

## Regeneration of dopaminergic neurons in goldfish retina

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### Summary

The conditions necessary to trigger regeneration of dopaminergic neurons were investigated in the goldfish retina. Intraocular injection of 6-hydroxydopamine (6-OHDA) was used to destroy dopaminergic neurons, and neuronal regeneration was monitored by injections of the thymidine analog bromodeoxyuridine (BUdR). Regenerated dopaminergic neurons, (identified by double-labeling with anti-tyrosine hydroxylase and anti-BUdR antibodies) were found within 3 weeks after 2 injections of 0.6 mg/ml 6-OHDA (estimated intraocular concentration), but *not* after injection of lower doses. All retinas with regenerated dopaminergic neurons also contained other types of regenerated neurons, including cones and ganglion cells, consistent with nuclear counts

which revealed non-selective cell loss (34-36%) in both the outer and inner nuclear layers after exposure to the high dose, but not lower doses of 6-OHDA. Regenerated neurons were produced by clusters of dividing neuroepithelial cells probably derived from rod precursors in the outer nuclear layer. These results demonstrate that dopaminergic neurons will not regenerate after they are selectively ablated but only as part of a developmental process that involves generation of multiple cell types.

Key words: 6-hydroxydopamine, differentiation, immunocytochemistry, BUdR, goldfish retinal regeneration.

### Introduction

The retina of teleost fish has a number of unique features that make it an attractive model for studying the role of cell-cell interactions in the determination of neuronal cell fate. In many species, the retina of the adult continues to grow by a process that includes neurogenesis (reviewed by Raymond, 1985). There are two morphologically and spatially distinct populations of residual dividing neuroepithelial cells (Boulder Committee, 1970) which contribute new neurons to the adult retina. In the germinal zone at the circumferential margin of the retina are neuroepithelial cells that give rise to annuli of new retina. These are spindle-shaped cells, with radially oriented processes that span the width of the retinal epithelium and with nuclei that display interkinetic nuclear migration (Müller, 1952; Raymond and Rivlin, 1987). Scattered across the differentiated retina within the layer of photoreceptor nuclei (the outer nuclear layer, ONL) are rod precursors. Rod precursors are modified neuroepithelial cells: they are globular or pleomorphic in shape (Johns and Fernald, 1981; Raymond and Rivlin, 1987; Fernald, 1989), and they divide in situ without nuclear migration (Johns, 1982; Raymond, 1985). They normally give rise only to rod photoreceptors, but their developmental

potential may not be as limited as their normal fate would suggest (Raymond et al., 1988; Raymond, 1991).

Following surgical lesions or cytotoxic destruction, the neural retina of adult goldfish will regenerate (Lombardo, 1968 and 1972; Maier and Wolburg, 1979), and rod precursors are thought to give rise to the regenerate. For example, if a small (<5 mm<sup>2</sup>) patch of retina is removed surgically, rod precursors flanking the wound proliferate and form a neuroepithelial "blastema" on the cut edge that resembles the marginal germinal zone. Gradually, over several weeks, the gap is filled with regenerated neurons (Hitchcock et al., 1991). Ouabain has also been used to destroy retinal neurons in goldfish, and again the retina regenerates within a few months from scattered clusters of elongated neuroepithelial cells (Maier and Wolburg, 1979; Raymond et al., 1988). Several observations led to the conclusion that rod precursors were the source of the regenerative clusters: (i) ouabain does not destroy rod precursors, (ii) migration of neuroepithelial cells from the marginal germinal zone into central retina is not seen, and (iii) if the amount of ouabain injected is reduced, neurons in the inner but *not* the outer retina are destroyed, and regeneration does *not* take place (Raymond et al., 1988). Thus, damage to the ONL appears to be needed in order to trigger regeneration,

which suggests the following hypothesis: when the cellular environment around the rod precursors is disturbed, their progeny change fate and differentiate into neurons other than rods (Raymond et al., 1988; Raymond, 1991).

If this hypothesis is correct, then deleting a single class of cell in the inner retina should *not* result in regeneration of the ablated cells. This prediction has been upheld in studies by Hitchcock (1989) in which propidium iodide was used to destroy ganglion cells in goldfish retina. Propidium iodide was inserted into the optic nerve where it was taken up by ganglion cell axons and transported retrogradely to the nucleus, subsequently killing cells. They never regenerated. Ablation of selective cells can also be accomplished with specific neurotoxins such as 6-hydroxydopamine (6-OHDA). When injected into the goldfish eye, 6-OHDA permanently destroys dopaminergic interplexiform cells (DAergic IPCs) [Negishi et al., 1982, 1985]. Recently, however, Negishi and colleagues made the surprising observation that if they injected supra-threshold amounts of 6-OHDA, the DAergic IPCs appeared to regenerate after approximately 2 months (Negishi et al., 1988). A possible explanation of this paradoxical result is that non-specific cellular damage occurs at high doses of 6-OHDA, which is autoxidized rapidly in solution to its quinone, forming hydrogen peroxide and various free radicals (Cohen and Heikkila, 1974). Since hydrogen peroxide and free radicals have been shown to be damaging to CNS tissue (Hall, 1989), generation of large quantities of these substances in solutions with high concentrations of 6-OHDA may be sufficient to cause non-specific cell loss.

