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REVIEW ARTICLE

Müller glia and phagocytosis of cell debris in retinal tissue

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Abstract

Müller cells are the predominant glial cell type in the retina of vertebrates. They play a wide variety of roles in both the developing and the mature retina that have been widely reported in the literature. However, less attention has been paid to their role in phagocytosis of cell debris under physiological, pathological or experimental conditions. Müller glia have been shown to phagocytose apoptotic cell bodies originated during development of the visual system. They also engulf foreign molecules that are injected into the eye, cone outer segments and injured photoreceptors. Phagocytosis of photoreceptor cell debris in the light-damaged teleost retina is primarily carried out by Müller cells. Once the microglial cells become activated and migrate to the photoreceptor cell layer, the phagocytic activity of Müller cells progressively decreases, suggesting a possible mechanism of communication between Müller cells and neighbouring microglia and photoreceptors. Additionally, it has been shown that phagocytic Müller cells acquire proliferating activity in the damaged teleost retina, suggesting that engulfment of apoptotic photoreceptor debris might stimulate the Müller glia to proliferate during the regenerative response. These findings highlight Müller glia phagocytosis as an underlying mechanism contributing to degeneration and regeneration under pathological conditions.

Key words: microglia; müller cells; paracrine factors; phagocytosis; retina.

Introduction

Phagocytosis has been considered to be the final stage of programmed cell death (PCD). PCD plays a crucial part during development of the vertebrate visual system (for a review, see Francisco-Morcillo et al. 2014). However, PCD also occurs in this region of the central nervous system (CNS) under pathological and experimental conditions. Cell debris generated during the degeneration process in the vertebrate retina is mostly removed through heterophagy by a variety of cells, including professional migratory phagocytes and non-professional stationary phagocytes. Professional phagocytes include cells of the macrophage lineage and microglial cells. Macrophages invade the vertebrate retina during early stages of development (Cuadros et al. 1991; Knabe et al. 2000; Rodríguez-Gallardo et al. 2005; Santos et al. 2008; Bejarano-Escobar et al. 2011). Later, microglial precursors enter the retina by different forms of migration, and differentiate into microglial cells (Navascués et al. 1995; Marín-Teva et al. 1999a,b,c; Santos et al. 2008; Bejarano-Escobar et al. 2011, 2013). These specialized phagocytes are thought to be the main cell types involved in clearing degenerating cells in the healthy, experimental and pathological retina (Egensperger et al. 1996; Rodríguez-Gallardo et al. 2005; Santos et al. 2008, 2010; Bailey et al. 2010; Bejarano-Escobar et al. 2011, 2012b). Retinal photoreceptor degeneration is accompanied by the migration of professional phagocytic cells into the outer nuclear layer (ONL). These phagocytes are derived from resident microglial cells, not from peripheral macrophages (Roque et al. 1996; Bailey et al. 2010; Santos et al. 2010; Bejarano-Escobar et al. 2012b). During this process, degenerating cells lose the 'do-not-eat-me' signals and then express soluble 'find-me' signals that activate resting microglia that become highly motile, migrating to the lesion area, and phagocytosing cell debris or damaged photoreceptors. The mechanics of phagocytosis is provoked by exposure of 'eat-me' signals on the surface of the injured neurons (for a review, see Ravichandran, 2011). In sum, clearance of cell debris by specialized phagocytes is well characterized.

On the contrary, much less is known about the molecular mechanisms involved in phagocytosis by non-professional phagocytes in the retinal tissue. Neuroepithelial cells have

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been described as participating in the removal of degenerating cells in undifferentiated tissue (García-Porrero & Ojeda, 1979; Martín-Partido et al. 1988; Francisco-Morcillo et al. 2004; Mellén et al. 2008; Bejarano-Escobar et al. 2011, 2013). Moreover, Müller cells, the principal glial cells of the vertebrate retina, are capable of phagocytosing fragments of retinal cells and foreign substances in the differentiating and mature retina (Table 1). It has been described that, under experimental conditions, different signalling molecules emanating from Müller glia and microglia may coordinate phagocytosis of cell debris in the retinal tissue. Phagocytic removal of tissue debris by microglia has an important function in creating an appropriate environment for the stimulation of regeneration in other regions of the CNS (Neumann et al. 2009). Therefore, reciprocal Müller cell–microglia interactions could be the basis of the acute retinal responses to injury and disease, and may be involved in stimulating the regenerative process in the retinal tissue.

This article reviews the current state of knowledge regarding the potential of Müller glia to phagocytose degenerating cells in developing, mature, pathological and injured retinas. We summarize recent findings regarding the secreted factors, signalling pathways and intrinsic factors that have been implicated in the phagocytic process and in the possible interactions between Müller glia and microglial cells. Moreover, we analyse the possible involvement of phagocytosis in other retinal events such as regeneration, degeneration and neuroprotection. This will be done through a review of the pertinent literature, as well as by using our own experience in recent studies of the phagocytosis of cell debris in retinal tissue.

Development and function of Müller glia

The retina is a laminated tissue in which the different neurons and their synaptic connections are arranged in different layers that have been remarkably conserved in vertebrate evolution (Fig. 1). Light-sensing neurons, the photoreceptors, occupy the outermost layer, the ONL, while the retinal projection neurons, the ganglion cells, are located in the innermost neuronal layer, the ganglion cell layer (GCL). The interneurons (horizontal, bipolar and amacrine cells) reside between the ONL and GCL, in the so-called inner nuclear layer (INL; Fig. 1). The cell bodies of Müller glia are also located in the INL (Fig. 1). These cells have a radial morphology, with two stem processes that extend in opposite directions, and span the entire retinal thickness (Fig. 1). Due to their ontogeny, morphology and distribution, these cells are considered analogous to radial glial cells of the cortex. The vitreal (inner) process of the Müller cells approaches the vitreal surface of the neural retina where it forms a so-called endfoot abutting the basal lamina, forming the inner limiting membrane (ILM). The scleral (outer) process reaches out the subretinal space, into which it sends numerous microvilli. These apical processes of Müller cells are attached to each other and to the inner segments of the photoreceptor cells by continuous heterotypic adherens junctions that collectively form the outer limiting membrane (OLM). Furthermore, scleral processes branch around the photoreceptor nuclei, removing neurotransmitters from the extracellular space of surrounding neurons (Newman & Reichenbach, 1996; Bejarano-Escobar et al. 2009, 2010). Therefore, these stem processes extend side branches that contact and/or ensheath different neuronal elements of the retina as well as the blood vessels in vascularized retinas.

