de novo* development (Raymond et al., 1988b); they are added late and continue to be produced well after the other retinal neurons are replaced. Therefore, the regeneration of rods might more accurately be characterized as a resumption of their normal development. Once new retina is regenerated, the rod precursors or their progeny cease being pluripotent and resume their rod-only fate. In the present study, the relatively small number of rods in the regenerated retina probably reflects the length of time the animals were allowed to survive postlesion. A longer survival time would have allowed more rods to be produced, and thereby reduced the difference in the planimetric densities in the regenerated and intact regions [Fig. 8(A)]. This interpretation also explains the high density of regenerated cones. During early development (Johns, 1982), cones are generated first at a high density, and then spread apart as rods are inserted into the photoreceptor layer. Again, in our experiments a longer survival time postlesion would have allowed the addition of more rods to the regenerated retina with a consequent reduction in the density of the regenerated cones.

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