The present study was designed to test the idea that the lesion produced by high doses of 6-OHDA spreads into the ONL and thereby triggers a regenerative response in the rod precursors. The strategy was to increase the amount of 6-OHDA injected into the eyes of adult goldfish until regeneration of DAergic IPCs was observed. If our hypothesis is correct, then we would expect to find loss of cells in the ONL correlated with regeneration of DAergic IPCs, regeneration of other neurons including photoreceptors in the ONL and various types of neurons in the inner retina (since non-specific damage should not be limited to the ONL), and finally, increased proliferation of the neuroepithelial cells putatively responsible for regeneration, i.e. the rod precursors.

## Materials and methods

Goldfish were obtained from a local pet store and were, on average, 3-4 cm in body length, with a nasotemporal eye diameter of 3.4-4.5 mm. All chemicals were from Sigma (St. Louis, MO) unless stated otherwise.

### *Intraocular injections*

#### *6-hydroxydopamine (6-OHDA)*

Fish were anesthetized in 0.2% tricaine methanesulfonate, wrapped in a wet paper towel and placed on the stage of a Wild stereomicroscope. The nasotemporal eye diameter was

measured with a caliper, and the ocular volume was estimated by spherical geometry (Easter et al., 1977). A slit was made in the nasal sclera at the limbus with a microknife (Tiemann, Plainview, NY), and a 5  $\mu$ l Hamilton microsyringe with a 33 gauge, fixed, blunt tip needle, about 1 cm long, was used to inject 6-hydroxydopamine hydrochloride in 0.9% NaCl; in solutions with higher concentrations of 6-OHDA, 3 mg/ml sodium ascorbate was added to retard the buildup of autoxidation products of 6-OHDA (quinones) [Jonsson and Sachs, 1975]. The concentration of 6-OHDA in the injection solution was either 3, 6 or 12 mg/ml, and the appropriate amount to be injected into each eye (0.9-1.7  $\mu$ l) was calculated from the ocular volume to yield estimated intraocular concentrations of 0.15, 0.3 or 0.6 mg/ml (0.7, 1.4 or 2.9 mM), respectively. In some cases both eyes were injected with the same amount and sometimes the injections were repeated on the following day. When only one eye was injected with 6-OHDA, the other eye was injected with an equivalent volume of the injection vehicle. In some cases, both eyes of control fish were injected with the vehicle alone.

#### *Bromodeoxyuridine (BUdR)*

To verify neuronal regeneration and to examine cell proliferation, fish treated with 6-OHDA (or controls) were also injected with BUdR.

To determine whether neurons had regenerated subsequent to the 6-OHDA injections, fish were given two intraocular injections of 0.4 mM BUdR in 0.9% saline (each one calculated to produce an estimated intraocular concentration of 20  $\mu$ M). The first injection was 6 days after 6-OHDA and the second was a week later. Fish were killed 8-14 days after the second BUdR injection and processed for double-label immunocytochemistry (various cell-specific antibodies paired with anti-BUdR) as described below. With this prolonged survival paradigm, newly generated or regenerated neurons will be labeled with BUdR.

To determine whether injection of 6-OHDA stimulated proliferation of rod precursors, both eyes were injected with BUdR (20-50  $\mu$ M). On the following day, eyes were harvested and processed for double-label immunocytochemistry as described below. With this short survival paradigm, only proliferating cells will be labeled with BUdR.

#### *Immunocytochemistry*

Fish were killed by decapitation, eyes were enucleated and fixed in 4% paraformaldehyde, 5% sucrose in 0.1 M phosphate buffer, pH 7.4. After 30 minutes, lenses were removed, the eyes bisected along the dorsoventral axis and fixed for an additional 30 minutes. Tissue was then rinsed, cryoprotected, frozen in a 2:1 mixture of 20% sucrose and OCT (Miles, Elkhart, IN), and radial sections were cut on a cryostat at 3  $\mu$ m (Barthel and Raymond, 1990). All subsequent steps were at room temperature unless otherwise noted.

For double-label immunocytochemistry, sections were blocked in 20% normal goat serum (NGS) in phosphate-buffered saline with 0.1% sodium azide and 0.5% Triton X-100 (PBSNT) for 30 minutes, and incubated overnight at 4°C in a polyclonal antibody against tyrosine hydroxylase (TH, diluted 1:150) [EugeneTech, Allentown, NJ], or in mouse monoclonal antibodies in ascites fluid produced by using goldfish retinal antigens (NN2, diluted 1:1000; RET1, diluted 1:500; Wagner and Raymond, 1991). Monoclonal antibody NN2 recognizes phagocytic cells (microglia and macrophages) and various vascular cells, including endothelial cells, in fish tissue. RET1 recognizes a nuclear antigen in several classes of retinal neurons including cones, horizontal cells, some