Müller cells could be identified and characterized in accordance with their location and morphological clues. However, the utilization of immunochemical markers facilitates the study of these glial cells in the developing and adult tissue (for a review, see Bejarano-Escobar et al. 2014). Due to the plasticity of Müller cells, they can be characterized by glial markers, cell cycle markers and stem cell markers (Roesch et al. 2008). Among them, the enzyme glutamine synthetase (GS) has long been considered a good molecular marker for Müller glia in the developing and mature retinal tissue (Peterson et al. 2001; Bejarano-Escobar et al. 2009, 2010, 2012a; Pavón-Muñoz et al. 2016). These cells also express selected classes of intermediate filament proteins, such as vimentin, glial fibrillary acidic protein (GFAP) and nestin (Raymond et al. 2006; Sánchez-Farías & Candal, 2016). Müller glia also express some genes involved in phagocytosis, suggesting that this cell type can phagocytose *in vivo*. Thus, lysosomal cathepsin B and D are highly expressed in specialized phagocytes such as macrophages and microglial precursors in the retina of mammals (Fig. 2A–C; Bejarano-Escobar et al. 2011). Abundant punctate cytosolic immunolabelling for cathepsin D is restricted to radially oriented cells in the fish retina (Fig. 2D,E). Phagocytic Müller cells could also be labelled by using deoxynucleotidyl transferase (TdT) dUTP nick-end labelling (TUNEL) histochemistry (see below).

The vertebrate retina is mainly composed of cells of neuroectodermal origin. Thus, Müller cells and retinal neurons are derived from a retinal progenitor cell that is multipotent at all stages of retinal histogenesis (Xiang, 2013). During retinogenesis, neurogenesis precedes gliogenesis, as elsewhere in the vertebrate CNS. Therefore, Müller cells are considered the last cell type generated during vertebrate retinogenesis (Prada et al. 1991; Rapaport et al. 2004; Francisco-Morcillo et al. 2006; Bejarano-Escobar et al. 2009, 2010, 2012a). These data disagree with the results described in the developing shark retina by Harahush et al. (2009). Their ultrastructural analysis revealed that Müller cells are present throughout the retina from very early stages of development, simultaneously with ganglion cell differentiation. However, these authors did not characterize these early differentiated Müller cells neurochemically. Morphological changes in the transition between young and mature Müller cells could be observed in the teleost retina

Table 1 Phagocytic activity of Müller cells.

Reference	Species	Technique	Most relevant events described
Fish			
Wagner & Raymond (1991)	Goldfish	Immunohistochemistry and cell culture	Müller cells are phagocytic for latex beads in culture but not *in vivo*
Morris et al. (2005)	Zebrafish	Immunohistochemistry and TUNEL histochemistry in transgenic zebrafish	Müller glia scavenge the cell debris from degenerating photoreceptors
Bailey et al. (2010)	Zebrafish	Immunohistochemistry and TUNEL histochemistry in transgenic zebrafish	A subset of Müller glia engulfed apoptotic photoreceptor cell bodies in light-damaged retinas. The Müller glia proliferative response is linked to phagocytic activity.
Bejarano-Escobar et al. (2012b)	Tench	Immunohistochemistry and TUNEL histochemistry in teleosts	Müller glia engulfed apoptotic photoreceptor cell bodies in light-damaged retinas during the first hours of intense light treatment. As activated microglial cells invade the ONL, the phagocytic activity of Müller cells progressively decreased
Reptiles			
Francisco-Morcillo et al. (2004)	Reptiles	TUNEL histochemistry	Müller cells phagocytose dying cells during retinal development
Birds			
Hughes & LaVelle (1975)	Chick	TEM study	Müller cells can phagocytose degenerating ganglion cells after tectal lesions during embryonic development
Hughes & McLoon (1979)	Chick	TEM study	Ganglion cell debris during retinal development is phagocytosed by Müller cells
Marín-Teva et al. (1999c)	Quail	TEM study, immunohistochemistry and TUNEL histochemistry	Müller cells can phagocytose cell debris in any layer of the developing retina
Thanos (1999)	Chick	Fluorescent retrograde staining of degenerating ganglion cells. Fluorescent ganglion cell debris is internalized by phagocytic cells	Müller cells perform phagocytosis at early stages of ontogenetic cell death. In more advanced stages, Müller cells are replaced by microglial cells
Mammals			
Friedenwald & Chan (1932)	Albino rabbit	TEM study	Müller cell phagocytic activity after the injection of melanin granules into the vitreous body
Blanks et al. (1972)	Retinal degenerative (rd) mouse	TEM study	Photoreceptor cell debris is phagocytosed by Müller cells
Caley et al. (1972)	Rodless CBA mouse	TEM study	Photoreceptor cell debris is phagocytosed by Müller cells
Kuwabara & Weidman (1974)	Rat	TEM study	Cell debris originated during retinal ontogeny is removed by Müller cells
Rosenthal & Appleton (1975)	Rabbit	Classical histological staining	Intravitreal copper foreign bodies are deposited in the Muller cell bodies
Algvere & Kock (1983)	Rabbit	TEM study	Müller cells penetrated the ILM and removed carbon particles from the vitreous body by endocytosis
Long et al. (1986)	Ground squirrel	TEM study	Phagocytosis of the outer segment discs shed from cones
Penfold & Provis (1986)	Human	TEM study	Müller cells phagocyte cell debris during ontogeny

