Degeneration and Regeneration of Ultraviolet Cone Photoreceptors during Development in Rainbow Trout

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ABSTRACT
Ultraviolet-sensitive (UVS) cones disappear from the retina of salmonid fishes during a metamorphosis that prepares them for deeper/marine waters. UVS cones subsequently reappear in the retina near sexual maturation and the return migration to natal streams. Cellular mechanisms of this UVS cone ontogeny were investigated using electroretinograms, in situ hybridization, and immunohistochemistry against opsins during and after thyroid hormone (TH) treatments of rainbow trout (Oncorhynchus mykiss). Increasing TH levels led to UVS cone degeneration. Labeling demonstrated that UVS cone degeneration occurs via programmed cell death and caspase inhibitors can inhibit this death. After the cessation of TH treatment, UVS cones regenerated in the retina. Bromodeoxyuridine (BrdU) was applied after the termination of TH treatment and was detected in the nuclei of cells expressing UVS opsin. BrdU was found in UVS cones but not other cone types. The most parsimonious explanation for the data is that UVS cones degenerated and UVS cones were regenerated from intrinsic retinal progenitor cells. Regenerating UVS cones were functionally integrated such that they were able to elicit electrical responses from second-order neurons. This is the first report of cones regenerating during natural development. Both the death and regeneration of cones in retinae represent novel mechanisms for tuning visual systems to new visual tasks or environments. J. Comp. Neurol. 499:702–715, 2006.

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Salmonid fishes represent effective model organisms for the study of retinal development. Observations over the past century have noted a loss of ultraviolet-sensitive (UVS) cones from salmonid retinae (Furst, 1904; Lyall, 1957; Bowmaker and Kunz, 1987; Hawryshyn et al., 1989; Kunz et al., 1994; Allison et al., 2003). At least in some salmonids, such as our model organism, O. mykiss, UVS cone disappearance may be associated with migration to deeper and/or marine waters (Hawryshyn et al., 1989; Beaudet et al., 1993; Novales Flamarique and Hawryshyn, 1996; Novales Flamarique, 2000; Deutschlander et al., 2001; Allison et al., 2003). The loss of UVS cones in rainbow trout is not complete, as a population remains in the dorso-temporal retina (Allison et al., 2003; Hawryshyn et al., 2003a). Notably, the UVS cones appear to regenerate in salmonid retinae near the time of sexual maturation and return migration to natal streams (Beaudet et al., 1997; Novales Flamarique, 2000). We are interested in the cellular mechanisms of UVS cone disappearance and reappearance. Understanding this process is relevant to how visual systems are tuned to the environment, as has been the case in studies of other migrating fishes (Sandy and Blaxter, 1980; Pankhurst, 1984; Evans and Fernald,
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1990; Fernald, 1990; Evans and Fernald, 1993; Helvik et al., 2001a,b; Mader and Cameron, 2004). Importantly, UVS cone death and regeneration could serve as an informative model of cellular or molecular events in vertebrate retinal development and repair.

Salmonids prepare for their migration to deeper waters through a metamorphic transition termed “smoltification.” Smoltification of O. mykiss can be induced with thyroid hormone (TH) treatment (Hoar, 1988), comparable to methods that induce tadpole metamorphosis. Previous reports have noted that TH treatment leads to a loss of UVS cones (Browman and Hawryshyn, 1992, 1994; Deutschlander et al., 2001; Hawryshyn et al., 2003a). Cessation of TH treatment results in UVS cones returning to the retina (Browman and Hawryshyn, 1994). TH treatments provide a controlled onset time for events of interest.

Here we used TH to investigate if UVS cone disappearance and reappearance occur through apoptosis and proliferation, respectively. These hypotheses have emerged from a wealth of data regarding retinal regeneration in teleosts following experimentally induced damage (Raymond et al., 1988; Raymond, 1991; Raymond and Hitchcock, 1997, 2000, 2004; Easter and Hitchcock, 2000; Wu et al., 2001; Otteson and Hitchcock, 2003). Following damage, proliferative cells in teleost retinae can regenerate all cell types including photoreceptors. Our experiments ask if similar proliferative events can replace UVS cones during the natural life history of salmonids.

