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RESEARCH ARTICLE

Light-induced degeneration and microglial response in the retina of an epibenthonic pigmented teleost: age-dependent photoreceptor susceptibility to cell death

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SUMMARY

Constant intense light causes apoptosis of photoreceptors in the retina of albino fish. However, very few studies have been performed on pigmented species. Tench (*Tinca tinca*) is a teleost inhabiting dimly lit environments that has a predominance of rods within the photoreceptor layer. To test the hypothesis that constant high intensity light can result in retinal damage in such pigmented epibenthonic teleost species, photodegeneration of the retina was investigated in the larvae and in juveniles of tench to assess whether any damage may also be dependent on fish age. We exposed both groups of animals to 5 days of constant darkness, followed by 4 days of constant 20,000 lx light, and then by 6 days of recovery in a 14 h light:10 h dark cycle. The results showed that the retina of the larvae group exhibited abundant photoreceptor cell apoptosis during the time of exposition to intense light, whereas that of juveniles was indifferent to it. Damaged retinas showed a strong TUNEL signal in photoreceptor nuclei, and occasionally a weak cytoplasmic TUNEL signal in Müller glia. Specific labelling of microglial cells with *Griffonia simplicifolia* lectin (GSL) histochemistry revealed that photoreceptor cell death alerts microglia in the degenerating retina, leading to local proliferation, migration towards the injured outer nuclear layer (ONL), and enhanced phagocytosis of photoreceptor debris. During the first days of intense light treatment, Müller cells phagocytosed dead photoreceptor cells but, once microglial cells became activated, there was a progressive increase in the phagocytic capacity of the microglia.

Key words: retina, photodegeneration, Müller glia, microglia, phagocytosis.

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INTRODUCTION

In teleost fish, light influences the entire life cycle from embryonic development to sexual maturation in adults (for a review, see Villamizar et al., 2011). However, it also generates potentially damaging reactive oxygen species within the eye. Thus, light has been an effective and frequently used environmental method to induce photoreceptor cell degeneration experimentally (Vihtelic and Hyde, 2000; Allison et al., 2006; Vihtelic et al., 2006; Bernardos et al., 2007; Fimbel et al., 2007; Kassen et al., 2007; Thummel et al., 2008a; Thummel et al., 2008b). Several factors affect the susceptibility of the fish retina to light damage, such as eye pigmentation, rod:cone ratio and the age of the animal.

Thus, constant intense light induces intense apoptosis of the photoreceptors in albino zebrafish (*Danio rerio*) and albino trout (*Oncorhynchus mykiss*), although the proliferative activity of neural stem cells replaces degenerating cells (Yurco and Cameron, 2005; Allison et al., 2006; Fausett and Goldman, 2006; Bernardos et al., 2007; Fimbel et al., 2007; Kassen et al., 2007; Thummel et al., 2008a; Thummel et al., 2008b). Similar studies on pigmented teleost fish are sparse, and have been conducted in species exposed to high light intensities in their natural habitat such as the goldfish (Marotte et al., 1979; Raymond et al., 1988) or rainbow trout (Allen and Hallows, 1997; Allison et al., 2006). In most pigmented teleost species, light damage is absent, although recent morphometric analysis in the retina of some of them reveal that, although pigmented teleosts have a higher threshold for damage from constant light, there is a reduction in the photoreceptor layer thickness under long, high intensity light exposure (Vera and Migaud, 2009).

In surface-dwelling diurnal albino fish retinas with a higher cone:rod ratio exposed to constant high intensity light, while some of the rod nuclei degenerate by apoptosis, the cones remain intact, even in areas where rod nuclei have degenerated (Allison et al., 2006). Additionally, it has been demonstrated that rod-dominated retinas, such as in nocturnal rodents, are damaged by forced exposure to moderate light (Wenzel et al., 2005; Santos et al., 2010). Therefore, pure-rod retinas could be more susceptible to damage than cone-rich retinas.

Most of the studies described above have been carried out on juvenile teleost fish where the retina is fully differentiated. However, there are differences between fish larval, juvenile and adult retinal morphology and cell composition (Kvenseth et al., 1996; Doldán et al., 1999; Helvik et al., 2001a; Helvik et al., 2001b; Evans and Browman, 2004; Bejarano-Escobar et al., 2009; Bejarano-Escobar et al., 2010; Bejarano-Escobar et al., 2012). Thus, most marine fish larvae have only pure-cone retina at their early developmental stages but later rods appear and the single-cone retina gradually transforms into a duplex retina (Blaxter, 1986; Kvenseth et al., 1996; Helvik et al., 2001a; Helvik et al., 2001b). Additionally, it has been described that light modulates retinal morphogenesis in fish at various stages of development. Thus, photoreceptor cell density is influenced by the experimental light conditions during larval stages (Raymond et al., 1988). Therefore, the threshold of light intensity

and the sensitivity to light vary between different stages of development.

The rod-dominated retinas of albino strains of rodents and the cone-diurnal retina of albino fish (Teleostei) are damaged by forced exposures to moderate light and this has led to their choice as animal models for the study of light damage to the human retina (Remé et al., 1998; Vihtelic and Hyde, 2000; Allison et al., 2006; Vihtelic et al., 2006; Bernardos et al., 2007; Fimbel et al., 2007; Kassen et al., 2007; Thummel et al., 2008a; Thummel et al., 2008b; Santos et al., 2010). Albino teleosts are rare in nature and most of the studies on light-induced photodegeneration in fish have focused on albino mutant zebrafish. Unfortunately, the cost of setting up and maintenance of a zebrafish facility is high and not all laboratories are in a position to undertake this type of study. Collaborations between aquaculture stations and research centres could represent an opportunity to solve this problem. Furthermore, the wide diversity of fish species suggests that alternative models for studying neurodegenerative processes might be found among lesser known species. The tench, *Tinca tinca* (Linnaeus 1758), has been widely cultivated in Europe and especially in Spain. It is a freshwater epibenthonic teleost, living near the bottom in waters with abundant vegetation in a dim light environment. It has been described as a strictly nocturnal species (Herrero et al., 2005). Previous studies in our laboratory have shown that there are abundant rods in the photoreceptor cell layer of larval and juvenile individuals (Bejarano-Escobar et al., 2009). Therefore, all these data led us to think that the rod-dominated retina of a nocturnal fish should be susceptible to light damage and could be a suitable model to study photoreceptor degeneration. The objectives of our study were to determine (1) the effects of high intensity light exposure on larval and juvenile fish retinas in teleost species adapted to a dim light environment, with abundant rods in the composition of their retinas, and (2) whether retinal degeneration occurs, and if so to analyse the time course of cell degeneration and phagocytosis of cell debris, and to identify the cell populations involved in these processes.

MATERIALS AND METHODS

Animal care and constant light treatment protocol

A total of N=153 larval (1 month post-hatch, total length 11 mm) and N=42 juvenile (12 months post-hatch, total length 9.18 cm) (Fig. 1) tench were kindly provided by the Centro de Interpretación Piscícola Las Vegas del Guadiana fish farm (Badajoz, Spain) and Tencazuaga s.c. fish farm (Azuaga, Badajoz, Spain). Photic injury to tench retina was carried out as described previously in the albino zebrafish retina (Vihtelic and Hyde, 2000) with some modifications. Tench were raised at 25°C under 14 h light:10 h dark conditions for 7 days. Prior to the light treatment, the tench were kept in constant darkness for 5 days to increase retinal sensitivity. Control group retinas were collected from the fish raised for 5 days in constant darkness. Larval and juvenile groups of experimental tench were transferred to 10 l clear polycarbonate tanks and exposed for 96 h to cool white light in a Versatile Environmental Test Chamber (MLR-350; Sanyo Electric Co., Osaka, Japan) with 15 standard fluorescent (40 W) lights. The spectrum of these fluorescent lamps showed three main wavelengths with peaks at 434.7, 545.5 and 576.4 nm. A hand-held luxometer (Datalogging Light Meter, 850008, Sper Scientific, Scottsdale, AZ, USA) measured a light intensity of 20,000 lx at the aquarium glass surface, at a water temperature of 25°C. Fish underwent 20, 31, 45, 55, 72 and 96 h of constant light treatment, followed by 6 days of recovery under a 14 h light:10 h dark cycle. The constant light treatment is summarized in Fig. 1. Animal care and experimental protocols followed the guidelines issued by the Animal Care and Use Committee of the University of Extremadura.

Tissue processing

The effect of the constant light treatment on tench retinas was analysed in semi-thin (morphological analyses) and cryostat (histochemical and immunohistochemical analyses) sections. For the morphological analyses, some larval and juvenile specimens were deeply anaesthetized with a 0.05% solution of tricaine methane sulphonate (MS-222; Sigma, St Louis, MO, USA) and immersed in a mixture of 2% glutaraldehyde and 2% paraformaldehyde (2% PFA) in phosphate buffer (PB, 0.1 mol l^{-1}, pH 7.4) for 8 h at 4°C. They were rinsed in PB, post-fixed in 2% osmium tetroxide for 2 h, dehydrated in graded acetone concentrations, rinsed in propylene oxide, and embedded in Spurr's resin. Serial frontal 3 μm sections were obtained with a Reichert Jung microtome. The sections were stained with alkaline 1% Toluidine Blue, washed three times in PB, dehydrated, and mounted with Eukitt (Kindler, Freiburg, Germany).