(continued)

Table 1 (continued)

Reference	Species	Technique	Most relevant events described
Mano & Puro (1990)	Human	Cell culture and TEM study	Müller cell cultures from *post mortem* eyes are able to phagocytose retinal fragments as well as latex beads
Stolzenburg et al. (1992)	Rabbit	TEM, brightfield light microscopy and fluorescence microscopy study	Müller cells show an intense phagocytosis of latex beads *in vitro*
Nishizono et al. (1993)	Rabbit	TEM study	Müller cells phagocytose egg-lecithin-coated silicone particles after intraocular injection
Egensperger et al. (1996)	Rabbit	TUNEL and lectin histochemistry	Fragmenting DNA is principally phagocytosed by microglia and Müller cells. Müller cells appear to be able to phagocytose dying cells in any retinal layer during development
Crafoord et al. (2000)	Albino rabbits	TEM study and immunohistochemistry	Implantation of melanin granules in the subretinal space may induce a cellular phagocytic response in macrophages and Müller cells
Francke et al. (2001)	Rabbit	TEM study	Retinal pigment epithelium melanin granules are phagocytosed by Müller glial cells in experimental retinal detachment
Ponsioen et al. (2007)	Human	Immunocytochemistry and cell culture	Müller cells in culture phagocytose collagen type II
Singh et al. (2014)	Human	Cell culture, phagocytosis assay, PCR analysis, measurement of intracellular metabolites, immunocytochemistry	Immortalized Müller glia can phagocytose and kill bacteria *in vitro*. Müller cells also produce a variety of antimicrobial molecules in response to bacterial challenge

Müller cells in the vertebrate retina are involved in cell debris removal during embryonic development and mature tissue homeostasis, but also under experimental and pathological conditions. ILM, inner limiting membrane; ONL, outer nuclear layer; PCR, polymerase chain reaction; TEM, transmission electron microscopy; TUNEL, TdT dUTP nick-end labelling.

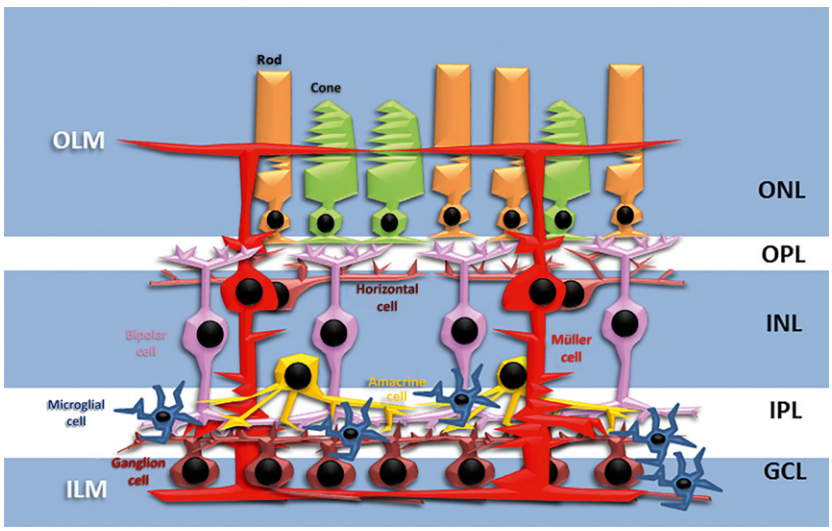

Fig. 1 Diagram of a vertebrate retina shows its typical multi-layered structure. The mature Müller cell is characterized by a centralized soma located in the INL, and processes extending to, and forming, the ILM and OLM. The Müller cell processes contain many branches that surround photoreceptor somata and neuronal processes. GCL, ganglion cell layer; ILM, inner limiting membrane; INL, inner nuclear layer; IPL, inner plexiform layer; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer.

(Bejarano-Escobar et al. 2009, 2010; Pavón-Muñoz et al. 2016). By using antibodies against GS, HNK-1 epitope and carbonic anhydrases, Peterson et al. (2001) defined immunohistochemically different stages of maturation for Müller cells in the developing zebrafish retina. They also recognized the first immature Müller cells very early in development, coinciding with the presence of the first newborn ganglion cells. More recently, an immunohistochemical analysis performed by Sánchez-Farías & Candal (2016) described a different multi-step Müller glia differentiation process in the developing shark retina. Thus, coinciding with early neurogenesis, neuroepithelial cells differentiate into early radial glial cells. With the subsequent increase of GFAP immunoreactivity, these authors were then able to define these cells as late radial glial cells, which, coinciding with the appearance of GS

immunoreactivity, acquire the typical morphology of young Müller cells with quite thick cell somata and processes. Finally, as cell maturation proceeds, Müller cells become long and slender and are identifiable as mature Müller cells. Therefore, the analysis of the processes underlying the transition from neuroepithelial cells to radial cells, and from radial cells to Müller cells, could shed some light on Müller glia differentiation.

Müller cells are noted for their wide range of roles in neural development and function. They exhibit many of the functions observed for radial glia, astrocytes and ependymal cells in other areas of the CNS. Excellent reviews on the role of Müller glia in homeostasis, retinal innate immunity, retinal diseases and regeneration of the visual system have been published in the last decade (Bringmann et al. 2006; de Melo Reis et al. 2008; Jadhav et al. 2009; Bringmann & Wiedemann, 2012; Wohl et al. 2012; Kumar et al. 2013; Reichenbach & Bringmann, 2013; Seitz et al. 2013; Gallina et al. 2014; Goldman, 2014; Gorsuch & Hyde, 2014; Lenkowski & Raymond, 2014; Hamon et al. 2016). In addition to all the functions described by these authors, Müller glia are known to phagocytose cell debris. The regulation of the phagocytic activity of retinal glial cells is poorly understood even though phagocytosis may play a role in retinal homeostasis, pathobiology, degeneration and regeneration. Recent findings indicate the presence of highly coordinated dynamic interactions between Müller cells and microglia that regulate the phagocytosis of cell debris. Furthermore, the proliferative response of Müller glia during photoreceptor degeneration could be induced by their phagocytic activity, which would suggest the possible involvement of phagocytosis in cell proliferation and tissue regeneration.