Proliferating cells in the retinae of postembryonic teleosts can be divided into several populations. Proliferation occurs at the retinal margins (Johns, 1977), termed the circumferential germinal zone (CGZ); here, new retinna is added in a manner analogous to adding growth rings on a tree. The CGZ gives rise to new retina that differentiates in a manner recapitulating embryonic retinal development (Perron et al., 1998; Olson et al., 1999; Otteson and Hitchcock, 2003). Other populations of proliferating retinal cells are observed in the central, mature retina. These include cells in the outer nuclear layer (ONL), often called “rod progenitors” since they are thought to exclusively produce rod photoreceptors during natural development (Johns and Fernald, 1981; Otteson and Hitchcock, 2003). Rod progenitors are replenished by PINCs, proliferating inner nuclear layer (INL) cells (Julian et al., 1998). Proliferation of PINCs, which may involve the support and/or dedifferentiation of Muller glia, is upregulated after experimental induced damage (Vihtelic and Hyde, 2000; Faillace et al., 2002; Allison et al., 2006a), and is thought to be the source of regenerating cones that appear after damage of goldfish retina (Wu et al., 2001). Each population of proliferating cells is robust in the quickly growing retina of postembryonic salmonids (Julian et al., 1998; Olson et al., 1999, 2000; Faillace et al., 2002; Candal et al., 2005; Allison et al., 2006a).

Our data support the hypotheses that UVS cones die by programmed cell death (PCD/apoptosis) and subsequently regenerate from proliferating cells of the central retina. We show that the regenerated UVS cones are functionally integrated into the retina such that they are able to trigger electrical responses from second-order neurons.

MATERIALS AND METHODS

Experimental animals and treatments

Rainbow trout (O. mykiss) (Walbaum, 1792) parr were acquired from the Vancouver Island Trout Hatchery (Duncan, BC, Canada) (5–12 g, 6–11 cm fork length, i.e., measured from the tip of the snout to the fork of the tail; note that smoltification occurs at ~25–30 g (Hawryshyn et al., 1989)). Smoltification is a term often reserved for ocean-going salmonids, which alter their physiology to osmoregulate during migration to marine environments. The developmental changes that occur in rainbow trout parr as they transition to smolts may not meet some definitions of smoltification; however, they do mimic many of the same physiological changes. Importantly, visual system changes at this time of development appear to be similar to those of ocean-going populations. Fish were maintained in 350-L flow-through tanks at the University of Victoria, with a mean water temperature of 15 ± 1°C under a 12L:12D photoperiod provided by standard fluorescent lights (color temperature 3500°K). TH treatment was performed under the same conditions, except that fish were transferred to standing water in 30-L aquaria; TH treatment was completed by adding 300 μg/L L-thyroxine (Sigma, St. Louis, MO) in 1.5 mL of 0.1 N NaOH to the water, changed daily. Fish maintained as controls for TH treatment were held in identical conditions with only vehicle (NaOH) added to the water, and were indistinguishable in the characters we measured from trout maintained in flow-through tanks described above. Fish were sampled after 4, 14, and 42 days of TH treatment. During TH treatment the entire volume of water was changed daily, whereas only half of the treatment water was changed in previous experiments (Browman and Hawryshyn, 1992, 1994). Care of fish and all procedures were in accordance with and approved by the University of Victoria Animal Care Committee under the auspices of the Canadian Council for Animal Care.

PCD of UVS cones during TH treatment

To determine if UVS cone disappearance involved PCD, a caspase inhibitor (blocks cell death) was applied during TH treatment. The distribution of UVS cones was assessed in retinnae with and without caspase inhibition. Caspase inhibitors were expected to block UVS cone disappearance if the disappearance involved PCD. Fish that had received 2 days of TH treatment were given an intracocular injection of the general caspase inhibitor zVAD-fmk (Calbiochem, San Diego, CA) dissolved in 0.1% dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS), pH 7.4, to produce a final concentration of ~300 μM. This caspase inhibitor has been used at similar concentrations to block cell death in zebrafish (Danio rerio) (Williams et al., 2000; Williams and Holder, 2000; Dong et al., 2002; Sanders and Whitlock, 2003). Atlantic salmon (salmo salar) (Fladmark et al., 1998), and rainbow trout (Moyes et al., 2002). These individuals, along with sham-injected negative controls, were maintained on TH treatment and sacrificed 2 days later. The timing of caspase inhibitor application was deemed appropriate because TH led to the disappearance of UVS cones within 4 days of treatment. Subsequent quantitative polymerase chain reaction (PCR) analyses showed that UVS opsin messenger RNA (mRNA) was measurably decreased within 2 days of TH treatment (Veldhoen et al., 2006).
from progenitor cells, bromodeoxyuridine (BrdU) was applied to fish that had received 42 days of TH treatment. This duration of TH treatment was sufficient to induce the loss of UV cones and UV sensitivity in a variety of experiments (although we can also observe the effects in a much shorter time period). BrdU treatments in preliminary experiments were performed by intracocular injection of 10 μL of 0.1 M 5-bromo-2’-deoxyuridine (Sigma, St. Louis, MO). Alternatively, fish were maintained in 1 L of water with 10 μM BrdU and clinoptilolite (a mineral used in aquaculture to absorb ammonia) for 48 hours. To sample the retinae, fish were deeply anesthetized in 300 mg/L MS-222 (tricaine methanesulphonate, Crescent Research Chemicals, Phoenix, AZ) until euthanized and retinae were fixed in 4% paraformaldehyde.

