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The inhibitor of phagocytosis, O-phospho-L-serine, suppresses Müller glia proliferation and cone cell regeneration in the light-damaged zebrafish retina

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Abstract

The damaged zebrafish retina replaces lost neurons through a regenerative response that initiates with the asymmetric cell division of Müller glia to produce neuronal progenitor cells that proliferate and migrate to the damaged retinal layer, where they differentiate into the lost neuronal cell types. Because Müller glia are known to phagocytose apoptotic retinal cells during development, we tested if Müller glia engulfed apoptotic rod cell bodies in light-damaged retinas. After 24 hours of constant intense light, damaged retinas revealed both a strong nuclear TUNEL signal in photoreceptors and a weak cytoplasmic TUNEL signal in Müller glia, although Müller glial apoptosis is not observed in the light-damaged retina. Light damage of a rod-specific transgenic reporter line, *Tg(XIRho:EGFP)^{fl}*, resulted in some Müller glia containing both TUNEL signal and EGFP, which indicated that this subset of Müller glia engulfed apoptotic photoreceptor cell bodies.

To determine if phagocytosis induced the Müller glial proliferative response in the light-damaged retina, we utilized O-phospho-L-serine (L-SOP), a molecule that mimics the phosphatidylserine head group and partially blocks microglial phagocytosis of apoptotic cells. Intravitreal injection of L-SOP immediately prior to beginning constant intense light treatment: i) did not significantly reduce light-induced photoreceptor cell death, ii) significantly reduced the number of PCNA-positive Müller glia, and iii) significantly reduced the number of cone photoreceptors in the regenerated retina relative to control retinas. Because L-SOP is also a specific group III metabotropic glutamate receptor (mGluR) agonist, we also tested if the more potent specific group III agonist, L-2-amino-4-phosphonobutyrate (L-AP4), the specific group III antagonist (*RS*)- α -Methylserine-O-phosphate (MSOP) or the specific group I antagonist, L-2-amino-3-phosphonopropanoic acid (L-AP3) affected Müller glial proliferation. We found no changes with any of these factors compared to control retinas, revealing that metabotropic glutamate receptors were not necessary in the Müller glia proliferative response. Furthermore, *ascl1a* and *stat3* expression were unaffected in either the L-SOP or MSOP-injected retinas relative to controls, suggesting L-SOP disrupts Müller glia proliferation subsequent to or in

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parallel with *ascl1a* and *stat3* activation. This implies that at least one signaling mechanism, in addition to the process disrupted by L-SOP, is required to activate Müller glia proliferation in the light-damaged retina.

Keywords

regeneration; Müller glia; retina; phosphatidylserine; type III metabotropic glutamate receptor; phagocytosis

INTRODUCTION

A wide variety of vertebrates (fish, amphibians, reptiles, and birds) regenerate various tissues that mammals cannot. Adult zebrafish can rapidly and accurately regenerate its damaged fin, heart and retina (Poss et al., 2007; Iovine et al., 2007; Vihtelic and Hyde, 2000), but the molecular signals that initiate these responses is largely unknown. Determining the source and identity of these signals in the adult zebrafish may reveal mechanisms that will facilitate tissue regeneration in humans.

Various models of retinal damage have been described in zebrafish (Bernardos et al., 2007; Fimbel et al., 2007; Sherpa et al., 2007; Otteson and Hitchcock, 2003; Yurco and Cameron, 2005; Fausett and Goldman, 2006), including constant intense light (Vihtelic and Hyde, 2000; Kassen et al., 2007). These insults result in the loss of different classes of retinal neurons. For example, intravitreal injection of a low concentration of ouabain causes the loss of ganglion cells and inner nuclear layer (INL) neurons (Fimbel et al., 2007), while constant intense light causes apoptosis of the rod and cone photoreceptors in the dorsal and central retina (Vihtelic et al., 2006). In all the retinal damage models, new neurons regenerate from a subset of Müller glia, which are induced to divide asymmetrically and produce a population of transit-amplifying neuronal progenitor cells that migrate to the proper retinal layer and differentiate into the retinal neurons that were lost (Bernardos et al., 2007; Vihtelic and Hyde, 2000; Kassen et al., 2007; Fimbel et al., 2007; Sherpa et al., 2007; Otteson and Hitchcock, 2003; Yurco and Cameron, 2005; Fausett and Goldman, 2006). In the light-damage model, the number of proliferating Müller glia is proportional to the amount of cell death (Vihtelic et al., 2006). Thus, the extent and location of photoreceptor cell death appears to be communicated to the associated Müller glia.

A variety of different molecules and pathways influence Müller glia proliferation either in the damaged or undamaged retina. For example, CNTF induces Müller glial proliferation in the absence of damage in the zebrafish retina (Kassen et al., 2009), while insulin and Fgf2 stimulates a similar response in the undamaged chick retina (Fischer et al., 2004). In contrast, some molecules increase Müller glial cell proliferation in the damaged retina, such as Wnt and Shh in the rodent retina (Osakada et al., 2007; Das et al., 2006; Wan et al., 2007; Close et al., 2006). Inhibition of either Wnt (Das et al., 2006) or Shh (Wan et al., 2007) signaling pathways reduces Müller glial cell-based regeneration in the rat. Inhibition of Notch signaling in the NMDA-damaged chick retina, however, either inhibits neuronal regeneration if blocked early or increases the numbers of regenerated neurons if blocked after neuronal progenitors are produced (Hayes et al., 2007).

In addition to responding to many extracellular signaling molecules, the Müller glia also phagocytose a variety of different substances. Foreign molecules that are injected into the vitreous of the rodent eye are phagocytosed and distributed throughout the whole Müller cell body (Friedenwald and Chan, 1932; Rosenthal and Appleton, 1975). Additionally, Müller glia phagocytose dying cells, such as RPE cell bodies upon disruption of the outer limiting

membrane (Francke et al., 2001), degenerated rod photoreceptors in a dominant transgenic zebrafish line (Morris et al., 2005), and during normal retinal development (Egensperger, et al, 1996). This suggests that Müller glia possess the necessary receptors and molecular pathways to phagocytose apoptotic cell bodies (Friedenwald and Chan, 1932; Rosenthal and Appleton, 1975; Wagner and Raymond, 1991).

We examined whether Müller glia engulf apoptotic photoreceptor bodies in the light-damaged zebrafish retina and if this process regulated Müller glial proliferation in regeneration. Specifically, we tested the role of the potential engulfment signal phosphatidylserine (PtdSer) (Fadok et al., 1992) by intravitreal injection of O-phospho-L-serine (L-SOP), which mimics the structure of the PtdSer headgroup. L-SOP was shown to significantly reduce the number of phagocytes that internalize apoptotic cell bodies (Witting et al., 2000). Because L-SOP also mediates group III metabotropic glutamate receptor (mGluR) function (Tizzano et al., 1995, Faden et al., 1997), we verified that the L-SOP effects were not mediated through mGluRs by injecting chemicals that stimulate or antagonize mGluRs. These studies demonstrated that L-SOP disrupts a process that is necessary for the maximal number of Müller glia to proliferate and regenerate cone photoreceptors in the light-damaged retina.

MATERIALS AND METHODS

Animal care and use

Adult *albino*^{b4} zebrafish (*Danio rerio*), which were 6–14 months of age (2.5–4 cm in length), were raised at 28.5°C in a 14 hours of light: 10 hours of dark regime under an average luminance of 200 lux using standard husbandry techniques (Westerfield, 1993). All experiments were conducted in accordance with the protocols approved by the animal use committee at the University of Notre Dame and the ARVO statement on the use of animals in vision research.

