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A journey into the retina: Müller Glia commanding survival and death

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Abstract

Müller Glial Cells (MGCs) are known to participate actively in retinal development and to contribute to homeostasis through many intracellular mechanisms. As there are no homologous cells in other neuronal tissues, it is certain that retinal health depends on MGCs. These macroglial cells are located at the centre of the columnar subunit and have a great ability to interact with neurons, astrocytes, microglia and endothelial cells in order to modulate different events. Several investigations have focused their attention on the role of MGCs in diabetic retinopathy, a progressive pathology where several insults coexist. As expected, data suggest that MGCs display different responses according to the severity of the stimulus, and therefore trigger distinct events throughout the course of the disease. Here, we describe physiological functions of MGCs and their participation in inflammation, gliosis, synthesis and secretion of trophic and antioxidant factors in the diabetic retina. We invite the reader to consider the protective/deleterious role of MGCs in the early and late stages of the disease. In light of the results, we open up the discussion around and ask the question: is it possible that the modulation of a single cell type could improve or even re-establish retinal function after an injury?

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Introduction

Since the industrial revolution, there has been a continuous development of new technology and communications, which has changed our lifestyle radically. The reduction in physical activities and the incorporation of high calorie food to the diet have had a negative impact on the metabolism, leading to an exponential increase in the number of people suffering from Metabolic Syndrome and Diabetes Mellitus (Kumar & Kelly, 2017). In developed countries, these pathologies top the ranking of chronic illnesses and therefore several global health policies have tried to reduce their incidence (mainly in children) as well as the severity of their complications in diagnosed patients. Diabetic patients are characterised by elevated levels of glycaemia, as a consequence of a primary pancreatic dysfunction or a reduction in the sensibility of target cells to insulin. Long-lasting hyperglycaemia can induce macro- and micro-vascular alterations in a wide range of organs. In the retina, the alteration of small calibre vessels leads to Diabetic Retinopathy (DR), a progressive pathology where the following two main stages can be clearly identified (Lechner *et al.*, 2017):

- Non-proliferative DR (NPDR): Characterized by microaneurysms, venous dilatations, intraretinal haemorrhage and lipid exudates.
- Proliferative DR (PDR): Characterized by the formation of new abnormal vessels (neovessels) towards the vitreous cavity, pre-retinal haemorrhages and retinal detachment, in addition to NPDR characteristics.

These two phases were defined by ophthalmologists many decades ago, when inspecting patients' eyes and for a long time this pathology was considered to be purely vascular. However, further investigations revealed that retinal functionality was altered (Phipps *et al.*, 2007) with neuronal and glial cells expressing a different pattern of proteins, even before vascular changes could be detected. Since then, researchers have focused their attention on the events occurring in nonvascular cells of the retina: neurons (photoreceptors, horizontal, bipolar, amacrine and ganglion cells), microglia (resident phagocytes) and macroglia (Müller cells and astrocytes). The fact that these types of cells reveal functional, biochemical and ultrastructural modifications, implies that the blood retinal barrier (BRB) is damaged and retinal tissue can be exposed to plasma proteins at abnormal concentrations (Loukovaara *et al.*, 2015). In this scenario, Müller glial cells (MGCs) were suggested to be key players in the maintenance of retinal homeostasis because they are able to interact with the rest of the cells that constitute this tissue and modulate multiple processes (Sorrentino *et al.*, 2016). In addition, MGCs together with endothelial cells (ECs) and

pericytes constitute the BRB, preventing any potential harmful molecules or pathogens contacting with the retinal cells. These macroglial cells can detect subtle changes in the environment and activate intracellular mechanisms that guarantee neuronal health. The first reaction to the external stimuli is called gliosis, which can be understood as a warning signal for neighbouring cells and a predisposition of MGCs to take required actions if the stimuli persist. Reactive gliosis is defined as the morphological and biochemical changes that glial cells undergo in the injured zone. Specifically, in MGCs, this includes hypertrophy, an augmented expression of intermediate filaments such as glial fibrillary acidic protein (GFAP), nestin and vimentin, and in some occasions, proliferation (Coorey *et al.*, 2012). When the damage is extensive or intense, MGCs may stay activated even after the stimuli have disappeared. This process of persistent gliosis is considered an indicator of a bad prognosis for retinal recovery (Liu *et al.*, 2016). Simultaneously, in the injured retina, MGCs participate in the inflammatory response through the secretion of cytokines and other mediators to the extracellular space and the vitreous cavity. One of the most relevant events of DR is the increased secretion of trophic factors mainly vascular endothelial growth factor (VEGF) and insulin like growth factor-1 (IGF-1), and metalloproteinases (MMPs) by the macroglia indicating that MGCs are involved in the formation of neovessels and the remodelling of the extracellular matrix during the proliferative stage (Rodrigues *et al.*, 2013). Recent proteomic studies are now beginning to establish a relationship between the type and concentration of the molecules detected in the vitreous and the progression of DR (Loukovaara *et al.*, 2015)

The active participation of MGCs in numerous processes, which exert direct and indirect effects on neighbouring cells, make MGCs a possible target for medical treatment by the modulation of their cellular mechanisms. In these sense, it would be interesting to have a better understanding of the time course of these events, the signalling pathways activated by MGCs and how they interact with neurons and ECs in this complex scenario.

MGCs in health

His vast experience in anatomy helped the physiologist Heinrich Müller to discover a radial cell in the retina, which he described as glial fibres that supported the tissue structure. Almost 150 years later, the knowledge about the functions of these cells in the retina has increased exponentially over time and it has been demonstrated that MGCs are essential for vision from their initial stages of development.

