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Selective regeneration of photoreceptors in goldfish retina

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SUMMARY

Previous work has shown that the neural retina in adult goldfish can regenerate. Following retinal damage elicited by surgical or cytotoxic lesions, missing neurons are replaced by foci of proliferating neuroepithelial cells, which previous studies have suggested are derived from rod precursors. In the intact retina, rod precursors proliferate but produce only new rods. The regenerative responses observed previously have involved replacement of neurons in all retinal layers; selective regeneration of specific neuronal types (except for rod photoreceptors) has not been reported. In the experiments described here, we specifically destroyed either cones alone or cones and rods with an argon laser, and we found that both types of photoreceptors regenerated within a few weeks. The amount of cone regeneration varied in proportion to the degree of rod loss. This is the first demonstration of selective regeneration of a specific class of neuron (i.e., cones) in a region of central nervous tissue where developmental production of that class of neuron has ceased. Selective regeneration may be limited to photoreceptors, however, because when dopaminergic neurons in the inner retina were ablated with intraocular injections of 6-hydroxydopamine, in combination with laser lesions that destroyed photoreceptors, the dopaminergic neurons did not regenerate, but the photoreceptors did. These data support previous studies which showed that substantial cell loss is required to trigger regeneration of inner retinal neurons, including dopaminergic neurons. New observations here bring into question the presumption that rod precursors are the only source of neuronal progenitors during the regenerative response. Finally, a model is presented which suggests a possible mechanism for regulating the phenotypic fate of retinal progenitor cells during retinal regeneration.

Key words: cell lineage, differentiation, argon laser, regeneration, photoreceptor, goldfish, retina

INTRODUCTION

The retinas of a number of vertebrates including embryonic chicks, frog tadpoles, adult urodele amphibians (newts and salamanders) and adult teleost fish are capable of neuronal regeneration (reviewed in Hitchcock and Raymond, 1992). In goldfish, following cytotoxic or surgical retinal lesions, proliferating cells intrinsic to the retina generate new retinal neurons (Raymond et al., 1988). In the intact retina, residual, dividing neuroepithelial cells in the outer nuclear layer, called rod precursors, give rise exclusively to rod photoreceptors (Johns and Fernald, 1981; Johns, 1982; Fernald, 1989). When a large fraction of the retinal neurons are destroyed in the goldfish by intraocular injections of ouabain, the retina regenerates within a couple of months from scattered clusters of elongated, dividing neuroepithelial cells (Maier and Wolburg, 1979; Raymond et al., 1988) which appear to derive from surviving rod precursors (Raymond et al., 1988). Similarly, if a small patch of goldfish retina is surgically removed, presumptive rod precursors along the cut edges of the retinal wound proliferate, forming a 'blastema' and, over the next couple of weeks, the retinal wound is gradually filled with regenerated neurons (Hitchcock et al., 1992; Hitchcock and Vanderyt, 1993). Intraocular injections of suprathreshold doses of the dopaminergic toxin, 6-hydroxydopamine (6OHDA) cause non-selective damage that destroys neurons in both the inner and outer nuclear layer, which also triggers a regenerative response (Braisted and Raymond, 1992). Interestingly, however, if retinal lesions are more selective, in that only a specific class of neuron (e.g., dopaminergic or serotonergic neurons or ganglion cells) is destroyed with a selective neurotoxin, or if cell loss is not selective but is confined to the inner retina, no regeneration occurs (Negishi et al., 1982, 1985, 1987, 1988; Raymond et al., 1988; Hitchcock, 1989; Braisted and Raymond, 1992). Paradoxically then, the more destruction, the better the regenerative response.

In summary, the above results suggest that specific types of neurons (at least those in the inner retina) cannot be replaced following their selective ablation. In all examples thus far, regeneration of cells in the inner retina was only observed when damage extended to the outer nuclear layer (ONL), that is, when photoreceptors were lost (Raymond, 1991; Braisted and Raymond, 1992). Since rod precursors are located in the ONL, this led to the hypothesis that alteration of the microen-

vironment surrounding rod precursors is necessary to provoke a regenerative response, i.e., a change in rod precursor fate (Raymond et al., 1988). Specific ablation of rod photoreceptors with tunicamycin, in combination with selective ablation of dopaminergic neurons, resulted in replacement of rods but failure to replace dopamine neurons, indicating that rod precursors, although they responded to the lesion by increased proliferation, did not alter their fate (Braisted and Raymond, 1993).

The motivation for the present study was to discover whether selective destruction of cones could trigger a change in rod precursor cell fate. Accordingly, we destroyed cones, or rods and cones, with an argon laser, alone or in conjunction with selective destruction of dopaminergic neurons with intraocular injections of 6OHDA. We found that, in all cases, cones and rods regenerated, but dopaminergic neurons did not. Unexpectedly, we also observed Müller glia proliferating and migrating into the ONL, a surprising finding which raises the possibility that Müller glia, or cells associated with them, might provide an alternative source of regenerative neuronal progenitors.

These results, taken together with earlier work, lead us to propose a model that suggests that the differentiated fate of progeny produced by retinal neuroepithelial cells, whether isolated rod precursors in the intact retina or neurogenic clusters in the regenerate, is regulated by signals within the ONL and by contact with the apical surface (outer limiting membrane, OLM, and/or subretinal space) and the basal surface (inner limiting membrane, ILM, and/or basal lamina) of the retina. This model offers an explanation as to why loss of photoreceptor cells in the ONL appears to be a necessary precondition for regeneration of neurons in both inner and outer retina.

MATERIALS AND METHODS

Overview

A summary of the experiments performed is in Table 1. We examined a total of 28 goldfish retinas, each with 2 to 4 laser lesions. In experiments 1 and 4, retinas were examined after short survival periods (a few days after lesioning) to measure various parameters associated with the lesions (expt. 1: amount of photoreceptor cell loss; expt. 4: stimulation of cell proliferation). Regeneration of photoreceptors was monitored in experiments 2 and 3 after multiple injections of bromodexoyuridine (to label newly generated cells) and survival periods of several weeks. In experiment 3, laser lesions were combined with injections of 6OHDA to determine whether ablation of photoreceptors could provoke regeneration of missing dopaminergic neurons under conditions in which they otherwise would not be replaced.

Animals

Goldfish (*Carassius auratus*), 7.5 to 10 cm in body length and with eye diameters of 5.0-6.0 mm, were purchased from Ozark Fisheries (Stoutland, MO). The diameter of the eye in the nasotemporal dimension was measured with a caliper on anesthetized fish. Unless otherwise stated, all chemicals were obtained from Sigma (St. Louis, MO).

Laser lesions

Lesions were made in goldfish retinas with an argon laser (System 920; Coherent, Palo Alto, CA) used in the ophthalmology clinic to perform human ocular laser surgeries. Because the optics of the fish eye are designed for vision underwater, when the fish is in air the lens must be removed to image the retina for focusing the laser beam. For lentectomies, fish were anesthetized in 0.2% tricaine methanesulfonate, a slit was made in the ventral cornea, and the lens was extracted; the cornea was not sutured. Both eyes were operated and allowed to heal for 2 to 3 weeks; fish with scarred corneas or cloudy vitreous were discarded.