Transgenic Lines

The transgenic line, *Tg(XIRho:EGFP)^{f11}*, a gift from Dr. James Fadool (Florida State University), was outcrossed into the *alb*^{b4} background (Fadool, 2003). The Müller glial-specific transgenic line, *Tg(gfap:EGFP)^{nt11}*, was described previously (Kassen et al., 2007). The 3.2 kb guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 2 (*gnat2*) promoter was amplified from a plasmid template (Kennedy et al., 2007) using Platinum Taq DNA polymerase High Fidelity (Invitrogen; Carlsbad, CA) and primers (5'-GGATCCTCCGGCAGCAACCTA-3' and 5'-CCATGGTCTGACGCTTCC-3') that incorporated *Bam*HI and *Nco*I restriction sites (underlined). The PCR product was cloned into the pCR8/GW/TOPO vector (Invitrogen) for DNA sequencing. A *Bam*HI/*Nco*I digest removed the *preproinsulin* (*insa*) promoter from the T2KXIG-*ins:nfsB-EGFP* plasmid (Pisharath et al., 2007) and the *gnat2* promoter was ligated upstream of *nfsB-EGFP*. The SP6 mMessage mMachine kit (Ambion; Foster City, CA) was used to *in vitro* transcribe Tol2 transposase mRNA from the pCSTZ2.8 plasmid (Kawakami et al., 1998). The *gnat2:nfsB-EGFP* plasmid and transposase mRNA were combined at a concentration of 25 ng/μl each and co-injected into 1–4 cell stage AB wild-type embryos. Injected F₀ adults were out-crossed to the AB strain and F₁ carriers were again out-crossed to establish independent transgenic lines. The *Tg(gnat2:nfsB-EGFP)^{nt21}* line was crossed into the *alb*^{b4} background for constant light damage experiments. NTR-EGFP was expressed in the majority of offspring of *Tg(gnat2:nfsB-EGFP)^{nt21}* out-crosses, consistent with multiple insertions of the transgene.

Intravitreal injections and constant intense light treatment

Intravitreal injections were described previously (Fimbel et al., 2007; Thummel et al., 2008). Briefly, 14-day dark-adapted adult *albino* zebrafish were anesthetized in 2-phenoxyethanol

and an incision was made in the cornea adjacent to the iris using a sapphire blade. A Hamilton syringe was used to intravitreally inject 0.5 μ L of either control saline (0.65% NaCl) or 0.65% saline containing either 20 mM O-phospho-L-serine (L-SOP), 20 mM (*RS*)- α -Methylserine-O-phosphate (MSOP), 20 mM L-2-amino-4-phosphonobutyrate (L-AP4), or 20 mM L-2-amino-3-phosphonopropanoic acid (L-AP3) into the left eye, while the right eye was not injected. Immediately following injection, the fish were revived in system water and subjected to constant intense light. All chemicals were obtained from Sigma (St. Louis, MO), excepting MSOP (Tocris Bioscience; Ellisville, MO). Light treatment was performed as previously described (Vihtelic and Hyde, 2000; Vihtelic et al., 2006; Kassen et al., 2007). Control and experimental groups were treated simultaneously in different tanks under the same lamps. Fish were subjected to either 12, 24 or 51 hours of constant light before they were anesthetized, enucleated and processed for further analysis. Some fish were injected with either 0.65% NaCl, 20 mM L-SOP, 20 mM MSOP, 20 mM L-AP4, or 20 mM L-AP3, but subjected to standard (14 hours light: 10 hours dark) light for either 24 or 51 hours of normal light as controls.

Cryosectioning

Eyes were fixed in either 3.7% formaldehyde in 1 \times phosphate-buffered saline (PBS) or a 9:1 mixture of ethanol:37% formaldehyde overnight at 4°C. The eyes were rinsed with 1 \times PBS, cryoprotected in 30% sucrose/PBS (pH 7.4) overnight at 4°C and frozen embedded in 100% Tissue Freezing Medium (Triangle Biomedical Sciences; Durham, NC). Serial sections (14–20 μ m) were cut tangentially through the central retina to obtain sections containing the optic stalk. Cryosections were mounted onto Superfrost/Plus glass slides, air dried for at least 20 minutes at 50°C and then stored at –80°C until use.

TUNEL

Frozen tissue sections were permeabilized with either Neuropore (R&D Systems, Inc.; Minneapolis, MN) for 75 minutes at room temperature or 0.1% sodium citrate buffer/0.1% Triton X-100 on ice for 2 minutes. The fragmented DNA was labeled enzymatically according to the manufacturer's protocol (TACS™ TdT Kit; R&D Systems, Inc.; Minneapolis, MN or ApoAlert DNA Fragmentation Assay Kit; Clontech; Mountain View, CA). Apoptotic cells were also labeled with biotinylated dUTP (NEB; Ipswich, MA) and detected by a 1:200 dilution of streptavidin-AlexaFluor conjugates (Invitrogen). Retinal TUNEL-positive nuclei were quantified at either 0 or 24 hours of constant, intense light treatment, when maximal retinal damage is observed (Vihtelic and Hyde, 2000). Positive and negative controls included adding TACS™-nuclease and omitting the TdT enzyme, respectively.

Immunohistochemistry

Retinal tissue sections were rehydrated in 1 \times PBS (pH 7.4), outlined with a Pap Pen (Ted Pella, Inc.; Redding, CA), and blocked with 1 \times PBS, 0.2% normal goat serum, 0.2% Triton X-100, and 1% DMSO for 60 minutes at room temperature. The sections were incubated overnight at 4°C with either a 1:1000 dilution of mouse anti-PCNA monoclonal antibody (clone PC10; Sigma Chemical), a 1:250 dilution of mouse monoclonal antibody 4C4 (kind gift of Dr. Jonathan Scholes, University College London), or a 1:500 dilution of rabbit anti-PCNA polyclonal antiserum (Abcam; Cambridge, MA) in blocking solution. Müller glia were immunodetected using a 1:500 dilution of mouse anti-glutamine synthetase monoclonal antibody (Chemicon International; Temecula, CA). Secondary antibodies, conjugated to either Alexa-Fluor 488 or 594 (Molecular Probes; Eugene, OR), were diluted 1:500 in PBS/2% normal goat serum/0.2% Triton X-100 and incubated for 1 hour at room temperature.

Images were collected with a Leica TCS SP5 Broadband Confocal Microscopy System (Leica Microsystems; Germany) using sequential laser scanning during colocalization analysis to eliminate spectral contamination.

5-ethynyl-2'-deoxyuridine (EdU) labeling of proliferating cells during light treatment

Light-damaged *albino* zebrafish were subjected to constant intense light treatment for 96 hours. The fish were intravitreally injected with 200 nmol EdU (Invitrogen) in 1× PBS after 48, 72 and 96 hours after starting the constant light treatment (Salic and Mitchison, 2008; Sugiyama et al., 2009). After the third EdU injection, the fish were transferred to normal lighting conditions until enucleation. Eyes were cryosectioned as described above and processed for EdU labeling by the Click-iT EdU Alexa Fluor 488 Imaging kit (Invitrogen) according to the manufacturers instructions and subjected to immunohistochemistry.

Statistical analysis

For both TUNEL and PCNA labeling, the central-most 700 μm of the dorsal retina, equidistant from the margin and the optic stalk, was quantified. Experiments were statistically compared by two-tailed Student's t-test assuming unequal variance. Due to variability in the light damage between groups of fish, experimental cohorts were compared only to saline-injected controls that were treated, with or without light, at the same time using the same light sources.

Quantitative real-time polymerase chain reaction analysis

Zebrafish retinas were collected after 12 hours of constant light treatment, dissected and RNA from a pool of three retinas for each test condition was isolated by TRIzol (Invitrogen). Single-stranded cDNA was generated with the SuperScript III reverse transcriptase (Invitrogen) using random hexamer primers. Transcript levels were analyzed using the SYBR Green PCR Master Mix on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems; Foster City, CA). Primers to detect *asc11a* (previously called *ash[a]*) and *stat3* were described previously (Kassen et al., 2007). The reaction mix contained 10 μl SYBR Green master mix (Applied Biosystems), various primer pairs (1.2 μl of each primer at 10 μM each), and several cDNA dilutions (7.6 μl) in a total volume of 20 μl. The reactions were incubated for 2 minutes at 50° C and 10 minutes at 95° C followed by 40 cycles of 15 seconds at 95° C, and 1 minute at 60° C. The comparative CT method was used for data analysis (Johnson et al., 2000; Vong et al., 2003). Two serial dilutions of cDNA from each of the time points were run in triplicate and the median CT value was compared against the corresponding median 18S rRNA CT values. The normalized CT values for each gene from each treated retina were compared to untreated control retinas to obtain the log₂-fold change in gene expression levels.