For the histochemical and immunohistochemical analyses, larval and juvenile tench were deeply anaesthetized and fixed by immersion in 4% PFA in 0.1 mol l^{-1} phosphate-buffered saline (PBS), pH 7.4, overnight at 4°C. The eyes of juvenile animals were removed and post-fixed for 12 h in the same fixative solution. For proliferating cell nuclear antigen (PCNA) immunohistochemistry, the animals were fixed in Bouin's fixative at 4°C for 36 h, then rinsed with 50% ethanol several times to remove traces of the fixative, and gradually hydrated.

Tissues were immersed in PBS, then cryoprotected in 10% sucrose solution in PBS and embedded in 10% gelatin:10% sucrose solution in the same buffer. The blocks were frozen and freeze-mounted onto aluminium sectioning blocks. Cryostat sections, 15 μm thick, were cut in the frontal plane. Sections through different retinal areas were thaw-mounted on SuperFrost-Plus slides (Menzel-Gläser, Braunschweig, Germany), air-dried and stored at –80°C until use.

TUNEL technique

Dead cells in the visual system were localized using terminal deoxynucleotidyl transferase (TDT)-mediated deoxyuridine triphosphate (dUTP) nick end labelling (TUNEL) (Gavrieli et al., 1992) with the *in situ* cell death detection kit POD (Roche, Basel, Switzerland). The TUNEL technique was performed on cryostat sections as described previously (Bejarano-Escobar et al., 2010).

Griffonia simplicifolia lectin histochemistry

We used B4 isolectin derived from *Griffonia simplicifolia* (GSL), which recognizes macrophages and microglial cells in the nervous system of several vertebrates (Ashwell, 1990; Streit, 1990; Kaur and Ling, 1991). Cryostat sections were washed several times in 0.05% Triton X-100 in PBS (PBS-T) and treated with 3% hydrogen peroxide in PBS solution for 45 min. After rinsing twice in PBS and once in PBS-T for 10 min, sections were incubated with biotinylated GSL (Sigma) at a concentration of 6 μg ml^{-1} in PBS-T overnight at room temperature (RT). The slides were rinsed twice in PBS for 15 min and incubated with a solution of 1:200 diluted ExtrAvidin-peroxidase (Sigma) or with ExtrAvidin-fluorescein isothiocyanate (FITC) (Sigma) for 2 h at RT. After rinsing twice in PBS for 15 min, the peroxidase reaction product was visualized with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.025% hydrogen peroxide in PBS for 10 min at RT. Sections developed with DAB were washed, dehydrated and mounted with Eukitt

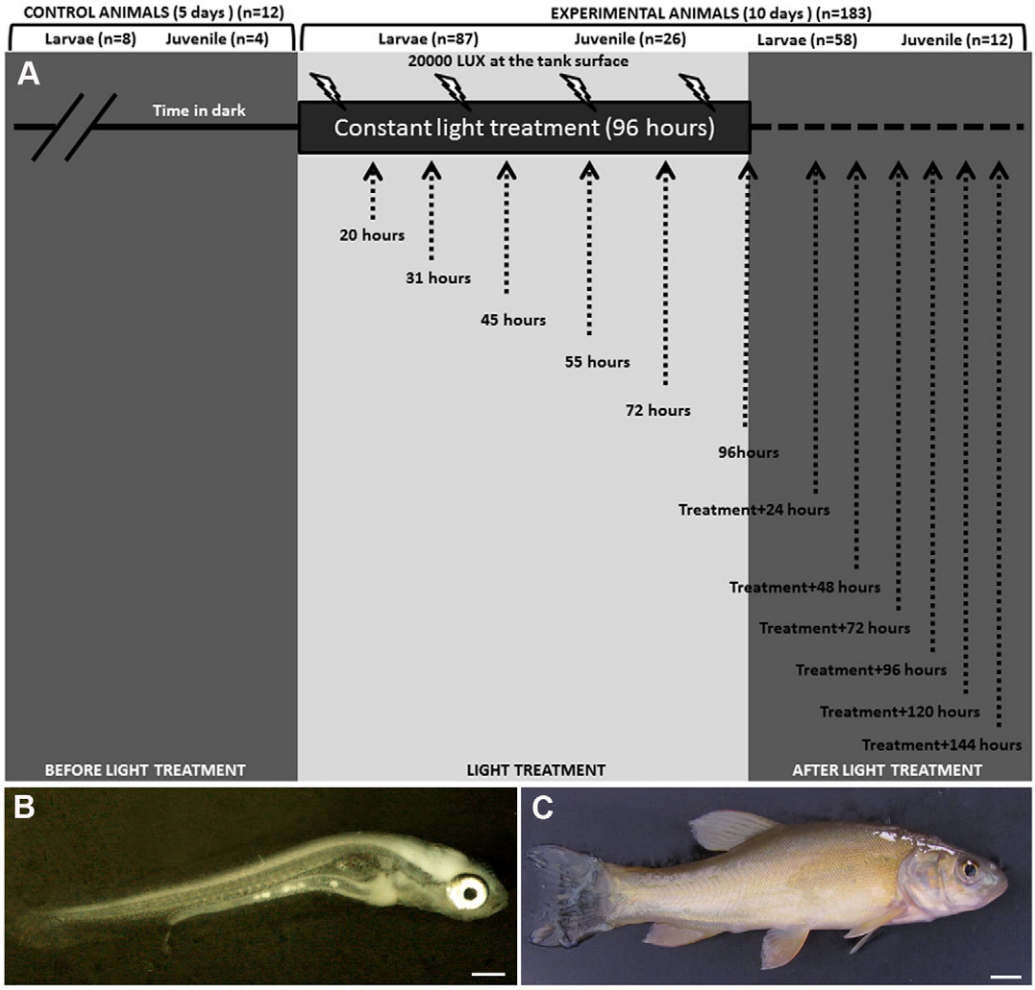

Fig. 1. (A) Schematic summary of the protocol used for analysing bright light-induced degeneration in the photoreceptor cell layer of the tench retina. Photograph of larval (B) and juvenile (C) tench included in the present study. Scale bars denote 1 mm in A, 1 cm in B.

(Kindler, Freiburg, Germany). Fluorescence labelled sections were coverslipped with Mowiol for observation. No signal was found in histochemical controls that were made omitting the GSL and using only the DAB solution.

Immunohistochemical studies

Working solutions and sources of primary antibodies used in the present study were as follows: rabbit anti-bovine rod opsin CERN-922 polyclonal antibody at 1:1000 (gift from Dr Willem J. DeGrip, Radboud University Nijmegen Medical Centre), mouse anti-glutamine synthetase monoclonal antibody at 1:200 (Chemicon MAB302, Millipore, Billerica, MA, USA) and mouse anti-PCNA monoclonal antibody at 1:500 (clone PC10, Abcam, Paris, France). Secondary antibodies used were Alexa Fluor 594 goat anti-mouse IgG antibody at 1:200, Alexa Fluor 488 goat anti-mouse IgG antibody at 1:200 and Alexa Fluor 488 goat anti-rabbit IgG antibody at 1:200 (Molecular Probes, Invitrogen, Carlsbad, CA, USA). Single and double labelling techniques were performed as described previously (Bejarano-Escobar et al., 2009; Bejarano-Escobar et al., 2010). Control experiments were performed by omitting the primary antibodies and were always negative. For double-labelling purposes, we first performed single immunohistochemistry followed by GSL histochemistry, and TUNEL histochemistry followed by single immunohistochemistry.

In all cases, sections were observed using an epifluorescence, brightfield Nikon Eclipse E600 microscope, and photographed using a digital camera (Axiocam HRc). Figures were assembled and images were corrected for brightness and contrast in Adobe Photoshop (v. CS4).

Quantification of TUNEL-positive nuclei, TUNEL-positive Müller cells and GSL-labelled microglial cells

Quantification was performed counting all TUNEL-positive nuclei, TUNEL-labelled Müller cells and GSL-labelled cells of the neural retina in sections from control and experimental tench. Cells were only counted in every second section to avoid any double counting. The surface of each section was measured on digital microphotographs using ImageJ free software (http://rsb.info.nih.gov/ij/). The density profiles were expressed as the mean ± s.e.m. number of apoptotic nuclei, Müller glial cells or microglial cells per square millimetre (an mm^{-2}, mgc mm^{-2} and mc mm^{-2}, respectively). Similar procedures have been described in the literature (Cook et al., 1998; Mayordomo et al., 2003; Francisco-Morcillo et al., 2004; Santos et al., 2010). Statistical analyses were performed using Student's two-tailed *t*-test. Differences between groups were considered as significant when $P<0.05$.