neurons in the inner nuclear layer (including the infrequent DAergic IPCs) and ganglion cells. Bound antibody was visualized by indirect immunofluorescence with secondary antibodies conjugated to Texas Red (TR) [or fluorescein isothiocyanate, FITC]. To visualize BUdR, sections were rinsed and treated with 2 N HCl in PBS with 0.5% Triton X-100 (PBST) for 30 minutes to denature the DNA and expose the BUdR antigen (Schutte et al., 1987). After rinsing, sections were again blocked and incubated overnight at 4°C in rat monoclonal anti-BUdR (Accurate Chemical, Westbury, NY) diluted 1:20. The following day, sections were rinsed and incubated for 30 minutes in secondary antibody conjugated to FITC (or TR). To avoid cross reactivity, all secondary antibodies were preabsorbed by the manufacturer (Jackson ImmunoResearch, West Grove, PA) against immunoglobins of the non-corresponding species. Controls included omission of the primary antibody and/or the secondary antibody. Slides were coverslipped with 60% glycerol in 0.1 M sodium carbonate buffer, with 0.4 mg/ml p-phenylenediamine to retard fluorescent bleaching (Johnson and Araujo, 1981), and viewed with a Leitz Aristoplan epifluorescent microscope, using narrow-band and wide-band FITC cubes (Leitz I3 and L3) and a TR cube (Leitz N2.1).

#### Nuclear counts

To determine the amount of cell loss, nuclei were counted in some of the double-labeled preparations. To visualize nuclei, 0.006 mg/ml of the fluorescent nuclear stain DAPI was added to the standard mounting medium (above), and sections were examined with the UV cube (Leitz A). Individual TH-immunoreactive (TH+) cells (labeled with FITC) were located and centered in the field of view at 160×. All nuclei in the INL and the ONL (except BUdR+ nuclei labeled with TR) were then counted in a 0.1 mm length of retina. Since we were interested in the amount of cell loss caused by a particular treatment, and BUdR+ nuclei represented cells that had regenerated after the treatment, they were not included in the nuclear counts. For each treatment, 11-30 fields were counted (2-3 retinas per treatment) and the averages calculated. In retinas in which no TH+ cells were found (see Results), random fields were examined at 160×. Data were analyzed statistically by the non-parametric Mann-Whitney U-Test (Krauth, 1983).

## Results

The only TH+ cells in the adult goldfish retina are DAergic IPCs (Dowling and Ehinger, 1978; Negishi et al., 1990a). These cells have large cell bodies located in the amacrine cell layer (inner part of the inner nuclear layer) and processes that synapse in both the inner and outer plexiform layers (Fig. 1). DAergic IPCs are sparsely distributed, with an approximate average density of 200 cells/mm<sup>2</sup> (Negishi, 1981; Negishi et al., 1985).

#### DAergic IPCs are destroyed after injection of 6-OHDA

Since regeneration of DAergic IPCs has been reported after high doses of 6-OHDA (Negishi et al., 1987, 1988), and we were interested in the parameters that might lead to their regeneration, increasing doses of 6-OHDA were administered, and fish were killed various times later.