RESULTS

Healthy Müller glia cell bodies become TUNEL-positive upon light-induced photoreceptor cell death

TUNEL-positive, apoptotic cells are detected in the outer nuclear layer (ONL) after only 6 hours of constant intense light treatment and increase in number until 12 hours, when they reach a maximum number that is maintained through 24 hours of treatment (Vihtelic and Hyde, 2000). In addition, a weak and diffuse TUNEL label was also observed in the inner nuclear layer (INL) and extended across both plexiform layers (Fig. 1, panels B and C, arrows). Furthermore, this INL labeling was most often found basal to a cluster of TUNEL-positive photoreceptors (Fig. 1C, asterisks) and exhibited the morphology of Müller glial cells. No labeling was detected in the absence of TdT enzyme (not shown), which confirmed that the INL signal resulted from specific labeling of fragmented DNA. Because this TUNEL labeling appeared to fill the cytoplasm of the Müller glial cells, including the processes (Fig. 1C, arrows), it likely did not represent apoptotic Müller glia.

To confirm that the diffuse INL TUNEL signal was associated specifically with Müller glia cells, we repeated the TUNEL analysis with light-damaged *Tg(gfap:egfp)^{nt11}* transgenic

zebrafish. The *gfap:egfp* transgene expresses EGFP under the transcriptional control of the Müller glial-specific *glial fibrillary acidic protein* promoter (Kassen et al., 2007). Non-light treated *Tg(gfap:egfp)^{nt11}* retinas displayed EGFP in Müller glia (Fig. 1D' and D''), but little TUNEL labeling (Fig. 1D and D''). After 24 hours of constant intense light, all TUNEL-positive INL cells colabeled with EGFP (Fig. 1E–E'', arrows), whereas not all EGFP-positive Müller cells colabeled with the TUNEL signal (Fig. 1E–E'', arrowhead). This confirmed that Müller glia are the predominant, if not the only, TUNEL-positive INL cell in the light-damaged retina. Furthermore, only a subset of the Müller glia were TUNEL-positive, which is similar to the previous observation that not all Müller glia proliferate during the regeneration response (Kassen et al., 2007; Thummel et al., 2008).

Müller glia engulf apoptotic, but not healthy, rod photoreceptor cell bodies

If the TUNEL-positive Müller glia were not dying, they could be phagocytosing apoptotic rod photoreceptor cell bodies. Therefore, we immunolabeled light-damaged retinas for various photoreceptor-specific antigens and analyzed their potential colocalization with TUNEL expression in the INL. The *Tg(XIRho:EGFP)^{fl1}* transgenic zebrafish line expresses EGFP throughout the rod photoreceptor cell body (Fadool et al., 2003). In undamaged *Tg(XIRho:EGFP)^{fl1}* controls, EGFP expression was restricted to the ONL and outer segments, even after signal amplification using an anti-GFP antibody (Fig. 2A). Further, there was no visible TUNEL labeling in either the INL or ONL in these undamaged retinas (Fig. 2B). In contrast, the light-damaged retinas exhibited the rod-derived EGFP in both the ONL and INL, where it displayed a characteristic Müller glial-like morphology (Fig. 2E). TUNEL labeling of the light-treated *Tg(XIRho:EGFP)^{fl1}* transgenic retinal sections revealed that EGFP colabeled with the TUNEL-positive cells (Fig. 2, panels F and H) and the Müller glial-specific glutamine synthetase immunolabeling (Fig 2, panels G and H). This confirmed the Müller glia in the light-damaged retina internalized remnants from the dying rod photoreceptors, which were likely apoptotic rod photoreceptor cell bodies that contained both rod-derived proteins and fragmented DNA.

We tested whether other photoreceptor proteins colocalized with the Müller glia in the light-damaged retina. Rhodopsin, normally restricted to the rod outer segments, but often mislocalized to the rod inner segments during photoreceptor damage was not detected in the INL of light-damaged retinas (Vihtelic, et al., 2000, Kassen, et al., 2007). Similarly, the zs-4 monoclonal antibody, which labels the rod inner segments (Trevarrow, et al., 1990, Vihtelic et al., 1999), did not label the INL of light-damaged retinas (Supplemental figure 1). We also tested if Müller glia engulfed apoptotic cone cells in the light-damaged retina by using the *Tg(gnat2:nfsB-EGFP)^{nt21}* transgenic zebrafish line that expresses EGFP specifically in cones under the control of the cone-specific transducin subunit promoter (Kennedy et al., 2007). We did not, however, detect cone cell-derived EGFP expression in the INL of light-damaged *Tg(gnat2:nfsB-EGFP)^{nt21}* transgenic fish (Supplemental figure 2).

Proliferating Müller glia colabel with TUNEL

To test whether the proliferating Müller glia had engulfed the apoptotic photoreceptor cell bodies, we labeled light-damaged retinal sections with TUNEL and immunostained for proliferating cell nuclear antigen (PCNA), which is associated with replicating DNA. After 24 hours of constant intense light, a subset of the Müller glia had entered S phase of the cell cycle based on PCNA immunostaining (Fig. 3, panels B and D). Strikingly, the majority of the PCNA-positive Müller glia co-labeled with the TUNEL signal (Fig. 3, panels A, B, and D, arrowheads), while a small percentage of the TUNEL-positive Müller glia did not coexpress PCNA (Fig. 3, panels A, B, and D, arrow). Thus, the same Müller glia responded to cell death by phagocytosing the TUNEL-positive apoptotic rod photoreceptor cell bodies and

proliferating. This suggests that the engulfment of apoptotic photoreceptor debris might stimulate the Müller glia to proliferate in the regenerative response.

Phosphatidylserine mimetic O-phospho-L-serine inhibits Müller glia proliferation in the light-damaged retina

To determine the role of engulfment of apoptotic debris on the proliferative response of Müller glia, we tested the ability of O-phospho-L-serine (L-SOP) to inhibit Müller glial cell proliferation. Apoptotic cells display phosphatidylserine (PtdSer) on the outer leaflet of the lipid bilayer as a signal to attract phagocytes and facilitate their engulfment and removal (Fadok et al., 1992). L-SOP mimics the structure of the PtdSer headgroup (Witting et al., 2000) and competes with PtdSer for binding to both PtdSer receptors and bridging proteins. Previous studies showed that L-SOP reduced microglial binding and phagocytosis of both apoptotic cells and lipid vesicles by 40% in cell culture (Witting et al., 2000).

We intravitreally injected either 0.65% NaCl or 20 mM L-SOP immediately prior to initiating the constant intense light treatment. We selected 20 mM L-SOP because higher doses proved neurotoxic in the retina (data not shown). After 24 hours of light, the control retinas possessed a statistically equivalent number of TUNEL-positive ONL cells as the L-SOP-injected retinas (Fig. 4, panels A–C, $p > 0.35$, $n=17$). After 51 hours of light treatment, however, the L-SOP-injected retinas exhibited significantly fewer ($69.5\% \pm 6.8$, $p < 0.005$, $n=25$) PCNA-positive INL cells relative to the saline-injected control retinas (Fig. 4, panels D–F). No change in the number of PCNA-positive ONL cells was observed between the control and the L-SOP-injected retinas after 51 hours of constant light (data not shown). These data suggest that L-SOP inhibited Müller glia proliferation, without affecting light-induced photoreceptor cell death.