**Electroretinograms (ERGs)**

ERGs were performed according to previously published protocols (Allison et al., 2003). The diameter of the stimulus beam was set to illuminate the full pupil, thus primarily stimulating the central retina. Additional details regarding UV adaptation experiments in our ERG protocol have been presented elsewhere (Hawryshyn et al., 2003b). ERGs were performed on a separate group of fish than the labeling protocols but received identical TH treatment.

**Immunohistochemistry and in situ hybridization**

To label UV cones, polyclonal antisera were raised against the 20 N-terminal amino acids predicted from *O. mykiss* UV opsins cDNA (access. no. AF425074) (Allison et al., 2003; Dann et al., 2004a). A cysteine residue was included at the carboxyl terminus of the synthesized peptide (synthesized by UVic-Genome BC Proteomics Centre, Victoria, BC, Canada) to allow conjugation to sulfhydryl-reactive inject maleimide-activated mariculture keyhole limpet hemocyanin (Pierce, Rockford, IL). Conjugated peptide was purified by dialysis and periodically injected with Freund’s adjuvant (Gibco-BRL, Gaithersburg, MD) into four BALB/c mice. The same antigen was used to produce polyclonal antiserum in a female Fisher 344 rat. These antisera were prepared by Immuno-Precise Antibodies (Victoria, BC, Canada).

Western blots of retinal homogenates were performed using 1:1,000 UVS opsins antisera raised in rat and 1:3,000 goat antirat secondary antibody conjugated to alkaline phosphatase (Vector Labs, Burlingame, CA). Serum was diluted in 3% nonfat milk (Becton Dickinson, Sparks, MD). Standard protocols were utilized as reported previously (Dann et al., 2004b). However, boiling the homogenate was omitted, PMSE was the sole protease inhibitor, and 1% octyl β-D-glucopyranoside (Sigma) was used as the detergent in the homogenization buffer.

Immunohistochemistry was carried out using standard protocols on retinal wholemounts or cryosections (10 μm). For BrdU immunohistochemistry, slides were incubated for 30 minutes in 2N HCl, washed in PBS with 0.1% Tween (PTW), fixed in 4% paraformaldehyde, and incubated with proteinase K for 10 minutes at 37°C (Allison et al., 2003). All antibodies were diluted in PTW with 2% heat-inactivated horse serum (Sigma). Primary antibodies: UVS opsin antisera raised in mouse, 1:5,000. UVS opsin antisera raised in rat, 1:100. SWS2 opsin antisera (Veldhoen et al., 1999), 1:500.
RESULTS

Characterization of antiserum against UVS opsin

Antiserum against a synthetic peptide representing the N-terminus of *O. mykiss* UVS opsin was developed. This antiserum was used to label UVS cones in *O. mykiss* retinas (see Fig. 2A–D for assessment of antibody specificity). Western blots of trout retina (not treated with TH) retinal homogenates labeled a single band (Fig. 2A), ~34 kDa, being the predicted molecular mass for UVS opsin. The adjacent lanes containing the same retinal homogenate showed no such band if the primary antibody was not included or if the antiserum was incubated with the cognate peptide before application (data not shown). With immunohistochemistry on sectioned or wholemount retinas, antiserum labeled approximately half the population of single cones, with no evidence of double cone labeling (Figs. 2B–D, 3A–C). Opsiop antiserum labeling was restricted to the outer segments of the single cones (Fig. 2D). Negative controls for all immunohistochemistry included omitting the primary antibody, which eliminated labeling. A preadsorption control of our UVS antiserum was performed by incubating the primary antibody with 10 μmol of peptide in 100 μL of diluted antiserum; this also eliminated labeling. Double-labeling with either of these sera and anti-SWS2 serum indicated that different populations of single cones were labeled. The UVS- and SWS-positive single cones occurred in equal numbers and in the expected repeating pattern of UV and SWS cone location (Fig. 2B). It is noteworthy that this antiserum against SWS2 opsin was previously colocalized to cells expressing SWS2 mRNA (Allison et al., 2003).