Morphometric analysis of microglial cells

In order to compare the morphology of microglial cells in control and experimental retinas, two different factors were used: circularity and aspect ratio. According to ImageJ software, circularity (C) is defined by the following equation: $C=4\pi$ area/perimeter^2. Circularity is a dimensionless parameter ranging

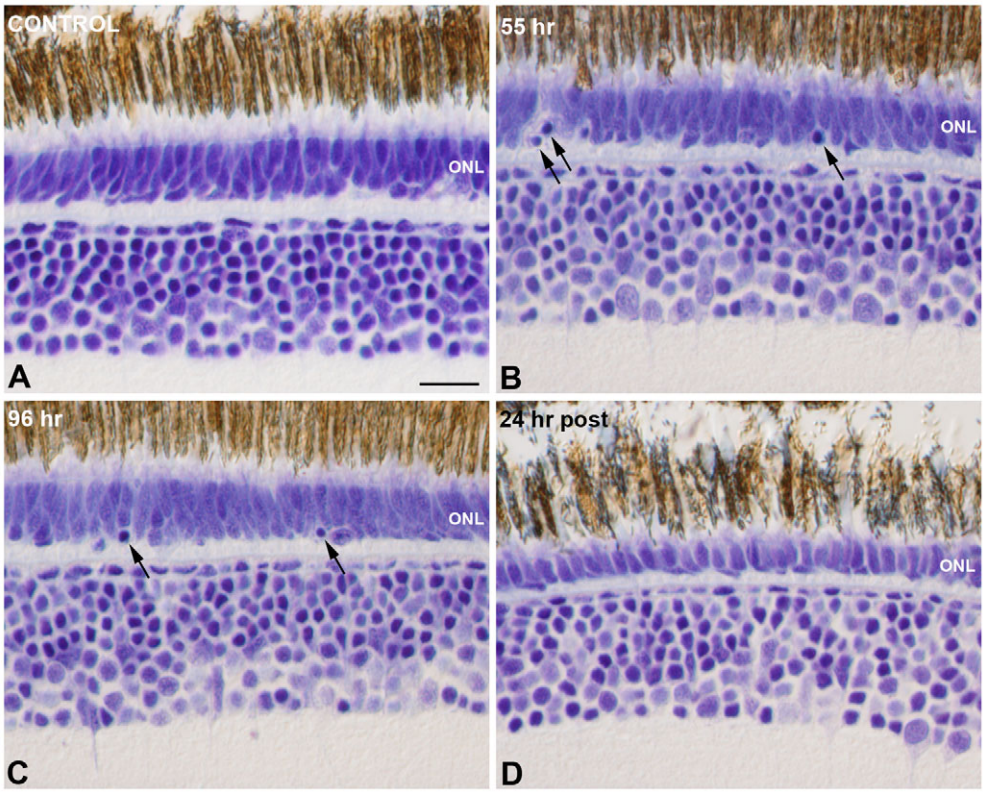

Fig. 2. Histological analysis of photoreceptor cell death during light-induced tench retinal degeneration in Toluidine Blue-stained transverse semi-thin sections. Micrographs were taken from the central region of tench larval retinas. (A) The non-light-damaged control retinas showed an intact outer nuclear layer (ONL) that contained stacks of 3–4 nuclei. No pyknotic nuclei were detected. (B,C) During the 96 h of constant light treatment, many pyknotic nuclei were observed in the ONL (arrows). (D) The ONL of 24 h post-treatment retinas was devoid of pyknotic nuclei. Photoreceptor cell somata were arranged in a single row. Scale bar denotes 25 μm.

from 0 (a perfect line) to 1 (a perfect circle). Aspect ratio (AR) describes the proportional relationship between the major and minor axes of microglial cells.

A total of 100 GSL-positive cells from control and experimental retinas were randomly chosen for this purpose. The mean ± s.e.m. number of *C* and AR values was calculated. Statistical analyses were performed using Student's two-tailed *t*-test. Differences between groups were considered as significant when $P<0.05$.

RESULTS

Light-induced loss of photoreceptors in the larval tench retina

We used classical histological procedures, histochemical and immunohistochemical methods to investigate whether light damage leads to photoreceptor cell death in tench. Pyknotic nuclei were absent in the outer nuclear layer (ONL) of control and experimental juvenile tench (not shown). Light microscope observations of Toluidine Blue-stained transverse semi-thin sections revealed that the incidence of cell death in the photoreceptor cell layer of control larvae was very low (Fig. 2A). However, intense light treatment (20,000 lx) resulted in histological signs of cell damage in the photoreceptor cells of the larvae group. Thus, pyknotic nuclei were localized in the vitreal half of the ONL of experimental animals during the 96 h of intense light treatment (Fig. 2B,C). Pyknotic nuclei were rarely found 24 h after the constant intense light treatment ceased (Fig. 2D). There was a progressive thinning of the ONL in the constant light-treated larval tench retinas (Fig. 2). CERN-922 polyclonal antibody has been found to be useful for labelling developing and mature photoreceptors in the retina of agnathans (Meléndez-Ferro et al., 2002; Villar-Cheda et al., 2008) and teleosts (Candal et al., 2005; Bejarano-Escobar et al., 2010), including the tench (Bejarano-Escobar et al., 2009). Immunoreactive photoreceptor cells were abundant in control retinas (Fig. 3A), but light damage led to the progressive loss of labelled cells as the experimental treatment advanced (Fig. 3B,C). In order to localize apoptotic nuclei and quantify the change in the number of degenerating photoreceptors in experimental animals, we used TUNEL histochemistry on frozen retinal sections. The ONL of control and experimental juvenile tench was devoid of apoptotic nuclei (not shown). Sparse TUNEL-positive nuclei were observed in the ONL of control larval tench retinas (24.3 an mm^{-2}) (Fig. 4A,B; Fig. 5). However, the density of TUNEL-positive nuclei was higher ($P<0.01$) during exposure to the bright light treatment, although it decreased ($P<0.01$) between 55 h (531.46 an mm^{-2}) and 72 h of exposure (226.05 an mm^{-2}) (Fig. 4C–H; Fig. 5). The density of apoptotic nuclei 24 h after the light treatment decreased ($P<0.01$) to values similar to those observed in control animals (17.03 an mm^{-2}) (Fig. 4I,J; Fig. 5). In addition, a diffuse TUNEL signal was also observed in cell somata and vitreal and scleral processes of radially oriented cells located in the inner nuclear layer (INL) (Fig. 4C–J). Occasional TUNEL labelling was also observed in the outer limiting membrane (Fig. 4C,D). No TUNEL labelling was detected in the absence of terminal deoxynucleotidyl transferase enzyme (not shown), which confirmed that the INL signal resulted from specific labelling of fragmented DNA. This TUNEL labelling located in the INL exhibited the morphology of Müller glial cells. In order to confirm that the diffuse INL TUNEL staining was associated with Müller glial cells, we combined TUNEL histochemistry with immunohistochemistry, using antibodies against glutamine synthetase, an excellent marker for Müller cells in the developing and mature fish retina (Linser and Moscona, 1979; Linser et al., 1984; Mack et al., 1998; Peterson et al., 2001; Bejarano-Escobar et al., 2009; Bejarano-Escobar et al., 2010; Bejarano-Escobar et al., 2012). Double labelling experiments revealed that TUNEL-positive INL cells co-labelled with glutamine synthetase (Fig. 9A–C). Because this TUNEL labelling appeared to fill the cytoplasm of the Müller glial cells, including the processes, it