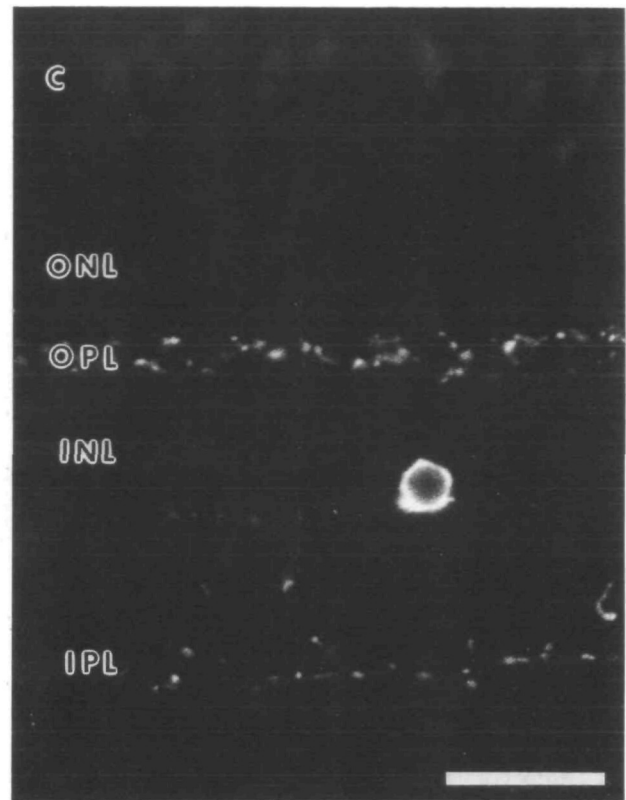
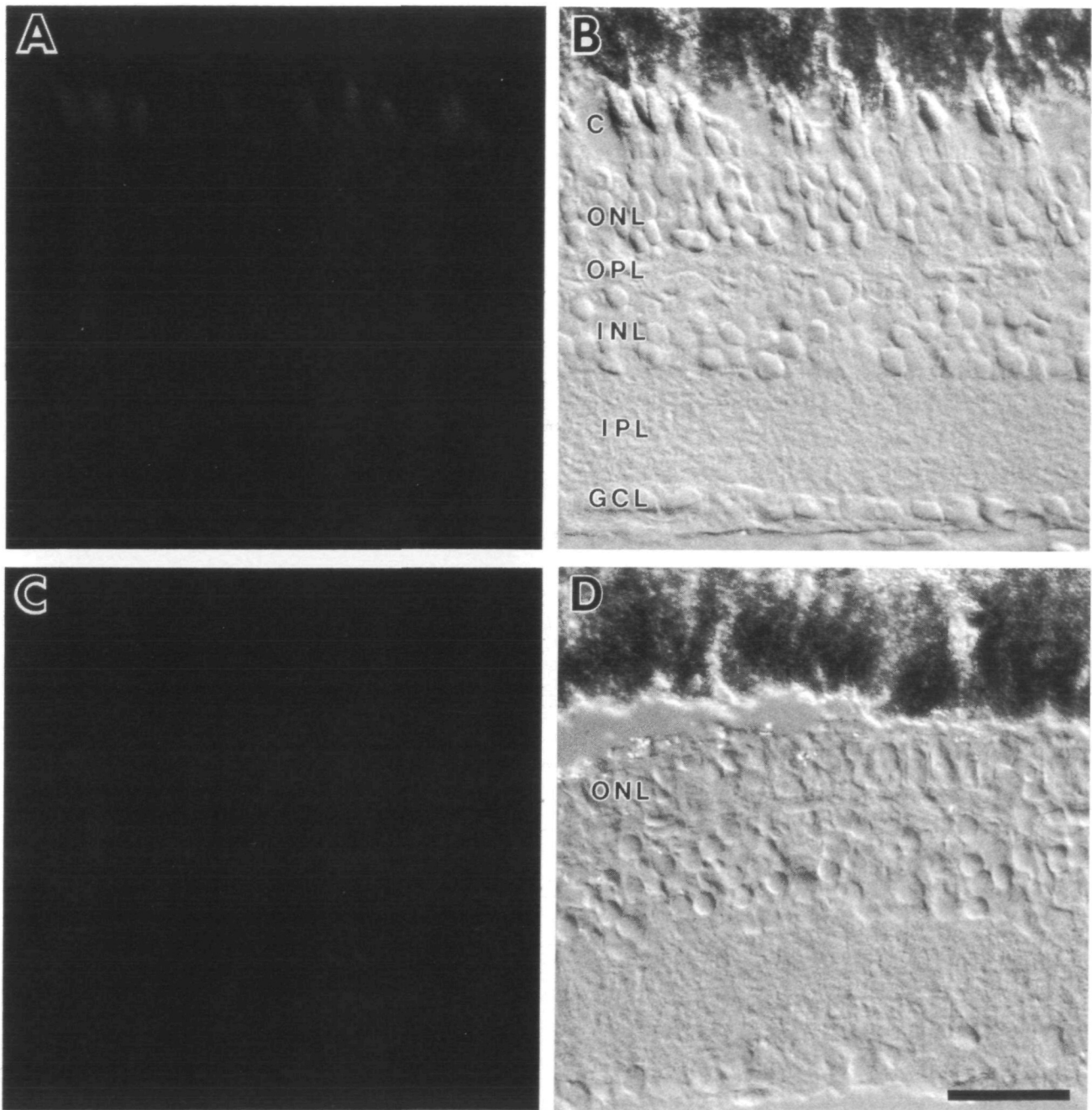


Fig. 1. Radial section showing a TH-immunoreactive DAergic IPC. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. The cone inner segments (C) are autofluorescent. Bar, 25  $\mu$ m.

With all doses of 6-OHDA tested DAergic IPCs were completely destroyed by one week. After either a single injection of 3 or 6 mg/ml 6-OHDA, or 2 injections of 6 mg/ml 6-OHDA (Fig. 2), no TH+ cell bodies or fibers were found in central retina up to 43 days later (Table 1). There were occasional TH+ somata and/or fibers in the far periphery, but these represent new (not regenerated) neurons added as part of the normal retinal growth that takes place in the interval subsequent to the 6-OHDA injections (Negishi et al., 1982). After 2 injections of 12 mg/ml 6-OHDA, no TH-immunoreactivity was detected at 7 days, but at 21 days, TH+ cell bodies were found in the central portions of all 4 retinas examined (Table 1). With this dose, there was also an obvious loss of nuclei in the outer nuclear layer and cones were absent in some portions of the retina (Fig. 2). Quantification of the cell loss is presented below. Intact TH+ cell bodies and fibers were found in all retinas from eyes injected with the vehicle alone (not illustrated). Addition of ascorbate had no effect on retinal histology.

#### DAergic IPCs regenerated 21 days after a high dose of 6-OHDA

To confirm that the DAergic IPCs that appeared by 21 days in the last experiment had regenerated, retinal sections from fish given multiple BUdR injections

