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Photic history modifies susceptibility to retinal damage in albino trout

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Abstract

Albino vertebrates exposed to intense light typically lose photoreceptors *via* apoptosis, and thus serve as useful models of retinal degeneration. In contrast, albino rainbow trout exposed to intense light maintain populations of rod and cone nuclei despite substantial damage to rod outer segments (ROS). The aim of this study was to differentiate between two hypotheses that could account for this divergent result: (1) trout rod nuclei remain intact during light damage, or (2) rod nuclei die but are replaced by cell proliferation. A further aim was to examine whether photic history modulates retinal damage, as in rodents. Albino and normally pigmented trout were moved from defined photic regimes into full daylight, while some were not moved to serve as protected controls. ROS were always maintained in pigmented fish and in albinos protected from full daylight. In albinos exposed to full daylight, ROS were removed over most of the central retina, whereas rod nuclei were maintained in the outer nuclear layer over 10 days. Pyknotic and TUNEL-labeled rod nuclei were abundant in affected albinos at all time-points tested. Rod death occurred without a decrease in the number of rod nuclei, confirming that proliferation must be replacing cells. Indeed a transient increase in proliferation was observed in retinal progenitors of albinos receiving 5 days of damaging light. This proliferative response was decreased with further damage. Cones remained intact even in areas where rod nuclei had degenerated. Pretreatment with light of moderate versus low intensity light affected the cell death and proliferative responses, and the ectopic localization of rod opsin. We conclude that apoptotic demise of rods, but not cones, occurred during light damage in retinas of albino trout and proliferative responses have a limited a capacity to replace lost rods.

Keywords: Apoptosis, Salmonid, Retinal light damage, Proliferating cell nuclear antigen, *Oncorhynchus mykiss*

Introduction

Various animal models, including rodents and teleost fish, have been valuable in understanding the toxicology of light damage in the retina. Light damage leads to photoreceptor programmed cell death in albino rodents (Shahinfar et al., 1991; Li et al., 1996). Damage can be caused by constant illumination of moderate intensity, or by cyclical illumination of higher intensity (Organisciak & Winkler, 1994). Factors affecting the susceptibility of photoreceptors to light damage include diet, circadian factors, ocular pigmentation, and prior exposure to light (Li et al., 2003). Recent progress has been made in identifying molecular pathways leading to photoreceptor apoptosis (Hao et al., 2002; Reme et al., 2003; Wenzel et al., 2005). Thus, despite their nocturnal habit and rod-dominated retina, the data from light-damaged rodent retinas have contributed greatly to our understanding of the mechanisms of photoreceptor cell death and retinal degeneration in humans.

Surface-dwelling diurnal fishes can be an attractive complement to the study of nocturnal rodents for several reasons. Their higher cone:rod ratio has evolved to function in a bright-light environment, and may be considered more representative of the human fovea. Furthermore, the retina continues to grow throughout the life of the fish, and this feature provides a regenerative potential (Easter & Hitchcock, 2000; Stenkamp & Cameron, 2002; Raymond & Hitchcock, 2004). The growth occurs through two primary mechanisms (Lyall, 1957; Ali, 1964; Johns, 1977): (1) addition of new retina by the circumferential germinal zone (CGZ) at the retinal periphery; and (2) stretching of the more central retina, with progenitor cells producing new rods to maintain their density (Johns & Fernald, 1981; Fernald, 1990).

Importantly, continuous ocular growth also gives the teleost retina a regenerative capacity that could facilitate understanding

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of repair mechanisms (reviewed in Raymond & Hitchcock, 1997, 2000; Stenkamp et al., 2001; Otteson & Hitchcock, 2003). Populations of progenitor cells are present in the central retina of fish, including rod progenitors in the outer nuclear layer (ONL) and the proliferating inner nuclear layer cells (PINCs). The latter replenish the former during natural development (Julian et al., 1998). The PINCs may be replenished by glia dedifferentiating to proliferating cells (Fischer & Reh, 2003). Thus, in addition to being effective models of photoreceptor degeneration, the light-challenged teleost retina might also contribute to understanding how stem cells can be signaled to replace lost photoreceptor cells.

Rainbow trout are an effective model for the study of light damage and regeneration in the retina. The cone photoreceptors, belonging to four spectral classes, are patterned in a mosaic (Browman & Hawryshyn, 1992; Beaudet et al., 1993; Beaudet et al., 1997; Allison et al., 2003) that can facilitate an understanding of mechanisms of cone differentiation (reviewed in Stenkamp & Cameron, 2002). The potential to regenerate photoreceptors in rainbow trout appears to be substantial, as indicated by the reappearance of ultraviolet-sensitive cones late in the fish's natural life history (Beaudet et al., 1997; Hawryshyn et al., 2003; Allison et al., 2005). The PINCs in rainbow trout increase their rate of proliferation in response to surgical injury (Faillace et al., 2002). Most importantly, raising albino rainbow trout in full sunlight or exposing them to 3000 lux constant incandescent light leads to the loss of rod outer segments (ROS) (Allen & Hallows, 1997; Allen et al., 1999).

Remarkably, the number of nuclei in the ONL, dominated by rod nuclei, did not decrease (Allen & Hallows, 1997). This result differed from observations on light damage in rodents and other fish, where the number of photoreceptor cells is reduced (Allen et al., 1999; Vihtelic & Hyde, 2000). It has been demonstrated that the loss of ROS in rainbow trout is localized to the central, mature retina (Allen et al., 2001). When albino trout were transferred to dim light conditions following damage, the ROS and scotopic visual sensitivity reappeared (Allen & Hallows, 1997). The data led to the formation of two hypotheses (Allen & Hallows, 1997), which are not mutually exclusive of each other: (1) that rods were surviving the light damage, that is, ROS were pruned from the rod nuclei, which elaborated new ROS when fish were transferred to dim-light conditions; and (2) that rods were dying but being replaced immediately by generation of new rods from progenitor cells. The primary objective of this work was to discriminate between these two hypotheses.

Our examinations of cell death and proliferation in the current work support hypothesis (2) above, that is, rods are dying and being replaced through the proliferation of retinal progenitors. During this experiment we found an unexpected decrease in the number of ONL nuclei in localized areas of the central retina of affected albinos, which had not been observed previously in trout. We hypothesized that this increased susceptibility resulted from preexposure to moderately intense cyclic light rather than to being reared in full daylight as in earlier studies. This hypothesis was verified under a repeated protocol using two different pretreatments.