In the mature retina, MGCs are situated transversally to all nuclear and plexiform layers, with their endfeet contacting the vitreous cavity and their microvilli projecting into the subretinal space. MGCs emit longitudinal cytoplasmic projections ensheathing neurons and vessels. Reichenbach et al. (1995) described MGCs as the centre of a columnar subunit, the smallest anatomical and functional structure needed for the transduction of the visual signals. The interactions established among the cells of a columnar subunit guarantee the maintenance of homeostasis and the initiation of a protective response in case of a focalized injury.

Neuronal-MGC interplay is considered a mutual-benefit relationship. This is based on the fact that certain processes are regulated by some enzymes present in MGCs and others in neurons. In

addition, specific signals require a feedback from other groups of cells. Although not every MGC action implies this intimate interaction, it is a common consensus that these cells cooperate in neuronal processing and the metabolism as described in the following examples:

- In fasting conditions, MGCs provide energy metabolites to neurons as they synthesize glycogen by gluconeogenesis, store it and then release glucose on demand. Results suggest that photoreceptors transform glucose into lactate, and this second metabolite may be the main source of carbons for gluconeogenesis in MGCs as they have a low expression of pyruvate kinase in the outer retina. The resultant glycogen deposits may be the source of glucose for neurons residing in the inner retina (Lindsay *et al.*, 2014; Hurley *et al.*, 2015).
- In the retina, most of the excitatory transduction signals are mediated by glutamate. MGCs participate in the uptake of this amino acid from the synaptic cleft, avoiding excitotoxicity. Moreover, glutamate is recycled to glutamine in MGCs and returned to neurons in order to provide them the substrate for neurotransmitter synthesis. GABA, an inhibitory neurotransmitter, is also degraded by MGCs via the citric acid cycle (Reichenbach & Bringmann, 2016).
- MGCs release neurotrophic and growth factors that may exert pro-survival and proliferative effects on neuronal and glial cells. In addition, MGCs synthesize glutathione, a molecule involved in the reduction of reactive oxygen species (ROS). Recently, it was described that glutathione also activates calcium mediated signalling (Freitas *et al.*, 2016).
- MGCs achieve hydro-electrolytic balance of retinal tissue through the regulation of the extracellular ion concentration and pH, thereby avoiding neuronal swelling in hypoosmotic stress. For this purpose, MGCs display ion channels, aquaporins and purinergic transporters, which facilitate water uptake and ion transport (Vogler *et al.*, 2016).
- MGCs actively participate in the recycling of photopigments from photoreceptors, catalysing the conversion of all-trans-retinal in 11-cis-retinol by retinaldehyde-binding protein. The product is then returned to photoreceptors to restart the visual cycle (Xue *et al.*, 2015).
- A key feature of the visual perception is how light gets across the retina and is detected by photoreceptors. MGCs are responsible for guiding the beam of light, as an optic fibre, directly to cones and rods. This mechanism is implicated in the reduction of light scattering and the improvement of visual acuity (Agte *et al.*, 2011).

At the same time, MGCs maintain a constant communication with other glial cells. Microglial and MGCs together may be responsible for assembling a pro-inflammatory response in chronic and acute pathological situations, including the synthesis of trophic factors necessary for neuronal survival after injury (Harada *et al.*, 2002). In addition, MGCs, pericytes, astrocytes, and ECs are the constituent cells of the BRB, a protective structure that provides immune privilege to the retina.

An interesting interplay may be established between MGCs and the endothelium. The radial disposition of MGCs cells enables the interaction with the vasculature in all plexus, where MGC

protrusions wrap up different calibre vessels. During development, there is a transient irrigation circuit called the hyaloid vasculature, which later regresses in order to give rise to the permanent vasculature. MGCs and ECs cooperate in remodelling of the extracellular matrix by secreting MMPs and in retinal angiogenesis by the secretion of trophic factors (Jacobo & Kazlauskas, 2015; Lorenc *et al.*, 2015). In addition, once the angiogenesis process has ended, MGCs secrete thrombospondin-1 to arrest endothelial proliferation (Yafai *et al.*, 2014).

When a flickering light stimuli is perceived by the retinal cells, neurons increase their activity and in consequence their metabolism. Apparently, neurotransmitters stimulate glial cells to initiate mechanisms that increase retinal blood flow. Taking in account that the vasculature of the neural retina lacks autonomic innervation, this endothelial-glial cell interaction seems to be the most important regulator of vessel tone. Later, this process was referred as neurovascular coupling by which MGCs and astrocytes release vasoactive substances that constrict/dilate vessels. Newman´s research group demonstrated that ATP released from glial cells was able to constrict arterioles, while calcium signalling exerted the opposite effect (Kur & Newman, 2014; Biesecker *et al.*, 2016).

The above evidence clearly demonstrates that the functions played by MGCs are necessary for the proper development and functioning of the retina. However, several processes taking place in MGCs are altered by DR.

Diabetic retinopathy

DR is an ocular consequence of chronic hyperglycaemia produced by a systemic imbalance in the metabolism of carbohydrates and lipids. Frequently, this pathology is diagnosed in advanced stages, since the initial phases do not manifest detectable symptoms. Although DR does not constitute a terminal disease, it impacts negatively on the quality of life of the patients because it leads to visual impairment or even blindness.