Fish were anesthetized, a coverslip was placed on the cornea, and the fish held manually so that its retina was positioned at the focal plane of the laser. With the laser settings at 130, 160 or 300 mw, 0.1 second duration and 500 μm diameter, 2 to 4 lesions were placed in ventral retina under visual control, taking care to avoid blood vessels and the optic nerve head. Lesions were visible as bleached spots with irregular borders and were larger and whiter with pulses of greater power. The laser settings were determined empirically, and resulted in lesions that were confined to the ONL (130 and 160 mw) or extended from the ONL into inner retinal layers (300 mw). The ONL of the retina in goldfish, as in other vertebrates, consists of the cell bodies of photoreceptors and the apical processes of Müller glia. The inner retina comprises two nuclear laminae: the inner nuclear layer, INL, containing second-order neurons and the cell bodies of Müller glia, and the ganglion cell/optic fiber layer, containing ganglion cells, glia and vascular cells.

Intraocular injections

Injections were done as previously described (Braisted and Raymond, 1992). Briefly, fish were anesthetized, a slit was made in the cornea near the limbus with a microscalpel, and a 33-gauge, blunt tip needle attached to a Hamilton microsyringe was used to inject 3 to 5.25 μl into the vitreous.

To destroy dopaminergic neurons selectively (Table 1, expt. 3), on 2 consecutive days both eyes were injected with 6-hydroxydopamine hydrochloride, 4.5 mg/ml in 0.9% NaCl with 3 mg/ml sodium ascorbate, to achieve an estimated intraocular concentration of 0.23 mg/ml (1.1 mM). The amount injected into each fish was calculated individually based on an estimate of eye volume from the measured eye diameter (Raymond et al., 1988; Braisted and Raymond, 1992). This paradigm destroys dopaminergic neurons without causing nonspecific damage that leads to their regeneration (Braisted and Raymond, 1992, and unpublished data).

To determine whether photoreceptors and/or dopamine neurons had regenerated (Table 1, expts 2 and 3), during the first few weeks after lesioning, multiple injections of the thymidine analogue, bromodeoxyuridine (BrdU) were given to label proliferating cells, and fish were allowed to survive for 1 to 5 weeks longer (a total of 4 to 9 weeks after laser lesions) to allow regenerated neurons to differentiate. Eyes were injected 5 or 6 times at 3-day intervals (beginning at 4 days or 19 days after lesions) with 0.4 mM or 1 mM BrdU in 0.9% NaCl (to produce an estimated intraocular concentration of 20 μM or 50 μM, respectively). The appropriate volume to be injected was determined for each fish, as described above. Radial cryosections (Table 1, expt. 2) and whole mounts (Table 1, expt. 3) were processed for immunocytochemistry with various cell-specific antibodies and with anti-BrdU antibodies as described below.

To determine whether laser lesions stimulated cellular proliferation (Table 1, expt. 4), fish were injected once with 50 μM BrdU at 3 days after laser lesions. Fish were killed the following day, and radial cryosections were processed for double-label immunocytochemistry (various cell-specific antibodies paired with anti-BrdU) as described below.

Immunocytochemistry

Cell-specific antibodies used included RET1, NN2, FGP2 and anti-tyrosine hydroxylase. RET1 is a mouse monoclonal antibody in ascites fluid produced against goldfish retinal antigens; it recognizes an unidentified nuclear antigen (M_r 50-70×10^3) in cones (but not rods), horizontal cells, most neurons in the INL, Müller glial cells and

ganglion cells (Wagner and Raymond, 1991). NN2 is another mouse monoclonal antibody produced against goldfish retinal antigens; it recognizes an uncharacterized cell-surface epitope on phagocytic cells (microglia and blood-born macrophages) and various vascular cells, including endothelial cells (Wagner and Raymond, 1991). FGP2 (a gift from M. Schwartz) is a rabbit polyclonal antibody produced against a goldfish intermediate filament protein, glial fibrillary acidic protein (GFAP), which is found in Müller glial cells (Bignami, 1984; Wagner and Raymond, 1991). A monoclonal antibody against tyrosine hydroxylase, TH (Incstar, Stillwater, NM), the rate-limiting enzyme in the synthesis of dopamine, was used to identify dopaminergic neurons (Braisted and Raymond, 1993).

Radial retinal cryosections cut at 3 μm were processed with standard methods for double-label immunofluorescence with the antibodies RET1 (1:500), NN2 (1:1000) or FGP2 (1:100), and rat monoclonal anti-BrdU (1:20; Accurate Chemical, Westbury, NY) as described previously (Barthel and Raymond, 1990; Braisted and Raymond, 1992). Cell-specific antibodies were visualized with Texas Red (TR) and anti-BrdU with fluorescein isothiocyanate (FITC).

Some retinas were isolated as whole mounts and processed for immunofluorescence with anti-TH (1;10,000), RET1 and anti-BrdU antibodies as described previously (Braisted and Raymond, 1992). For triple-labeled preparations, anti-TH was visualized with TR, RET1 with 7-amino-4-methlycoumarin-3-acetic acid (AMCA) and anti-BrdU with FITC. Some retinal whole mounts were later cryoprotected, frozen and sectioned at 3 μm. The TR, AMCA and FITC fluorescence survives the freezing procedure.

Retinal whole mounts and radial cryosections were coverslipped with 60% glycerol in 0.1 M sodium carbonate buffer, containing 0.4 mg/ml p-phenylenediamine to retard fluorescent bleaching (Johnson and Araujo, 1981), and viewed with a Leitz Aristoplan epifluorescent microscope, using narrow and wide band FITC cubes (Leitz L3 and I3), a TR cube (Leitz N2.1) and an AMCA cube (Leitz A).

Morphometrics

To examine the extent of cell damage (Table 1, expt. 1), 3-4 days after laser lesions, eyes were enucleated, corneas were removed and eyes cups were fixed in mixed aldehydes, processed for methacrylate embedding and sectioned radially at 3 μm as described (Braisted and Raymond, 1993). Lesion diameter was defined as the maximum retinal length devoid of cone nuclei. Camera-lucida drawings were made from 3 sections at or near the center of the lesion, and the outer limiting membrane (OLM) within the lesion (i.e., the region devoid of cone nuclei) was traced at 640× magnification. These tracings were digitized and the linear extent of the lesion was measured using the graphics package SigmaScan (Jandel Scientific, San Raphael, CA).

Although cones were completely, or nearly completely, destroyed by the laser, variable numbers of rods survived. To estimate the extent of loss of rod nuclei, areas of the ONL within the lesion in which surviving rod nuclei were present were traced with camera lucida at 1600× magnification. Typically, groups of surviving rods were found in patches of variable size, although the density of rod nuclei within a patch was comparable to surrounding intact regions. The drawings were digitized, and areas containing surviving rods were measured with SigmaScan, summed and designated as A_l. The % rod loss in each lesion was calculated as $(A_c\text{-}A_l)/A_c\times100$, where A_c is the area containing rod nuclei in a segment of intact retina of length equivalent to the lesion length. A_c was determined from camera-lucida drawings of ONL approximately 200 μm away from the lesion, in a region of equivalent rod density (Powers et al., 1988).