Materials and methods

Fish handling and dissections

Fish were maintained at Kamas State Fish Hatchery in Summit County, UT. The albino trout (*Oncorhynchus mykiss*) utilized in this study have an autosomal recessive mutation in the tyrosinase gene (Bridges & von Limbach, 1972).

Series 1

On July 22, 2001, we transferred albino and normally pigmented rainbow trout from a covered raceway (where they had been raised completely protected from direct sunlight) into an open raceway with little opportunity for behavioral avoidance of sunlight. This methodology differed from previous experiments where trout were raised in outdoor raceways and sampled at various times of the year (Allen & Hallows, 1997; Allen et al., 1999, 2001). Fish were transferred serially such that eyes could be sampled after periods of 0, 2, 5, 10, and 20 days of exposure. Normally pigmented fish ranged from 6.5 cm to 9.0 cm in standard length, whereas albinos ranged from 9 cm to 13 cm and were 2 months older than the normally pigmented fish. This minor difference in age is unlikely to affect the qualitative outcomes of the experiment (as confirmed by our results from fish used in Series 2). Regardless, we do not statistically compare results between pigmented and albino fish.

Series 2

On July 26, 2002, fish which had been kept under indoor cyclic fluorescent (relatively dim) light for 1 month remained in place (not-pretreated) or were placed outdoors in shaded (moderately intense) daylight for 10 days (pretreated). Fish from both groups were identified by fin-clip and then placed in full sunlight such that pretreated and not-pretreated fish were sampled after 0, 2, 5, and 10 days. The 10-day duration of damage was selected because this represented the time-point with the most dramatic change in proliferating cell nuclear antigen (PCNA) and TUNEL labeling during the previous experiment. Several of the albino fish assigned to the pretreated 0-day treatment group were lost to garter snake predation, leaving fewer individuals per time-point for examination. In these experiments (Series 2), albino and normally pigmented trout were age-matched and ranged from 9 cm to 12 cm at time of sampling.

Light levels were measured at the water surface with a Gamma Scientific spectroradiometer. Daily fluctuation in daylight intensity was measured with a recording pyroheliometer on site. Indoor fish were on a 12-h light/12-h dark cycle under fluorescent lights, whereas fish outdoors were exposed to 13.7-h light/10.3-h dark during treatment. The light levels for outdoor exposed fish, measured at about noon on a clear day, was approximately 4×10^{16} Photons$\cdot$cm$^{-2}\cdot$nm$^{-1}\cdot$s^{-1} integrated over 400–700 nm. The pretreated trout (in covered raceways) received light levels approximately two orders of magnitude less than the exposed fish. The not-pretreated trout (maintained indoors) were exposed to light levels approximately half the intensity of pretreated trout.

Enucleated eyes were immediately placed into ice-chilled fixative as appropriate to each method (below). The care and use of animals conformed to the principles adopted by the American Physiological Society and the Society for Neuroscience and the University of Victoria Animal Care Committee under the auspices of the Canadian Council on Animal Care.

Histology and labeling

Semithin sectioning was performed on eyes fixed in 2% glutaraldehyde–2% paraformaldehyde in phosphate buffer (pH 7.2) and embedded in epoxy resin as described previously (Allen & Hallows, 1997; Allen et al., 1999, 2001). The sclera was sliced open to admit fixative and in a manner to define the orientation of the eye.

Other eyes from each treatment were processed for TUNEL labeling. They were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) and held on ice. These eyes were shipped overnight to Victoria, BC. They were rinsed in PBS and dissected into four quadrants. The embryonic fissure, which reaches the peripheral retina at the ventral-nasal aspect of the eye, was used as a landmark. Eyes were cryopreserved, frozen, and sectioned (10-μm thickness) as described previously (Allison et al., 2003). Each of the four quadrants was frozen such that radial sections were examined from a known location in the retina. After rehydration, the sections were treated with 10 μg/ml proteinase K at 37°C and postfixed in 4% paraformaldehyde, using nuclease-free solutions. TUNEL labeling and negative controls were performed as per manufacture's protocols (Roche Biochemicals, Nutley, NJ). The signal produced by TUNEL labeling was a green fluorescent product, which was visualized and documented as described previously (Allison et al., 2003).

Other eyes from each treatment were processed for PCNA immunohistochemistry to mark proliferative cells. Eyes were placed in cold fixative composed of 38% formaldehyde diluted in 9 parts ethanol. These eyes were also shipped to Victoria, BC. The eyes were hemisected along the nasal-temporal axis and cryopreserved as above. Sectioning produced radial sections (10 μm) along the nasal-temporal axis. Sections were processed for PCNA immunohistochemistry using standard protocols as reported previously (Allison et al., 2003). Primary antibody was mouse-anti-PCNA (PC-10; Santa Cruz Biotechnology, Santa Cruz, CA) and secondary antibody was goat-anti-mouse conjugated to alkaline phosphatase (Vector Labs, Burlingame, CA). Both antibodies were diluted 1:1000 in PBS with 1:50 heat-inactivated horse serum (Sigma, St. Louis, MO). Signal was visualized using BCIP/NBT as per manufacturer's protocol (Gibco/BRL, Gaithersburg, MD), which produces a dark purple precipitate.

Other immunohistochemistry was performed on sections adjacent to those used for TUNEL labeling. Primary antibodies were mouse-anti-bovine rhodopsin (K16-155C, provided by Dr. Hargrave, University of Florida, Gainesville (Adamus et al., 1991)), previously shown to label rods and one member of the double cones in rainbow trout (Veldhoen, 1996). Presumably, the double cone labeling represents the medium-wavelength sensitive cone that expresses a subclass of the rhodopsin gene, Rh2 (Allison et al., 2003; Dann et al., 2004). We also employed mouse monoclonal antibodies zpr-1 and zpr-3 (Zebrafish International Resource Center, Eugene, OR) raised against zebrafish retinal antigens (Larison & Bremiller, 1990), that label double cones and rods of zebrafish, respectively. These antibodies were diluted 1:250 and visualized with 1:1000 goat-anti-mouse conjugated to Alexa-Fluor-594 (red fluorescent signal; Molecular Probes, Eugene, OR). Labeled cells were counted in each section and the length of the section measured using image analysis software (Northern Eclipse, Empix Imaging, Inc., Cheektowaga, NY). For PCNA quantifications the labeled cells of the CGZ were not included in the counts. To assess proliferation in the central retina, we also counted the number of PCNA labels in a single field of view (approximately 300 μm) of nasal retina that was one field of view removed from the optic nerve. We examined eyes from three individuals per treatment for the Series 1 TUNEL measurements, and four individuals for all other analyses. In each instance we counted labeled cells from four retinal sections. We compared values amongst treatments in SPSS software using analysis of variance with a *post-hoc* pair-wise Tukey test ($\alpha = 0.05$).