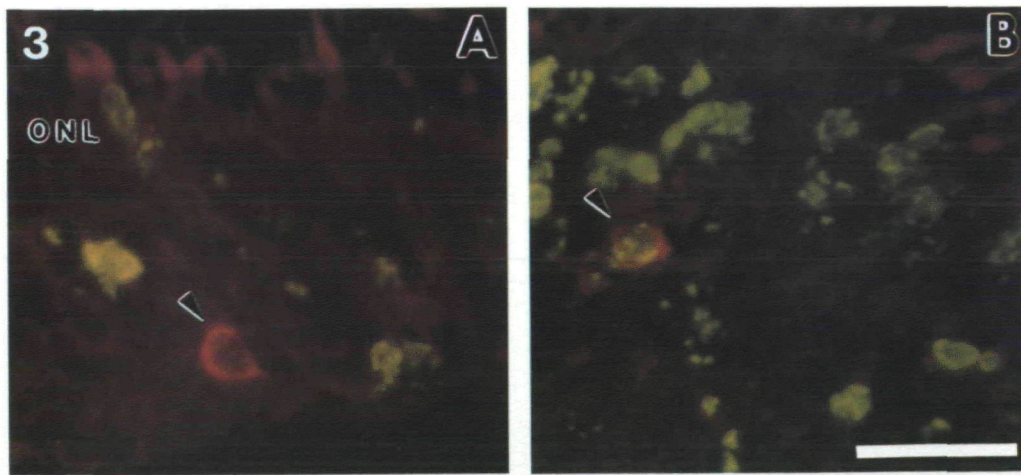


**Fig. 2.** No specific staining with anti-TH antibodies was detected in radial sections 7 days after either (A) 6 mg/ml  $\times$  2 or (C) 12 mg/ml  $\times$  2 of 6-OHDA. (B) and (D) are paired Nomarski interference contrast views of sections shown in A and C, respectively. Note the presence of autofluorescent cone inner segments in A that are absent in panel C. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; C, cones. Bar, 25  $\mu$ m.

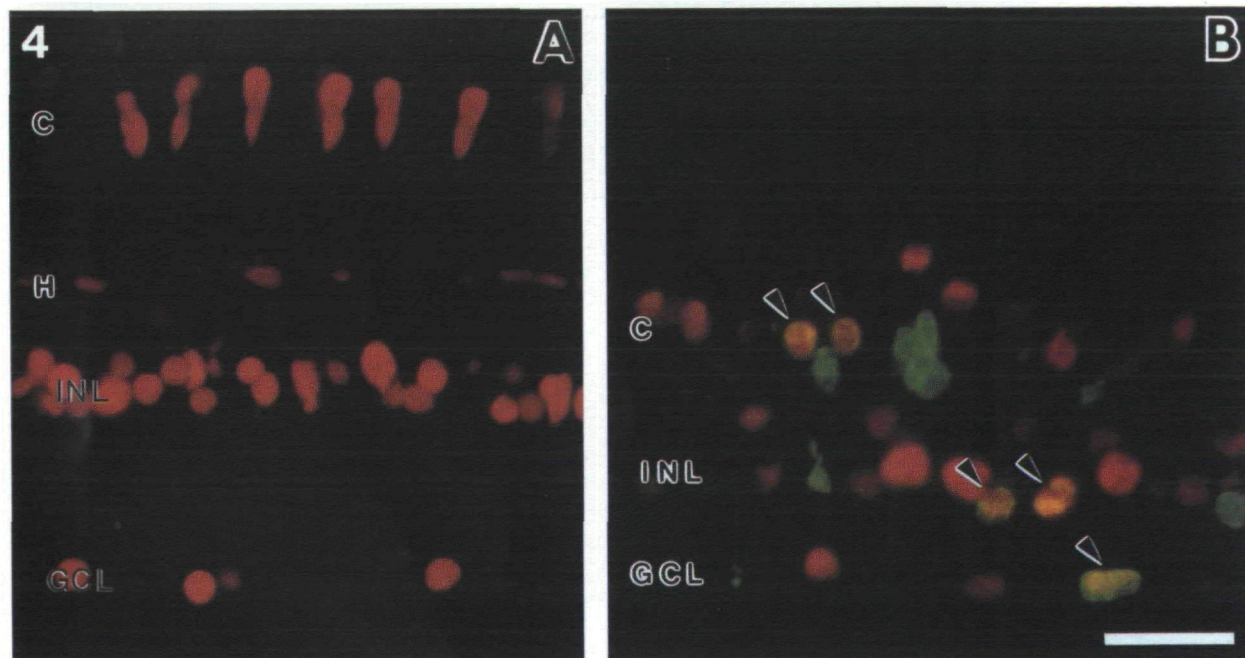
during the period of presumed regeneration were double-labeled with anti-TH and anti-BUdR antibodies. Any TH+ cells that were also BUdR+ must be DAergic IPCs born subsequent to the BUdR injections.

In all 4 retinas examined 21 days after two injections of 12 mg/ml 6-OHDA, TH+/BUdR+ somata were found in central retina (Fig. 3). In a total of 124 sections examined, 61 TH+ cells were found and of these 7% (4/61) were also BUdR+. The low percentage of TH+