To determine the extent of photoreceptor regeneration after laser lesions (Table 1, expt. 2), fish were given multiple injections of BrdU and allowed to survive for 4 weeks. The lesion was identified by an interruption in the regular row of cone nuclei (labeled with RET1) in the ONL; within this segment there were regenerated cone nuclei (BrdU-labeled and RET1-labeled = BrdU+/RET1+), and regenerated rod nuclei (BrdU+/RET1−). To estimate the degree of cone regeneration, all RET1+ cone nuclei within the lesion were counted at 320× magnification in 10 to 18 sections through each of 4 lesions (2 at 130 mw and 2 at 160 mw), and the average number of cones per unit retinal length (C_l), measured along the OLM in camera-lucida drawings, was determined for each. Cone nuclei were also counted within a 330 μm length of retina 100-200 μm outside each lesion, and the number per unit retinal length determined (C_c). The percentage of cones that regenerated in each lesion was $C_l \div C_c \times 100$. New rods are added to intact retina as part of the normal growth process (Johns, 1982), but the rate of rod addition is enhanced in the lesioned area. This response was quantified by counting BrdU+ rod nuclei per unit length within the lesion (R_l) and in the intact region (R_c), and determining the fold-increase ($R_l \div R_c$).

To determine the time course of cone regeneration (Table 1, expt. 3), 7 to 9 weeks after lesioning, cones (BrdU+ or −) were counted within lesions (2-6 sections per lesion) from eyes injected 5 times with BrdU at 3-day intervals either early in the regeneration process (between 4 and 16 days, 4 lesions) or later (between 19 and 31 days, 5 lesions). The fraction of BrdU+ cones within the lesions was compared for early or late BrdU injections to determine how many cones were being produced in each interval.

The unpaired Student's *t*-test was used to evaluate significance; the Chi-squared test was used to evaluate correlations.

RESULTS

Low power laser lesions selectively destroyed photoreceptors

The pigmented epithelial layer at the back of the neural retina selectively absorbs the laser energy (L'Esperance, 1983), and retinal cells are destroyed by heat; since cell bodies of cone photoreceptors are in closest proximity (Fig. 1A), they are the most susceptible. 4 days after 130 mw laser lesions (Table 1, expt. 1), cones were destroyed in a patch 224±35 μm in diameter (mean of seven lesions, ±s.d.). In three of these lesions no rod loss was apparent, in three other lesions a few rods were lost, and in one lesion, 25% of rods were destroyed (Fig. 1A,B). In all cases, the greatest rod loss was in the center of the lesions. After 160 mw lesions, cones were destroyed over a larger extent (313±44 μm diameter, n=4 lesions), but substantial rod loss occurred in all cases and averaged 61±11%. Rod loss was complete or nearly so in the center of the lesions at this power (Fig. 1D), but in the periphery only cones were destroyed, thus resembling the center of the 130 mw lesions. In both 130 mw and 160 mw lesions, the INL appeared intact (Fig. 1), although cell counts were not done. Raising the power of the laser to 300 mw resulted in obvious destruction of neurons in inner retinal layers (both INL and ganglion cell layer; data not shown).

Photoreceptors regenerated within lesioned areas

Regenerated cells were identified unambiguously by BrdU-labeling. Fish were injected 6 times with BrdU (20 μM estimated intraocular concentration) at 3-day intervals beginning at 4 days and allowed to survive for 4 to 8 weeks after laser lesions, to allow regenerated cells to differentiate (Table 1, expt. 2; Fig. 2). Because rod precursors in the goldfish retina continually add new rod photoreceptors, even in intact fish there are scattered BrdU+ rods in the ONL; however, within the lesion, the number of BrdU+ rods was increased. Rod nuclei do not label with RET1, so BrdU+/RET1− nuclei

in the ONL were considered rods. At 4 weeks after lesioning, there was a 4-fold increase in number of BrdU+ rods in the 130 mw lesions, and a 34-fold increase in the 160 mw lesions, compared to an equivalent length of undamaged retina nearby. Since more rods were destroyed within 160 mw compared to 130 mw lesions (see above), this suggests that the missing rods were being replaced.

Regenerated cones were also present in all lesions at 4 weeks, and they tended to be concentrated in the center of lesions where rod loss was greatest (Fig. 2). Regenerated cones were identified as BrdU+/RET1+ nuclei (Fig. 2A). Since RET1 labeling is not a unique feature of cone nuclei (other cells in the inner retina also express RET1), their identification was confirmed by morphological features (as viewed in non-counterstained cryosections with Nomarski optics, Fig. 2B). The distinctive morphological features of teleost cones include nuclear position (the cone nuclei abut or protrude through the OLM) and the presence of an apical process consisting of an inner and outer segment. Regeneration of cones was incomplete in the 130 mw lesions at 4 weeks; only 10% to 25% of the cones were replaced in the 2 lesions quantified (Fig. 3). In the 160 mw lesions, in contrast, cones were overproduced (by 15% to 20% in 2 lesions; Fig. 3). Retinas examined 8 weeks after laser lesions resembled those examined at 4 weeks, with only sparse regenerated cones in 130 mw lesions and substantial numbers in 160 mw lesions (2 lesions examined at each energy; data not shown).

The presence of regenerated cones in a given retinal section was strongly correlated with the presence of regenerated rods (Chi-squared test, $P<0.001$). In 36 sections through 130 mw lesions, 11 (30%) contained no BrdU+ rod or cone nuclei, 21 (60%) had both BrdU+ rod and cone nuclei, 2 (5%) had BrdU+