Results

Histology of light-damaged retinae

In pigmented fish, regardless of light treatment, sections in radial plane demonstrated that rod outer segments (ROS) and normal retinal structure were maintained (Fig. 1A), although ROS were shortened (Table 1). ROS were also maintained in albinos protected from sunlight (Fig. 1B) in both years. This was consistent with previous light-damage paradigms on these fish (Allen & Hallows, 1997; Allen et al., 1999, 2001).

ROS were lost from central retinas of albino trout in full daylight (Fig. 1C), as expected. Rod nuclei were maintained except in limited portions of the central retina of some individuals from day 2 to day 10 (Table 1, Fig. 1D). This limited ONL loss had not been observed in albino trout during previous experiments (Allen & Hallows, 1997; Allen et al., 1999, 2001) where the fish had been raised in outdoor raceways. However, in much of the damaged albino retina, rod nuclei were maintained despite ROS elimination. Also, rods close to the peripheral margins (typically within the most peripheral 0.5 mm of a 5–7 mm retinal circumference) maintained ROS, as observed previously (Allen et al., 2001). Cones remained intact in all retinal samples, including those in which rod nuclei had degenerated (Fig. 1D). Pyknotic nuclei were observed in all light-damaged albinos. Pyknotic nuclei were localized to the vitread half of the ONL in the central retina (Fig. 1).

TUNEL labeling increased during light damage

TUNEL labeling provided a clear demonstration that many rod photoreceptors were dying during light treatment of albino fish in Series 1 experiments (Figs. 2A & 2B). The TUNEL-positive nuclei were localized to the vitread half of the ONL, and thus were rod nuclei. This was the same location where pyknotic nuclei were observed in semithin sections (Fig. 1). Furthermore, the increases in TUNEL-positive rod nuclei were limited to the central retina, and were almost absent in the peripheral retina where ROS were maintained (Figs. 2A & 2B). Some TUNEL-positive nuclei were observed in the peripheral retina, as expected in developing retinal areas (Biehlmaier et al., 2001; Candal et al., 2005). TUNEL-positive nuclei did not change in abundance in pigmented fish despite light treatment (Fig. 3A). Low levels of TUNEL-positive nuclei were observed in untreated albinos, similar to pigmented fish. However, each of the 12 albino fish treated with damaging light had elevated TUNEL-positive nuclei in some location of the retina. TUNEL-positive nuclei in albino trout treated for 10 days were significantly higher than 0 days ($P = 0.030$). There was an obvious qualitative increase in cell death at the earliest duration of treatment we examined (2 days). It is noteworthy that the increases in TUNEL-positive label were observable coincident with the disappearance of rod nuclei in central locations of histological samples (Table 1). The significant increase in TUNEL labeling was confirmed in Series 2 experiments, described below.

PCNA labeling transiently increased with light damage

PCNA labeling (Fig. 4) revealed the expected populations of dividing cells based on previous examinations of rainbow trout (Julian et al., 1998) and other salmonids (Candal et al., 2005). The peripheral retina (CGZ) was strongly labeled and clusters of dividing cells were apparent in both the outer and inner nuclear layers (ONL and INL) of the central retina. Because histological