UVS cone PCD

Retinal maps of UVS cone distribution based on UVS opsin antiserum labeling showed that UVS cones disappeared from the retina when trout were treated with TH (Fig. 3). This result was confirmed using in situ hybridization labeling of UVS opsin mRNA (Fig. 3). UVS opsin in situ hybridization was quantified by counting UVS and SWS opsin labels on adjacent sections to control for tissue stretching and to ensure that opsin mRNA remained detectable (Allison et al., 2003).

Previous data from electron-microscopic examination of Atlantic salmon (*S. salar*) indicated that UVS cones were generated in the CGZ of large fish and were immediately removed from the retina by apoptosis (Kunz et al., 1994). This process created mature retina that lacked UVS cones. It has been demonstrated that similar populations...
of UVS cones are generated and immediately removed from most of the peripheral retina of *O. mykiss* (large fish, not treated with TH, where UVS cones have disappeared from most of the retina during natural development). However, UVS cones do not disappear from the dorso-temporal smolt retina (Allison et al., 2003). This regional difference (Fig. 1) was exploited to examine if *O. mykiss* UVS cones disappear by PCD similar to regional difference (Fig. 1) was exploited to examine if PCD was occurring. PCD was inhibited using a caspase inhibitor. Mapping the distribution of UVS cones using in situ hybridization demonstrated that fewer UVS cones disappeared when TH and caspase inhibitor were applied together, as compared to when TH was applied alone (compare Fig. 3H, 3G). This experiment represents within-fish data, and was repeated in three individuals. In all three cases TH eliminated UVS cones from the retina (Fig. 3G), whereas the disappearance of UVS cones was notably reduced by caspase inhibitor (Fig. 3H). Negative controls for this experiment included sham injection (i.e., no caspase inhibitor, physiological saline, and DMSO; see Materials and Methods) into the contralateral eye. Other negative controls included fish where both eyes received sham injection. Negative controls were indistinguishable from the results reported in Figure 3G, where UVS cones were absent from the central retina due to the effects of TH.

**Intrinsic retinal progenitor cells are the source of UVS cones**

Several weeks after termination of TH treatment, UVS cones reappeared in much of the retina, often at densities similar to untreated fish (Fig. 4). Because there was an almost complete loss of UVS cones during TH treatment, we concluded that these UVS cones were not residual but reappeared in the retina after cessation of TH treatment. This provided a controlled onset time for UVS cone regeneration, which we considered prerequisite to any study of the mechanisms. For example, if our studies were limited to natural development only, the appropriate timing of BrdU application would have been difficult to estimate, and impractical to replicate, due to expected variation in the growth trajectories of salmonids. Most importantly, a negative result using BrdU in such experiments would have been difficult to interpret with certainty.

In several fish BrdU was applied during the days following the termination of TH treatment. Double-labeling for UVS opsin and BrdU was completed on retinal sections (Fig. 4F,G). Localization of BrdU demonstrated that proliferating cells in the central retina differentiate into cones that express UVS (i.e., SWS1) opsin mRNA and protein. Thus, the source of reappearing UVS cones was proliferating cells that had proliferated following our application of BrdU. The time between the termination of TH treatment and examination of regenerated cones (42 days) was sufficient for stem cells to differentiate into cones, which can occur in less than 20 days in goldfish (Wu et al., 2001). Fourteen cells were identified with UVS opsin mRNA colocализed with BrdU within the central region of the retina in five fish. No such colocalization was observed with SWS2 opsin mRNA, nor was BrdU found in the nuclei of double cones.
After TH removal, UVS cones regenerated in the central retina, at considerable distances from the CGZ. The position that had been occupied by the CGZ at the time when TH treatment was terminated was approximated by the abundance of cells labeled by BrdU, in the vicinity of the new CGZ. This position was noted in each section and integrated into our maps of UVS cone distribution (green line in Fig. 4D). There was a sizeable distance (e.g., 1 cm) between UVS cones that had regenerated in the central retina and the CGZ. This indicates that proliferating cells of the central retina were most likely the source of the reappearing UVS cones.