Photoreceptor apoptosis and Müller glial cell proliferation in light-damaged retinas are not regulated by the metabotropic glutamate receptors

In addition to binding phosphatidylserine receptors, L-SOP is also an agonist of group III metabotropic glutamate receptors (group III mGluRs), although less potent than the structurally related L-2-amino-4-phosphonobutyrate (L-AP4) (Faden et al., 1997). Both compounds are neuroprotective during stress, whereas group I mGluR antagonists, such as L-2-amino-3-phosphonopropanoic acid (L-AP3), exacerbate neurotoxicity (Maiese et al., 2000). We tested whether the L-SOP-mediated suppression of Müller glial proliferation was due to potential interactions with mGluRs by comparing the effects of L-AP4 (group III agonist), (RS)- α -Methylserine-O-phosphate (MSOP, group III antagonist), and L-AP3 (group I antagonist) relative to 0.65% NaCl. We intravitreally injected L-AP4, MSOP, L-AP3, or saline into dark-adapted *albino* zebrafish and immediately introduced them to constant intense light for 24 hours. Retinal sections were then analyzed for neuroprotection of photoreceptor death by TUNEL (Figure 5, panels AC). L-AP4, MSOP, and L-AP3 did not induce photoreceptor cell death in non-light treated retinas (data not shown) and were not neuroprotective in light-treated retinas relative to the saline-injected control (Figure 5D, L-AP4, $p > 0.85$, $n = 13$; MSOP, $p > 0.95$, $n=17$; L-AP3, $p > 0.45$, $n = 20$, respectively). Similar to L-SOP, these three compounds had neither a neurotoxic effect on the non-light-treated retina nor a neuroprotective effect in the light-treated retina.

To determine whether Müller glia-mediated proliferation was dependent on mGluR signaling, L-AP4, MSOP, and L-AP3-treated fish were submitted to 51 hours of constant light treatment and analyzed for PCNA expression (Fig. 5, panels E–H). At this time, the majority of Müller glia that will respond to light damage are PCNA-positive. Light-treated L-AP4, MSOP, or L-AP3 retinas showed no significant differences in the number of PCNA-positive Müller glia relative to saline-injected light-damaged control retinas (Fig. 5H, L-AP4, $p > 0.11$, $n = 37$;

MSOP, $p > 0.63$, $n = 27$; L-AP3, $p > 0.64$, $n = 33$, respectively). At this same time point, L-SOP significantly reduced the number of PCNA-positive Müller glia relative to the saline-injected control retinas. Thus, the L-SOP-mediated suppression of Müller glial proliferation in the light-damaged retina is not due to the action of L-SOP on the group III mGluRs.

Distribution of microglia in L-SOP-treated retinas

We also examined if the L-SOP-mediated suppression of Müller glia proliferation in the light-damaged retina was through the microglia, a known pleiomorphic phagocytic cell type in the retina (Hume, et al., 1983; Egensperger, et al., 1996). Microglia are detected in the retina by the 4C4 monoclonal antibody (Becker and Becker, 2001.). In the undamaged retina, microglia were present at relatively low numbers primarily in association with the rod outer segments, the INL, and the GCL (Fig. 6A, arrowheads). By 16 hours of constant light, the morphology of the 4C4-positive cells suggested that the microglia had become activated (Fig. 6B, arrowhead). However, large numbers of PCNA-positive rod precursor cells in the ONL were not associated with any microglia (Fig. 6B, arrow). By 31 hours of constant light, when the Müller glia began proliferating, the majority of the Müller glia were still not associated with the microglia (Fig. 6C, arrow). Only by 96 hours of constant light did we find increased numbers of activated microglia in the INL, as well as near the rod outer segment region (Fig. 6D, arrowheads).

To examine if L-SOP mediated its suppression of Müller glia proliferation through the microglia, we observed the microglia after 24 hours of constant light, when maximal rod photoreceptor cell death occurred and immediately before Müller glia proliferation began. We utilized dark-adapted *albino Tg(XIRho:EGFP)^{fl}* transgenic line, which expresses EGFP from the *Xenopus* rod opsin promoter (Fadool, 2000). Light-treated saline-injected *albino Tg(XIRho:EGFP)^{fl}* control retinas possessed activated microglia near the rod outer segments and in both the ONL and GCL (Fig. 6E). While many of these microglia were present in the region of the rod outer segments, they did not appear to possess any significant amounts of engulfed EGFP (Fig. 6, panels F and G). Intravitreal injection of either 20 mM MSOP (Fig. 6, panels H–J) or 20 mM L-SOP (Fig. 6, panels K–M) did not affect either the relative number or distribution of the 4C4-positive microglia, as well as their potential engulfment of EGFP. Thus, the L-SOP-mediated suppression of Müller glia proliferation did not appear to be through the microglia, although the microglia could regulate Müller glia proliferation through a paracrine-mediated mechanism.

L-SOP disrupts Müller glia proliferation subsequent to or in parallel with the activation of *ascl1a* and *stat3* expression in the light-damaged retina

Because Müller glia proliferation is dependent on increased *ascl1a* and *stat3* expression in light-damaged retinas (Fausett et al., 2008; Kassen et al., 2007), we tested whether L-SOP affected transcription of these genes. Dark-adapted, *albino* zebrafish were injected with either 0.65% NaCl, 20 mM L-SOP, or 20 mM MSOP and subjected to 12 hours of constant intense light and compared to undamaged *albino* retinas. The retinas were isolated by dissection and mRNA levels were evaluated by quantitative real-time PCR (Figure 7). Control undamaged retinas had low levels of *ascl1a* and *stat3* transcripts when compared with light-treated retinas. Light treatment of 0.65% NaCl-injected retinas caused a significant increase in the expression of *ascl1a* ($p < 0.00005$, $n = 4$) and *stat3* ($p < 0.0005$, $n = 4$) relative to untreated controls. Treatment with MSOP at the start of constant light treatment had no effect on the levels of either *ascl1a* ($p > 0.76$, $n = 4$) or *stat3* ($p > 0.26$, $n = 4$) relative to saline-injected control retinas. Similarly, treating light-damaged retinas with L-SOP failed to affect the increased expression of either *ascl1a* ($p > 0.12$, $n = 4$) or *stat3* ($p > 0.84$, $n = 4$) compared to saline-injected control retinas. Thus, the L-SOP-mediated suppression of Müller glial proliferation in the light-damaged retina is

likely acting downstream or in parallel of the activation of *ascl1a* and *stat3* transcription and upstream of PCNA expression.

L-SOP inhibits cone cell regeneration in the light-damaged retina

To assess the effects of L-SOP-mediated inhibition of Müller glial cell proliferation on photoreceptor cell regeneration, we injected dark-adapted *albino* zebrafish with 0.65% NaCl, 20mM MSOP, or 20mM L-SOP. The fish were intravitreally injected with EdU at 48, 72, and 96 hours of constant light to label dividing cells and their progeny. After the third EdU injection, the fish were transferred to normal light conditions for an additional 10 days. Eyes were enucleated and processed for cryosectioning to assess cone cell regeneration. Reduced numbers of both short single cones expressing UV opsin (Fig. 8, panels A–C) and long single cones expressing blue opsin (Fig. 8, panels D–F) were found in L-SOP-treated retinas relative to controls (Fig. 8, panels G and H, respectively).

This suppressed regeneration of cones could result from either reduced numbers of Müller glia, as observed by fewer PCNA-positive Müller glia (Fig. 4), or L-SOP acting directly on PCNA, which would result in the Müller glia undergoing apoptosis (Thummel et al., 2008). To examine the latter possibility, we immunostained the retinas described above for glutamine synthetase expression (Fig. 8, panels J–M). We found no statistical difference in the number of glutamine synthetase-positive Müller glia in the L-SOP-treated retinas relative to controls ($p > 0.1$, $n = 10$). Thus, L-SOP did not directly affect PCNA expression or activity, which would have resulted in the loss of Müller glia. Therefore, we examined EdU incorporation to confirm that L-SOP suppressed the number of proliferating Müller glia. Surprisingly, a statistically equivalent number of EdU-labeled cells was observed in the INL, as well as the ONL, of all three treatments (Fig. 8, panels A–F and I). We examined if these EdU-labeled cells were microglia that had invaded the INL upon light damage. However, the majority of the EdU-labeled cells in both the L-SOP-treated and the control retinas did not colabel with the anti-4C4 antibody (data not shown), suggesting that these EdU-labeled INL cells were not residual microglia. While the identity of the EdU-labeled INL cells in the L-SOP-treated retina remains unknown, it is clear that L-SOP suppressed the regeneration of cone photoreceptors in the light-damaged zebrafish retina.