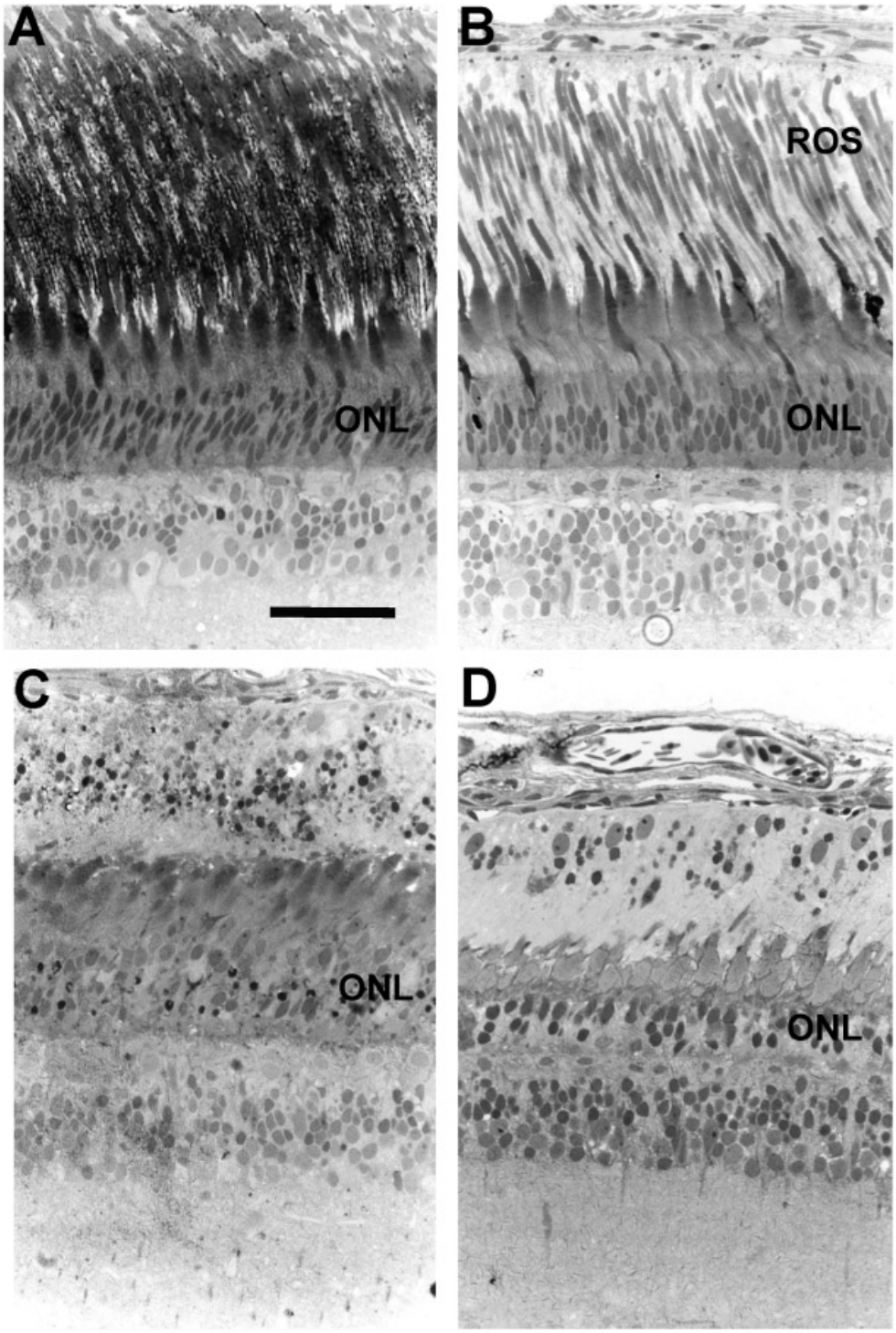

Fig. 1. Histology of retina from albino and normally pigmented trout during light damage. A: Plastic sections reveal that there is no alteration to retinal structure after 20 days of light treatment on pigmented trout. B: Albino trout protected from intense light (0-day controls) also show normal retinal structure, except a lack of melanin. C: Light damage leads to loss of rod outer segments (ROS) but no change in the number of outer nuclear layer (ONL) nuclei in albino trout treated with light for 10 days, except in limited areas of the retina. Note darkly stained pyknotic nuclei. D: After 20 days of light damage in albino trout some areas of retina were damaged to an unexpected degree, in that rod nuclei had been lost from the ONL. Scale bar in A = 50 μm, and is representative for panels A–D.

assessments of light-induced damage reveal that the damage was greatest in the central retina, we quantified the number of PCNA-positive ONL and INL nuclei in a central area of retina. We also quantified PCNA labeling across the entire span of the retina (excluding the CGZ). PCNA labeling did not change significantly in pigmented trout regardless of light treatment (Figs. 3B & 3C). PCNA label had similar abundance in untreated albinos and pigmented trout (Figs. 3B & 3C). PCNA-positive INL nuclei in the

Table 1. *Quantification of retinal morphology in albino and normally pigmented trout during various light regimes*[a]

	ONL (nuclei/100 μm)	Number of ROS (per 100 μm)	ROS Length (μm)
	Series 1		
Days exposure after transfer from covered raceways to full daylight			
0: Normal	57.0 ± 4.44 (3)	30.2 ± 1.28 (3)	47.5 ± 6.70 (3)
0: Albino	54.6 ± 3.42 (4)	29.9 ± 2.14 (4)	2.5 ± 8.43 (4)
2: Albino	46.2 ± 0.59 (4)	7.35 ± —	4.89 ± 2.53 (4)
5: Albino	40.2 ± 4.87 (4)	—	—
10: Albino	25.4 ± 2.98 (4)	—	—
20: Albino	22.9 ± 7.82 (2)	2.80 ± 3.16 (2)	1.25 ± 1.76 (2)
20: Normal	54.7 ± 3.80 (3)	25.5 ± 1.68 (3)	40.8 ± 5.36 (3)
	Series 2		
Days after transfer from indoors to full daylight (not-pretreated)			
0: Normal	47.6 ± 5.86 (3)	34.0 ± 4.37 (3)	38.2 ± 2.71 (3)
0: Albino	51.5 ± 2.32 (4)	36.5 ± 2.12 (4)	37.5 ± 6.70 (4)
2: Albino	36.0 ± 4.00 (3)	8.22 ± 8.50 (3)	3.33 ± 2.20 (3)
5: Albino	34.9 ± 5.82 (5)	4.48 ± 4.70 (5)	2.30 ± 3.07 (5)
10: Albino	27.7 ± 7.45 (5)	1.88 ± 2.16 (5)	1.05 ± 1.22 (5)
10: Normal	53.5 ± 4.13 (5)	26.9 ± 10.9 (5)	25.4 ± 10.8 (5)
Days after transfer from shaded to full daylight (pretreated)			
0: Normal	55.0 ± 1.41 (2)	31.2 ± 1.06 (2)	34.0 ± 1.32 (2)
0: Albino	49.7 ± 1.06 (2)	28.0 ± — (2)	14.7 ± 5.74 (2)
2: Albino	46.2 ± 7.07 (2)	8.4 ± 5.09 (2)	3.01 ± 0.70 (2)
5: Albino	32.5 ± 8.26 (3)	1.25 ± 0.65 (3)	1.22 ± 1.12 (3)
10: Albino	31.7 ± 13.1 (2)	—	—
10: Normal	46.8 ± 2.58 (2)	30.6 ± 5.65 (2)	21.6 ± 16.4 (2)

[a]Outer nuclear layer (ONL) reflects number of rod nuclei, and rod outer segment (ROS). "—" Not measurable.

central retina (Fig. 3B) of albinos receiving 5 days of light treatment increased significantly as compared to untreated albinos ($P = 0.005$) or albinos exposed to 2 days of light ($P < 0.001$).

PCNA labeling of the ONL decreased at 10 days of light treatment, whether the quantification focused on the central retina (Fig. 3B) or the entire retina (Fig. 3C). The number of labeled nuclei in the entire ONL was significantly lower at 10 days compared to 5 days of light treatment ($P = 0.010$). The number of labeled nuclei at this location significantly increased between 10 and 20 days of light treatment ($P = 0.010$).