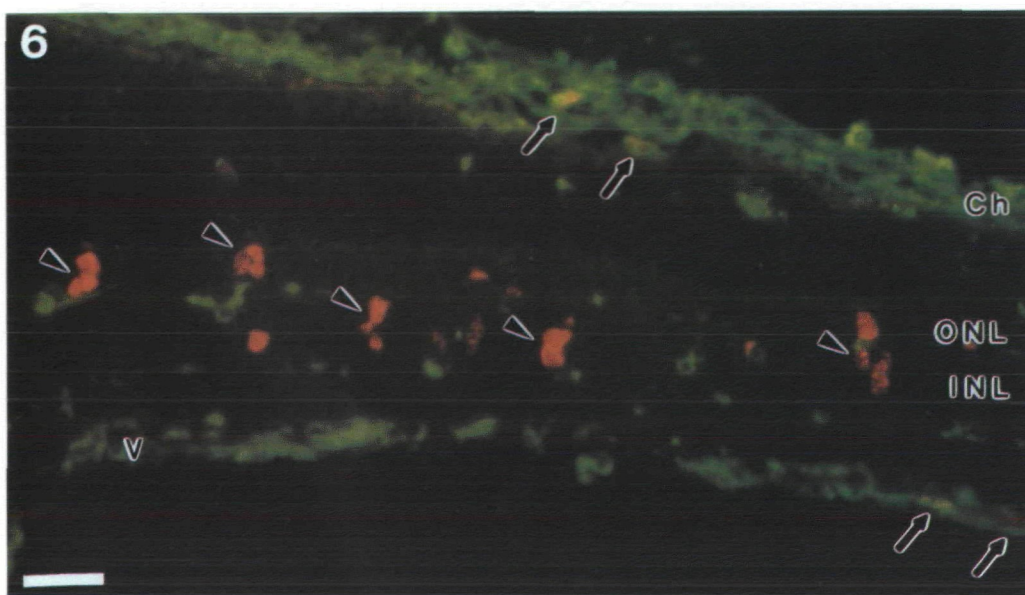
cells that were labeled with BUdR+ is explained by the fact that the fish in this experiment were given 2 pulse injections of BUdR, 1 week apart. Since the BUdR is only available for a limited time, probably less than one day (Johns, 1982), not all of the regenerated cells would have incorporated the label. The two pulses of BUdR could be detected in the growth zone at the far periphery (the region of new retina added as a result of mitotic activity in the germinal zone), where two



**Fig. 3.** (A and B) Radial sections of retinas from fish injected with BUdR 6 and 13 days after injection of 12 mg/ml  $\times$  2 of 6-OHDA, and killed 8 days later. Arrowheads indicate two double-labeled and therefore regenerated DAergic IPCs. The lamination of the regenerated retina is somewhat disorganized, but the ONL is at the top. Many other nuclei are BUdR+/TH-. Anti-TH visualized with Texas Red; anti-BUdR visualized with FITC. Bar, 25  $\mu$ m.



**Fig. 4.** (A) Immunofluorescent micrograph of a radial section of an intact goldfish retina stained with the monoclonal antibody RET1. (B) Immunofluorescent micrograph of a radial section of a retina from a fish injected with BUdR 6 and 13 days after 12 mg/ml  $\times$  2 of 6-OHDA, and killed 8 days later. Arrowheads show double-labeled and therefore regenerated neurons, including two cones, two inner nuclear layer neurons and what is most likely a ganglion cell. Notice that the nuclei of young regenerated cones in B are round rather than peanut-shaped as the nuclei of mature cones in A. The change in cone nuclear morphology observed during regeneration recapitulates that seen during development (Raymond et al., 1988). RET1 visualized with Texas Red; anti-BUdR visualized with FITC. C, cones; H, horizontal cells; INL, inner nuclear layer; GCL, ganglion cell layer. Bar, 25  $\mu$ m.



**Fig. 6.** Immunofluorescent micrograph of a radial section of a retina from a fish injected with BUdR 6 days after 12 mg/ml  $\times$  2 of 6-OHDA and killed 1 day later. Clusters of dividing cells in both the inner and outer nuclear layers (arrowheads) label with an anti-BUdR antibody (red), but not with a non-neural monoclonal antibody, NN2 (green). Arrows show dividing non-neural cells in the choroid and retinal vascular layer (at the vitreal surface) which label with both the anti-BUdR and the NN2 antibody. Anti-BUdR visualized with Texas Red; NN2 visualized with FITC. ONL, outer nuclear layer; INL, inner nuclear layer; Ch, choroid; V, vascular layer. Bar, 25  $\mu$ m.

Table 1. Regeneration of DAergic IPCs

| Experiment | 6-OHDA (mg/ml) | No. retinas examined | Survival after 6-OHDA (days) | No. retinas with TH+ cells |
|------------|----------------|----------------------|------------------------------|----------------------------|
| 2.21.90    | 3              | 3                    | 6                            | 0                          |
|            |                | 3                    | 11                           | 0                          |
|            |                | 3                    | 16                           | 0                          |
|            |                | 2                    | 24                           | 0                          |
| 3.31.90    | 3              | 2                    | 7                            | 0                          |
|            |                | 3                    | 14                           | 0                          |
|            |                | 4                    | 23                           | 0                          |
|            | 6              | 3                    | 7                            | 0                          |
|            |                | 3                    | 14                           | 0                          |
|            |                | 4                    | 23                           | 0                          |
| 4.30.90†   | 6 (×2)*        | 3                    | 7                            | 0                          |
|            |                | 5                    | 43                           | 0                          |
| 6.26.90†   | 6 (×2)*        | 4                    | 7                            | 0                          |
|            |                | 4                    | 21                           | 0                          |
|            |                | 4                    | 36                           | 0                          |
|            |                | 4                    | 36                           | 0                          |
|            | 12 (×2)*       | 4                    | 7                            | 0                          |
|            |                | 4                    | 21                           | 4                          |

\*The (×2) denotes that the 6-OHDA was injected on 2 consecutive days. For these retinas, survival after 6-OHDA is given in days after the second injection of 6-OHDA.