DISCUSSION

In the retina, a rapid and robust regeneration response is detectable within a few hours after retinal damage, as Müller glial cells hypertrophy and begin to express *ascl1a* (Fausett et al., 2008) and *stat3* (Kassen et al., 2007). A subset of these Müller glia proliferate to produce neuronal progenitor cells that migrate radially into the damaged nuclear layer and differentiate into the lost neurons. Little is known about the molecular mechanisms that initiate this regeneration response in the Müller glia. Here we show that some Müller glia either phagocytosed apoptotic photoreceptors or that they, in fact, were apoptotic. This latter hypothesis was rejected as the TUNEL labeling was cytoplasmic and no pyknotic nuclei were found in the INL, in striking contrast to the strongly TUNEL-labeled and pyknotic nuclei of the apoptotic photoreceptors. Furthermore, a loss of Müller glial cells during the normal regeneration response was not found (Fig. 8; Vihtelic et al., 2000; Kassen et al., 2007). Finally, we found that rod-specific EGFP in the transgenic *Tg(XIRho:EGFP)^{fl1}* zebrafish colocalized with the TUNEL signal in a subset of Glutamine synthetase-expressing Müller glial cells (Fig. 2A, panels E–H) only after light damage. These data demonstrate selective endocytosis of dying rod photoreceptors by Müller glia and support previous studies that Müller glia engulfed RPE debris and apoptotic neurons in both developing and damaged retinas (Egensperger et al., 1996; Francke et al., 2001; Morris et al., 2005; Friedenwald and Chan, 1932).

Surprisingly we did not detect either rhodopsin or the inner segment zs-4 antigen colabeling with the Müller glia (data not shown and Sup. Fig. 1, respectively). The lack of Müller glial engulfment of rod inner and outer segments likely results from these regions lying apical to the outer limiting membrane (OLM), which limits their contact with the Müller glia. It is more likely that they are phagocytosed by the retinal pigment epithelial cells and the highly-phagocytic and mobile microglia, which remove dying photoreceptor cell debris (Hume et al., 1983; Egensperger et al., 1996). While rhodopsin and other proteins become mislocalized in dying photoreceptors, engulfment of these proteins by the Müller glia may be at levels lower than those detectable by immunohistochemistry. The extremely high levels of rod-derived EGFP in the *Tg(XIRho:EGFP)^{fl1}* transgenic fish is at sufficiently large amounts in the regions basal to the OLM to be detected in the Müller glia if it was endocytosed. Although this EGFP expression in the Müller glia could be due to inadvertent expression of the *XIRho:EGFP* transgene in the Müller glia, the EGFP expression was often limited to the apical regions of the Müller glia, matching the subcellular distribution of TUNEL labeling rather than the expected expression throughout the cell body of autonomously expressed EGFP. Thus, we conclude it is more likely that the EGFP was engulfed by Müller glia.

We also failed to detect cone-derived EGFP in the Müller glia from the light-damaged *Tg(gnat2:ntr-EGFP)^{nt21}* transgenic line (Sup. Fig. 2). Indeed, no colabeling of TUNEL and cone-derived EGFP was found, despite obvious cone death and INL streaming of TUNEL label (likely from rod cells) at 51 hours of light damage (Sup. Fig. 2). This suggests that either these cone cell-specific proteins were below our level of detection or the Müller glia selectively engulfed dying rod cell bodies. If only dying rods were engulfed by the Müller glia, then a different signal must be produced by the dying cones to ensure their regeneration. The necessity of multiple signals is consistent with the inability of L-SOP to suppress the increased expression of the *ascl1a* and *stat3* genes, which were shown to be required for both Müller glial cell proliferation and regeneration (Fausett et al., 2008; Kassen et al., 2007; Kassen et al., manuscript in preparation). Thus, another, yet to be identified, signal must be required to induce the expression of the *ascl1a* and *stat3* genes and only more detailed molecular analysis will elucidate all of the signals required to regenerate neurons in the damaged zebrafish retina.

We found that the 4C4-labeled microglia infiltrated and hypertrophied in response to the light damage (Figure 6). However, they did not amplify in number and become the predominant phagocytic cell in the retina until later in the constant light treatment (Fig. 6D). Their presence in association with the rod outer segments is consistent with previous reports that the microglia engulfed dying photoreceptors in the light-damaged retina (Ng and Streilein, 2001). However, we failed to detect 4C4-positive microglia in close proximity to the Müller glia that were either engulfing dying rod cell debris or proliferating (Figs. 2 and 6, respectively). While this suggests that L-SOP was acting directly on Müller glial cell proliferation, it does not eliminate the possibility that L-SOP suppressed microglial phagocytosis of the dying rods, which in turn activated the proliferation of the Müller glia through a paracrine-mediated signaling mechanism.

If phagocytosis of dying rod cells signals the Müller glia to proliferate, then all PCNA-positive Müller glia should coexpress TUNEL signal. We found that all PCNA-positive Müller glia colabeled with TUNEL, however, not all Müller glia expressed PCNA at 24 hours of constant light (Fig. 3). This is consistent with our previous finding that approximately 50% of the Müller glia were PCNA-positive in the light-damaged retina (Thummel et al., 2008). This suggests that either only a subset of Müller glia are capable of proliferating or that a regenerative paracrine-like signal, which acts over a limited spatial range, induces a specific predetermined subset of Müller glia to proliferate. Because all of the PCNA-positive Müller glia had phagocytosed TUNEL-positive rod cell debris (Fig. 3), it is more likely that proximity to the cell death, not intrinsic qualities, determined which Müller glial cells responded to damage.

One important engulfment signal of apoptotic cell bodies is phosphatidylserine (PtdSer), which is normally maintained on the inner leaflet of healthy cells and exposed to the extracellular environment upon apoptosis (Fadok et al., 1992; Fadok et al., 2001). Blocking PtdSer-based cell-cell interaction with small molecules inhibits microglial phagocytosis of apoptotic cells *in vitro* (Witting et al., 2000). We found that intravitreal injection of the phosphatidylserine headgroup mimetic, L-SOP, significantly reduced the number of PCNA-positive Müller glia after 51 hours of constant intense light. In contrast, L-SOP failed to reduce the number of EdU-labeled INL cells 10 days after terminating the constant light treatment (Fig. 8I). EdU, a thymidine analog, was shown to label the same proliferating cells and their progeny as BrdU (Salic and Mitchison, 2008; Sugiyama et al., 2009). Immunolabeling EdU-treated retinas with the microglial-4C4 monoclonal antibody revealed that most of the EdU-labeled INL cells were not microglia. If the EdU-labeled INL cells represent Müller glia and their neuronal progenitor progeny, it is possible that the L-SOP-mediated suppression of Müller glial cell proliferation was only transient. Thus, the number of EdU-labeled cells in the L-SOP-treated retinas may have reached control levels by 10 days after terminating the light treatment (Fig. 8), but the L-SOP-treated retinas were still delayed in differentiating the regenerated short and long single cone cells relative to the control retinas (Fig. 8, panels A–H).

Because L-SOP binds both the phosphatidylserine receptor and the group III metabotropic glutamate receptor (mGluR), we examined if L-SOP exerted its effects through the group III mGluRs by comparing L-SOP to another agonist and two antagonists of mGluRs. While L-SOP is a group III mGluR agonist, it is only one-tenth as efficient as the related L-AP4 agonist (Tizzano et al., 1995, Faden et al., 1997). Group III mGluR agonists attenuate *in vivo* neurotoxicity of foreign substances introduced into the brain (Zhou et al., 2006) and *in vitro* neurotoxicity of physical trauma in mixed neuron and glia cultures (Faden et al., 1997). However, neither L-SOP nor L-AP4 were neuroprotective in the light-damaged retina. The Group I selective antagonist L-AP3, which protects against nitric oxide-induced apoptosis (Maiese et al., 2000) and hypoxia-induced apoptosis (Opitz et al., 1991), also was not neuroprotective in the light-damaged retina. In contrast, the Group III mGluR antagonist, MSOP, exacerbates neurotoxicity (Faden et al., 1997; Zhou et al., 2006), but was not neurotoxic in the zebrafish retina at the concentration we used, whereas a higher concentration (100 mM) was neurotoxic to all retinal cells (data not shown).