Once the pathology is installed, a strict glycaemic control can slightly improve the retinal function but this guarantees neither regression nor the arrest of the progression of the symptomatology (Holfort *et al.*, 2011). This could be indicative of the complexity of the pathophysiology, where many mechanisms activated in response to hyperglycaemia are not inhibited when the blood glucose levels return to normal. The rise in the systemic glucose concentration is known to increase the polyol and hexosamine pathways in most cells, and also to produce the glycosylation of circulating proteins that can extravasate to retinal tissue. Moreover, metabolic stress induces an increment in the production of ROS and mitochondrial dysfunction, leading to bioenergetic imbalance (Wan *et al.*, 2015). If protective mechanisms, such as unfolded protein response and autophagy, cannot give a solution to cellular injury, the cell death machinery is activated. In the early stages of DR, subtle biochemical and functional alterations occur mostly in ECs and other components of the BRB including pericytes and MGCs, due to prolonged contact with the bloodstream. *In vitro* studies in ECs have shown a reduction in cell viability and an increase in VEGF production proportional to the glucose concentration (Betts-Obregon *et al.*, 2016). In similar conditions, interleukin-6 (IL-6) treatment promotes the signal transducer and activator of transcription 3 (STAT3) signalling in ECs seeming to be responsible for the decline in tight junctions

and consequently vascular leakage (Yun *et al.*, 2017). The above-mentioned glucose mediated alterations have also been described in pericytes. However, the secretion of pro-inflammatory cytokines by the microglia cooperates in the generation of oxidative stress and subsequent loss of pericytes (Ding *et al.*, 2017).

In MGCs, hyperglycaemia was reported to up-regulate GFAP, modify the expression pattern of aquaporins, and to reduce the interaction between insulin receptor and insulin receptor substrate 1, despite the fact that the number of insulin receptors and their phosphorylation capacity was normal (Fukuda *et al.*, 2010; Ola, 2014). Moreover, the loss of MGC functionality was correlated with a decrease in tight junctions between ECs and a consequent increase in vascular permeability (Shen *et al.*, 2010a). Although MGCs are highly resistant cells, they can activate the caspase-1 pathway, leading to pyroptosis, an infrequent cell death program. The Mohr group suggested that pyroptosis could be a mechanism to eliminate MGCs secreting IL-1β, in order to decrease retinal inflammation (Coughlin *et al.*, 2017).

The overexpression of connective tissue growth factors causes basal lamina thickening (Kuiper *et al.*, 2008) and strong arteriolar constriction in diabetic patients. These alterations in the BRB added to hypertension produce changes in blood flow, leucostasis as well as increase in the cytokines release and hypoxia. The mechanisms underlying hypoxia are not completely elucidated, but it is thought to be a consequence of multiple factors such as vascular alterations, decreased arrival of erythrocytes and the increased use of the oxygen in oxidation pathways. This deprivation of oxygen could also activate other harmful mechanisms and reinforce retinal injury.

In the following sections we will describe the participation of MGCs in the main cellular processes associated with the pathophysiology of DR.

Inflammatory Response

Glial cells detect even subtle modifications of the retinal environment and communicate messages to distant cells through cytokines and chemokines. MGCs are immunocompetent cells and together with microglia and astrocytes, they are in charge of retinal defence and the maintenance of the pro- and anti-inflammatory balance. *In vitro* studies have shown that the receptor of the advanced glycation end product was overexpressed in MGCs exposed to high glucose levels. When this receptor comes into contact with a ligand, it can trigger a mitogen-activated protein kinase (MAPK) signalling pathway which enhances pro-inflammatory cytokine synthesis (Zong *et al.*, 2010). Moreover, GeneChip Rat Genome oligonucleotide array studies carried out in MGCs isolated from diabetic rats induced by streptozotocin (STZ) detected the upregulation of more than 70 genes related to pro-inflammatory and acute-phase response proteins (Gerhardinger *et al.*, 2005). As MGCs are in contact with the vitreous cavity, these proteins can be quantified in this accessible fluid to determine DR severity and to design a personalized treatment. However, special care should be taken when analysing molecules in vitreous space in advanced stages of DR, because the breakdown of the BRB allows the extravasation of plasma proteins. Under healthy conditions, α_2Macroglobulin (α_2M) is an abundant protein in blood, but not in retinal tissue. It has been proposed that this protein is a marker of increased permeability of the BRB. However, recent

studies have shown that α_2M can also be synthesized in retinal cells, including MGCs (Liu *et al.*, 2014; Barcelona *et al.*, 2016). In proliferative retinopathies, α_2M regulates extracellular matrix remodelling, modulates the availability of trophic factors and cooperates in the enhancement of the innate immune response (Federici Canova *et al.*, 2015), which led to the assumption that this acute-phase protein might be considered an interesting biomarker of DR progression (Barcelona *et al.*, 2016).

Because diabetes is a multifactorial pathology, dyslipidemia must be considered a relevant risk factor in the development of type 2 diabetes and an aggressive insult for cells. Recently, new animal models are trying to reproduce systemic alterations suffered in Metabolic Syndrome, as this pathology frequently leads to Diabetes Mellitus. Unpublished data from our group show that Apo E KO mice fed with a high fructose diet for four months develop early DR vascular and neuronal alterations, including augmented permeability and a decreased electroretinographic response. An imbalance in lipid metabolism is correlated with chronic inflammation, which disturbs the homeostasis of most tissues including the retina. Unlike astrocytes, IL-6 and IL-8 secretion by MGCs increased exponentially when incubated with free fatty acids, in particular, linoleic acid (Capozzi *et al.*, 2016). In this sense, advanced lipoxidation end products (ALEs) are responsible for IL-6 and tumour necrosis factor alpha (TNFα) synthesis in MGCs (Yong *et al.*, 2010). Despite the signalling pathways responsible for these effects have not been completely elucidated yet, there is growing interest in the study of lipids as cellular modulators, since they have been implicated in the activation of diverse mechanisms.