Müller glia and phagocytosis

Müller cell phagocytic activity

Müller cells are capable of phagocytosing debris from dead cells, pigment epithelial cells and diverse foreign bodies under physiological, pathological and experimental conditions (Table 1). The first evidence of phagocytic activity by Müller cells was reported by Friedenwald & Chan (1932). Melanin granules injected intravitreally in albino rabbits were engulfed by these glial cells. These results were confirmed many years later by different techniques, including transmission electron microscopy (TEM). Thus, following the intravitreal injection of different foreign bodies, these particles appeared inside radial processes of Müller cells in TEM sections (Rosenthal & Appleton, 1975; Nishizono et al. 1993). They also engulfed intravitreally injected erythrocytes (Miller et al. 1986). Under these experimental conditions, the inner limiting lamina was thinned, interrupted or had disappeared, allowing Müller cell processes to make contact with the foreign bodies. Furthermore, they engulf melanin granules injected into the subretinal space (Crafoord et al. 2000) and erythrocyte debris from subretinal or vitreous haemorrhages (Koshibu, 1978; Miller et al. 1986). Surprisingly, fish Müller cells were found to be able to phagocytose latex beads *in vitro*, but not *in vivo* (Wagner & Raymond, 1991). Cultured human (Mano & Puro, 1990; Ponsioen et al. 2007) and rabbit (Stolzenburg et al. 1992) Müller cells are capable of phagocytosing latex beads. More recently, *in vitro* studies using immortalized human retinal Müller glia showed that they can phagocytose and kill bacteria in a time-dependent manner (Singh et al. 2014). Additionally to the engulfment of external substances, Müller cells have also been reported to be active in the phagocytosis of cellular debris during the permanent renewal of photoreceptor outer segments in the mammalian retina (Long et al. 1986). They also phagocytose melanin granules derived from retinal pigment epithelial cells in models of experimental retinal detachment, where pigment epithelium is occasionally detached together with the neural retina (Francke et al. 2001). Recent evidence suggests that this phagocytic clearance following injury is more than simple tidying-up, but instead plays a fundamental role in facilitating the reorganization of neuronal circuits and triggering repair.

The phagocytic activity of Müller cells becomes more relevant with the clearance of cell debris during development and retinal injury. TEM examination revealed that apoptotic neurons are removed by Müller cells during human (Penfold & Provis, 1986), rat (Kuwabara & Weidman, 1974), chick (Hughes & McLoon, 1979) and quail (Marín-Teva et al. 1999c) retinal development. Egensperger et al. (1996) studied the spatiotemporal patterns of cell death and phagocytic cells in the developing retina of several mammals. They used the TUNEL technique that has been designed to detect apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis (Gavrieli et al. 1992). The method is based on the ability of TdT to label blunt ends of double-stranded DNA breaks independent of a template, allowing the detection of fragmenting chromatin in degenerating nuclei. The technique showed intense labelling in the nuclei of degenerating cells, in cell fragments containing condensed chromatin, and in intracellular chromatin fragments (micronuclei). Surprisingly, there was also diffuse TUNEL labelling within the cytoplasm of radially oriented cells. Similar results have been found by our group in the developing retina of fish (Bejarano-Escobar et al. 2013), reptiles (Francisco-Morcillo et al. 2004) and birds (Francisco-Morcillo et al. 2014). Furthermore, cytoplasmic TUNEL labelling is also found in cells with the same morphology in the teleost retina when photoreceptor degeneration is induced by treatment with constant intense light (Fig. 2F; Thummel et al. 2008; Bailey et al. 2010; Bejarano-Escobar et al. 2012b) and in a transgenic model of rod degeneration in zebrafish (Morris et al. 2005). Radially oriented TUNEL-positive cells have a morphology typical of Müller cells, and labelled cells also express GS, a typical