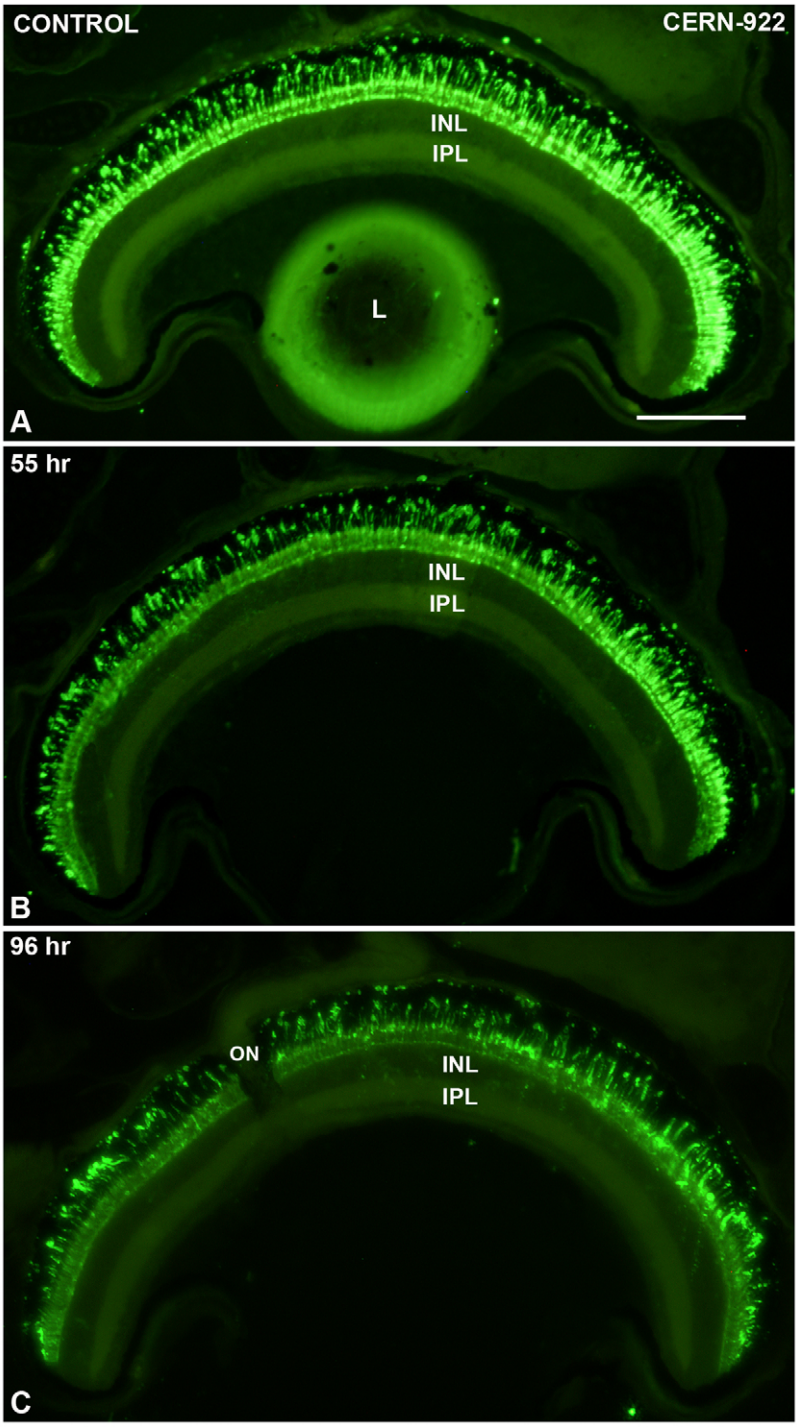

Fig. 3. Distribution of CERN-922-positive photoreceptors in the retina of control (A) and experimental tench larvae (B,C). In all panels dorsal is to the right. (A) The control retina displays robust photoreceptor labelling. (B,C) During the 96 h of light treatment the immunochemical signal was progressively reduced compared with the control section shown in A. INL, inner nuclear layer; IPL, inner plexiform layer; L, lens; ON, optic nerve. Scale bar denotes 200 μm.

probably did not represent apoptotic Müller glia. The number of TUNEL-positive Müller cells progressively diminished as the constant light treatment advanced (Fig. 4C–H). Thus, the density of TUNEL-positive Müller glial cells was high after 31 h of light exposure (493.46±17.05 mgc mm^{-2}) but decreased significantly after 72 h of constant light treatment (60.62±9.10 mgc mm^{-2}, P<0.01).

Microglial response in light-damaged larvae retinas

The marker used throughout the present study to identify macrophages and microglial cells is the B4 isolectin derived from *G. simplicifolia* (GSL). This recognizes these types of cells in the nervous systems of several vertebrates (Ashwell, 1990; Streit, 1990; Kaur and Ling, 1991). Sparse (22.07 mc mm^{-2}) and homogeneously distributed weakly labelled GSL microglial cells were observed in the inner plexiform layer (IPL) and INL in the control group (Fig. 6A–C; Fig. 7). They had elongated cell bodies (C=0.17; AR=3.52) and few slender processes (Fig. 6A–C). However, during constant intense light treatment, these cells changed in distribution, number and shape (P<0.01), showing amoeboid morphological features (C=0.55; AR=2.16) with short and thick processes (Fig. 6D–L). Activated microglial cells could be distinguished in all retinal layers under experimental conditions (Fig. 6G–L). The ONL, devoid of microglial cells in control retinas (Fig. 6A–C; Fig. 7B), was colonized by these cells in conditions of photoreceptor

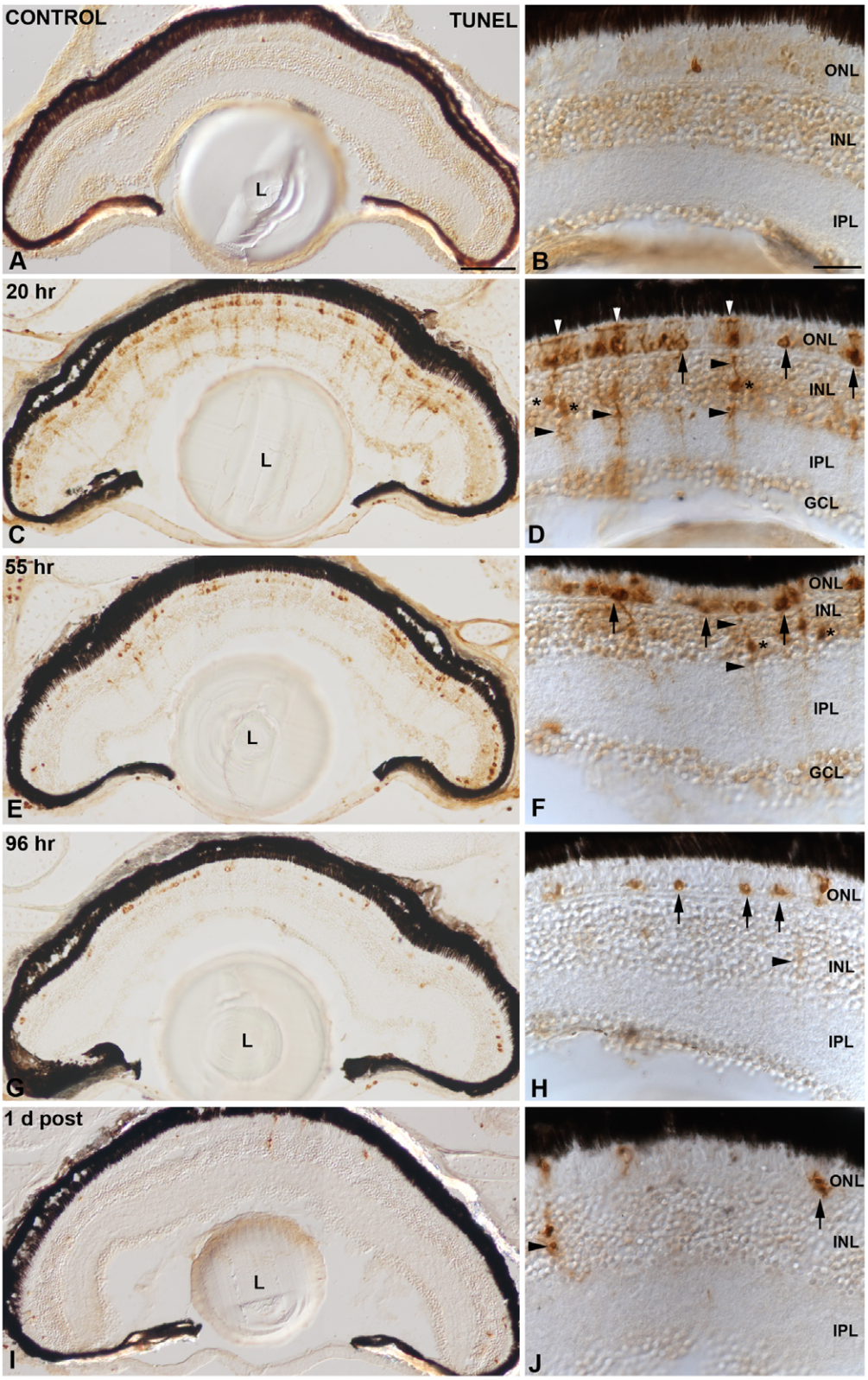

Fig. 4. TUNEL histochemistry showing the progression of retinal cell death in the photoreceptor layer in the light-damaged larval tench retina. In all panels, dorsal is to the right. (A,B) No significant TUNEL signal was detected in the retinal section of a pre-constant-light control eye. (C,D) After 20 h, the light-damaged tench retina exhibited abundant TUNEL-positive nuclei in the ONL (arrows). Müller cell somata (asterisks) and processes (black arrowheads) were also stained with TUNEL histochemistry. The outer limiting membrane (OLM) occasionally appeared labelled (white arrowheads in D). (E,F) After 55 h, abundant degenerating photoreceptors were still observed in the ONL (arrows), although fewer TUNEL-positive somata (asterisks) and processes (arrowheads) were observed in the INL. (G,H) Apoptotic nuclei in the ONL were still abundant at the end of the experimental procedure (arrows). TUNEL-positive elements in the INL were sparse (arrowhead). (I,J) TUNEL staining almost disappeared from the ONL (arrow) and the INL (arrowhead) in experimental retinas 24 h after light exposure. GCL, ganglion cell layer. Scale bars denote 100 μm in A,C,E,G,I, 25 μm in B,D,F,H,J.