**Functional integration of UVS cones**

ERGs demonstrated that UV sensitivity disappeared after 14 days of TH treatment (Fig. 5). The resulting spectral sensitivity was similar to that measured in smolts (Allison et al., 2003). Forty-two days after cessation of TH treatment, UV sensitivity returned (Fig. 5C) to levels similar to that observed in untreated fish. The return of UV sensitivity represented an independent UVS cone mechanism as determined by repeating ERGs under different photic background conditions that used UV light to specifically light adapt (decrease the sensitivity of) the UVS cone mechanism (Fig. 5D shows a difference spectrum as a result of UV chromatic adaptation illustrating a strong UV component).

**DISCUSSION**

**Characterization of antiserum against UVS opsin**

An antiserum was developed to label UVS cones; the specificity of this label is discussed here. The UVS opsin antiserum labels only a single band in a Western blot of trout retinal homogenates. This band was not present in negative control experiments including omitting the serum and preadsorption control with the cognate peptide.

Cells labeled by UVS opsin antisera were identified as UVS cones based on their morphometry: UVS cones are known to be the accessory corner cones in rainbow trout retina (reviewed in Allison et al., 2003), thus, in cross-section the partitioning membrane of double cones points toward central single (SWS) cones, and the remaining single cones are UVS cones. In tangentially sectioned material, UVS opsin antiserum labeled only accessory corner cones (Fig. 2C; note that not all outer segments were captured in this plane of section). In radially sectioned material the row of labeled single cones was interspersed with double cones where the partitioning membrane was bisecting the double cone, indicating that the labeled single cones were in the accessory corner cone position (Fig. 2D). The UVS opsin antibody labeling was localized to the outer segments of single cones, a cellular compartment known to contain opsin protein. Control experiments for UVS opsin antiserum labeling consisted of omission of the serum, and a preadsorption control using the cognate peptide representing the UV opsin N-terminal. No labeling was observed in any such control experiments.

Double-labeling experiments also confirmed the specificity of UVS opsin antisera. The antisera labeled only single cones, never double cones that were identified by zpr1 immunolabeling and by morphology. Two populations of single cones are present in rainbow trout retina, expressing either UVS or SWS opsin as defined by microspectrophotometry and in situ hybridization (Hawryshyn and Harosi, 1994; Hawryshyn et al., 2001, 2003a; Allison et al., 2003). To confirm that our antiserum was labeling UVS cones, it was used to double-label retinae with an SWS opsin antiserum (Fig. 2B). Previous work confirmed the specificity of the SWS antiserum by localizing it to cells that express SWS2 opsin mRNA via in situ hybridization (Allison et al., 2003). The results demonstrate that UVS and SWS antisera each label half of the single cones and each occurs in the repeated, alternating, spatial pattern that is well established for all salmonid single cones (Bowmaker and Kunz, 1987; Hawryshyn et al., 1989; Beaudet et al., 1993; Kunz et al., 1994; Forsell et al., 2001; Allison et al., 2003). This pattern of alternating UVS and SWS cones was confirmed previously by in situ hybridization for their respective opsin mRNA on rainbow trout retina (Allison et al., 2003). Similarly, it has been established that zebrafish have two populations of single cones that express either UVS or SWS opsin and occur in a repeated alternating pattern as defined by microspectrophotometry, in situ hybridization, and immunohistochemistry (Nawrocki et al., 1995; Raymond et al., 1993, 1996; Vihtelic et al., 1998; Cameron, 2002; Allison et al., 2004; Raymond and Barthel, 2004). UVS opsin antisera applied in combination with zebrafish SWS opsin antiserum (Vihtelic et al., 1999) labeled alternating single cones of adult zebrafish retina (data not shown). Labeling with our UVS opsin antiserum has also been colocalized to zebrafish UVS cones that express green fluorescent protein under the control of the zebrafish SWS1 opsin promoter (Takechi et al., 2003, data not shown).