†3 mg/ml sodium ascorbate was added to the 6-OHDA injection solution.

distinct annuli of BUdR+ nuclei, with unlabeled nuclei intervening, were seen (data not shown). We believe that the TH+ somata located in central retina that were not labelled with BUdR had also regenerated.

Many BUdR+ cells that were not immunoreactive for TH were found in retinas with regenerated DAergic IPCs (Fig. 3). To help identify these cells, sections were double-labeled with BUdR and the RET1 monoclonal antibody, which, as mentioned in Materials and methods, labels the nuclei of cones, horizontal cells, some INL cells (including DAergic IPCs) and ganglion cells (Fig. 4). In all 4 retinas with regenerated TH+ cells, many RET1/BUdR double-labeled nuclei were found, including cones, cells in the INL and an occasional ganglion cell (Fig. 4). Furthermore, in these retinas, there was an obvious disruption of retinal lamination and a decrease in retinal width, with many areas devoid of cones and horizontal cells (Fig. 4). In contrast, no RET1+/BUdR+ cells were found in the central areas of the retinas with no regenerated TH+ cells, and the location and number of RET1+ cells in these retinas was qualitatively similar to intact retinas, and retinal lamination and width appeared normal (data not shown).

#### Cells are lost from both INL and ONL after injection of a high dose of 6-OHDA

In order to verify that cells in addition to DAergic IPCs were lost following exposure to a high dose of 6-OHDA, we counted cells in the ONL and INL in retinas 21 days after 2 injections of 6 or 12 mg/ml 6-OHDA or vehicle. A significant decrease ( $P < 0.01$ ) in number of cell nuclei per 0.1 mm retinal length was found in both the INL (34%) and ONL (36%) of retinas

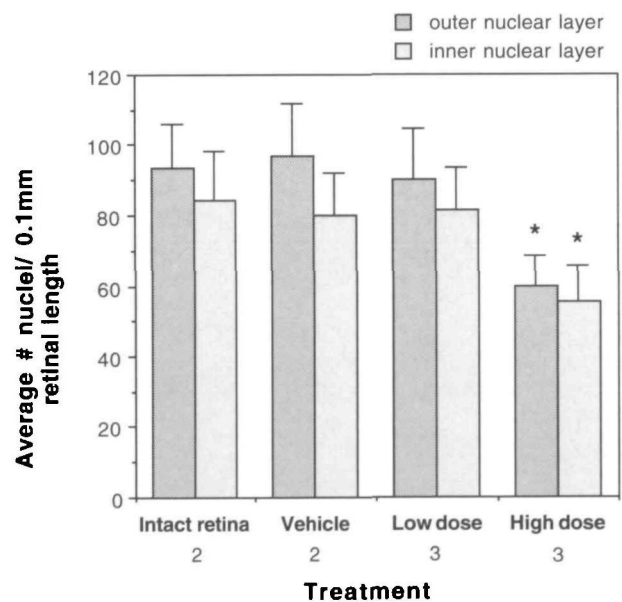


Fig. 5. Nuclear counts (see Materials and methods) from radial sections of retinas from fish given 2 injections of either 6 mg/ml (low dose) or 12 mg/ml (high dose) of 6-OHDA on 2 consecutive days, and killed 21 days later. The \* indicates a significant difference in cell counts compared to control (untreated) retinas. The error bars indicate one standard deviation. The number of retinas examined in each treatment group is indicated.

after treatment with 12 mg/ml × 2 of 6-OHDA, compared to untreated retinas (Fig. 5). We excluded BUdR+ cells from the counts (see Materials and methods) because they represented newly regenerated cells. However, other regenerated cells were not labeled with BUdR (see above), so that our estimate of

cell loss represents a minimum value. Almost certainly the actual amount of cell loss in these retinas was substantially greater.

After treatment with 2 injections of 6 mg/ml of 6-OHDA or the vehicle, there was no decrease in cell number compared to untreated retinas (Fig. 5). Of course DAergic IPCs were missing, but they represent such a small fraction (approximately 0.3% of the amacrine cell layer) that their loss has an insignificant impact on total cell number in the INL.

#### *Injection of a high dose of 6-OHDA leads to the formation of clusters of dividing neuroepithelial cells*

In retinas in which regeneration of DAergic IPCs and other neurons including RET1+ neurons had occurred, an increase in proliferation of neuroepithelial cells located in central retina, i.e. rod precursors, would be expected in the period prior to the appearance of regenerated cells. Our next goal was to demonstrate the presence of these proliferating cells.

Radially elongated clusters of BUdR+ cells (Fig. 6) were found in all 4 retinas from fish injected with 12 mg/ml  $\times$  2 of 6-OHDA and killed 7 days later (Table 2). These proliferating clusters were only found in the regenerating retinas, and were never seen in untreated ( $n > 10$ ) or vehicle-injected ( $n = 6$ ) retinas. Of the 15 retinas examined from fish injected with doses of 6-OHDA *not* associated with regeneration, only one had clusters of BUdR+ nuclei (Table 2). We assume that in this single retina, which had received 6 mg/ml  $\times$  2 of 6-OHDA, damage was more extensive than typical for this dose and neuronal regeneration was occurring.