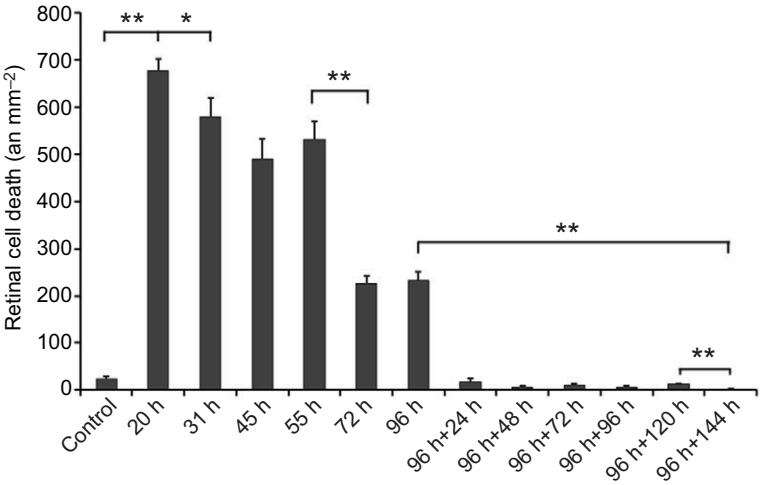

Fig. 5. Quantitative analysis of the density of TUNEL-positive nuclei in the ONL of control and experimental larval tench retinas. In control animals, the outer retina contained a low density of TUNEL-positive nuclei. However, a significant increase in the density of TUNEL-positive nuclei was observed following 20 h of bright light exposure and was maintained until 55 h. The density of TUNEL-positive nuclei decreased significantly after 72 h, although it remained higher than in control retinas until the end of the treatment, returning to almost normal levels after the light exposure ceased. Data are expressed as means ± s.e.m. Statistical significance is indicated by asterisks (*P<0.05, **P<0.01). an mm^{-2}, apoptotic nuclei per square millimetre.

degeneration (Fig. 6G–L; Fig. 7B). Finally, the density of activated microglial cells progressively increased during the experimental procedure in the entire retina, reaching the highest value (425.78 mc mm^{-2}) after 96 h of constant light treatment (Fig. 7A). In the ONL, the density profiles of microglial cells increased during the first 31 h (P<0.05) and remained at the same level between 31 and 96 h of exposition to bright light (Fig. 7B). In the experimental animals 24 h after light exposure ceased the density of microglial cells greatly diminished in the entire retina (90.79 mc mm^{-2}, P<0.01) (Fig. 7A) and in the ONL (13.17 mc mm^{-2}, P<0.01) (Fig. 7B), although the density values were higher than those observed in control animals until 5 days later for the entire retina (P<0.01) (Fig. 7A), and until 4 days later in the case of the ONL (P<0.01) (Fig. 7B). Moreover, during the 5 days after light treatment, microglial cells retained a more activated phenotype, still different from their morphology in the control retinas (Fig. 6M–O). They were mainly located in more vitreal layers (Fig. 6M–O), although a few of them were still detected in the ONL (Fig. 6O; Fig. 7B).

Proliferative cells in the experimental larval retinas

We performed immunohistochemical detection of PCNA, an antigen expressed in the nuclei of cells during DNA replication, that has previously been used to identify proliferating precursor cells in the teleost fish retina (Mack and Fernald, 1997; Velasco et al., 2001; Cid et al., 2002; Candal et al., 2005; Bernardos et al., 2007; Bejarano-Escobar et al., 2009; Bejarano-Escobar et al., 2010), and to investigate whether cell proliferation is affected by the incidence of constant high intensity light in experimental animals. Many PCNA-positive nuclei were found in the ciliary marginal zone (CMZ) in both control and experimental tench (Fig. 8A,C,E). In control retinas, sparse radially oriented proliferating nuclei that had a fusiform morphology were observed in the INL (Fig. 8A,B). However, many PCNA-positive nuclei showing different morphologies were distributed throughout the different retinal layers in experimental retinas (Fig. 8C–F). Some of them, mainly located in the INL, showed their main axis radially oriented (Fig. 8C–F). Furthermore, rounded and elongated tangentially oriented proliferative nuclei were dispersed throughout the ONL, the IPL and ganglion cell layer (Fig. 8C–F). Double immunolabelling experiments demonstrated many PCNA-positive cells that were also labelled with GSL histochemistry (Fig. 9D–F). However, proliferative nuclei located in the CMZ and some of the PCNA-positive nuclei dispersed throughout the ONL and INL were not labelled with GSL (Fig. 9D–F).

DISCUSSION

Constant light-induced cell death in the pigmented larval tench retina

The major goal of the present study was to develop a pigmented teleost model suitable for studying neurodegeneration-induced processes in the vertebrate retina. Albino teleosts are a better choice than pigmented animals for investigating the mechanisms by which light damages the retina because of their relatively high susceptibility to light damage. Thus, constant intense light exposure of albino zebrafish causes widespread photoreceptor cell death (Vihtelic and Hyde, 2000; Vihtelic et al., 2006; Thummel et al., 2008a; Thummel et al., 2008b; Bailey et al., 2010), and rod outer segment degradation ('solar pruning') and photoreceptor loss in the albino rainbow trout (Allen and Hallows, 1997; Allen et al., 1999; Allison et al., 2006) and albino oscar *Astronotus ocellatus* (Allen et al., 1999). However, albino teleosts are rare in nature. Allison and colleagues have shown that, while extensive rod death occurred in the retina of experimental albino fish species, the cones remained intact even in areas where rod nuclei degenerated (Allison et al., 2006). Therefore, the susceptibility to light damage seems to be higher in rods than in cones. As for mammals, rod-dominant nocturnal fishes can be an attractive complement to the study of photoreceptor cell death. A recent study conducted in our laboratory has shown that rods are numerous in the photoreceptor layer of tench (Bejarano-Escobar et al., 2009) as a result of the adaptation to light conditions in its epibenthonic habitat. All these data led us to think that the tench retina should be susceptible to light damage and could be a suitable model for studying photoreceptor degeneration.

However, we found that the juvenile tench retinas remained undamaged after constant light treatment, in agreement with previous studies conducted on pigmented surface-dwelling diurnal adult fish species (Raymond et al., 1988; Allen and Hallows, 1997; Allen et al., 1999; Allison et al., 2006). What makes the retina so resistant to light damage in pigmented fish species? Ocular melanin protects the retina against light-induced cell toxicity, acting as an anti-oxidant adjacent to the rod outer segments (Sanyal and Zeilmaker, 1988). Moreover, unlike the mammalian retina, in response to light, melanin granules migrate in an apical direction within processes of the retinal pigment epithelium and enshroud photoreceptors (Allen and Hallows, 1997). Additionally, photoreceptors are capable of sliding into or out of the deep recesses of the retinal pigment epithelium (Wagner, 1990). Recently, some authors have found differential severity and sensitivity to high intensity light exposure between the retinas of