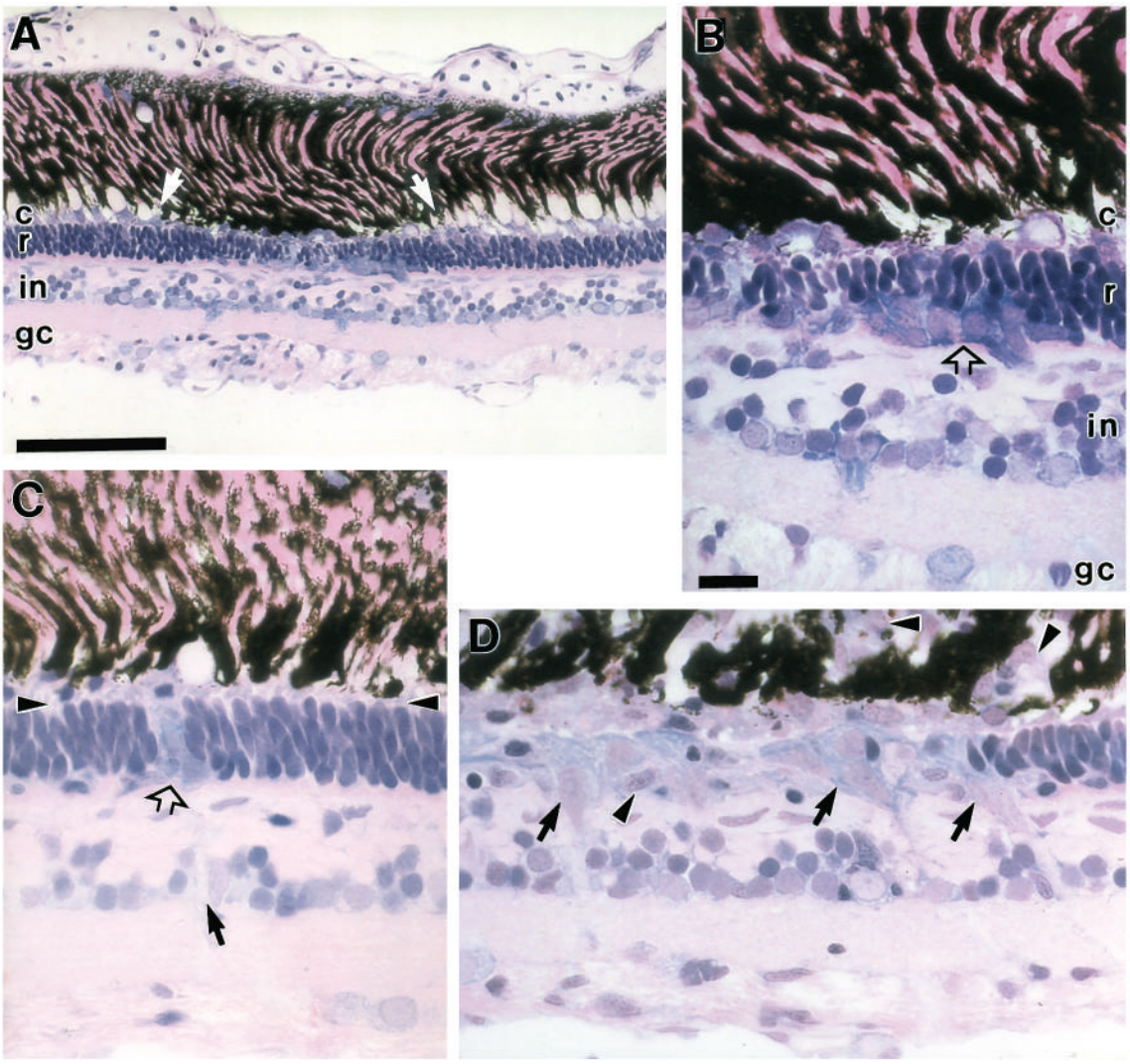


Fig. 1. Radial methacrylate sections of retinas 4 days after laser lesions. (A) 130 mw laser lesion showing partial rod loss in the center. The white arrows indicate surviving cones that define the borders of the lesion. (B) The lesion depicted in A, shown at higher magnification. A cluster of rod precursors is indicated by the open arrow. (C) Another 130 mw lesion. Note the cluster of rod precursors (open arrow) in contact with a radial Müller fiber (black arrow) and the outer limiting membrane (arrowheads). (D) 160 mw laser lesion. Note the ectopic Müller cells in the outer plexiform and outer nuclear layers (black arrows), and microglia/macrophages in the outer nuclear layer and subretinal space (arrowheads). c, layer of cone nuclei; r, layer of rod nuclei; in, inner nuclear layer; gc, ganglion cell layer. Bar, 100 μm (A) and 20 μm (B, also applies to C and D).

Table 1. Summary of experiments

Experiment	Laser power (mw)	No. lesions (No. retinas)	Survival time	Histological method
1	130	7 (4)	ST	MS
	160	14 (5)	ST	MS
2	130	4 (4)	LT	CS
	160	4 (3)	LT	CS
3*	160	28 (7)	LT	WM**
	300	8 (2)	LT	WM
4	160	7 (3)	ST	CS

*Eyes were injected with 6OHDA 4 and 5 days before laser lesions.
**4 of 7 whole mounts were cryosectioned and regenerated cones were quantified in 9 lesions.
ST, short-term survival (3 or 4 days after laser lesion); LT, long-term survival (4 to 9 weeks after laser lesion; MS, methacrylate sections; CS cryosections; WM, whole mounts.

rods but not cones, and 2 (5%) had BrdU+ cones but not rods within the lesion.

Regenerated cones were also visualized in retinal whole mounts at 7 or 9 weeks after 160 mw laser lesions (Table 1, expt. 3; these eyes also received 6OHDA injections as described below). 'Hot spots' of BrdU+ nuclei, which represented regenerated photoreceptors (Fig. 4A), were found in all 7 retinas examined. In intact retina the cone nuclei, labeled with RET1, were in a regular mosaic array, but within lesions this pattern was disorganized (Fig. 4B). A discontinuous annulus of retina virtually devoid of RET1+ cones separated the regenerated from the intact cones, consistent with observations from radial sections that most regenerated cones were concentrated in the center of the lesion (Fig. 2). When these whole mounts were subsequently sectioned, both BrdU+/RET1− rods and BrdU+/RET1+ cones were found in the ONL within lesions (data not shown), as described above.

To determine the time course of cone regeneration, the fraction of cones labeled when BrdU was administered during the first two weeks after lesion was compared to the fraction labeled by BrdU injected during the third and fourth weeks. As in experiment 2, multiple injections of BrdU were given but, in experiment 3, the intraocular concentration of BrdU was increased from 20 μM to 50 μM in an attempt to label all regenerated cones. (In experiment 2, not all of the putative regenerated cones in the lesion were BrdU+.) For this analysis, the retinas previously prepared as whole mounts and subsequently cryosectioned were used (Table 1, expt. 3), and the survival times were 7 to 9 weeks. The cell counts showed that 90% of cone nuclei within the lesion were labeled with BrdU administered within the first 2 weeks and 10% were labeled when BrdU was given during the third and fourth weeks (Fig. 5). Thus, all of the presumptive regenerated cones within the lesion were indeed regenerated in that they could be labeled with BrdU administered within 4 weeks of the lesion, with the vast majority generated in the first 2 weeks. Substantial numbers of labeled rod nuclei (BrdU+/RET1−) were also found in the lesions in retinas injected with BrdU either early or late (data not shown).

Dopaminergic neurons in the INL do not regenerate if they are selectively destroyed along with photoreceptors

The only TH+ cells in the goldfish retina are dopaminergic interplexiform cells (Dowling and Ehinger, 1978; Negishi et al., 1990). These cells have large cell bodies in the amacrine

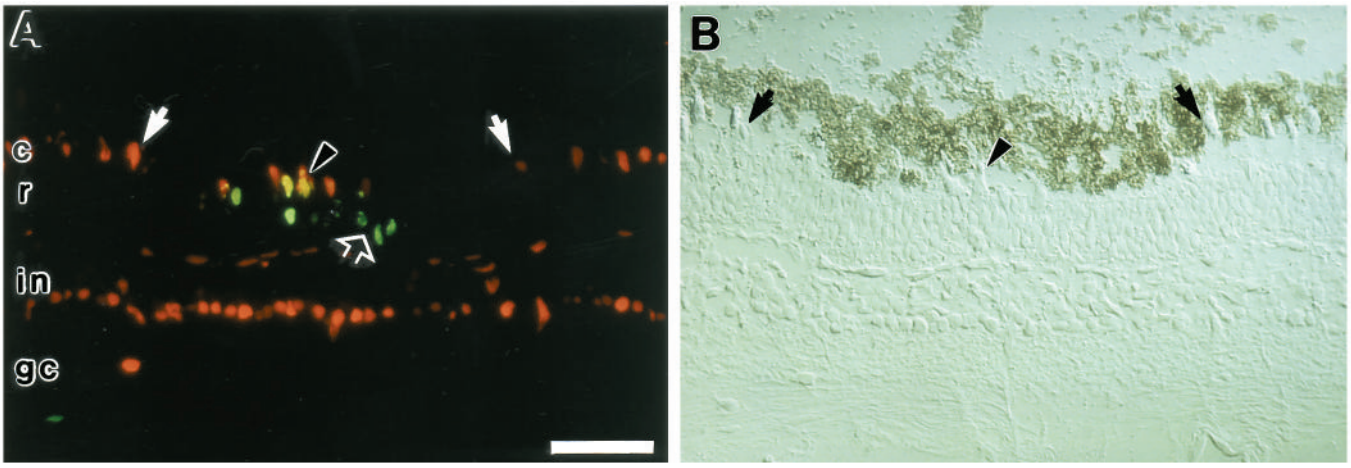


Fig. 2. Radial cryosection of a retina 26 days after 130 mw laser lesion labeled with RET1 (visualized with TR) and anti-BrdU (visualized with FITC). (A) Immunofluorescence; (B) Nomarski optics. The evenly spaced row of RET1+ cone nuclei (red in A, indicated by arrows in A and B) is interrupted at the boundaries of the lesion. Other nuclei in the inner nuclear and ganglion cell layers are also RET1+. Double-labeled, regenerated cones (RET1+/BrdU+) are yellow (in A; arrowhead in A and B) and regenerated BrdU+ rods are green (in A; open arrow in A). Both are concentrated in the center of the lesion. Abbreviations as in Fig. 1. Bar, 50 μm.