To date, the inflammatory response has not been observed to have been produced by a single cell type. Indeed, macroglial and microglial cells join together in the inflammatory response. Many researchers have described that interactions among resident monocytes, MGCs and astrocytes are essential for modulating gene expression and for mediating distinct responses of glial cells. MGCs secrete the glial cell line-derived neurotrophic factor, leukaemia inhibitor factor and cytokines, and experience morphological changes when co-cultured with activated microglia (Wang *et al.*, 2011). Additionally, *in vitro* studies have revealed that high glucose conditions induce IL-1β secretion by ECs, and this molecule then upregulates IL-1β expression in an autocrine but also paracrine way in MGCs and astrocytes (Liu *et al.*, 2012a). Many researchers have postulated IL-1β to be key regulator of the retinal inflammatory response. In agreement, IL-1β is the only cytokine with the ability to stimulate IL-8 production in MGCs through the activation of the p38 MAP kinase pathway (Liu *et al.*, 2014). IL-8, at the same time, is responsible for the amplification of the inflammatory response, as it is a potent activator and recruiter of leucocytes to the injured tissue. Research on primary cultures of MGCs obtained from rat retinas demonstrated a novel mechanism of down regulation of glutamate uptake mediated by IL-1β under hypoxic conditions. It was proposed that IL-1β decreases the expression of the inward rectifying potassium channel (Kir) 4.1 and glutamate-aspartate transporter (GLAST) in MGCs in order to avoid the conversion of the excessive glutamate released from dying cells into glutamine (Chen *et al.*, 2014). Thus, IL-1β is involved in the inflammation response and also in the modulation of glutamate transport. Interestingly, *in vitro* studies have shown that increased levels of glutamate, in the extracellular

space, induce neuronal cell death, but this does not even stress the MGCs. The subsequent gliosis observed in excitotoxic conditions may be mediated by unknown molecules secreted by neurons (Heidinger *et al.*, 1999).

IL-17A secreted by lymphocytes T helper 17 was found in plasma samples of type 1 and 2 diabetic patients of different ages (Hang *et al.*, 2014; Labikova *et al.*, 2014), and also in vitreous samples (Takeuchi *et al.*, 2015), together with other pro-inflammatory cytokines. Retinal microglial cells, but not MGCs or astrocytes, were observed to produce IL-17A when exposed to hypoxia. In contrast, MGCs express IL-17A receptor and their interaction induces VEGF secretion. The intravitreal injection of IL-17A in Ins2Akita diabetic mice increased GFAP expression, decreased glutamine synthetase enzyme and aggravated BRB breakdown (Qiu *et al.*, 2016). Moreover, neutralization of IL-17A with a monoclonal antibody administrated intraperitoneally prevented ganglion cell death and reduced vascular abnormalities in a model of oxygen-induced retinopathy (OIR) (Talia *et al.*, 2016).

Undoubtedly, the inflammation response plays an important role in the establishment and the progression of DR. Here, we have only described the participation of the most relevant cytokines implicated in this pathology. Nevertheless, we consider that to determine the actual contribution of inflammation in the disease, a general overview of the actions mediated by individual cytokines should be taken in account since many of these could induce opposite effects.

Gliosis

MGCs are characterized by a strong ability to perceive fluctuations in homeostatic conditions. Moreover, they are surprisingly resistant to injuries due to their active metabolism, which facilitates the depuration of altered proteins and organelles, the repair of damaged structures and neutralization of ROS. The first response to an external harmful stimulus is reactive gliosis. This process takes place in early stages of DR, when initial alterations in the retina are detected by MGCs, and is considered to be a protective response. As we mentioned earlier, a key feature of gliosis is the upregulation of GFAP and vimentin. The increase in intermediate filaments is an unspecific response to a wide variety of stimuli and is accompanied by other cellular events that reflect a change in the status of the glial cells. In particular, the rise in intermediate filaments is a strategy to prevent mechanical lesions in the neuroretina.

In a rat model of diabetes type 1 induced by STZ, GFAP was increased in retinas of treated animals compared to control. These results were corroborated by *in vitro* experiments where MGCs were exposed to a high glucose concentration. The increase in GFAP levels was reverted by the addition of insulin, suggesting that hyperglycaemia is a strong stimulus for glial activation (Layton *et al.*, 2006). Asnaghi *et al*.(2003) demonstrated that the polyol pathway may be mediating the rise in GFAP. In this study, diabetic rats showed increased levels of GFAP and aldose reductase, an enzyme of polyol pathway that catalyses the reduction of glucose to sorbitol. Moreover, the oral administration of an inhibitor of aldose reductase (sorbinil) prevented gliotic changes. An increase in retinal vimentin expression was observed in STZ-induced diabetic rats, in MGCs cell cultured under high glucose conditions and in epiretinal membranes of diabetic patients (Zhou *et al.*, 2017).

Advanced glycation end products (AGEs) are also identified by MGCs as potentially dangerous molecules. *Ex vivo* experiments in retinal explants incubated with glycated albumin revealed an increase in the expression of GFAP in MGCs (Lecleire-Collet *et al.*, 2005). On the other hand, *in vitro* studies in a human cell line of MGCs (MIO-M1) demonstrated that α_2M induces GFAP overexpression via the activation of the membrane receptor low-density lipoprotein-related protein 1 (LRP1), a multi-ligand receptor expressed in MGCs and other retinal cells. These results were confirmed *in vivo*, by the intravitreal injection of α_2M at a similar concentration to those reported in diabetic patients (Barcelona *et al.*, 2011). LRP1 is a signalling and endocytic receptor that increases its expression in the inner nuclear layer and inner limiting membrane (where MGCs are located) in retinas of diabetic patients (Barcelona *et al.*, 2010) and OIR rats (Sanchez *et al.*, 2006), and plays different roles in DR. At the initial stages, the α_2M/LRP1 system may activate reactive gliosis as a first response to retinal injury. Nevertheless, in the proliferative stage, increased levels of MMP-2, MMP-9 and activated α_2M were detected in vitreous samples of PDR patients, evidencing that α_2M plays a critical role in regulating the proteolytic activity during the neovascular phase (Sanchez *et al.*, 2007).