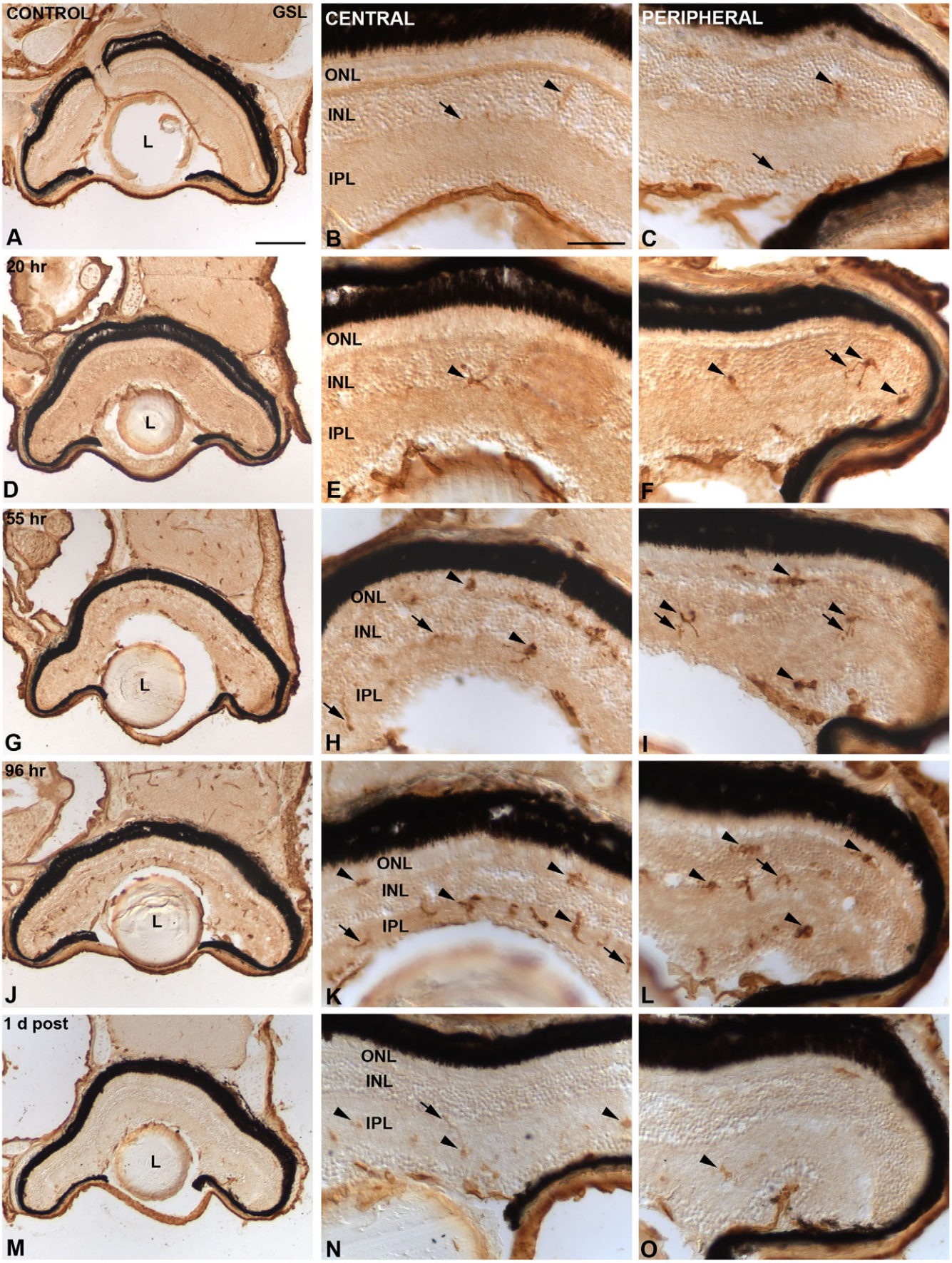

Fig. 6. See next page for legend.

Fig. 6. Changes in distribution pattern and microglial morphology in control (A–C) and light-exposed animals during 20 h (D–F), 55 h (G–I) and 96 h (J–L) of light exposure, and 24 h after light exposure (M–O), as shown by *Griffonia simplicifolia* lectin (GSL) histochemistry. Overviews of retinas (A,D,G,J,M) and higher magnifications from the central (B,E,H,K,N) and peripheral regions (C,F,I,L,O) are shown. In all panels, dorsal is to the right. (A–C) In control tench retinas, sparse elongated somas (arrowheads) and thin processes (arrows) were faintly labelled in both the IPL and the INL. (D–L) Constant intense light for 96 h resulted in an increase in the intensity of GSL staining and in the number of labelled cells. Microglial cells became larger (arrowheads) and showed thicker processes (arrows) after activation in response to photoreceptor degeneration. During the experimental period, microglial cells progressively invaded the ONL. (M–O) The intensity of GSL staining and the number of labelled cells greatly diminished 24 h after the constant light treatment. The microglial cell distribution resembles that observed in the control animals. Scale bars denote 200 μm in A,D,G,J,M, 50 μm in B,C,E,F,H,I,K,L,N,O.

wild pigmented species, in particular European sea bass, Atlantic cod and Atlantic salmon juveniles (Vera and Migaud, 2009). These authors exposed each species to intense constant light and, using morphometric analyses, found a reduced thickness of the photoreceptor layer in the three species, with cod being the most affected of all. Some of these species were exposed to constant high intensity light for 14 and 25 days. The effects of longer exposures to high intensity light in the juvenile tench retina therefore merit further investigation.

In contrast, TUNEL labelling provided a clear demonstration that many photoreceptors were dying during light treatment of larval tench. The early larval retinas of nearly all teleost fish species either contain only cone photoreceptors (no rods) or are highly cone dominated, and differentiate rods at later stages (Sandy and Blaxter, 1980; Kvenseth et al., 1996; Helvik et al., 2001a). The tench retina differentiates its rod cells very early during the prolarval period, during the first postnatal day (Bejarano-Escobar et al., 2009). This fact increases the susceptibility of the larval tench retina to light damage. Our results clearly demonstrated that photoreceptor sensitivity to bright light varies with the age of the animal.

A time course analysis revealed that the TUNEL-positive nuclei in the ONL appeared within the first 20 h of constant light, suggesting that apoptotic pathways were activated in the photoreceptors during the first 24 h of light treatment, in agreement with a previous study conducted on albino zebrafish (Vihtelic and Hyde, 2000). The authors of the latter study also found that in the albino zebrafish retina the greatest reduction in the photoreceptor layer occurred during the first 3 days of constant light (Vihtelic and Hyde, 2000), consistent with our observations that the greatest incidence of cell death in the larval photoreceptor cell layer occurred during the first 55 h of treatment. Finally, Vihtelic and colleagues have found that while extensive photoreceptor cell death occurs in the central and dorsal regions of the albino zebrafish retina, many rods and cones located in ventral regions survive the light treatment (Vihtelic et al., 2006). In the present work, we found that photoreceptor degeneration in experimental tench larvae is not extensive and seems to occur homogeneously over the entire retina.

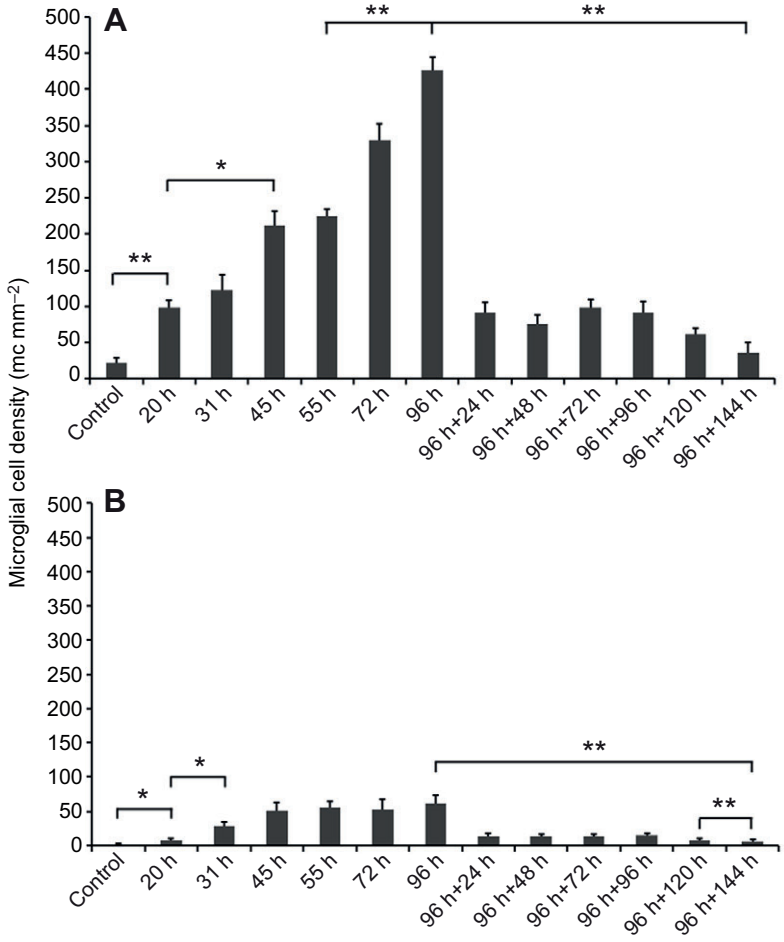

Fig. 7. Quantitative analysis of the density of GSL-positive microglial cells in the entire retina (A) and in the ONL (B) of control and experimental larvae. In both cases the density of labelled microglial cells progressively increased until 96 h of bright light exposure, and decreased abruptly once the treatment ceased. Mean values after light treatment were significantly greater than in control retinas. Data are expressed as means ± s.e.m. Statistical significance is indicated by asterisks (*P<0.05 or **P<0.01). mc mm^{-2}, microglial cells per square millimetre.