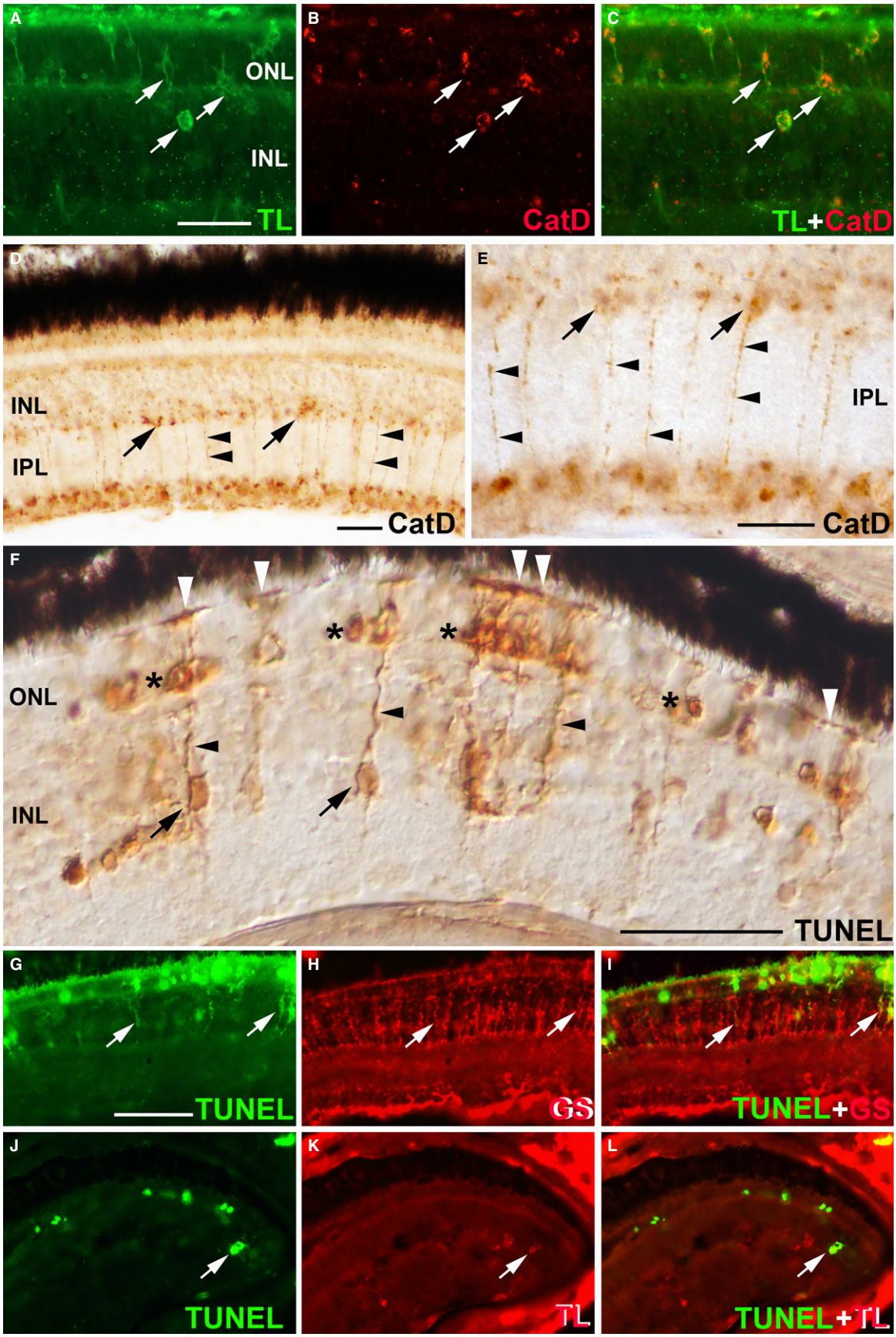

Fig. 2 Müller glia and phagocytosis. (A–C) Microglial cells can be detected with tomato lectin (TL) histochemistry in the developing mouse retina (arrows in A,C). These phagocytic cells also express high levels of cathepsin D (Cat D; arrows in B,C). (D,E) (E) is a magnification of (D). Strong immunoreactivity against cathepsin D is detected in the cell somata (arrows) and processes (arrowheads) of a sub-population of Müller cells in the teleost retina. (F) TdT dUTP nick-end labelling (TUNEL) histochemistry shows retinal cell death in the photoreceptor layer (asterisks) in the light-damaged larval teleost retina. The cell somata (arrows) and processes (black arrowheads) of a sub-population of Müller cells are also stained with TUNEL histochemistry. The OLM occasionally appeared labelled (white arrowheads). (G–I) TUNEL-positive Müller cells are also labelled with antibodies against glutamine synthetase (GS) (arrows) in the light-damaged larval teleost retina. (J–L) TUNEL analysis also reveals cytoplasmic signal in TL-positive microglial cells in the light-damaged larval teleost retina (arrows). Scale bars: 25 μm (A–C); 50 μm (D–F; in G–L). INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer.

Müller cell marker (Fig. 2G–I; Bejarano-Escobar et al. 2012b). Some authors suggest that this TUNEL labelling is specific of cell death and therefore identifies degenerating Müller cells (Thummel et al. 2008). However, various morphological changes occur in apoptotic cells. Thus, during early stages of apoptosis, when cell shrinkage occurs, cells show a smaller size, which means that the cytoplasm is dense and the organelles are more tightly packed. Furthermore, extensive plasma membrane blebbing occurs, followed by destructive fragmentation of the nucleus and separation of cell fragments into apoptotic bodies during a process called 'budding' (Kerr et al. 1972). These morphological changes of apoptotic cells have been observed in the degenerating photoreceptors located in the ONL (Morris et al. 2005; Thummel et al. 2008; Bailey et al. 2010; Bejarano-Escobar et al. 2012b). The apparent intact healthy morphology of radially oriented TUNEL-positive cells and the absence of apoptotic nuclei in the INL 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 cell death process (Egensperger et al. 1996; Francisco-Morcillo et al. 2004; Morris et al. 2005; Bailey et al. 2010; Bejarano-Escobar et al. 2012b). Phagocytosing microglia may occasionally also be labelled with the TUNEL technique (Fig. 2J–L). Therefore, TUNEL staining may generally be regarded as a method for the detection of DNA fragmentation (DNA damage) and, under the appropriate circumstances, more specifically as a method for identifying apoptotic cells. Bailey et al. (2010) also detected weak cytoplasmic TUNEL labelling in a subset of Müller cells that engulf apoptotic photoreceptor bodies in the light-damaged zebrafish retina, and that all proliferating Müller cells co-labelled with TUNEL, suggesting that phagocytosis could be involved in the Müller glial proliferative response after injury. Photoreceptor degeneration induces the activation of microglia, contributing to the de-differentiation and proliferation of the Müller cells (Fischer et al. 2014). By using O-phospho-L-serine (L-SOP), a molecule that blocks microglial phagocytosis of cell debris, Bailey et al. (2010) found a reduction in the number of proliferating Müller glia, suggesting that the mechanism disrupted by L-SOP is required to activate Müller glia proliferation in the injured retina. Several hypotheses have been advanced to explain the mechanisms of L-SOP-mediated suppression of Müller glia proliferation. One of these hypotheses is that L-SOP-suppressed microglial phagocytosis of degenerating photoreceptors could affect the paracrine activation of the Müller cell proliferation by microglial cells. Therefore, Müller glial proliferation processes and phagocytosis could be linked, with both being regulated by microglia.