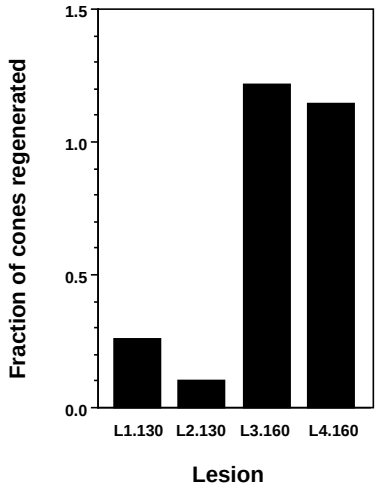


Fig. 3. Fraction of regenerated cones calculated from cone nuclear counts at 26 days after laser lesion (see Methods for additional information). The lesion numbers (L1, L2 etc.) and laser power settings (130 or 160 mw) are indicated on the abscissa.

cell layer (inner part of the INL), and processes that synapse in both the inner plexiform layer and outer plexiform layer. Regeneration of dopaminergic neurons has been observed previously, but only in the context of regeneration of other retinal neurons, including cones. Here we asked whether the proliferating cells that have been triggered to regenerate photoreceptors also respond to signals from inner retina indicating that dopaminergic neurons are missing, and replace them, too.

The same retinas used to estimate time course of cone regeneration (Table 1, expt. 3) were used to examine whether dopaminergic neurons also regenerated; these fish had been injected intraocularly with 6OHDA and then given laser lesions (160 mw) 4 days later. Following multiple BrdU injections as described above, retinal whole mounts were processed for immunocytochemistry with anti-TH, RET-1 and anti-BrdU antibodies. At 7 to 9 weeks, no regenerated dopaminergic neurons (TH+/BrdU+) were found in central retina, either within the laser lesions or in the surrounding regions. TH+ cells and processes were found only in the most peripheral retina adjacent to the germinal zone (in 7 of 7 retinas; data not shown). These represent dopaminergic neurons generated by the germinal zone as part of the ongoing process of retinal

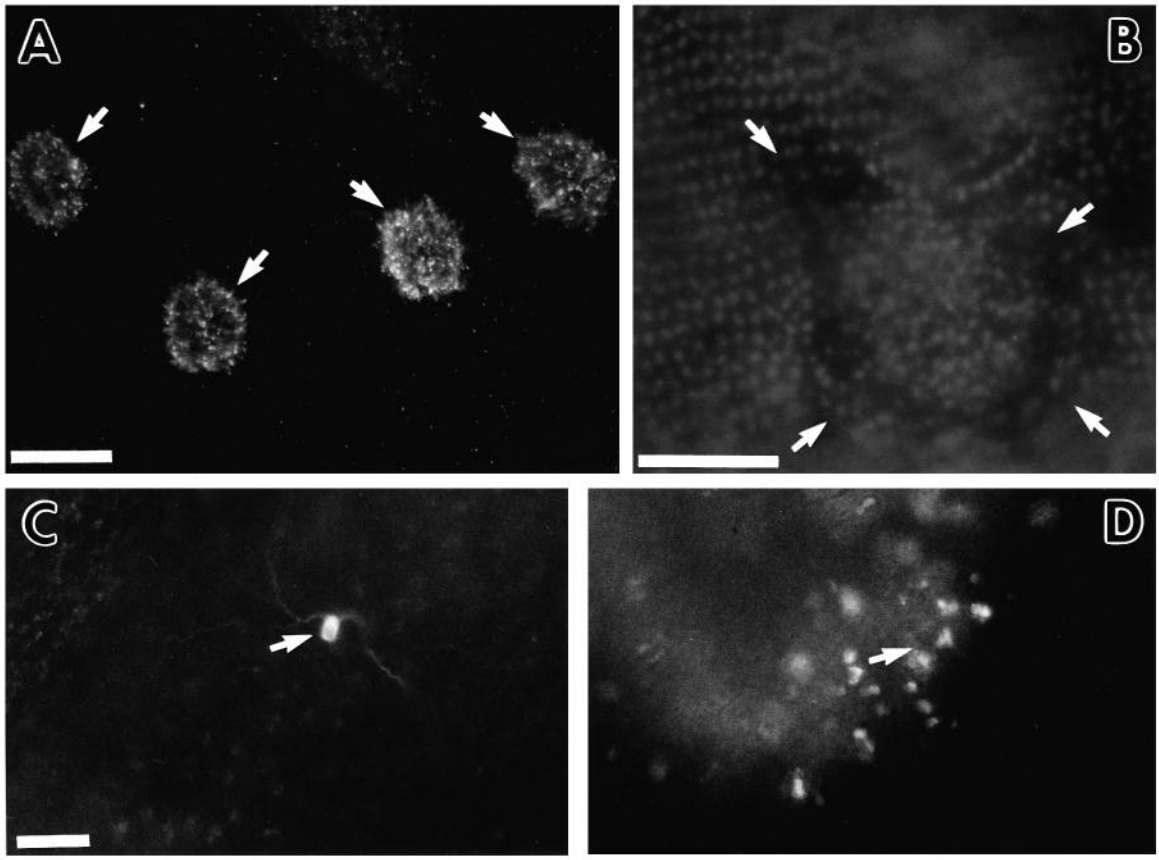


Fig. 4. (A) Retinal whole mount 70 days after 300 mw laser lesions showing 'hot spots' of BrdU+ nuclei representing lesions (arrows). (B) Retinal whole mount 49 days after 160 mw laser lesions showing disruption of RET1+ cone mosaic within a lesion. The approximate edges of the lesion are indicated by the arrows. (C) The retinal whole mount shown in A was subsequently frozen and sectioned. A regenerated dopaminergic neuron, labeled with anti-TH (arrow), is visualized with the TR filter cube. (D) Same microscopic field as C, but visualized with the FITC filter cube to show BrdU+ nuclei. The location of the TH+ cell in C is indicated by the arrow; this cell is not double-labeled. Bar, 300 μm (A), 100 μm (B), 50 μm (C, also applies to D).

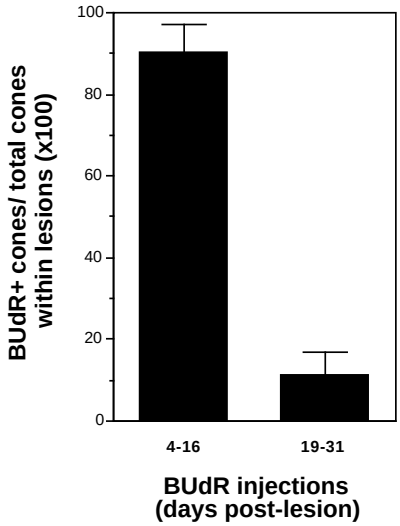


Fig. 5. Proportion of regenerated (BrdU+) cones following BrdU injections at different times after laser lesions. The interval (in days) over which BrdU was injected is indicated on the abscissa. The error bars indicate one s.d.

growth and do not represent a regenerative response (Negishi et al., 1982; Braisted and Raymond, 1993).