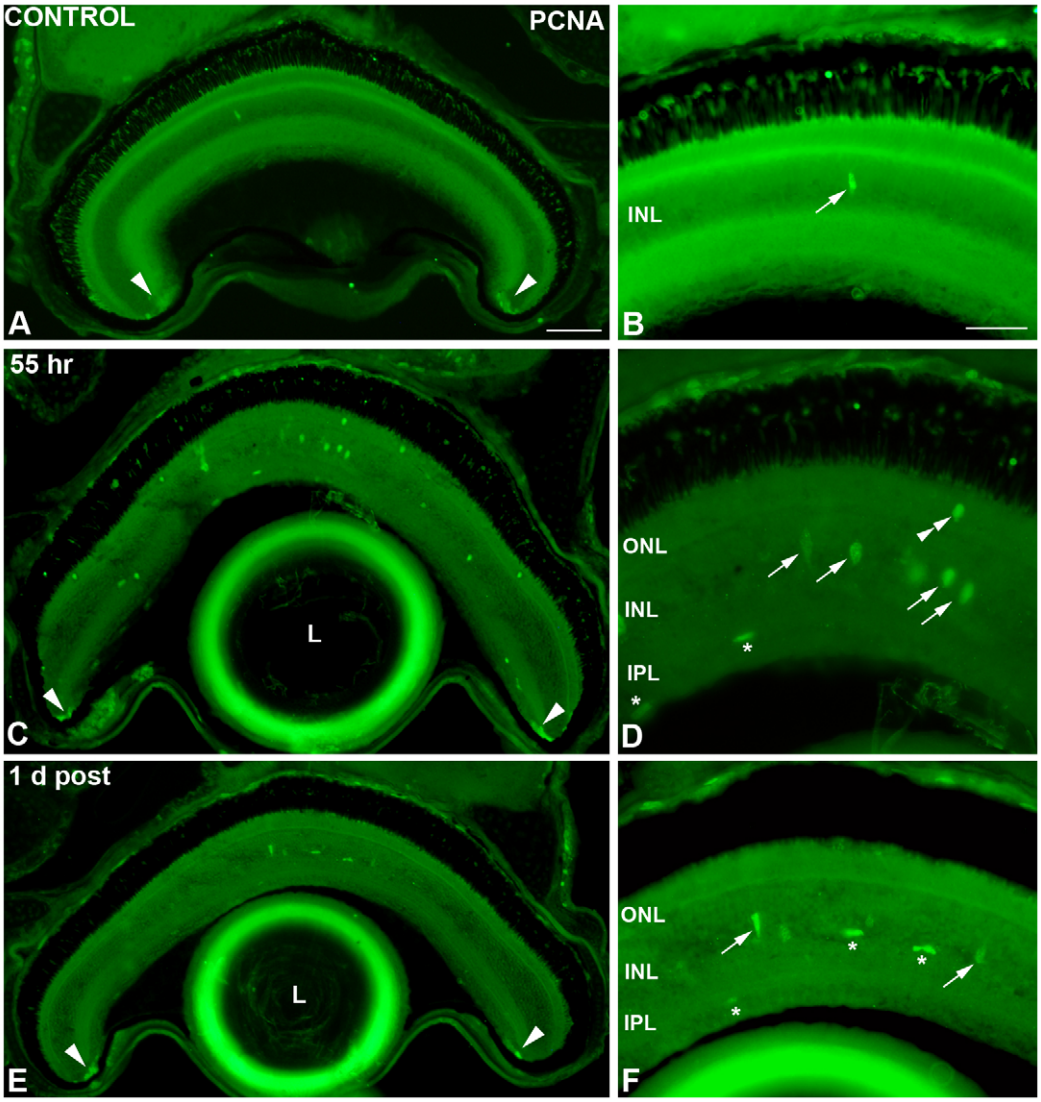

Fig. 8. Distribution of proliferating cell nuclear antigen (PCNA)-positive nuclei in the retina of control (A,B) and experimental tench (C–F). Overviews of retinas (A,C,E) and higher magnifications from the central regions (B,D,F) are shown. Arrowheads in A,C,E illustrate proliferative cells in the ciliary marginal zone (CMZ). In all panels, dorsal is to the right. (A,B) Sparse PCNA-positive nuclei located in the INL were detected in control retinas (arrow). (C,D) During intense light treatment, retinal sections possessed an increased number of PCNA-positive nuclei randomly distributed throughout the retina. Many of these nuclei had a morphology that is consistent with Müller glia (arrows). Tangentially oriented PCNA-positive nuclei (asterisks) were also observed in different retinal layers. PCNA-positive nuclei were also observed in the ONL (double arrowhead). (E,F) The number of PCNA-positive nuclei diminished in experimental animals 24 h after treatment. Radially (arrows) and tangentially oriented (asterisks) positive nuclei could be distinguished. Scale bars denote 100 μm in A,C,E, 25 μm in B,D,F.

Therefore, we consider the larval tench retina to be an appropriate model in which to study several aspects of neurodegenerative processes in the vertebrate visual system.

Müller cells in the experimental tench retinal tissue

Müller cells and proliferation

Destruction or significant damage to the photoreceptor cells can stimulate retinal cell regeneration in the teleost retina (Vihtelic and Hyde, 2000; Vihtelic et al., 2006; Bernardos et al., 2007; Thummel et al., 2008a; Thummel et al., 2008b; Bailey et al., 2010). These studies demonstrate that injury stimulates cell proliferation in the retinal tissue, in agreement with the results described in the present study. We were able to distinguish four different populations of proliferative cells in the experimental tench: retinal progenitors in the CMZ, a population of dividing cells located in the INL, photoreceptor precursors in the ONL, and microglial cells dispersed throughout the different layers of the retina (see below). All these proliferating populations of cells were also observed in control retinas, and had previously been characterized in our laboratory in the developing, juvenile and adult tench retina (Bejarano-Escobar et al., 2009). The morphological features and spatial distribution of many proliferative nuclei located in the INL were highly coincident with those described for Müller cells. This finding is in agreement with previous studies that have shown that selective damage in the photoreceptor layer induces a significant increase in the number of proliferating Müller glia to produce neuronal progenitors that continue to undergo cell division and accurately differentiate into photoreceptors to replace those that have been lost (Vihtelic and Hyde, 2000; Fausett and Goldman, 2006; Bernardos et al., 2007; Fimbel et al., 2007; Thummel et al., 2008a; Thummel et al., 2008b; Bailey et al., 2010). However, proliferative Müller cells seemed to be a small fraction of the proliferative cells labelled in the experimental tench retinal tissue. Most of the proliferating cells corresponded to microglial cells (see below). In contrast, constant intense light treatment in albino zebrafish, which selectively kills rod and cone photoreceptors (Vihtelic and Hyde, 2000), induces approximately 50% of the Müller glial cells to divide and produce neuronal progenitor cells (Thummel et al., 2008b). This difference in the proliferative response of Müller cells in the larval tench retina with respect to that observed in the albino zebrafish may be because (1) while intense light causes widespread photoreceptor cell death that affects all rods and most of the cone cells in the albino zebrafish (Vihtelic and Hyde, 2000), in the tench retina this treatment affected fewer photoreceptor cells, and/or (2) the growth rate of larval tissues is greater than that observed in the adult zebrafish, and new photoreceptors are constantly being added to the ONL, the situation being one of homeostatic reposition of these cells by this stage.

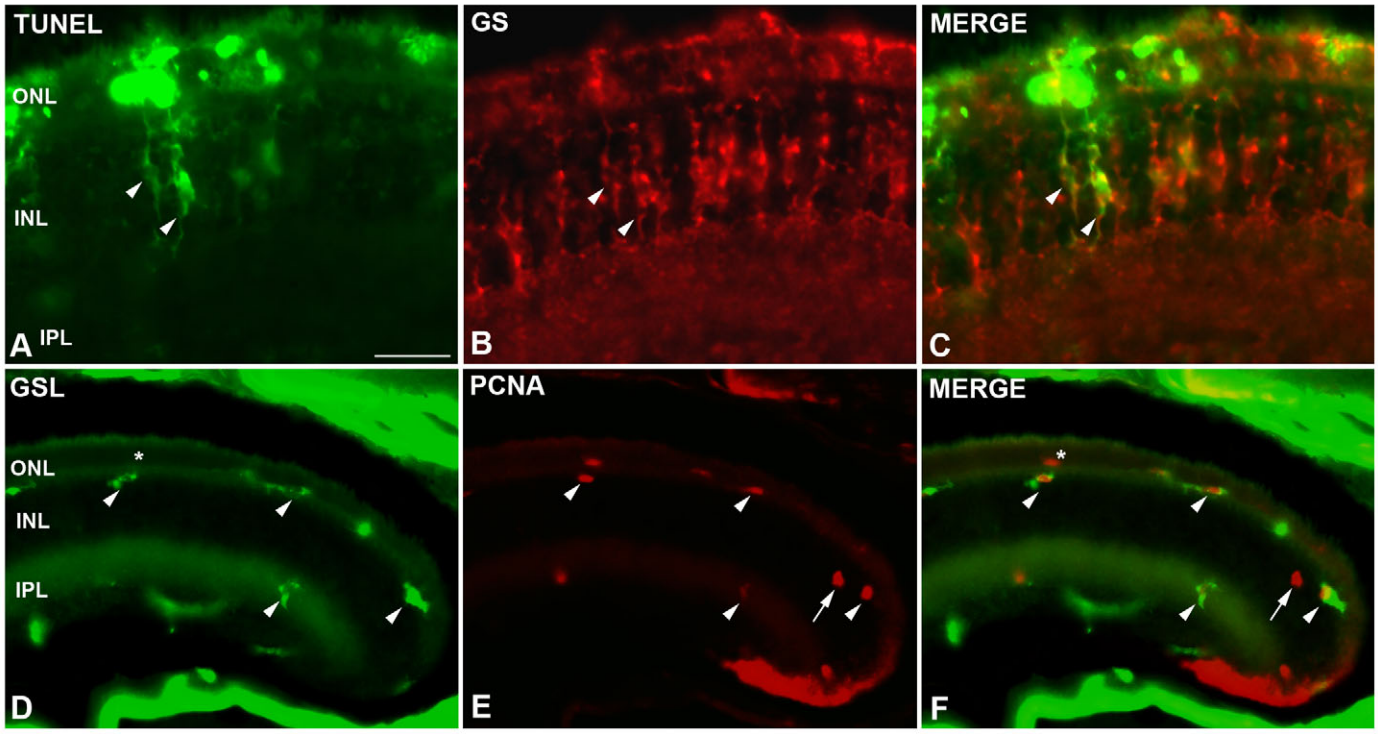

Fig. 9. Characterization of TUNEL-positive cells located in the INL (A–C), and PCNA-positive cells dispersed throughout the entire retina (D–F), in light-damaged tench retinas after 72 h of treatment. (A–C) Experimental retinas exhibited TUNEL-labelled cells in the INL, which also expressed glutamine synthetase (arrowheads). (D–F) Several populations of PCNA-positive cells were distributed in different retinal layers (E). Many proliferating cells were also identified with GSL histochemistry (arrowheads in D–F). However, other PCNA-positive populations located in the ONL (asterisks in D, F) and the INL (arrows in E, F) did not co-label with GSL. Scale bar denotes 25 μm.