Hypertrophy is another non-specific gliotic manifestation of MGCs, often detected in oedematous retinas. The MGC's plasmatic membrane contains ion channels and transmembrane water transporters which regulate the influx and efflux of water, potassium and sodium to restore extracellular space homeostasis and prevent neuronal swelling. In the diabetic retina, water accumulates in the tissue because of uptake alterations and further release of water to circulating compartments. The downregulation in Kir 4.1 channels in perivascular processes of MGCs, which mediate K^+ efflux to blood, seems to be the main reason for hydroelectrolytic imbalance. In addition, in humans with PDR, there is a mislocalization of Kir 4.1 channels that reduces potassium currents in retinal cells, which in turn alters synaptic activity and threatens cell survival (Bringmann *et al.*, 2002). Similarly, changes in the distribution of aquaporins 1 and 4 (water transporters) lead to an increased number of apoptotic neurons, thereby contributing to retinal dysfunction (Fukuda *et al.*, 2010).

As described above, gliosis is a beneficial reaction in the presence of a slight injury because glial cells activate protective mechanisms for neurons. In this situation, no large alterations are observed in MGCs, and therefore this condition is referred as "conservative" gliosis. However, persistent gliosis (known as "massive gliosis") is considered to be a sign of extensive damage and an excessive response to the stimulus, which can be detrimental for retinal tissue. For example, widespread damaged caused by hypoxia induces an increase in GFAP and reduction in glutamine synthetase in MGCs, with a corresponding progression of the pathology in the OIR mouse model. This correlates with an the increase in the number of TUNEL positive cells then observed over time, suggesting a contribution of reactive gliosis events in neuronal degeneration (Ridano *et al.*, 2017).

Chronic hyperglycaemia induces neuronal death and the disorganization of retinal layers with a loss of glia-neuron interactions. This massive gliotic response includes the proliferation and migration of MGCs, with the purpose of making new connections with remaining nerve cells. In

the proliferative stages of DR, the MGC phenotype changes (acquiring characteristics similar to those of fibroblasts) and they proliferate resulting in the formation of epiretinal membranes. This annexed tissue alters retinal elasticity, exerts tractional forces, and recurrently causes retinal detachment and intravitreal haemorrhage. Although the mechanisms responsible for glial proliferation are not completely elucidated, the X-linked inhibitor for apoptosis protein (XIAP) has been implicated in the induction of this process (Sun *et al.*, 2013). While the migratory capacity is regulated by stimuli of a different nature, such as protease inhibitors (α_2M), trophic factors (IGF-1) and lipids (Sphingosine-1-Phosphate) (Barcelona *et al.*, 2013; Lorenc *et al.*, 2015; Simon *et al.*, 2015).

Once MGCs have proliferated and migrated, some of these can dedifferentiate to progenitor cells and originate new neurons in the damaged layers. Heterogeneity in the MGC population has been detected in zebrafish retinas. Three types of MGCs were identified, with different expressions of the transcriptional factors (STAT3, Ascl1a) and RNA binding proteins (Lin28a) implicated in retinal regeneration. These proteins may originate a particular gene expression profile that enables MGCs to re-enter the cell cycle (Nelson *et al.*, 2012). It has been proposed that mammalian retinas become enriched with quiescent MGCs, so they fail to renew neurons. In this sense, studies in zebrafish have proved extremely useful for determining the master regulators of this complex process; therefore, further investigations are now being focused on the modulation of these proteins in mammalian or avian retinas in order to stimulate MGC reprogramming (Reyes-Aguirre & Lamas, 2016; Beach *et al.*, 2017). Thus, inducing changes in the expression of certain proteins or epigenetic modifications is considered to be a possible therapy for restoring the pool of neurons in the retina.

In summary, the macroglial response is mainly considered neuroprotective as long as the mechanisms related to glial activation give rise to rescue/repair signals in a reasonable time period. However, the response should be silenced independently of the resolution of the pathology by highly controlled mechanisms; otherwise it will contribute to retinal damage. In addition to unspecific reactions, there are some glial functions that can only be activated by a particular stimulus, including the secretion of trophic factors, variations in glutamine synthetase activity, or nitric oxide production, among others. Some of these will now be described in the following sections.

Trophic factors

The survival and the good performance of nerve cells in the adult retina are subject to the presence of small amounts of trophic factors, mainly secreted by the macroglia and microglia. The actions exerted by these molecules on neurons and ECs can be either direct or indirect, with the interaction with specific receptors. However, the abnormal concentration of mature trophic factors, the presence of their pro-forms or changes in their receptors can all cooperate in the development of DR. In fact, the currently available treatments try to reverse the damage induced, mainly by VEGF. The pattern and the levels of secreted trophic factors changes in early stages of DR and vary throughout the progression of the disease. Unfortunately, medical devices to

measure the concentrations of VEGF do not exist, so the therapy is mostly administered when macroscopic alterations are detected.

Next, we will briefly describe some of the actions mediated by trophic factors and how MGCs synthesize them and regulate their availability in the extracellular space. The most studied trophic factor involved in the pathophysiology of DR is VEGF, a heparin binding glycoprotein, with five isoforms (VEGF-A, -B, -C, -D and -E) originated by alternative splicing of a common gen. These molecules interact with membrane receptors (mainly VEGFR-1, VEGFR-2, VEGFR-3) and co-activators to transduce intracellular signals, which mediate distinct effects depending on the VEGF isoform and the receptor involved (Wang *et al.*, 2015).