As a control to demonstrate that dopaminergic neurons are not prevented from regenerating by the laser lesioning process, both eyes of one fish were injected with 6OHDA (4.5 mg/ml) and then given 300 mw lesions (4 in each eye), to destroy cells in the inner retina as well as the ONL, followed by 6 BrdU injections beginning at 4 days, as above (Table 1, expt. 3). 10 weeks after lesioning, retinal whole mounts were processed for immunocytochemistry with anti-TH and anti-BrdU. TH+ cells with labeled processes were found in 2 of 8 lesions (both examples were in the same retina, and there was a single TH+ cell per lesion; Fig. 4C). Unfortunately, neither of these presumed regenerated TH+ cells were labeled with BrdU (Fig. 4D), probably because they were generated after the last BrdU injection (at 19 days). We do not believe that these TH+ cells survived the 6OHDA for the following reasons: (1) no TH+ cells were found in central regions of retina from eyes injected with the same dose of 6OHDA and given 160 mw laser lesions (7 of 7 lesions, Table 1, expt. 3); (2) rare, surviving TH+ cells found after injection of similar doses of 6OHDA were weakly TH+ and never possessed TH+ processes (Braisted and Raymond, 1993), and (3) the only TH+ cells found in 6OHDA/laser lesioned retinas were within BrdU+ 'hot spots'. These results show that destruction of retinal cells with laser lesions does not preclude regeneration of dopaminergic neurons.

Rod precursors are found within laser lesions

The next question is the source of regenerated photoreceptors. In the residual ONL of the lesion at 3 to 4 days (Table 1, expt. 1) were cells with the cytological characteristics of rod precursors (Raymond and Rivlin, 1987). These cells had pleiomorphic, medium-sized nuclei, intensely basophilic cytoplasm (Fig. 1B) and occasionally appeared to be in contact with the processes of Müller cells and/or the OLM (Fig. 1C). The number of rod precursors per section within 130 mw lesions increased with increasing rod loss. In lesions with no apparent rod loss (3 of 7), 90% of sections examined (n=83) possessed at most 1 rod precursor. Within lesions sustaining a small amount of rod loss (<25%; 3 of 7), 75% of sections examined (n=8) possessed 2 to 4 rod precursors per section. Within the single lesion sustaining 25% rod loss, rod precursors were abundant in virtually all sections examined (Fig. 1A). These histological observations support the BrdU data discussed above, which demonstrated an up-regulation of mitotic activity in the lesioned area. The entire ONL (where the rod precursors are normally located) appeared to be destroyed in the center of 160 mw lesions, but surviving rod precursors were also occasionally present in the periphery (not shown).

Unfortunately, no antibodies that selectively label rod precursors are available. Rod precursors, like rod nuclei, do not label with RET1, but because they proliferate, they can be labeled with BrdU at short survival times. At 1 day following a single injection of BrdU (50 μM) given at 3 days after 160 mw lesions (Table 1, expt. 4), approximately 30% (21% to 45% within three individual lesions; 5 to 6 sections examined per lesion) of the BrdU+ nuclei in the ONL within 160 mw lesions were RET1–. While these are potentially rod precursors, it is certain that not all of them are. These other proliferating cells, including the 70% that were RET1+, are discussed in the next section.

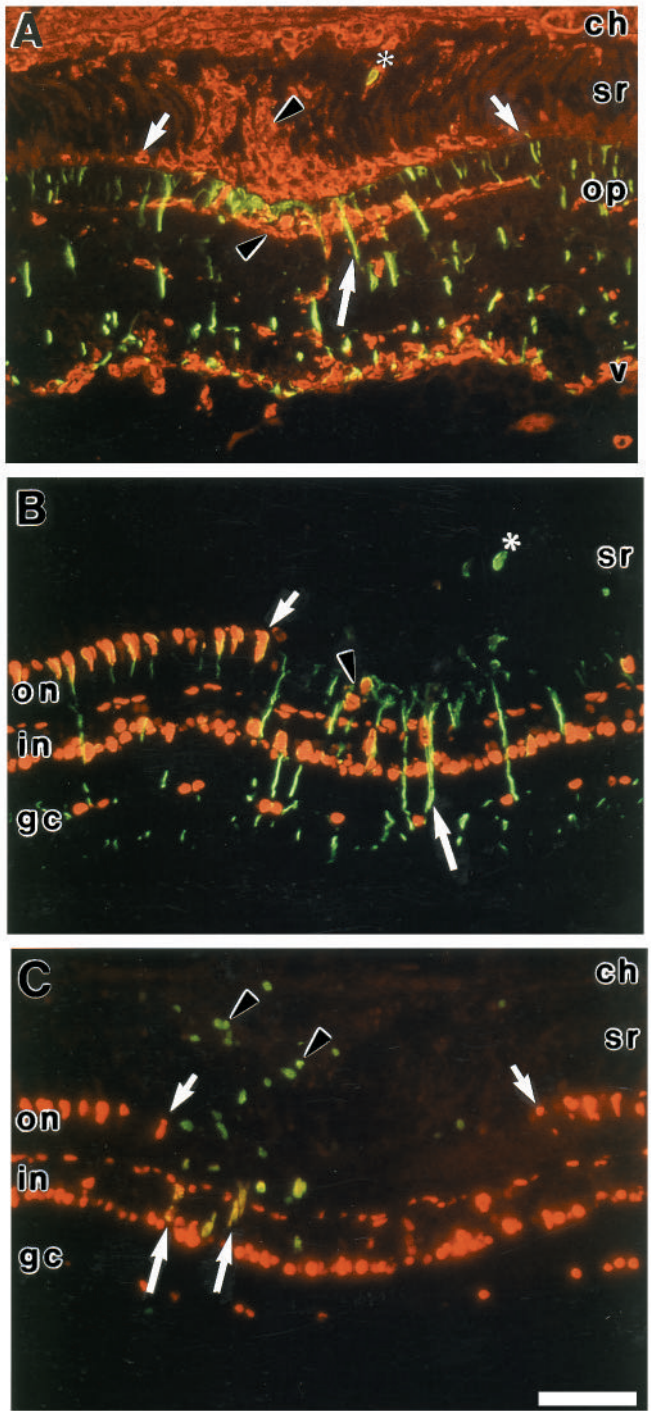
Laser lesions cause migration of glial cells and macrophages into the ONL

The issue of the source of regenerated photoreceptors is complicated by the presence of other proliferating cells in the lesioned region. Resident microglia in teleost retina are characterized by small, round or irregular, darkly stained nuclei, and cells with these features were found in the lesion and in the adjacent subretinal space at 3 to 4 days (Table 1, expt. 1, Fig. 1D). The identity of these cells was confirmed with the monoclonal antibody NN2 (Table 1, expt. 4). At lesion sites, NN2+ cells accumulated in the vascular membrane, outer plexiform layer, ONL and subretinal space (Fig. 6A). These cells are phagocytic (Wagner and Raymond, 1991) and are attracted to the wound where they are involved in scavenging cellular debris. Microglia/macrophages do not express the RET1 antigen, so the RET1–/BrdU+ cells present in the lesions at 3 to 4 days (30% of the BrdU+ cells, see above) probably reflect a combination of rod precursors and microglia/macrophages.