In order to confirm that these clusters of dividing cells did not represent an inflammatory response to the neuronal degeneration, retinal sections were double-labeled with BUdR and the monoclonal antibody, NN2, which, as mentioned in Materials and methods, labels macrophages, microglia and various vascular cells in the fish retina. Although many BUdR+ cells in the retina also labeled with the NN2 antibody, the proliferating clusters did not (Fig. 6).

In summary, injection of the highest dose (12 mg/ml  $\times$  2) of 6-OHDA always (4/4) leads to the formation of clusters of dividing, non-vascular cells, and always (4/4) leads to regeneration of DAergic IPCs. The similarity of these clusters of proliferating cells to the neurogenic

foci that give rise to regenerated neurons in ouabain-treated retinas (Raymond et al., 1988) and surgically damaged retinas (Hitchcock et al., 1991) suggests that these cells are generating new neurons to replace those destroyed by the 6-OHDA.

## Discussion

Results from the present study confirm earlier reports that when DAergic IPC are selectively ablated they are not replaced (Negishi et al., 1982; Negishi et al., 1985). The idea that loss of a specific class of retinal cell is insufficient to trigger a regenerative response is further supported by studies in which suicide transport of propidium iodide injected into the optic nerve leads to death of retinal ganglion cells in adult goldfish: the ganglion cells do not regenerate (Hitchcock, 1989). Furthermore, serotonergic neurons in the inner nuclear layer of adult goldfish retina do not regenerate after they are specifically ablated with 5,7-dihydroxytryptamine (Negishi et al., 1988). However, the results of the present study show that when retinal cell loss is more extensive, and includes both the INL and the ONL, DAergic IPCs do regenerate along with other retinal neurons.

Which cells must be destroyed and how much destruction is needed to trigger regeneration of DAergic IPCs? Although we cannot be sure of the minimum amount of retinal damage needed to trigger regeneration, in our experiments more than one third of the cells in both the INL and ONL were destroyed. In order to pinpoint more precisely the site and amount of cell loss needed to provoke regeneration of DAergic IPCs, we plan to combine 6-OHDA injections with other neurotoxins that will specifically target cells in either the ONL or the INL. We predict that cell loss in the ONL is necessary since we believe that the neurogenic clusters that give rise to the regenerated neurons following both 6-OHDA and ouabain lesions derive from rod precursors which are located there, and since the conditions necessary to provoke rod precursors to regenerate retinal neurons following ouabain lesions include loss of cells in the ONL (Raymond et al., 1988).

What is the origin of the regenerated DAergic IPCs? Clusters of dividing (BUdR+) cells were consistently found in retinas from fish in which DAergic IPCs later regenerated. Cells in these clusters did not label with a non-neural antibody, NN2, and this, in addition to their radially elongated shape, suggests that these are neuroepithelial cells probably derived from rod precursors in the ONL. Since these cells were always found in retinas with regenerated DAergic IPCs, and not in those with no regeneration, it is likely that they are the source of regenerated DAergic IPCs and other neurons. Similar clusters of dividing cells in adult goldfish retinas after intraocular injection of 6-OHDA have also been described (Negishi et al., 1990b) by using antibodies to proliferating cell nuclear antigen (PCNA) which is present in the nucleus of dividing cells (Almendral et al., 1987).

**Table 2.** Neuroepithelial cell proliferation

| 6-OHDA (mg/ml)    | No. retinas examined | No. with BUdR+ clusters |
|-------------------|----------------------|-------------------------|
| 3                 | 5†                   | 0                       |
| 6                 | 3                    | 0                       |
| 6 ( $\times$ 2)*  | 7                    | 1                       |
| 12 ( $\times$ 2)* | 4                    | 4                       |

\*The ( $\times$ 2) denotes that the 6-OHDA was injected on 2 consecutive days. In addition, 3 mg/ml sodium ascorbate was added to the 6-OHDA injection solution.

†3 of the 5 retinas were examined 6 days after 6-OHDA injection; all others on this table were at 7 days.

Once regeneration commences, it is likely that additional mechanisms come into play to regulate choice of cell fate so that the proper number of neurons are produced and placed in the proper layers. For example, there is evidence for feedback regulation of cell number based on neurotransmitter identity. Studies in goldfish (Negishi et al., 1982) and frog tadpole (Reh and Tully, 1986) retinas have shown that after ablation of dopaminergic neurons with 6-OHDA under conditions that do not trigger regeneration of the cells lost from central retina, the density of newly generated dopaminergic neurons is selectively increased in the growth zone (the annulus of new retina added as a result of mitotic activity in the marginal germinal zone). These results suggest that neuroepithelial cells in the marginal germinal zone are able to up-regulate production of dopaminergic neurons to compensate for their loss in central retina. The authors hypothesized that feedback regulation from previously differentiated cells controls the number of cells of each type being produced in the germinal zone (Reh and Tully, 1986). Similar mechanisms may operate in the regenerating retina, but the source and nature of these interactions remain to be determined.

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