In the intrauterine life, VEGF commands systemic vasculogenesis by inducing proliferation and differentiation of ECs in order to establish the arterial and venous plexus. The acquisition of the correct architecture of the vascular tree depends on the spatiotemporal expression of VEGF and its concentration. High levels of VEGF stimulate arterial differentiation while lower levels are involved in the formation of venous vessels. Moreover, a strong reduction in VEGF signalling by blocking the VEGF receptor results in EC death and the arrest of vessel growth (Casie Chetty *et al.*, 2017). In the retina, the primary hyaloid vasculature is replaced by the definitive one following a physiological hypoxic gradient generated by the increase in metabolic activity. Macroglial cells detect low oxygen concentrations and secrete transiently this vasoformative molecule which responds to oxygen demand (Stone *et al.*, 1995). In addition to MGCs, other retinal cells that contribute to the synthesis and release of VEGF are ECs, astrocytes, retinal pigmented epithelium and ganglion cells (Wang *et al.*, 2010).

Autocrine and paracrine VEGF signalling has been described as a key factor in the development and progression of several pathologies. In the DR context, VEGF is the most harmful protein due to its pro-angiogenic properties. This factor can be primarily secreted by MGCs after prolonged exposure to high levels of glucose and fatty acids (oleic and linoleic acid), two harmful stimuli frequently observed in the Metabolic Syndrome (Capozzi *et al.*, 2016). In this inflammatory environment, ROS levels are increased and they oxidise low density lipoprotein molecules, which have been shown to increase VEGF synthesis (Fu *et al.*, 2016).

VEGF gradually increases and reaches high levels in PDR. Rodent models have been shown to reproduce most aspects of the early stages of DR, but do not develop neovascularization as humans probably owing to the short lifespan of the animals. However, a valid approach to vascular proliferative events and neuronal injury is represented by the OIR model, where the acute hypoxic stimulus induces neovessel formation towards the vitreous cavity, as well as neurodegeneration and retinal functional damage. Thus, is considered a versatile platform for anti-angiogenic drug tests, and great advances have been made with regard to the understanding of the last phase of DR. Recently, we demonstrated that anti-VEGF treatment selectively controls neovascularization but not any other pathologic components in the OIR mouse model such as neuronal damage or MGC activation, and cannot improve retinal functional impairment (Ridano *et al.*, 2017). On the other hand, a recent study in retinal explants of mice demonstrated that VEGF prevents neuronal

apoptosis shortly after exposure to various harmful stimuli frequent in diabetics including hyperglycaemia, AGEs and oxygen peroxide. However, this neuroprotective effect was eliminated when the *ex vivo* retinas were preincubated with a VEGF trapping protein or after many days of stimuli (Amato *et al.*, 2016). Thus, the following dilemma arises: how to correctly modulate VEGF levels in each patient in order to produce regression of neovascularization without inducing neuronal demise? Some new strategies have proposed the suppression of VEGF specifically secreted by MGCs. This VEGF is thought to mediate pathological changes observed in DR because it induces the expression of inflammatory markers, including TNFα, intercellular adhesion molecule (ICAM)-1 and the leucocytes adhered to the vessel wall (Wang *et al.*, 2010). Minor contributions from other retinal cells are insufficient to trigger the above-mentioned alterations. Implementing a CRE/lox system it was possible to obtain mice VEGF KO only in MGCs, which showed reduced levels of retinal VEGF when following the OIR model. These mice also developed significantly fewer neovessels and less vascular leakage, but had a similar retinal function and morphology that control (Bai *et al.*, 2009).

Some researchers have supported the idea of combined treatments. In the hyperglycaemic and hypoxic milieu with an imbalance not only altering VEGF levels but also various trophic factors, each one of which can contribute to gliosis, neurodegeneration and/or neovascularization. An important contributor to the pathogenesis of DR is IGF-1, a hormone with metabolic, proliferative and survival properties. New studies have examined its participation at the early and late phases of the disease. An indispensable tool was a transgenic mouse model overexpressing intraocular IGF-1, which did not show metabolic alterations. Chronic elevated levels of IGF-1 resulted in the progressive degeneration of nerve cells as evidenced by decreased functionality, structural alterations and increased cleaved caspase-3 positive cells (Villacampa *et al.*, 2013). In addition, the same model revealed IGF-1 dependent vascular alterations, including a loss of pericytes, the presence of acellular capillaries and preretinal neovascularization (Ruberte *et al.*, 2004).

During the proliferative stage of retinopathy, IGF-1 participates in neovessel formation by:

a) Enhancing VEGF synthesis: The interaction of IGF-1 with its membrane receptor (IGF-1R) is able to activate the PI3-Kinase and MAPK signalling pathways, which stabilize and accumulate hypoxia-inducible factor (HIF) -1α in the cytosol. Then, HIF-1α translocates to the nucleus and induces gene expression, including VEGF (Treins *et al.*, 2005).
b) Stabilizing neovessel: IGF-1 mediates the persistent activation of the ERK pathway in order to avoid newly formed neovessel disorganization (Jacobo & Kazlauskas, 2015).
c) Remodelling the extracellular matrix: After ligand binding, IGF-1R transduces intracellular signals that increase MMP-2 activity in the proliferative phase, thereby contributing to the establishment of neovessels in the retina (Lorenc *et al.*, 2017). There is a cooperative effect of ECs and MGCs to achieve remodelling, since both synthesize and secrete MMP-2 to the milieu.