Other nuclei within the residual ONL at 3 to 4 days after lesion were identified as Müller glia. This was completely unexpected, since Müller nuclei are normally located in the INL. Müller nuclei were found at the level of the ONL within 160 mw lesions (13 of 14 examined) but, in the 130 mw laser lesions, they were only in the lesion that sustained 25% rod loss (1 of 7). In methacrylate sections (Table 1, expt. 1), these cells had large, oval or lobulated, lightly stained nuclei, often with prominent nucleoli, and basophilic, radially oriented cytoplasmic processes (Fig. 1C). To confirm their identification as Müller glia, we used RET1 and the goldfish GFAP antibody FGP2 (Table 1, expt. 4). In lesioned areas, ectopic, often radially oriented RET1+ nuclei were seen spanning the outer plexiform layer or within the ONL but below the OLM (Fig.

6B). These ectopic RET1+ nuclei were associated with intensely stained FGP2+ processes. We believe these are Müller cells migrating from the INL into the ONL. Other alternatives are that the RET1+ nuclei in the ONL within lesions are surviving cone nuclei, or another type of RET1+ neuron migrated from INL to ONL, or perhaps expression of the RET1 antigen is induced in cells (e.g., rods) which are typically RET1–. However, in methacrylate sections, these nuclei have characteristics of Müller cells (Fig. 1D; Erickson et al., 1983; Raymond and Rivlin, 1987).

In the three lesions at 4 days in which BrdU+ nuclei were quantified (see previous section), 70% of the BrdU+ cells were these RET1+, ectopic Müller nuclei. Of the ectopic Müller nuclei in the ONL and outer plexiform layer, approximately 76% (58% to 95%) were also BrdU+. These results show that, unlike Müller cells in unlesioned retina, which are quiescent, Müller cells in the region of the lesion were proliferating (Fig. 6C). These proliferative effects were narrowly confined to the lesioned area, with no evidence of lateral spread to adjoining retina.



DISCUSSION

The results of this study demonstrate that, in the adult goldfish retina, photoreceptors can be selectively replaced when they are destroyed with a laser lesion. We previously showed that rod photoreceptors were replaced following their selective destruction with the antibiotic, tunicamyin (Braisted and Raymond, 1993), but that result was not too surprising, given that rod photoreceptors are continuously being generated in the adult teleost retina, and so their regeneration merely involved an enhancement of proliferative activity in rod precursors without a change in cell fate. In the present study, however, cone photoreceptors regenerated, implying that proliferating neuroepithelial cells in the central, differentiated retina responded to signals (as yet unidentified), indicating that cones were missing, and selectively replaced them. Although regeneration of retinal neurons in goldfish has been demonstrated previously by a number of laboratories using a variety of techniques to destroy neurons (reviewed by Hitchcock and Raymond, 1992), in all previous cases (except the tunicamycin studies), the regenerative response involved the generation of multiple neuronal types in all retinal layers (Raymond, 1991).

Fig. 6. (A) Radial cryosection 4 days after 160 mw lesion double-labeled with NN2 (red, visualized with TR) and anti-GFAP antibody FGP2 (green, visualized with FITC). The boundaries of the lesion are indicated by the short white arrows. NN2+ microglia (arrowheads) are collected in the outer plexiform layer and in the subretinal space within the lesion. Radial Müller fibers are labeled with FGP2 (long white arrow); note that, within the lesion, the GFAP-immunoreactivity is enhanced. A clump of FGP2-immunoreactivity in the subretinal space is encased within an NN2+ cell (asterisk). (B) Another section from the same retina as in A, but double-labeled with RET1 (red, visualized with TR) and FGP2 (green, visualized with FITC). The lesion boundary on one side is indicated by the short white arrow, where the row of RET1+ cone nuclei ends. Note again, the enhanced GFAP immunoreactivity in radial Müller fibers within the lesion (long white arrow). Ectopic RET1+ Müller cell nuclei (arrowhead), are in the outer nuclear layer. Note the FGP2-immunoreactivity in the subretinal space (asterisk), similar to A. (C) Another section from the same retina as in A and B, now double-labeled with RET1 (red, visualized with TR) and anti-BrdU (green, visualized with FITC). Again, the lesion boundaries are indicated with short, white arrows. Note the radially oriented double-labeled (and therefore yellow) nuclei, which represent proliferating Müller cells that appear to be migrating from the inner to the outer nuclear layer (long white arrows). Arrowheads indicate (green) BrdU+ cells, presumably macrophages, in the subretinal space and choroid. ch, choroidal vessels; on, outer nuclear layer; op, outer plexiform layer; sr, subretinal space; v, vascular membrane on the vitreal surface; other abbreviations as is Fig. 1. Bar, 50 μm and applies to all panels.

The phenomenon described here is quite different, in that a highly selective phenotypic choice was made by the proliferating neuroepithelial cells in response to the removal of a specific neuronal cell population.

The observations made in the present study encourage a re-evaluation of our working hypotheses. First, the notion that retinal regeneration in goldfish recapitulates development, which we had previously advanced (Raymond, 1991), must be qualified, since selective regeneration of cones (perhaps in association with rods) in the absence of regeneration of retinal neurons in inner layers has been demonstrated here. The outer retina, specifically the photoreceptors, may be privileged in this regard, however, since the conditions that trigger regeneration of inner retinal neurons appear to differ. The premise (Raymond et al., 1988) that regeneration of inner retinal neurons only occurs in association with regeneration of photoreceptors is still upheld, but present results imply that while photoreceptor loss may be necessary for regeneration of inner retinal neurons, it is not sufficient. Second, a careful study of histological and immunofluorescent sections from laser-lesioned retinas provided evidence of ectopically located cells in the damaged ONL: (1) cells with the cytological characteristics of rod precursors were sometimes in contact with the OLM in regions where cone and rod nuclei had been destroyed in substantial numbers, and (2) Müller nuclei, often proliferating, were displaced into the outer plexiform and ONL. These observations suggest that (1) interactions with extracellular signals in the subretinal space or interactions among proliferating cells, perhaps in the context of junctional contacts at the apical surface, may be more important than cell-cell interactions within the ONL in triggering a regenerative response, and (2) Müller cells should be considered as a potential source of neuroepithelial cells from which regenerated neurons derive. These ideas are incorporated into a working model of inductive interactions that might regulate choice of cell fate during retinal regeneration in adult goldfish (Fig. 7).

The model in Fig. 7 hypothesizes that, in the intact retina or in retinas sustaining loss of cells from inner layers only, local signals in the ONL provide an environment conducive to proliferation of rod precursors and the differentiation of their progeny into rod photoreceptors. The nature of these signals is uncertain; soluble and contact-mediated mitogens and differentiation factors are all possibilities. Since serial electron microscopic reconstructions of rod precursors have shown that they are isolated, mitotic cells wholly contained within the ONL, and they do not have processes that reach the apical surface or extend into inner retina (Raymond and Rivlin, 1987), it is likely that signals that direct rod precursors to produce exclusively rods under normal conditions arise within the ONL (Fig. 7).

When cone photoreceptors are destroyed (or better, if both rods and cones are destroyed), rod precursors gain access to the apical surface (the OLM); this surface is normally occupied primarily by cone nuclei. Following damage, mitotic activity is enhanced and isolated rod precursors become clusters of proliferating cells adjacent to the OLM (Figs 1, 7). The model proposes that contact with the apical surface exposes rod precursors directly to apically derived signals which alter their fate resulting in the production of new cones in addition to rods. Although the nature of these putative signals is unknown, there are a number of possibilities. For example, s-laminin, a variety of the extracellular matrix molecule laminin, is localized to the subretinal space at the apical surface of the retina and may exert a stimulatory effect on photoreceptor commitment or differentiation (Hunter et al., 1992). The retinal pigmented epithelium is also a source of photoreceptor differentiation factors (Hewitt et al., 1990; Tombran-Tink et al., 1991).