Pretreatment light regimes affect cellular responses

We repeated the above experiments using fish that were maintained in two different photic regimes to assay if this affected the cell death and proliferative responses. Our TUNEL-labeling experiment on retinae in Series 2 confirmed the Series 1 results. TUNEL-positive cells were qualitatively increased in each fish and were localized to the vitread portion of the ONL (i.e. they were rod nuclei). TUNEL-positive nuclei were significantly higher during light damage (Fig. 5A) in the retina for not-pretreated ($P < 0.002$) and pretreated albino trout ($P = 0.002$). TUNEL-positive nuclei were significantly lower in pretreated compared to the not-pretreated fish ($P < 0.005$). PCNA-positive ONL nuclei (Fig. 5B) were significantly less abundant after ten days of treatment in Series 2 ($P = 0.007$), very similar to Series 1; however, this was limited to the pretreated retina. Given that reductions in rod nuclei reached about the same final level at day 10 (Table 1) in both pretreated and not-pretreated groups, a lower death rate must have been coincident with a lower replacement rate. Indeed, PCNA-positive nuclei were significantly less abundant in the pretreated as compared to the not-pretreated fish ($P = 0.010$). Thus, pretreatment with moderate intensity daylight led to decreases in both TUNEL-positive and PCNA-positive labeling of nuclei in the ONL during subsequent exposure to full daylight, such that the decline in number of rod nuclei were about the same as in the not-pretreated group.

Rod opsin localization as an index of pretreatment effect

We examined the immunohistochemical localization of rod opsin during light damage as an additional index to measure the effect of pretreatment photic conditions. Previous work has demonstrated that opsin mislocalization increases during light damage in teleosts (Vihtelic & Hyde, 2000). This was not completed on fish from Series 1 due to lack of materials. In normally pigmented fish, our immunohistochemistry revealed the expected localization of the label to the inner and outer segments of rods and one member of the double cones (Fig. 2C). We observed rod opsin expression to be mislocalized in ONL of light-damaged albinos (Fig 2D). The mislocalization of rod opsin (Fig. 4C) was significantly higher in the light-damaged central retinae of pretreated ($P < 0.002$) and not-pretreated fish ($P < 0.015$) when compared to controls. There was negligible mislocalization in the peripheral retina. The mislocalization of rod opsin was also significantly higher in the pretreated albinos than in the not-pretreated albinos ($P < 0.007$). A parallel to this was the observation of several rod opsin immunoreactive cells in the INL: mislocalization of rod opsin in the INL was observed in the pretreated albinos (Fig. 2D), whereas no such INL mislocalization was observed in the not-pretreated group. The ONL and INL rod opsin labeling appears to represent mislocalized expression in rods, not double cones, (i.e. it repre-

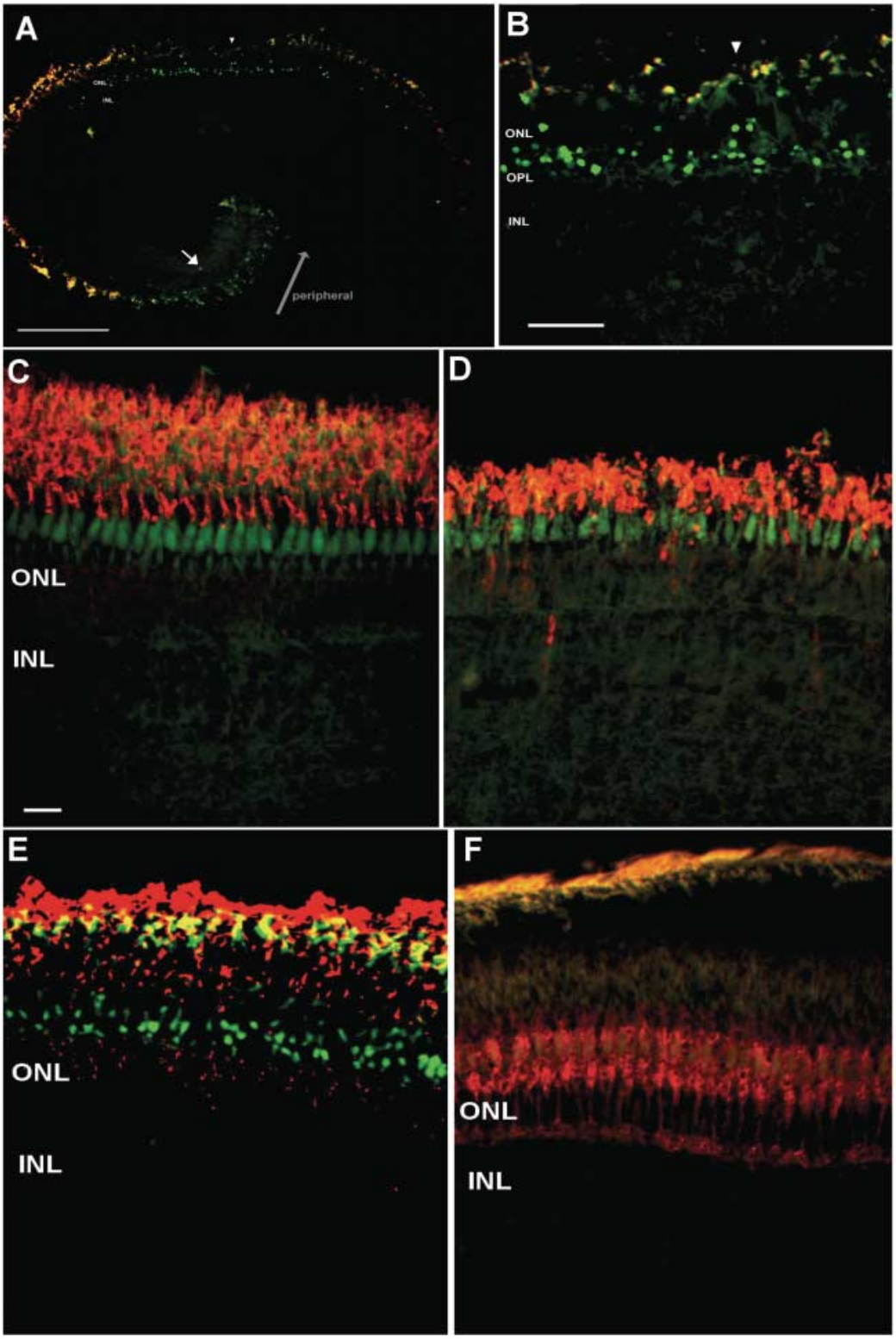

Fig. 2. Immunohistochemistry and TUNEL labeling during light damage. A & B: TUNEL labeling (green fluorescent signal) was localized to the central retina and the outer nuclear layer in light-damaged albino trout (these examples are from Series 1, 10 days treatment). Arrowhead in A indicates location of panel B. Grey arrow indicates peripheral retina (CGZ). White arrow points to a rare TUNEL label in the peripheral retina. Red autofluorescence is also visible. C: In normally pigmented fish and undamaged albinos, immunohistochemistry for rod opsin revealed the expected localization of the label (red fluorescent signal) to the inner and outer segments of rods and one member of the double cones (bar = 25 μm). Green autofluorescence represents double cone inner segments. D: Rod opsin expression was reduced from the rod outer segments (ROS), and mislocalized in both the outer and inner nuclear layers (ONL & INL) of light-damaged albinos. E: Similar mislocalization was apparent for zpr-3 immunoreactivity (red fluorescent signal) that labels rods (and is not normally in the ONL). F: No such mislocalization was apparent for zpr-1 immunoreactivity (red fluorescent signal) that labels double cones. Scale bar in A = 100 μm; in B = 25 μm. Scale bar in C is representative for panels C–F = 25 μm.