Coordinated phagocytic activity between Müller cells and microglia

Retinal neurons, microglia and macroglia exchange functionally significant signals under both uninjured and pathological conditions (for a review, see Vecino et al. 2016). In the healthy retina, inhibitory and excitatory neurotransmission modulates ATP release from Müller glia, regulating microglial motility (Uckermann et al. 2006; Fontainhas et al. 2011). When retinal neurons are damaged, Müller cells and microglia undergo a quite substantial functional and structural phenotype change. Thus, microglial cells are activated – from a 'resting' to an 'activated' phenotype – during retinal inflammation, injury or disease (Roque et al. 1996; Zeiss & Johnson, 2004; Bailey et al. 2010; Santos et al. 2010; Bejarano-Escobar et al. 2012b). Moreover, under these pathological conditions, Müller cells also demonstrate some degree of activation and reactive gliosis (Bringmann et al. 2006). The activation of both types of cells promotes functional interactions between them, regulating photoreceptor cell survival (Harada et al. 2002). In other cases, photoreceptor degeneration attracts and recruits microglia into the ONL. Activated microglial cells phagocytose cell debris and increase the secretion of inflammatory cytokines, either acting directly on rod photoreceptors (Scuderi et al. 2015), or indirectly via the pro-inflammatory activation of Müller cells that induce photoreceptor degeneration (Liu et al. 2015). In the case of glaucoma, the change of both cell types to a reactive phenotype initiates signalling cascades that may serve a neuroprotective role, but may also proceed to promote damaging effects on retinal neurons, especially in the ganglion cells (Seitz et al. 2013). It has also been described that activated Müller cells upregulate inflammatory factors, including monocyte chemoattractant protein-1, recruiting microglial cells to the injured area in a positive-feedback manner (Nakazawa et al. 2006; Hollborn et al. 2008). *In vitro* studies have shown that Müller cells change shape, and decrease the expression of gliosis markers when they are co-cultured with activated microglia (Wang et al. 2011). They also present reduced proliferative

activity and express higher protein and mRNA levels of trophic factors such as glial cell-derived neurotrophic factor and leukaemia inhibitory factor, and increase the protein expression of pro-inflammatory factors capable of inducing microglia activation. Moreover, co-cultured Müller cells also show increased expression of chemotactic cytokines and cell-adhesion molecules, allowing microglial cell processes to adhere closely with Müller cell processes forming fascicles that may serve as a scaffold for the radial migration of microglia (Wang et al. 2011), as occurs during embryonic development (Marín-Teva et al. 1998; Sánchez-López et al. 2004). These results indicate that, in situations of pathology or injury, Müller cells and microglia can perform mutual and reciprocal signalling that amplifies local inflammation, adaptive neuroprotection and physical interaction (Wang et al. 2011).

With respect to phagocytosis, although Müller glia participate in the clearance of cell debris, amoeboid microglia seem to be the principal phagocytic cell for retinal cells that die during development and under experimental conditions (Hume et al. 1983; Ashwell et al. 1989; Cuadros et al. 1991a; Egensperger et al. 1996; Moujahid et al. 1996; Thanos et al. 1996; Thanos, 1999; Rodríguez-Gallardo et al. 2005; Santos et al. 2008; Bailey et al. 2010; Bejarano-Escobar et al. 2011, 2012a). The phagocytic role of Müller glia becomes relevant in degenerative regions where microglial cells are absent. Different phases of PCD have been described during retinal development (for reviews, see Valenciano et al. 2009; Francisco-Morcillo et al. 2014). During these phases, there has been observed occasional spatiotemporal coincidence of dying cells with macrophages and microglial precursors in the developing avian retina (Cuadros et al. 1991), and in both the embryonic and postnatal mammalian retina (Hume et al. 1983; Ashwell et al. 1989; Egensperger et al. 1996; Rodríguez-Gallardo et al. 2005; Santos et al. 2008; Bejarano-Escobar et al. 2011), suggesting that cell death is the stimulus attracting specialized phagocytes migrating into the developing visual system. However, other researchers have reported that, in some stages of retinal development, there is no chronotopographical coincidence between specialized phagocytes and PCD. Thus, in the developing quail retina, microglial