While L-SOP significantly reduced the number of PCNA-positive Müller glia in the light-damaged retina (Fig. 4F), L-AP4, L-AP3, or MSOP did not have any significant effect (Fig. 5H). This suggests that L-SOP was not acting through the mGluR to repress Müller glia proliferation. The inability to detect either engulfed rod cell debris in the microglia or increased numbers of activated microglia closely associated with the PCNA-positive Müller glia at 31 hours of constant light, further suggests that L-SOP is not acting on the microglia, which in turn, stimulated the Müller glia in a paracrine-like fashion.

Surprisingly, L-SOP suppressed Müller glial proliferation without decreasing the number of TUNEL-positive Müller glia in L-SOP-treated retinas relative to saline-injected retinas (data not shown). We also found no difference in the number of EGFP-positive Müller glia in the light-damaged *Tg(XlRho:EGFP)^{fl}* transgenic retinas treated with saline, MSOP, or L-SOP (data not shown). Because L-SOP did not suppress the number of Müller glia engulfing dying rod cell debris, we tested if L-SOP decreased the relative amount of debris engulfed by the Müller glia. Due to the high variability in TUNEL labeling intensity in the tissue sections, however, we were unable to distinguish any difference in the amount of TUNEL signal in the L-SOP-treated Müller glia and the controls. Because we were unable to detect a difference in the number of Müller glia that engulfed dying rod cell debris or the amount that was engulfed, we tested if another compound that blocks the endocytic pathway had an analogous effect as L-SOP. We found that chlorpromazine, which inhibits clathrin-mediated endocytosis (Wang

et al., 1993), also reduced the number of PCNA-positive Müller glia in the light-damaged retina (Bailey and Hyde, manuscript in preparation). This leaves open the mechanism of L-SOP-mediated suppression of Müller glia proliferation. First, L-SOP (and chlorpromazine) may block Müller glial cell proliferation by inhibiting the quantity of dying rod cell debris that is engulfed by Müller glia. Second, L-SOP (and chlorpromazine) may suppress microglial engulfment of dying rod cell debris, which activate Müller glial cell proliferation in a paracrine-like manner. Third, L-SOP may act through an unidentified process (possibly related to or different from chlorpromazine) in reducing Müller glial cell proliferation. We are currently performing experiments to try to differentiate between these three possibilities.

Increased expression of both *ascl1a* and *stat3* are required for the proliferation of Müller glia in the regenerating retina (Fausett et al., 2008; Kassen et al., 2006). However, neither L-SOP nor MSOP altered the increased expression of *ascl1a* and *stat3*, which suggests that the L-SOP-mediated repression of Müller glia proliferation is either downstream or in a parallel pathway to *ascl1a* and *stat3* transcription activation. Preliminary results in our lab suggest that apoptotic photoreceptors produce a diffusible molecule that induces this regeneration response (Nelson and Hyde, personal communication). This is consistent with an unknown signaling pathway activating the expression of *ascl1a* and *stat3* either upstream of or in parallel with the Müller glia phagocytosis of apoptotic rod cell bodies. Further studies are needed to determine if either of these hypotheses correctly describes the underlying mechanism involved in activating the Müller glia proliferation response in the light-damaged retina.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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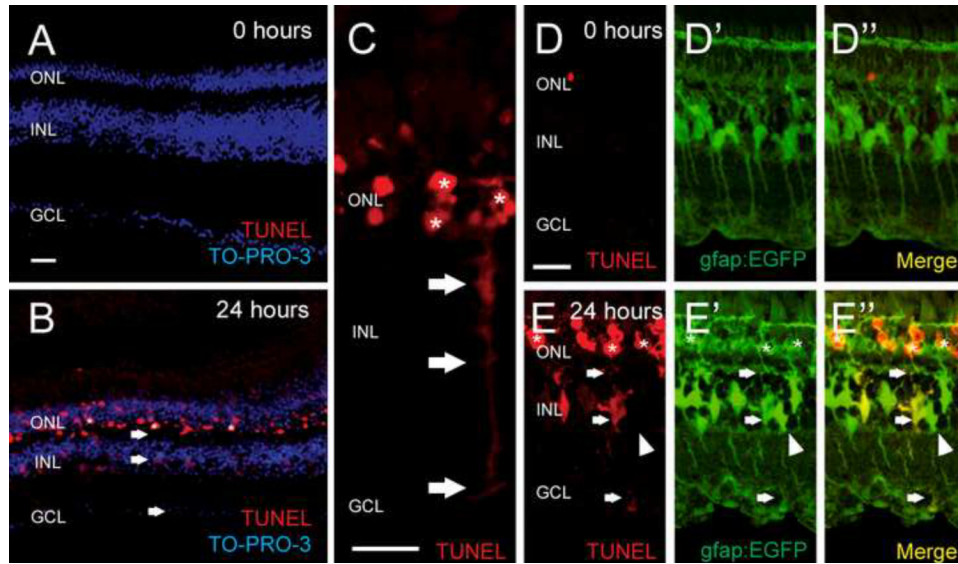


Figure 1. Light-induced TUNEL labeling of dying photoreceptors are detected in both the outer nuclear layer and Müller glia

Both *albino* (A–C) and *albino* $Tg(gfap:EGFP)^{m11}$ transgenic (D–E) zebrafish were treated with constant intense light for either 0 (A and D) or 24 hours (B, C, and E). Retinal sections were stained for DNA fragmentation (TUNEL, red) and nuclei (TO-PRO-3, blue). At 0 hours, no significant TUNEL signal was detected (A, D). After 24 hours, the light-damaged *albino* retina exhibited TUNEL signal in both the ONL and INL (B, arrows). Higher magnification of a 24-hour light-damaged retina shows a TUNEL-positive INL cell with Müller glia morphology (C, arrows) directly adjacent apoptotic photoreceptors (asterisks). Sections of 24-hour light-treated *albino* $Tg(gfap:EGFP)^{m11}$ fish, which express EGFP in Müller glia (E'), revealed TUNEL signal (E) colabeled with a subset of Müller glia in only the light-damaged retina (E'', arrows). The remaining EGFP-positive Müller glia lacked the TUNEL signal (arrowheads). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars represent 25 μ m.