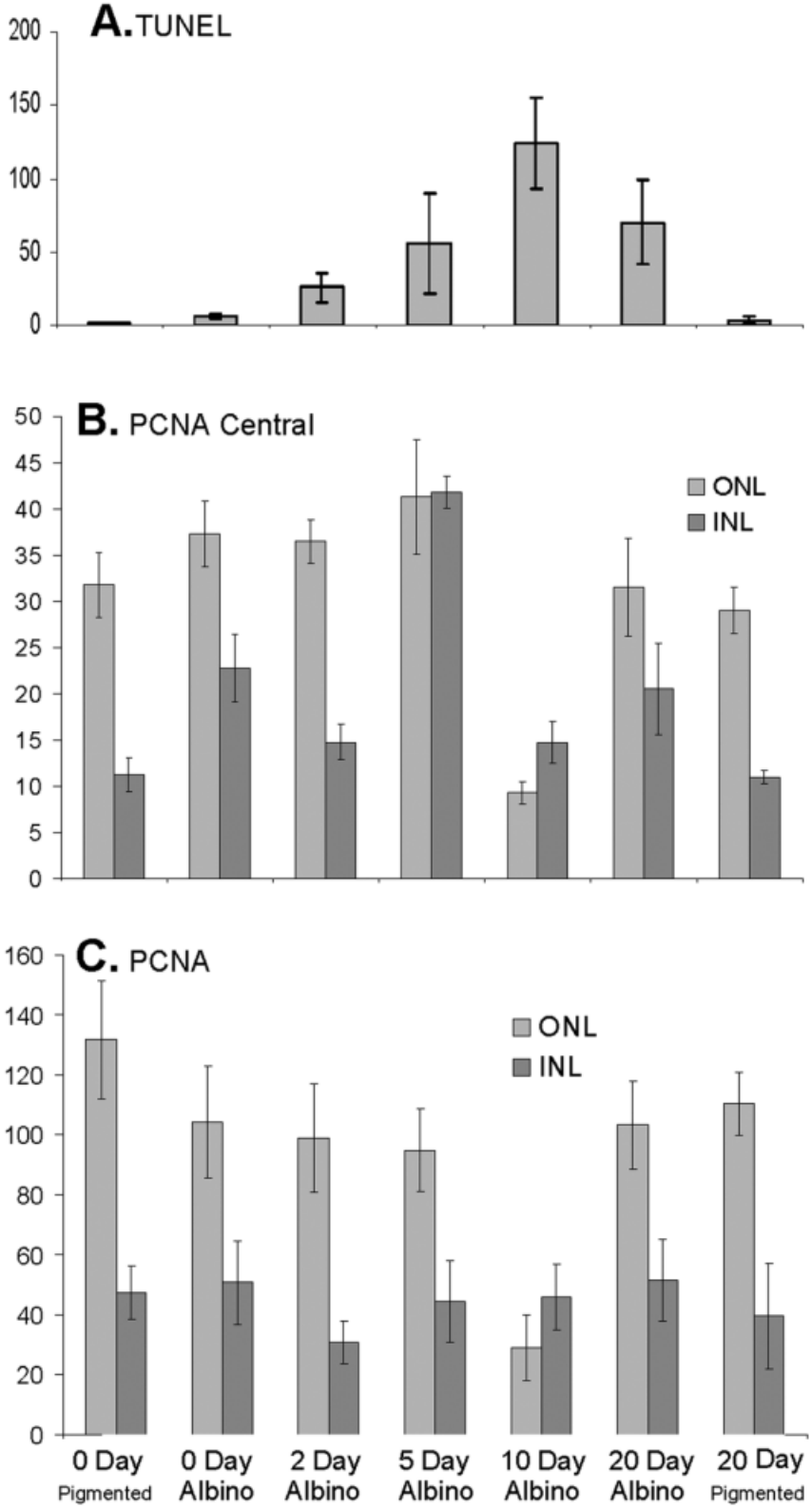

Fig. 3. Light modulates cell death and proliferation in albino trout (Series 1 experiment). TUNEL and proliferating cell nuclear antigen (PCNA) labeling, indicating cell death and proliferation, respectively, quantified in retinae of albino and normally pigmented rainbow trout treated with various durations of light. A: Quantification of TUNEL labeling in rod nuclei. No significant differences were measured for a given location; however, when the total labels were summed for a given individual there was a significant difference between 0 and 10 days of light damage in albino trout. Normally pigmented trout showed no such increase regardless of light treatment. B: Quantification of PCNA labels in outer and inner nuclear layers (ONL & INL) in the central retina. One field of view (FOV, approximately 300 μm) was quantified per section. Among albino trout the number of labels significantly increased in the INL when 5 days treatment was compared to earlier time-points. Labels in the ONL decreased significantly at 10 days compared to earlier time-points, and significantly increased between 10 and 20 days. C: Quantification of PCNA labeling in the ONL and INL of the entire retinal section (excluding the CGZ). In albino trout, PCNA labeling in the ONL significantly decreased at 10 days compared to all other time-points.

sents RH1, not RH2 immunoreactivity) because we noted similar mislocalization of zpr-3 immunoreactivity (Fig. 2E). We also believe that the mislocalized rod opsin expression does not represent RH2 opsin because we observed no mislocalization of zpr-1 immunoreactivity (neither to the cytoplasm nor to the INL) that labels double cones (Figs. 2E & 2F). The zpr-1 labeling confirms the observation from semithin sections that cones are not damaged substantially during these light treatments.