**UVS cone degeneration**

Mapping UVS cone densities using immunohistochemistry and in situ hybridization demonstrated that TH led to a disappearance of UVS cones. The data are consistent with previous findings (Browman and Hawryshyn, 1992, 1994; Deutschlander et al., 2001) and our ERG results (below). TH treatment of *O. mykiss reduced the distribution of UVS cones in a manner very similar to natural development (Allison et al., 2003; Hawryshyn et al., 2003a). This resulted in a complete loss of UVS cones from the central retina, similar to natural development of other salmonid species, including sockeye salmon *O. nerka* (Navales Flamarique, 2000) and Atlantic salmon (Kunz et al., 1994). The distribution of UVS cones was assessed in trout using these methods after 4, 14, and 42 days of TH treatment. No difference was detected between these retinæ, consistent with our observations by quantitative PCR that UVS opsin was reduced within 2 days of TH treatment and remains reduced at later timepoints examined, i.e., 9 and 22 days of treatment (Veldhoven et al., 2006). This is also consistent with our observations that 9 days of TH treatment can induce changes in opsin protein levels as measured by isotope-coded affinity tag labeling followed by tandem mass spectroscopy (Allison et al., 2006b).

Evidence presented in this study supports the hypothesis that the disappearance of salmonid UVS cones occurs through PCD. The data demonstrate that PCD of UVS cones occurs in naturally developing retina. The proliferating CGZ gives rise to UVS cones that are removed from the ventral retina immediately after they differentiate (Allison et al., 2003) in a “conveyor belt fashion” (Kunz et al., 1994). This contrasts dorsoventral areas of *O. mykiss*
Figure 3
retina, where UVS cones were not removed (Allison et al., 2003; see also Fig. 1). In this study, differences in TUNEL and Annexin V labeling between these regions demonstrated that UVS cones disappear by PCD. The generation and immediate death of UVS cones from the retina previously led us to hypothesize that their generation and differentiation was required to signal proper differentiation of adjacent photoreceptors or second-order neurons (Allison et al., 2003). This hypothesis remains untested.

TH treatment in experiments combined with caspase inhibitors also demonstrate loss of UVS cones by PCD. Whereas TH treatment led to the loss of UVS cones from the entire central retina, caspase inhibitor significantly limited UVS cone disappearance in the contralateral eye. Because caspase inhibitor blocks PCD and specifically blocked UVS cone disappearance, we conclude that UVS cones disappear by PCD. This caspase inhibitor has been used at similar concentrations to block cell death in zebrafish (Williams et al., 2000; Williams and Holder, 2000; Dong et al., 2002; Sanders and Whitlock, 2003), Atlantic salmon (Fladmark et al., 1998), and rainbow trout (Moyes et al., 2002). The conclusion that UVS cones disappear by PCD confirms a detailed electron micrographic study that examined UVS cone apoptosis in a salmonid from a more basal genus, Salmo salar (Kunz et al., 1994), and this conclusion is likely general to the Pacific salmonids (genus Oncorhynchus).

Recent publications also acknowledge that apoptosis is the primary mechanism of UVS cone loss in salmonids (Novales Flamarique, 2000, 2005; Cheng and Novales Flamarique, 2004), although they propose other events prior to UVS cone death that are not supported by the available data. These articles argue that all single cones in small salmonids express UVS (SWS1) opsin mRNA and subsequently all single cones express SWS2 opsin mRNA. Methodological details requisite for this interpretation were not presented, including hybridization stringencies and riboprobe lengths that impart labeling specificity. Importantly, the interpretation of other (microspectrophotometry) data in those studies required the assumption that recently produced opsin be confined to the base of the outer segment (Cheng and Novales Flamarique, 2004; Novales Flamarique, 2005). Although this is true in rods, cone opsin shows no such spatiotemporal pattern because it diffuses quickly throughout the topologically continuous cone outer segment (Young, 1969, 1971, 1976; Besharse, 1986; Eckmiller, 1987, 1993, 1997). Regardless, the conclusion that pink salmon O. gorbuscha single cones all express UVS opsin early in ontogeny, and subsequently all express SWS2 opsin (Cheng and Novales Flamarique, 2004; Novales Flamarique, 2005), is not true for rainbow trout (Allison et al., 2003; current work) or S. salar (Forsell et al., 2001) as determined by labeling cone-specific mRNA and protein. UVS and SWS opsin have been cloned from the retinal mRNA of pink salmon smolts (and other salmonid species, Dann et al., 2004a), indicating that UVS expression is not lost entirely in these species.