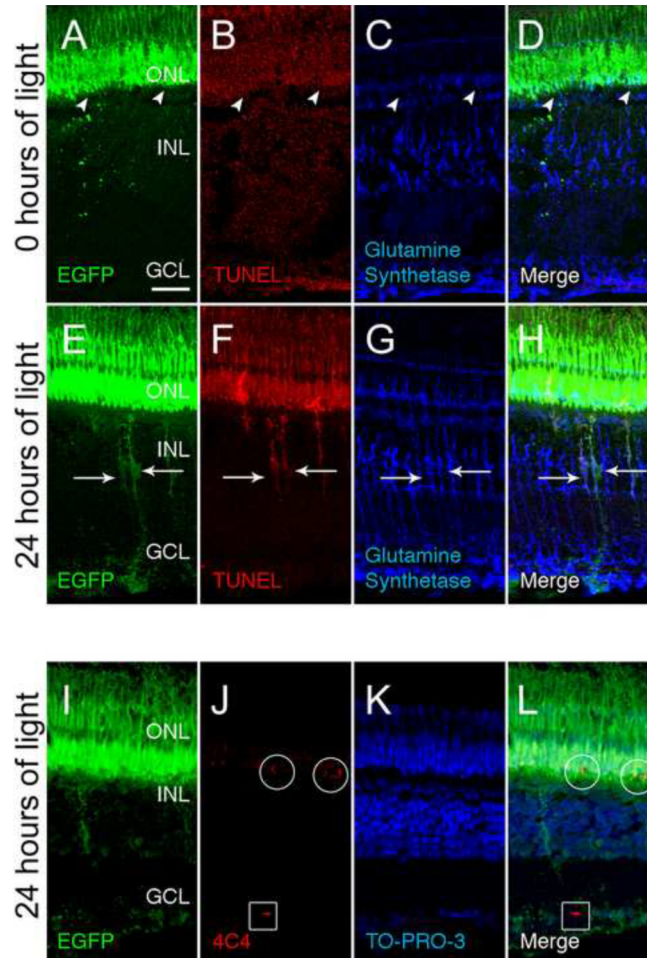


Figure 2. Müller glia engulf dying photoreceptor cell bodies in the ONL

The *Tg(XIRho:EGFP)^{fl}* transgenic fish express EGFP in rod photoreceptors from the *Xenopus rhodopsin* promoter. At 0 hours of light (A–D), control retinas exhibited EGFP expression (A and D, green) only in rods in the ONL (arrowheads) and the outer segments. There was no detectable TUNEL labeling (B) and low levels of Glutamine synthetase was restricted to the Müller glia (C and D). After 24 hours of light, EGFP was detected in both the ONL and INL (E and H, arrows), as was TUNEL labeling (F and H, arrows). Glutamine synthetase, which is expressed in Müller glia (G and H), colocalized with the rod-derived EGFP and TUNEL signal in the INL (H, arrows). An adjacent tissue section to that of panels E–H revealed that mAb 4C4-positive microglial/macrophage cells (J and L) were detected in both the ONL (circles) and GCL (boxes). These microglial/macrophage cells were EGFP-positive (I and L) in the ONL, but not in the GCL. The nuclear layers were identified by TO-PRO-3 staining (K and L). GCL ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar represents 25 μm .

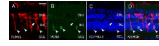


Figure 3. Proliferating Müller glia engulf dying photoreceptor cell bodies

Dark-adapted *albino* zebrafish were exposed to constant intense light for 24 hours and retinal sections were analyzed for cell death by TUNEL (A and D, red), proliferation by PCNA expression (B and D, green), and stained for cell nuclei by TO-PRO-3 (C and D, blue). D represents the merged image of panels A–C. All of the PCNA-positive Müller glia (B, arrowheads) were also TUNEL-positive (D), while some TUNEL-positive Müller glia (A, arrow) were not PCNA-positive (D). GCL ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar represents 25 μ m.

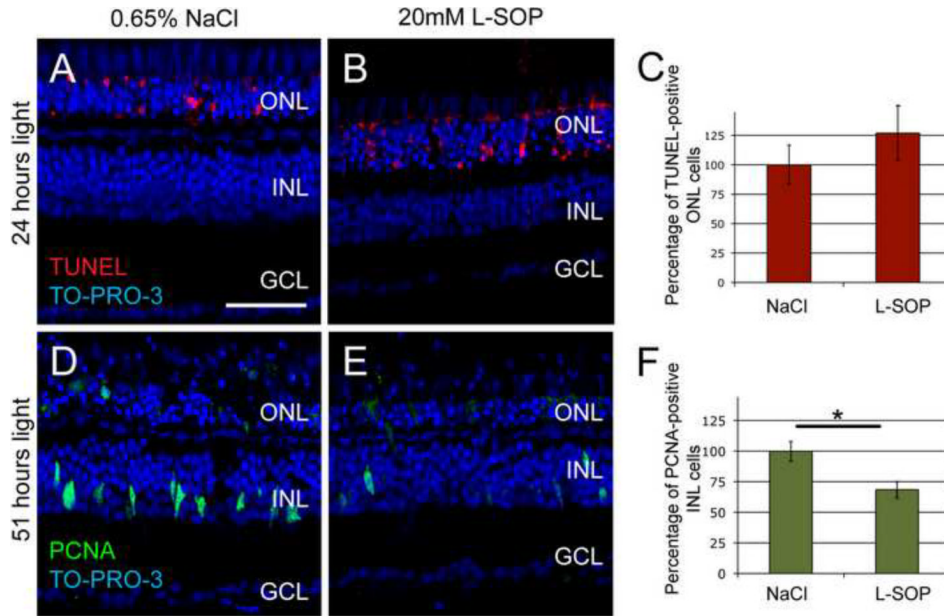


Figure 4. O-phospho-L-serine (L-SOP) inhibits Müller glial cell proliferation in the light-damaged retina

Dark-adapted *albino* zebrafish were injected intravitreally with either 0.65% NaCl (A and D) or 20 mM L-SOP (B and E) immediately prior to starting constant intense light treatment. After 24 hours of constant light (A and B), the retinas were assayed for cell death by TUNEL (red) and counterstained with TO-PRO-3 (blue). The number of TUNEL-positive ONL photoreceptors in the L-SOP-injected retina was normalized to the 0.65% NaCl control retinas (C). There was no statistically significant difference in TUNEL-positive photoreceptors between control and L-SOP-injected retinas ($p > 0.35$, $n = 17$). After 51 hours of constant light (D and E), the retinas were assayed for cell proliferation by PCNA immunostaining (green) and counterstained with TO-PRO-3 (blue). The L-SOP-injected retinas contained significantly less PCNA-expressing Müller glia ($69.5\% \pm 6.8$) than the 0.65% saline-injected eyes (F, $p < 0.005$, $n = 25$). GCL ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar represents 50 μm .

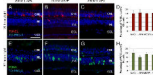


Figure 5. Metabotropic glutamate receptor agonists and antagonists fail to affect either light-induced photoreceptor cell death or Müller glial cell proliferation

Dark-adapted *albino* zebrafish were injected intravitreally with either 0.65% NaCl (Fig. 4, panels A and D), 20 mM L-2-amino-4-phosphonobutyrate (L-AP4, A and E), 20 mM (RS)- α -Methylserine-O-phosphate (MSOP, B and F) or 20 mM L-2-amino-3-phosphonopropanoic acid (L-AP3, C and G) immediately prior to 24 hours of constant light treatment and then assayed for cell death by TUNEL (A–C, red) and counterstained with the nuclear dye TO-PRO-3 (blue). (D) The number of TUNEL-positive ONL bodies, which was normalized to 0.65% NaCl-injected controls, was not statistically different between either L-AP4, MSOP, or L-AP3 injected eyes and the control (D, L-AP4, $p > 0.85$, $n = 13$; MSOP, $p > 0.95$, $n = 17$; L-AP3, $p > 0.45$, $n = 20$). Zebrafish, similarly treated, but exposed to constant intense light for 51 hours (E–G) were assayed for Müller glia proliferation by immunohistochemistry using anti-PCNA antibodies (green) and counterstained with TO-PRO-3 (blue). The number of PCNA-expressing Müller glia, which was normalized to 0.65% NaCl-injected control eyes, was not statistically different between either L-AP4, MSOP, or L-AP3 retinas and the control (H, L-AP4, $p > 0.11$, $n = 37$; MSOP, $p > 0.63$, $n = 27$; L-AP3 $p > 0.64$, $n = 33$). GCL ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar represents 50 μm .