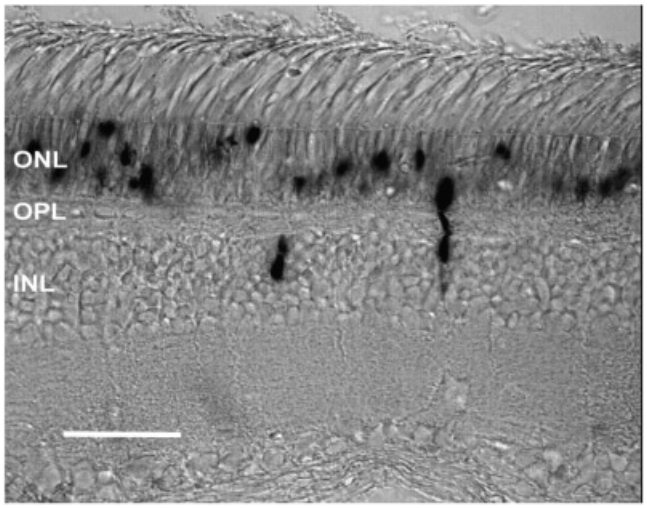

Fig. 4. Immunohistochemistry for proliferating cell nuclear antigen (PCNA) on radial retinal section of albino rainbow trout. Scale bar = 50 μm.

Discussion

Previous investigations of light damage in albino rainbow trout retinae demonstrated that despite rod outer segment (ROS) degradation, there was no decrease in the number of ONL nuclei (Allen & Hallows, 1997; Allen et al., 1999, 2001). This result distinctly contrasts other teleost and rodent models, wherein insults that induce ROS loss also lead to loss of the rod and cone photoreceptor (ONL) nuclei (Marotte et al., 1979; Penn, 1985; Raymond et al., 1988*a*; Allen et al., 1999) through apoptosis (Shahinfar et al., 1991; Li et al., 1996; Vihtelic & Hyde, 2000). Two mechanisms for these observations on albino trout were considered (Allen & Hallows, 1997): (1) that the rod nuclei were protected from death; and (2) rods were dying and being replaced by proliferating cells.

Proliferation and photoreceptor regeneration may occur in rainbow trout retina at rates that support rod replacement. Ultraviolet-sensitive (UVS) cones reappear in the trout retina during natural ontogeny (Beaudet et al., 1997; Hawryshyn et al., 2003) by regenerating from retinal progenitor cells (Allison et al., 2005). High levels of cell proliferation have been identified in rainbow trout retinae (Julian et al., 1998; Olson et al., 1999) that can be modulated by surgical injury (Faillace et al., 2002). Indeed proliferation was up-regulated, following light-induced apoptosis, in similar experiments using albino zebrafish (Vihtelic & Hyde, 2000). However, the data from zebrafish do not speak directly to

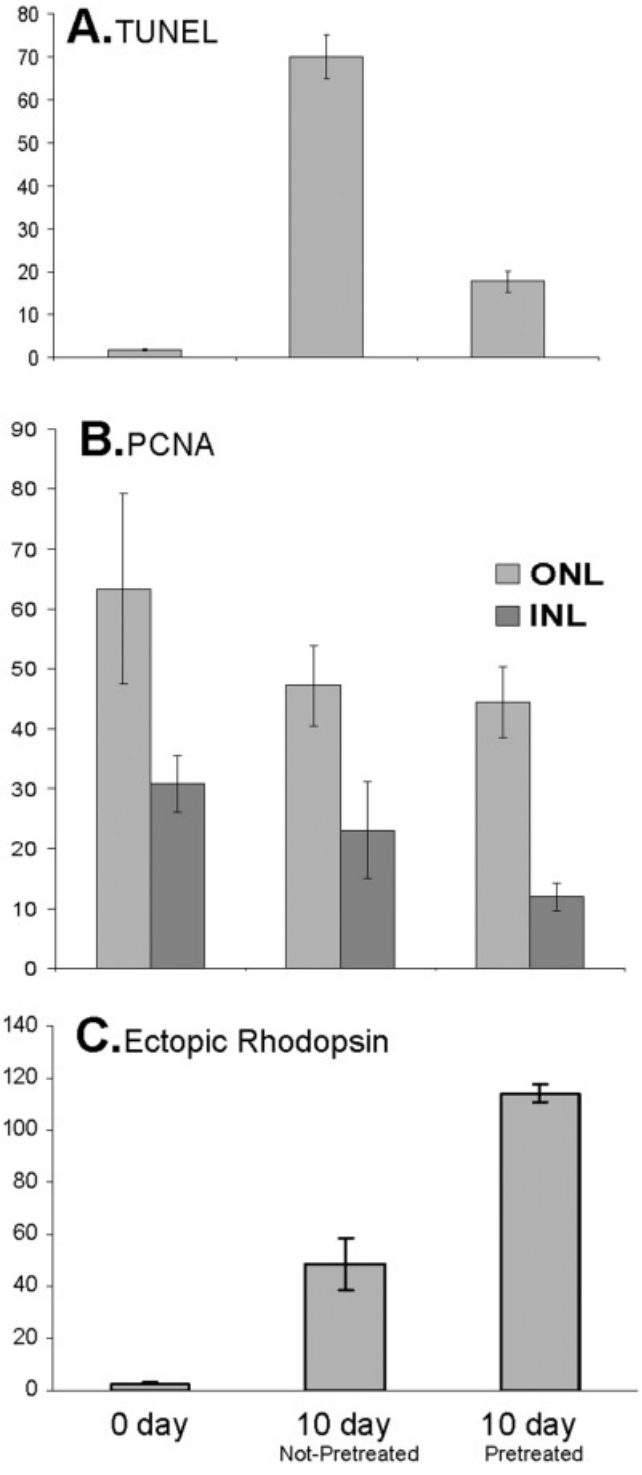

Fig. 5. Photic history modulates the effects of damaging light in albino trout (Series 2 experiment). Details similar to Fig. 3, but fish were either maintained in dim light (not-pretreated) or exposed to moderately intense light (pretreated) prior to exposure to high-intensity damaging light. A: TUNEL labeling indicates rod cell death was significantly increased after 10 days of damaging light in albino trout that were not-pretreated as compared to fish not exposed to damaging light (0 days) and compared to albinos that were pretreated with moderate light. B: Proliferating cell nuclear antigen (PCNA) labeling, indicating proliferation, in the outer and inner nuclear layers (ONL & INL) of albino trout retina. After exposure to 10 days of damaging light, proliferation was significantly decreased in the INL of pretreated albino trout as compared to unexposed (0 day) albino trout and compared to albino trout that were not-pretreated. C: Immunohistochemical detection of rod opsin expression in light-damaged albino trout revealed ectopic, disorganized expression amongst the rod nuclei (see Fig. 2). The occurrence of this was significantly higher in the retina from all fish treated after 10 days of damaging light as compared to unexposed (0 day) fish. The number of occurrences of ectopic rod opsin was modulated by photic history, and was significantly different amongst pretreated and not-pretreated albinos in all but the ventral retina. Legend as per panel A.