The TH-induced loss of UVS cones is similar to natural ontogeny of rainbow trout UVS cones (Allison et al., 2003). In natural ontogeny, a surge of serum TH at metamorphosis is coincident with UVS cone loss. In species where UVS cones do not disappear during natural ontogeny, such as zebrafish, TH treatment does not lead to loss of UV cones (as determined by opsin immunocytochemistry; W.T. Allison and C.W. Hawryshyn, unpubl. results) despite causing a shift in zebrafish visual pigments (Allison et al., 2004). We are interested in how TH signaling leads to death of a specific class of cone photoreceptor. It has been proposed that UVS cone death involves transcriptional regulation of the UVS opsin. Transcription factors NF-κB and c-jun bind the UVS opsin promoter, but not the other opsin promoters in O. mykiss retina (Dann et al., 2004b). These transcription factors are involved both in photoreceptor apoptosis (Hafezi et al., 1999; Krishnamoorthy et al., 1999; Wenzel et al., 2002) and in TH-regulated pathways (Perez et al., 1993; Lee et al., 2000) in other organisms. Our recent work supports TH having an effect on opsin mRNA abundance (Veldhoen et al., 2006), although a causal role for opsin abundance in cell fate remains untested.

**Regeneration of UVS cones**

When TH treatment was discontinued, the distribution of UVS cones returned to a state similar to that of untreated fish. BrdU was incorporated into regenerating UVS cones, but not into other cones, and only if BrdU was applied during the period when the UVS cones regenerated. The differentiation of proliferating retinal cells into
Figure 4
UVS cones was demonstrated at the levels of opsin mRNA and opsin protein expression. Thus, it was observed that: 1) BrdU was incorporated in UVS cones only during periods when UVS cones were reappearing, and is not from DNA repair; 2) BrdU appears exclusively in UVS cones, not other cones. It is unlikely that our analysis of BrdU-labeled cells detected cell types other than UVS cones, because we did not see these UVS cones in retinae where UVS cones had not regenerated (e.g., fish not treated with TH, or fish sacrificed before TH removal). Further, it is unlikely that the BrdU-labeled cells were rod photoreceptors, as new rods are known to move to the vitreal portion of the ONL within days of their birth (Mack and Fernald, 1995, 1997; Mack et al., 2003; Henderson and Fernald, 2004). Thus, the data demonstrate that proliferating cells were producing UVS cones.

The source of regenerating cells in damaged teleost retina is not the CGZ but the centrally located proliferating cells in the mature retina (Raymond et al., 1988; Raymond and Hitchcock, 1997; Wu et al., 2001; Otteson and Hitchcock, 2003). Proliferating cells move radially, within a few cell diameters, to their final location. Considering the sizeable distance (>0.5 cm) between the periphery and regenerated UVS cones, it is unlikely that the CGZ was the source of new UVS cones. Proliferating cells known to occur in the central, mature salmonid retina (Ahlbert, 1976; Julian et al., 1998; Faillace et al., 2002; Candal et al., 2005) are the source of regenerating UVS cones. It is not clear from our data if the source of the regenerating UVS cones is in the inner or outer nuclear layer.

Our conclusion that UVS cones are regenerated from retinal progenitor cells after cessation of TH treatment can probably be extended to unperturbed salmonid ontogeny. UVS cones return near the time of sexual maturity in *Oncorhynchus* species (Beaudet et al., 1997; Novales Flamarique, 2000) and in *S. salar* (Ahlbert, 1976). One alternate hypothesis is that UVS cones remain in the retina in some form of dormancy, such that they would not be recognized by morphology, gene expression, or visual sensitivity. We believe this is unlikely because during natural development the disappearing UVS cones undergo apoptosis in both *O. mykiss* and a basal salmonid genus (Kunz et al., 1994; current study). A switch in opsin expression cannot explain the return of UV sensitivity, as it is clear from several studies that the UVS (corner) cones are disappearing from the retina, not merely switching their opsin expression. Regeneration from proliferating cells is the only plausible mechanism for UVS cone regeneration observed at this time. Indeed, dormant photoreceptors have never been reported during natural development, and hypotheses regarding salmonid photoreceptor dormancy following damage (Kurz-Islider and Wolburg, 1982; Allen and Hallows, 1997) have not been supported by subsequent data (Raymond et al., 1988; Allen et al., 2002; Allison et al., 2006a). The population of proliferating cells is substantial in the quickly growing trout retina (Julian et al., 1998).