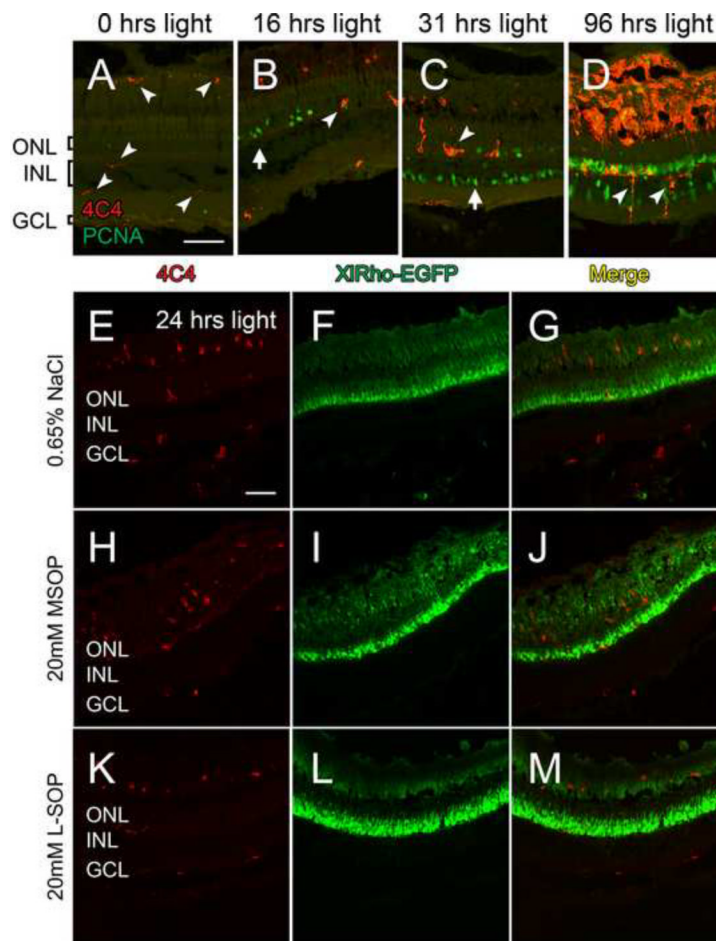


Figure 6. Microglial activity during light damage

Dark-adapted *albino* zebrafish were placed in constant intense light for various lengths of time, sacrificed, and retinal sections were colabeled with the 4C4 monoclonal antibody, which detects microglia (red), and anti-PCNA monoclonal antibody (green). Undamaged retinas possessed a limited number of microglia in the plexiform layers and associated with the rod outer segment region (A, arrowheads). After 16 hours of constant-intense light, larger, activated microglia (arrowhead) were found in the ONL (B). At 31 hours of constant light, activated microglia were restricted to the apical regions of the INL and the ONL (C, arrowhead), whereas PCNA-positive Müller glia were present in the basal INL (C, arrow). At 96 hours of constant light, activated microglia filled much of the ONL and the space formerly occupied by the rod outer segments, as well as some infiltration in the INL (D, arrowheads). (E–M)

Immunohistochemistry of microglia in *albino* $Tg(XIRho:EGFP)^{fl1}$ transgenic retinas. Dark-adapted *albino* $Tg(XIRho:EGFP)^{fl1}$ zebrafish were treated with either 0.65% NaCl (E–G), 20 mM MSOP (H–J), or 20 mM L-SOP (K–M), subjected to 24 hours of constant intense light, sectioned, and labeled with 4C4 monoclonal antibody (E, G, H, J, K, and M, red). EGFP was detected throughout the rods (F, G, I, J, L, and M, green). Despite abundant numbers in the outer segments, an average of fewer than 5 4C4-positive microglia were observed in 400 μ m of the ONL and INL of the L-SOP, MSOP, and saline-injected retinas. GCL ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar represents 50 μ m and is the same for panels A–D and E–M.

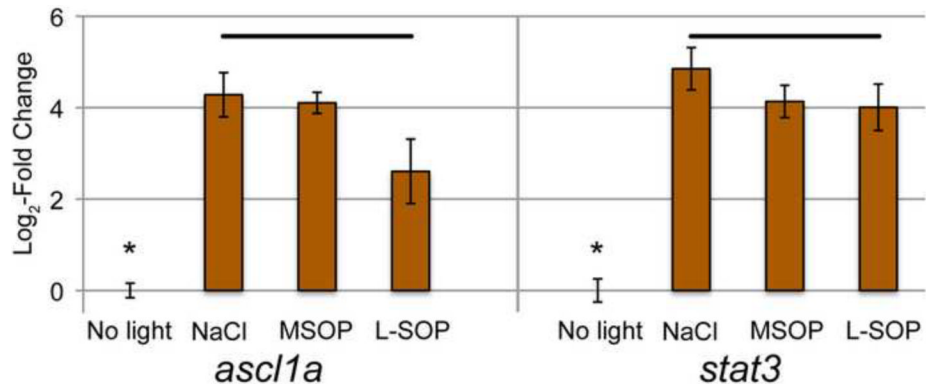


Figure 7. Expression levels of *ascl1a* and *stat3* are unaltered in the L-SOP-treated retina
 Dark-adapted *albino* zebrafish were either untreated and housed at normal lighting conditions (no light) or injected intravitreally with either 0.65% NaCl, 20 mM L-SOP or 20 mM MSOP and subjected to 12 hours of constant intense light. RNA was isolated from the retinas and expression of *ascl1a* and *stat3* were analyzed by quantitative real-time PCR. Constant intense light treatment caused a statistically significant increase in both *ascl1a* ($p < 0.05$, $n = 4$) and *stat3* ($p < 0.005$, $n = 4$) expression, regardless of whether they were injected with saline, L-SOP, or MSOP, relative to undamaged controls (asterisks). There was no significant difference, however, between L-SOP and either saline or MSOP-treated control retinas in light-damaged eyes ($p > 0.10$, $n = 4$ for *ascl1a*, $p > 0.26$, $n = 4$ for *stat3*). Each n represents three pooled retinas harvested from different fish.

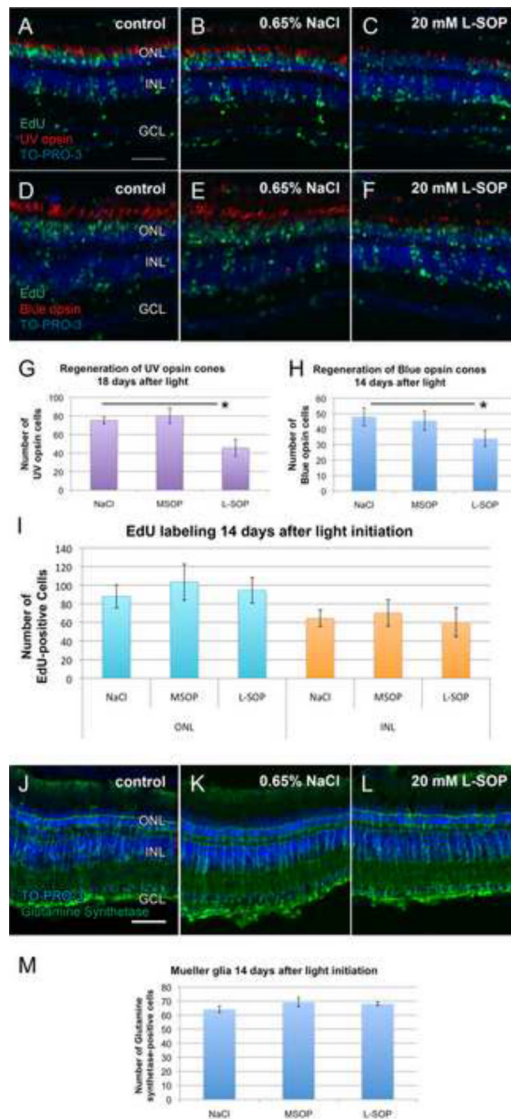


Figure 8. L-SOP-mediated inhibition of UV and blue cone cell regeneration

Dark-adapted *albino* zebrafish were intravitreally injected with EdU after 48, 72, and 96 hours of constant intense light. After the third injection, the fish were placed in standard light conditions for 10 days and then sacrificed. Retinal tissue sections were immunostained for EdU incorporation (A–F, green) and either the short single cone UV opsin (A–C, red) or the long single cone blue opsin (D–F, red). Eyes were either uninjected (A and D) or injected with either 0.65% NaCl (B and E) or 20 mM L-SOP (C and F) prior to initiating the constant light treatment. All sections were costained with TO-PRO-3 (A–F, blue). Statistically significant fewer short single cones ($p < 0.05$, $n = 9$) and long single cones ($p < 0.05$, $n = 12$) were present in the L-SOP-injected retinas relative to the control retinas (G and H, respectively). There was no significant difference in the number of EdU-labeled ONL and INL cells between the L-SOP-treated eyes relative to the controls ($p > 0.05$, $n = 19$). The same retinas described above were immunolabeled with anti-Glutamine synthetase monoclonal antibody (J–L, green) and counterstained with TO-PRO-3 (J–L, blue) to quantify the number of Müller glia present in the regenerated retinas (M). There was no statistically significant difference between the number of glutamine synthetase-positive Müller glia over a 400 μm linear retinal distance in the L-SOP-treated retinas relative

to the controls (M, $p>0.10$, $n=12$) GCL ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar represents 50 μm and is the same for panels A–F and J–L.