IGF-1 is also known to regulate MMP-2 activity in MGC filopodia to facilitate the migration of the macroglial cells to the injured area. This process, mediated by PI3-Kinase/AKT signalling pathway,

is necessary for tissue repair and scar formation (Lorenc *et al.*, 2015). The evidence provided above clearly points to the IGF-1/IGF1-R system being an interesting target for DR treatment. In fact, a combined delivery of antibodies against VEGF and IGF-1 could be an excellent approach to reduce the neuronal damage derived from neovascularization and to alleviate effects produced by an excessive concentration of trophic factors.

Treatment is even more complex because alterations in other trophic factors have been detected. Brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3) and -4 (NT-4) are part of a family of neurotrophins, with all these synthesized from a common gen. They are involved in many processes related to neuronal development, creation of the synaptic network and neuronal plasticity (Abu El-Asrar *et al.*, 2013). In order to become active, their pro-forms should be cleaved by proteolytic enzymes. However, recent studies have reported derived functions from the interaction with their longer peptides. Results suggest that alterations in both, the activity and synthesis of enzymes in charge of protein cleavage lead to the accumulation of pro-forms and to a reduction in the level of the cleaved proteins. Ali *et al.* (2011) observed that oxidative stress reduces MMP-7 activity, a protease secreted by MGCs, thereby misbalancing the proNGF/NGF ratio and thus contributing to neurodegeneration and BRB breakdown. In fact, high levels of proNGF were observed in aqueous humour of patients with PDR (Ali *et al.*, 2011) as well as in serum samples (Mohamed & El-Remessy, 2015).

For decades, it was thought that pro-enzymes were inactive, but today there is growing evidence demonstrating their activity. Unlike the mature form of NGF which interacts with its receptor TrkA (Tropomyosin receptor kinase A), proNGF prefers to bind with $p75^{NTR}$. This receptor and the events mediated by proNGF were found upregulated in MGCs of diabetics resulting in profound damage to retinal neurons. MGCs are the only retinal cells that express $p75^{NTR}$ receptor in adulthood, so the effects of the non-cleaved NGF are attributed to the activation of MGC cellular pathways. In this sense, a study in non-diabetic mice showed that the proNGF/$p75^{NTR}$ interaction induced TNFα release from glial cells and subsequent retinal ganglion cell death (Lebrun-Julien *et al.*, 2010). Promising results were obtained from pharmacological experiments carried out in animal models of DR by two different approaches: inhibition of the receptor $p75^{NTR}$ by a small molecule antagonist and the use of anti-proNGF blocking monoclonal antibody; with both approaches avoiding retinal degeneration (Barcelona *et al.*, 2016). Overexpression by electroporation of proNGF plasmid in rat retinas increased BRB permeability, as assessed by the increased extravasation of plasma proteins (Matragoon *et al.*, 2012). Furthermore, *in vitro* experiments in human retinal ECs revealed angiogenic functions of proNGF when interacting with TrkA (Elshaer *et al.*, 2013), and *in vitro* studies in astrocytes also demonstrated the ability of proNGF to increase VEGF (Kim *et al.*, 2013).

The above evidence points to proNGF being an important player in the development of vascular, neuronal and inflammatory alterations of DR. It has been previously demonstrated that α_2M-NGF complexes bind to the TrkA receptors but do not induce TrkA dimerization or activation, resulting in deficient trophic support. In addition, α_2M makes stable complexes with proNGF, conveying resistance to proteolysis that results in more proNGF but less NGF. Finally, α_2M-proNGF complexes

bind $p75^{NTR}$, inducing the synthesis of TNFα. These mechanisms are operative *in vivo*, with α_2M having the ability to cause neurodegeneration by $p75^{NTR}$ in a proNGF-dependent manner (Barcelona & Saragovi, 2015).

NT-3 and NT-4 trophic factors and the shedding form of their receptors TrkA and TrkB, but not TrkC, were incremented in vitreous samples of DR patients. Similar results were observed by immunohistochemical analysis of epiretinal membranes (Abu El-Asrar *et al.*, 2013). With regard to BDNF, a decrease in its concentration was detected in serum and vitreous samples of diabetic patients, signifying a reduction in the neuroprotection provided (Kaviarasan *et al.*, 2015).

Some recent investigations have focused on the study of a neuroprotective and antiangiogenic trophic factor discovered in the retina: the pigment epithelium-derived factor (PEDF), with the first cells known to synthesize and secrete this soluble molecule being the pigmented epithelium cells. The secretion of PEDF by MGCs is known to activate the NF-κB signalling pathway, which avoids ganglion cell apoptosis in oxygen deprivation conditions (Unterlauft *et al.*, 2014). At the vascular level, PEDF is able to prevent the formation of neovessels towards the vitreous cavity, and the breakdown of the BRB by upregulating the proteins that constitute the tight junctions between ECs, including zonula occludens-1 (Ibrahim *et al.*, 2015). Moreover, PEDF is able to reduce VEGF levels in a significant manner, thereby contributing to the restoration of vascular homeostasis. The mechanisms underlying this process involve the interaction of PEDF with its plasmatic membrane receptor (PEDF-R), which activates gamma secretase activity. This enzyme cleaves the transmembrane domain of VEGF-R1, impairing VEGF signalling (Cai *et al.*, 2006). However, these beneficial functions are decreased in DR due to a reduced synthesis and secretion of PEDF (Yoshida *et al.*, 2009; Saidi *et al.*, 2011).