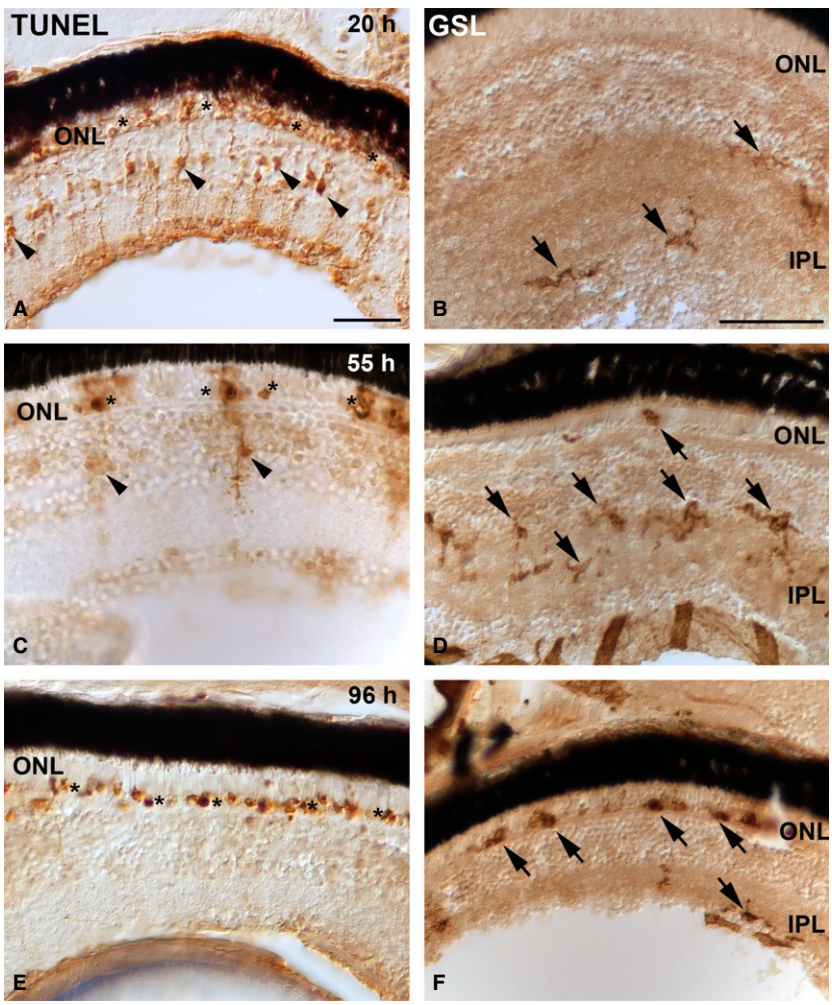

Fig. 3 TdT dUTP nick-end labelling (TUNEL) histochemistry (A,C,E) and *Griffonia simplicifolia* lectin (GSL) histochemistry (B, D, F) showing the progression of retinal cell death in the photoreceptor layer and changes in distribution pattern and microglial morphology, respectively, in larval teleost after 20 h (A, B), 55 h (C, D) and 96 h (E, F) of light exposure. TUNEL labelling shows that abundant photoreceptors are dying during light treatment (A, C, E) (asterisks). In the first hours of constant light treatment, the number of TUNEL-positive Müller cells is high (arrowheads in A). However, during the experimental period, cytoplasmic TUNEL staining in the INL became progressively restricted to fewer cells (arrowheads in C, E). In the first hours of constant light treatment, microglial cells are mainly located in the inner border of the INL and in the IPL (arrows in B). As the treatment advances, they progressively become larger and show thicker processes after activation in response to photoreceptor degeneration, and they migrate towards the ONL (arrows in D). Microglial cells invade the photoreceptor layer phagocytosing cell debris (arrows in F). Scale bars: 25 μm (A); 50 μm (B–F). IPL, inner plexiform layer; ONL, outer nuclear layer.

precursors arrive to the INL only after PCD has ceased in this layer (Marín-Teva et al. 1999c). Furthermore, no evident correlation is found between the chronotopographical distribution patterns of TUNEL-positive bodies and of macrophages/microglial cells during early and late stages of visual system development in sharks (Bejarano-Escobar et al. 2013). During ontogeny in these species, increased phagocytic activity is observed in such non-specialized phagocytes as neuroepithelial cells and Müller glia. Thanos (1999) observed staining of vital and dying ganglion cells in the developing chick retina following the intraocular injection of carbocyanine dyes. Phagocytic cells that remove the fluorescent debris become fluorescently labelled themselves. Radial Müller glia are the only class of cells to become phagocytic between embryonic day 9 (E9) and E16. They are replaced exclusively with microglial cells from E17 onwards, suggesting an interaction between Müller cells and immigrating microglia that regulates the phagocytic process. Similar results were obtained in our laboratory in the teleost retina following light-induced photoreceptor degeneration. Microglial cells are not present in the mature ONL of vertebrates (Santos et al. 2008; Bejarano-Escobar et al. 2013). Therefore, if photoreceptor degeneration occurs, microglial cells invade this layer from inner regions of the retina (Harada et al. 2002; Bailey et al. 2010; Santos et al. 2010; Bejarano-Escobar et al. 2012b). Microglial cells, located mainly in the inner plexiform layer (IPL), become activated and undergo a program of morphological and molecular changes, becoming deramified and developing an enlarged cell body with several short, thickened processes. Later, microglial cells develop an amoeboid appearance and become phagocytic. Depending on the species, this morphological transformation may occur within days or even hours of the initial activation. Our own laboratory's