the above hypotheses regarding rainbow trout. In zebrafish, light induced a substantial loss of ONL nuclei and led to cone cell death (Vihtelic & Hyde, 2000); therefore the mechanisms that maintain the number of ONL nuclei in trout remain unknown.

Our results demonstrate that cell death occurs in rods of albino trout exposed to damaging levels of light. Observations during both experiments (Series 1 and 2) demonstrated a qualitative increase in TUNEL-positive rod nuclei in each of the 20 fish receiving light damage. Both TUNEL-positive and pyknotic nuclei were located in the vitread portion of the ONL, and were observed rarely in the peripheral regions of the retina, where light damage is less pronounced (current study, Allen et al., 2001). In Series 1, TUNEL label was increased at all time-points, including a significant increase at 10 days of light damage. In Series 2, TUNEL label was significantly increased in fish that were either pretreated or not-pretreated with light. Thus, three separate trials over 2 years demonstrated a significant increase in TUNEL labeling in rod nuclei during 10 days of damaging light treatment.

The number of ONL nuclei did not decrease within the first 10 days of light damage. This constant number of ONL nuclei was observed, except in limited portions of the central retina, despite increases in pyknotic and TUNEL-positive rod nuclei, and a substantial loss of ROS. We observed no increase in TUNEL labeling nor ROS loss in normally pigmented trout. Taken together, these data confirm the hypothesis that rods are dying and being replaced by the progeny of proliferating cells present in the retina.

Consistent with the above conclusion, PCNA labeling demonstrated that light can modulate proliferation in albino fish. Light had no effect on PCNA in pigmented fish at the time-points tested. PCNA labeling in the INL doubled (a significantly increase) at 5 days of damaging light treatment in the central retina. This increase in proliferation occurred at a time when rods were dying (at 2 & 5 days) but when the number of rods was not decreasing. The result is consistent with data (Julian, 1997, also described in Faillace et al., 2002) that showed increases in PCNA label in the same brood stock of albino trout as in the current work, and using previously described light treatment regimes (Allen & Hallows, 1997; Allen et al., 1999, 2001). A similar increase in PINCs has been observed previously in trout after surgical damage (Faillace et al., 2002) and zebrafish following light damage (Vihtelic & Hyde, 2000).

When the entire span of the retina was considered (excluding the CGZ) significant decreases in PCNA labeling were measured after 10 days of light damage (Fig. 3B). This was coincident with the peak of TUNEL labeling, and could represent a level of damage that includes progenitors. Such speculation would require the effect to be transient and include repair mechanisms, because the PCNA labeling subsequently increased in a significant manner. This increase in proliferation at 20 days could also be viewed as a response to the large loss of rod nuclei that occurred by day 10.

Although significant increases in PCNA labeling were observed, the effect of light on proliferation in albino trout did not always mirror the dramatic increase in cell death. The methods we used allow only a "snap-shot" of the proliferative events that occurred. For example, proliferation in the trout retina varies in a circadian rhythm (Julian et al., 1998), and an increase in proliferative rate may be more or less apparent during another portion of the day. Further experiments should incorporate considerations of circadian rhythms in proliferation (Julian et al., 1998) and utilize cell fate mapping to demonstrate that the rate of rod addition is modulated by light dosage. Similarly, our hypothesis predicted an increase in ONL proliferation that was not apparent following the increase in INL proliferation. Similar observations of increased INL, but not ONL, proliferation were made by Julian (1997) in a similar experiment. It may be that our infrequent sampling regime missed events in the ONL response. Alternatively, it cannot be excluded that the INL progenitors are directly replacing lost rods without a substantial increase in ONL proliferation. Although current models (Raymond et al., 1988*b*; Raymond & Hitchcock, 2000; Otteson et al., 2001) emphasize PINCs as replacing ONL progenitors, no data exist to eliminate INL progenitors directly replacing lost rods in the absence of an amplifying proliferative event in the ONL.

Some rod nuclei were lost in albino trout by 20 days of light exposure (Series 1), although cones remained intact. This result differed from previous examinations of albino trout raised in full sunlight which had shown no decrease in the number of ONL nuclei at the time of sampling (Allen & Hallows, 1997; Allen et al., 1999, 2001). The result has several interesting implications: (1) it is a further demonstration that some rods in the central retina of albino trout can be eliminated by the current light regime, that is, the treatments used led to rod cell death; (2) increases in proliferative events were insufficient to replace all of the dying rods in some central locations; (3) maintaining fish in moderately high intensity light has a protective effect and/or enhances the regenerative capacity of the retina.

The latter implication was supported by experiments in Series 2. Both PCNA and TUNEL labeling were significantly different between pretreated and not-pretreated groups. We also observed disorganized and ectopic rod opsin expression in the ONL and INL, reminiscent of ectopic opsin observed in light-damaged zebrafish (Vihtelic & Hyde, 2000). The ectopic rod opsin expression may represent degenerating rods and/or new rods that are being generated to replace rods and maintain the number of ONL nuclei. Regardless, the TUNEL, PCNA, and rod opsin data all support the hypothesis that photic history of the fish can modulate the retina's susceptibility to damage and/or its regenerative capacity. The TUNEL labeling supports a protective effect of pretreatment, as there was significantly less rod apoptosis in the pretreated group. Furthermore, the PCNA data are not consistent with the pretreated fish having increased regenerative capacity. This represents the first report we are aware of where pretreatment with light modulated the effects of light damage in a nonmammalian vertebrate.

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