The most important aspect of retinal regeneration is the restoration of visual function (Raymond and Hitchcock, 1997). Our ERG data indicate that TH reduced UV sensitivity, consistent with previous analyses (Browman and Hawryshyn, 1992, 1994; Deutschlander et al., 2001). The loss of UV sensitivity was greater in TH-treated fish than in smolts, consistent with the greater loss of UVS cones described herein by quantification of labeling. This loss of UV sensitivity occurred earlier than that observed previously in this lab (Browman and Hawryshyn, 1992), although changes and reductions of UV sensitivity are evident in the previous study at the earlier timepoint investigated (21 days treatment). TH treatment used here probably resulted from exposure to TH because the entire volume of TH was changed daily, thus being approximately twice as much TH as in previous experiments where only half the water was changed. Another important difference in our experiments is the position of stimulating light: the central retina was illuminated in the present experiments, whereas only the ventral retina was stimulated previously (Browman and Hawryshyn, 1992). Furthermore, substantial differences in recording UV sensitivity exist, and the ability to directly compare ERG and behavioral measures is questionable. Regardless, the in situ hybridization and immunohistochemical results presented here, along with quantitative PCR data (Veldhoen et al., 2006) and proteomic data (Allison et al., 2006b), were consistent with our TH treatment leading to death of UVS cones over the course of days, not weeks.

Several weeks after the cessation of TH treatment, the UV sensitivity returned (Fig. 5C) to levels similar to that observed in untreated fish. This is consistent with UVS cone distribution maps above, and with previous psychophysical experiments (Browman and Hawryshyn, 1994). Our ERGs demonstrate that the regenerated UVS cones were functional. This is consistent with psychophysical measurements in similar experiments (Browman and Hawryshyn, 1994). Adaptation with UV light demonstrated that the return of UV sensitivity represents an independent UVS cone mechanism. Importantly, these results were derived from the b-wave of the evoked response and primarily represent bipolar cell activity and possibly some activity of third-order neurons (Stockton and Slaughter, 1989; Awatramani et al., 2001). Therefore, regenerated UVS cones made functional connections such as direct bipolar cell inputs.
that they are able to transmit information to higher-order neurons.

Thyroid hormone is known to affect retinal development in several vertebrates (reviewed in Harpavat and Cepko, 2003). Mutation of a thyroid hormone receptor, THRβ-2, also results in an increase in UVS cones, at the expense of M-cones (Ng et al., 2001; Shibusawa et al., 2003; Roberts et al., 2005) and may lead to a dorsoventral asymmetry in photoreceptor identity in mice (Applebury et al., 2000). A dorsoventral asymmetry in Xenopus retinal deiodinases that metabolize TH in an organ-specific manner controls proliferation during tadpole metamorphosis (Marsh-Armstrong et al., 1999). These dorsoventral asymmetries are reminiscent of the asymmetry in UVS cone loss in rainbow trout (see Fig. 1 and Allison et al., 2003).

Our conclusions refine previous interpretations on the role of proliferating cells in the central teleost retina. The pluripotent character of these cells has been recognized from experiments inflicting retinal damage. It is widely held, however, that during natural development these cells give rise solely to rods (Johns and Fernald, 1981; Johns, 1982; Raymond and Hitchcock, 1997; Faillace et al., 2002; Otteson and Hitchcock, 2003). Our results strongly implicate a broader potential for retinal progen-[505x239]A: Spectral sensitivity of small trout (parr, data points represented by diamonds) has UV sensitivity similar to the sensitivity to light at 420 nm. The solid line represents the best-fit weighting of UVS and SWS cone mechanisms. During natural development UV sensitivity decreases in larger fish (smolts, dashed line). This panel is modified from a previous presentation (Allison et al., 2003). The coefficients of cone mechanisms in the best-fit weighting of the parr were KUVS = 0.556, and KSWS = 0.963. B: Spectral sensitivity of small parr treated with thyroid hormone (TH) for 2 weeks shows a lack of UV sensitivity, very similar to the sensitivity of larger fish during natural development (compare to the dashed line in A). n = 3, KUVS = 0.237, and KSWS = 1.125. C: Spectral sensitivity of fish treated with TH and subsequently maintained without TH treatment for 10 weeks. UV sensitivity was regained. n = 6, KUVS = 0.522, and KSWS = 1.066. D: This difference spectrum confirms that the UV sensitivity was mediated by an independent UVS cone mechanism. The spectral sensitivity of three of the fish that were examined in C was determined again using UV chromatic adaptation. The difference spectrum is shown and the solid curve represents a template of the UVS cone sensitivity (λmax = 360 nm) fit by hand.
itor cells during natural development, suggesting that they can also give rise to UVS cones during normal ontogeny in salmonid fishes and possibly in other species.

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LITERATURE CITED