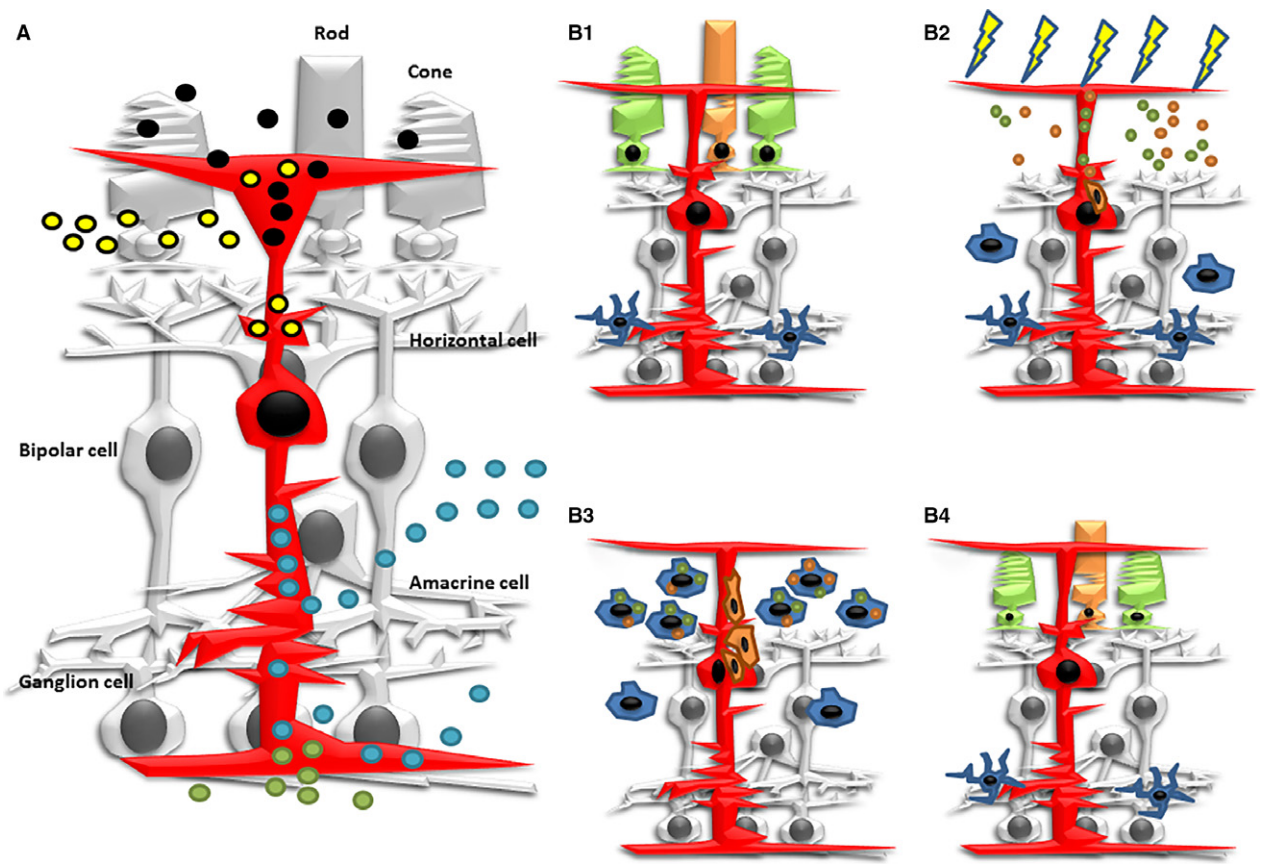

Fig. 4 Schematic diagrams showing several aspects of phagocytosis in Müller glia. (A) Müller cells (in red) phagocytose melanin and outer segment discs (black circles) under physiological conditions. They also engulf photoreceptor cell debris (yellow circles) under pathological and experimental conditions. They participate in the removal of cell debris during development of the retina (blue circles). They also engulf foreign molecules that are injected into the eye (green circles). (B1) Microglial cells in the fish retina are mainly observed in the IPL. (B2) During the first hours of constant intense-light treatment, cell debris from photoreceptor degeneration is phagocytosed by Müller cells. Signalling molecules activate proliferation activity in phagocytic Müller cells. These signals also activate microglia activation and migration to the photoreceptor layer. (B3) As microglial cells invaded the photoreceptor layer, they become highly phagocytic and participate in the removal of cell debris. However, phagocytic activity of Müller cells progressively decreased. (B4) Migrating precursors from Müller cells proliferate and differentiate into rod and cone photoreceptors, regenerating the missing neurons.

studies in the teleost retina show that the first amoeboid microglial cells appear in the ONL 55 h after the initiation of intense light exposure (Bejarano-Escobar et al. 2012b). During this period, abundant TUNEL-positive Müller cells are observed (Fig. 3A), while microglial cells, mainly located in the IPL, clearly show an activated morphology in response to photoreceptor degeneration (Fig. 3B). The number of TUNEL-positive Müller cells decreases (Fig. 3C), coinciding with the arrival of microglial cells to the ONL (Fig. 3D). Phagocytic Müller cells disappear after 96 h of bright light exposure (Fig. 3), while abundant microglial cells are located in the ONL, coinciding chronotopographically with abundant TUNEL-positive nuclei (Fig. 3,F). Our results thus clearly show that cell debris that originates during the first hours of constant light treatment is phagocytosed by Müller cells, coinciding with the activation of microglial cells. As activated microglial cells invade the photoreceptor layer, the phagocytic activity of Müller glia progressively decreases (Bejarano-Escobar et al. 2012b). However, Santos et al. (2010) show the mouse ONL to be invaded by microglial cells immediately after the light exposure. This difference in the timing of microglial activation and migration may be attributable to inter-specific differences and/or attenuation of the microglial activation under hypothermic conditions. Hypothermia has been described as reducing microglial activation in a temperature-dependent manner both *in vivo* and *in vitro* (Seo et al. 2012). Indeed, those workers demonstrate that hypothermia below 29 °C has major inhibiting effects on microglia. Fish are ectotherms, meaning that they rely on the environment to control their temperature. The water temperature in our experimental study was 25 °C, perhaps low enough to attenuate microglial activation and migration in the damaged teleost retina.

We therefore hypothesized that there are bidirectional feedback signals between microglia and Müller cells that may constitute a coordinated phagocytic response following the induction of retinal damage (Fig. 4).

Conclusions

Müller cells are involved in the phagocytosis of neuronal debris under both physiological and pathological conditions. Phagocytosis has been shown to play a key role in retinal regeneration. In particular, disruption of phagocytic activity significantly reduces both the proliferation of Müller cells in response to injury and the regeneration of photoreceptors. New evidence suggests that upregulation of Müller cell/microglial cell cross-talk occurs during phagocytosis in response to photoreceptor degeneration in the teleost retina. These bidirectional interactions between macroglial and microglial cells may mediate adaptive responses within the retina following injury, and photoreceptor degeneration could induce phagocytic activity in Müller cells and the activation of microglial cells located in more internal regions. Furthermore, activated Müller cells release soluble factors that play a crucial role in driving microglial activation and migration. Müller cells may also form an adhesive cellular scaffold that guides the migration of microglia through the layers of the retina. In sum, therefore, phagocytic activity of Müller cells could be involved in processes of degeneration, proliferation and regeneration in the retina of vertebrates. Understanding the cellular mechanisms regulating retinal cell degeneration and regeneration is crucial for the development of treatments for neurodegenerative diseases. In other regions of the CNS, insufficient clearance by microglia, prevalent in several neurodegenerative diseases and increasing with ageing, is associated with an inadequate regenerative response (Neumann et al. 2009). Thus, gaining a clearer understanding of the mechanism behind and the functional significance of Müller cell/microglial-mediated clearance of retinal cell debris following injury may help to open up exciting new approaches to therapy.

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Author contributions

R.B.E., H.S.C. and J.O.A. performed immunohistochemical and histochemical studies. G.M.P. and J.F.M conceived and designed the experiments and constructed. J.F.M. wrote the paper.

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