Another potential signalling event is the reestablishment of adherens junctions by the proliferating neuroepithelial cells when they contact the apical surface. Hinds and Hinds (1979) proposed that adherens junctions were critical to the differential choice of cell fate by the primitive neurons in the embryonic mouse retina: they showed by serial electron micro-

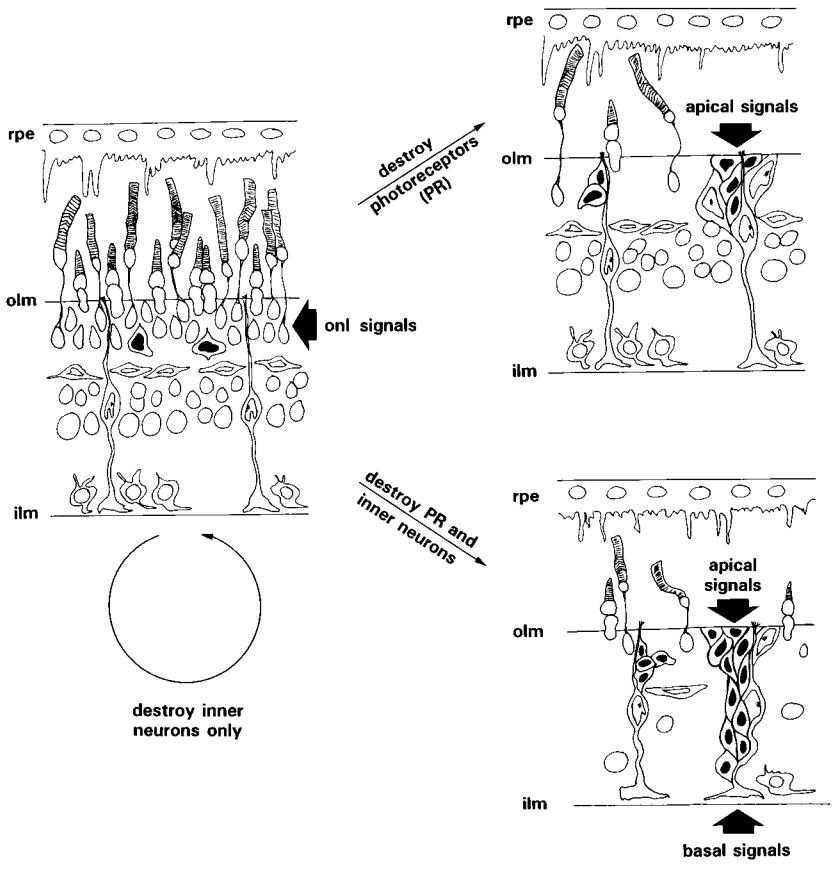


Fig. 7. Model of inductive interactions that might regulate cell fate in the teleost retina during regeneration. Rod precursors and regenerative neuroepithelial cells are indicated with filled nuclei; Müller cell nuclei are lobulated and have prominent nucleoli. Microglia and macrophages are omitted for clarity. ilm, inner limiting membrane; olm, outer limiting membrane, onl, outer nuclear layer; rpe, retinal pigmented epithelium. See text for further explanation.

scopic reconstructions that neuroepithelial cells that retained an adherens junction and an association with the OLM and failed to regrow a vitread process differentiated as cone photoreceptors. Following this logic, the trigger that leads to a switch in rod precursor fate could be apical junctional contact. In the embryonic retina of most vertebrates, including goldfish (Johns, 1982), cones are produced before rods, and hence the interpretation is that altered rod precursors revert to an earlier developmental state and produce cones. The idea that re-establishment of epithelial polarity associated with junctional specializations and the expression of specific adhesion molecules might be a critical determinant of phenotypic choice is appealing, since such processes are critical to cell differentiation in a number of tissues (Ben-Ze'ev, 1991) including retina (Takeichi, 1990; Geiger and Ayalon, 1992).

When retinal cell loss is more extensive, and involves cells in both outer and inner retina (Fig. 7), clusters of proliferating neuroepithelial cells extend across the entire retina (Raymond et al., 1988; Hitchcock et al., 1991; Braisted and Raymond, 1992). It is under these conditions, and only these, that inner retinal neurons regenerate. The model speculates that, when inner retinal cells are destroyed, the neuroepithelial cells come under the influence of different signals localized in or near the basal lamina at the vitreal surface (Morest, 1970; Hinds and Hinds, 1979; Reh and Nagy, 1987) which trigger other pathways of phenotypic differentiation. The nature and origin of these signals remains to be determined, but clearly there is a hierarchical process at work, such that events at the apical surface are obligatory for those at the basal surface.

Previous studies have suggested that the proliferating neuroepithelial clusters in regenerating goldfish retina originate from rod precursors (reviewed in Hitchcock and Raymond, 1992), and the discussion to this point has continued with that presumption. However, there are two other possible sources that should be considered. First, clusters of proliferating cells that resemble neuroepithelial cells have been observed in the INL, the ONL or spanning these two layers within a day following retinal damage that results in minimal cell loss (Raymond et al., 1988; Negishi et al., 1991a,b) or following injections of various mitogenic growth factors into the goldfish eye (Negishi and Shinagawa, 1993). Second, the present study showed that Müller nuclei also proliferate and migrate into the space vacated by lost photoreceptors following laser lesions. During embryonic and larval retinal development in goldfish, the residual neuroepithelial cells from which rod precursors derive are initially sequestered in the INL, apposed to Müller cells, and they later migrate along radial Müller fibers, crossing the outer plexiform layer into the ONL (Raymond and Rivlin, 1987). It is likely that the physical association between Müller cells and the persistent neuroepithelial cells that give rise to rod precursors in the fish retina reflects a relationship by lineage, as has been demonstrated in the postnatal rodent retina, where clones composed of rods and Müller cells occur during the last stages of neurogenesis (Turner and Cepko, 1987). It is further possible that quiescent neuroepithelial cells remain in the INL or the ONL of the adult retina, perhaps in association with Müller fibers; the existence of these cells is difficult to demonstrate as there are no specific markers for them, and they do not proliferate under normal conditions. If they are present, however, they could contribute to the regenerative process.

The possibility that Müller cells might dedifferentiate, proliferate and give rise to new neurons must also be considered. There is evidence that the Müller glial phenotype is unstable (Linser and Irvin, 1987), especially in the absence of neurons (Moscona and Linser, 1983; Linser and Perkins, 1987). More recently, Linser and colleagues have shown that glial cells in vitro can express phenotypic characteristics of both neurons and glia, when differentiated neurons are removed from the cultures (Galileo and Linser, 1992). Arguing against the idea that Müller cells might give rise to regenerated neurons in the damaged fish retina, however, is that cytological changes identical to those seen here (proliferation, migration, increased expression of GFAP) have also been observed in Müller cells in mammalian retinas following damage, but in the absence of neuronal regeneration (Bignami and Dahl, 1979; Eisenfeld, et al., 1984; Erickson, et al., 1987; Ishigooka, et al., 1989). Only further study will allow us to identify definitively the source of regenerating neurons in the goldfish retina.

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