Müller cells and phagocytosis

We observed cytoplasmic TUNEL labelling in cells located in the INL of retinal sections of larval tench. These cells presented long and thin processes that stretched across the retina. The detailed examination of these cells revealed the typical morphology of Müller cells. Double labelling techniques showed that TUNEL-labelled cells located in the INL also expressed glutamine synthetase, a typical marker of Müller cells in the tench retina (Bejarano-Escobar et al., 2009). Cytoplasmic TUNEL labelling in Müller cells has been reported previously during normal vertebrate retinal development (Egensperger et al., 1996; Marín-Teva et al., 1999c; Francisco-Morcillo et al., 2004) and in the injured zebrafish retina (Morris et al., 2005; Thummel et al., 2008b; Bailey et al., 2010). Some authors claim that these TUNEL-positive Müller cells are apoptotic (Thummel et al., 2008b). However, most of the studies cited above suggest that cytoplasmic TUNEL labelling results from the dispersion of photoreceptor DNA into the cytoplasm of Müller cells, which engulfed cell debris that originated during the degeneration process (Egensperger et al., 1996; Marín-Teva et al., 1999c; Francisco-Morcillo et al., 2004; Morris et al., 2005; Bailey et al., 2010). The apparent intact healthy morphology of Müller cells and the absence of apoptotic nuclei in the INL, in contrast with the strongly TUNEL-positive apoptotic bodies labelled in the ONL, support the second hypothesis. Furthermore, no loss of Müller cells during the normal regeneration response in the zebrafish retina occurs (Vihtelic and Hyde, 2000; Kassen et al., 2007; Bailey et al., 2010). We also found that the number of TUNEL-positive Müller cells is high during the first hours of constant light treatment. However, cytoplasmic TUNEL staining in the INL became progressively restricted to fewer cells, coinciding with the microglial invasion of the ONL. This event appears to be a consequence of the fact that microglial cells have not yet arrived at the degenerating ONL when intense cell death is taking place during the first hours of constant light treatment. However, as activated microglial cells invaded the ONL, the phagocytic activity of Müller cells progressively decreased.

Microglia in the experimental tench retinal tissue

Macrophages and microglial cells are present in the developing and mature retina, and their distribution through the retinal layers has been well documented in vertebrates (Ashwell, 1989; Marín-Teva et al., 1998; Marín-Teva et al., 1999a; Marín-Teva et al., 1999b; Rodríguez-Gallardo et al., 2005; Santos et al., 2008; Bejarano-Escobar et al., 2011). Abundant microglial cells, identified with nucleoside diphosphatase and tomato lectin histochemistry, can also be observed in the retina, optic nerve and optic tectum of adult tench (Velasco et al., 1995; Velasco et al., 1999). In the retina, they are mainly distributed throughout the optic fibre layer (OFL), IPL and outer plexiform layer (OPL). Sparse microglial cells can also be observed in the INL (Velasco et al., 1999). In the present study we found a similar distribution of microglial cells in the control larval retina, but they were less abundant than in the adult tench retina.

During constant light treatment, variations in the number, morphology and distribution of GSL positive cells were observed. Thus, after 20 h of constant light intensely GSL-stained microglial cells were restricted to the same retinal layers as in control retinas, although they were present in a higher number. These cells showed large rounded somata with short thick processes, the typical

morphology of activated microglial cells. They progressively invaded the ONL and were found near the nuclei of degenerating photoreceptors, suggesting that they were phagocyting cell debris released during photoreceptor degeneration. Macrophages and microglial cells are highly phagocytic and participate in the removal of cell debris during development of the visual system (Hume et al., 1983; Martín-Partido and Navascués, 1990; Egensperger et al., 1996; Moujahid et al., 1996; Rodríguez-Gallardo et al., 2005; Santos et al., 2008; Bejarano-Escobar et al., 2011). Furthermore, retinal microglia are activated in response to nearly all pathological stages of the retina (Langmann, 2007), with microglial cells migrating to the layers affected by degeneration. Thus, the adult ONL which is normally devoid of microglial cells (Velasco et al., 1999; Santos et al., 2008; Bejarano-Escobar et al., 2011), is colonized by these cells in conditions of photoreceptor degeneration during normal development (Bejarano-Escobar et al., 2011) or under pathological or experimental conditions (Roque et al., 1996; Ng and Streilein, 2001; Harada et al., 2002; Hughes et al., 2003; Zeiss and Johnson, 2004; Zeng et al., 2005; Bailey et al., 2010; Santos et al., 2010).

Double labelling techniques showed that many of the activated microglial cells were also labelled with the proliferation marker anti-PCNA. Therefore, the increase in microglial cells in the experimental tench retina results from local proliferation, in agreement with previous studies performed on the tench visual system (Velasco et al., 1995; Jimeno et al., 1999). Similar results have been described in the *rd-1* mouse, in which a population of proliferating cells in the ONL accompanies photoreceptor death. These dividing cells have been identified as microglial cells originating from the inner retina (Zeiss and Johnson, 2004). However, other studies have not detected microglial proliferation in experimentally induced neurodegeneration in mammals (Rogove et al., 2002; Santos et al., 2010). Some authors suggest that, after retinal degeneration induced by exposure to bright light, microglial cells invade the ONL from inner regions of the retina (Roque et al., 1996; Ng and Streilein, 2001; Santos et al., 2010). These authors show that microglial cells disappear from the OFL, IPL and INL coincidentally with microglial invasion of the ONL. However, other possible origins for activated retinal microglia can be envisaged, such as the vitreous body (Santos et al., 2010) or blood-borne macrophages from blood vessels located in adjacent tissues such as the choroid, the ciliary body or the optic nerve (Joly et al., 2009; Santos et al., 2010). Therefore, our observations support the hypothesis that the increase in microglial cells after photodegeneration in the larval tench retina results from local proliferation from pre-existing microglial cells dispersed throughout the inner layers of the retinal tissue, but we cannot rule out the entry of some microglial cells from other regions of the central nervous system, the vitreous body or the choroid.

One day after light treatment, retinal microglia almost disappeared from the ONL. However, during the next 5 days following the intense light treatment, the density of microglial cells was higher than the control values, and, although they progressively became more ramified, these cells retained some morphological features typical of activated microglia, suggesting the persistence of a certain degree of activation, in agreement with previous studies (Santos et al., 2010).

Therefore, microglial cells seem to be intimately engaged with the degenerative process. However, the role of microglial cells in neurodegeneration remains controversial. Thus, *in vitro*, microglial cells have been shown to produce neurotrophic factors (Mallat and Chamak, 1994). The protective effects of neurotrophic factors on photoreceptors may be mediated both directly (in the case of fibroblast growth factor FGF2) and indirectly (in the case of brain-derived neurotrophic factor BDNF and ciliary neurotrophic factor CNTF) through the activation of Müller cells and inner retinal neurons (Kirsch et al., 1997; Wahlin et al., 2000; Wahlin et al., 2001). However, microglial cells have also been shown to produce potentially cytotoxic compounds (Théry et al., 1991; Flavin et al., 2000; Combs et al., 2001). Roque and colleagues have shown that retina-derived microglial cells induce apoptosis of photoreceptors *in vitro*, thus lending support to the hypothesis that microglia accelerate the death of dystrophic photoreceptors (Roque et al., 1999). *In vivo*, Frade and Barde have demonstrated that during the early phase of cell death in the chick retina, microglial cells are the source of nerve growth factor NGF, which causes cell death in the retinal neurons by activating the neurotrophin receptor p75 (Frade and Barde, 1998). Functional evaluation of microglial behaviour during photodegeneration in the tench retina should provide further insight into the mechanisms that regulate photoreceptor degeneration and/or regeneration.

LIST OF ABBREVIATIONS

an	apoptotic nuclei
CMZ	ciliary marginal zone
GCL	ganglion cell layer
GSL	*Griffonia simplicifolia* lectin
INL	inner nuclear layer
IPL	inner plexiform layer
mc	microglial cells
mgc	Müller glial cells
OFL	optic fibre layer
OLM	outer limiting membrane
ONL	outer nuclear layer
OPL	outer plexiform layer
PCNA	proliferating cell nuclear antigen
TUNEL	terminal deoxynucleotidyl transferase (TDT)-mediated deoxyuridine triphosphate (dUTP) nick end labelling

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