An interesting method to cope with the trophic factor imbalance is gene therapy, which enables with high efficiency the overexpression of PEDF by episomal constructs (Calado *et al.*, 2016). In addition, PEDF has anti-inflammatory properties, since it reduces the secretion of pro-inflammatory cytokines such as IL-6, IL-3, interferon gamma (INFγ) and TNFα by MGCs and inhibits microglial reactivity (Liu *et al.*, 2012b). In addition, PEDF also decreases IL-1β and consequently the glutamine synthetase activity increases (Shen *et al.*, 2010b). As described above, this pleiotropic factor was demonstrated to have beneficial effects on different aspects of DR, decreasing the damage caused in neurons, glia and vessels. Thus, studies with intravitreal, subretinal or eye drops administration of PEDF, carried out in diverse animal models of DR, have demonstrated this treatment to be a promising pharmacologic tool for this ocular pathology.

The final results will be the consequence of single and/or synergic actions directed by trophic factors, among other molecules, which may be positive or negative for DR. The role of trophic factors in angiogenesis and neurodegeneration is unquestionable. However, trophic factors might not increase/decrease in a similar way in every diabetic patient. Thus, pharmacogenomic studies are essential for the development of an optimum therapy for each patient at specific stages of DR, which needs to consider individual requirements.

Antioxidant pathways

Although diabetic patients manifest many systemic symptoms since the onset of the disease, the loss of vision is not an early sign. In fact, a main characteristic of this ocular pathology is its slow progression and we consider that there are two relevant factors involved in the protection of the retinal tissue:

- The BRB as the first defence front against systemic insults, which preserves the retinal environment, providing a relative isolation to post-mitotic cells.
- The presence of compensatory mechanisms displayed by stressed cells when potentially harmful stimuli are perceived.

Once the BRB is damaged and insult is sensed by retinal cells, the restoration of homeostasis will depend on the intracellular compensatory events. Antioxidant pathways are a frequent response to oxidative stress in metabolic and neurodegenerative diseases and are an emerging therapeutic target. They control ROS neutralization, and also the elimination of subcellular structures, proteins and lipids altered by those reactive molecules, which can lead to cellular demise.

In vitro studies have shown that hyperglycaemia is able to increase oxygen peroxide levels in MGCs (Giordano *et al.*, 2015). However, novel isolation protocols and proteomic studies of MGCs have revealed a high resistance to oxidative stress, probably due to an increased expression of the perodoxins -1, - 4 and -6, enzymes that participate in oxygen peroxide removal (Grosche *et al.*, 2016). In oxidative conditions, the MGC protective response is mainly mediated by the activation of the transcriptional factor Nrf2 (nuclear factor eritroyd -2-related factor 2), and the subsequent increased expression of the proteins involved in ROS elimination (Xu *et al.*, 2014).

Surprisingly, certain signalling pathways can be modulated by ROS, as these molecules are phosphatase inhibitors. They are able to trigger the phosphorylation of ERK 1/2, thus increasing neuronal survival (Groeger *et al.*, 2012). The concept of the regulation of intracellular processes by ROS opens up an intriguing field of study, where these molecules are not only considered detrimental but also necessary for the response to an insult.

In MGCs, the degradation systems are altered under high glucose conditions. Autophagy is an intracellular process by which modified or no longer needed proteins and organelles are eliminated from the cytoplasm in a double-membrane vesicle (Villarejo-Zori & Boya, 2017). However, this catabolic process cannot reach the final stage (degradation of the cargo) because of an increase in the lysosomal pH and a decrease in lysosomal enzyme activity. Treatments with autophagy inductors, such as rapamycin, have restored the normal flux and prevented the activation of apoptosis (Lopes de Faria *et al.*, 2016).

Finally, *in vivo* studies in diabetic rats have demonstrated that oral administration of green tea and resveratrol (both compounds with antioxidant activity) reversed the alteration of glutamine synthetase expression and activity, as well as the expression of the glutamate transporter GLAST (Silva *et al.*, 2013; Zeng *et al.*, 2016). The enhancement of these natural antioxidant mechanisms

could be a complementary strategy to cope with oxidative stress and excitotoxicity in the diabetic retina. If MGCs can neutralize free radicals and clear cellular waste, they will be able to carry out actions related to neuronal care.

Conclusions

Trying to unravel the events that occur in the pathology of DR requires an integrated view, taking into account all its aspects. Unfortunately, DR is still not completely understood mainly because it is multifactorial origin, where many insults coexist. In this review, we have compiled information related to MGC participation in DR. The interest in these abundant macroglial cells arises from all the functions that they perform related to health and disease. MGCs have shown a strong involvement in the maintenance of homeostasis and development of retinal tissue. Although, they do not transduce the visual information directly, they ensure that neurons are in perfect conditions to carry this out. MGCs are the main suppliers of trophic factors and survival signals for post-mitotic cells, they cooperate in the recycling of photopigments and neurotransmitters, and remove neuronal waste. In fact, MGCs are one of the most active cells in the retina, even when our eyes are closed.

Although MGCs also contribute to the establishment and progression of the disease (Figure 1), this should not be considered as a harmful effect, but rather the consequence of a chronic activation of a protective response to injuries. Dyslipidemia, hypoxia, hyperglycaemia, ROS and altered proteins are some of the insults that stress and damage retinal cells in DR. Under this scenario, MGCs quickly react to avoid the loss of retinal homeostasis by activating warning signals. Consequently, they increase GFAP, stimulate the migration of phagocytes for further protection, and release trophic factors to preserve neuronal wellbeing. These above- mentioned processes are necessary for tissue reparation in the case of slight damage. However, an exacerbated and persistent response to the stimuli leads to retinal demise, with chronic inflammation, neurodegeneration, neovascularization and fibrosis, which are reflected in the loss of vision.

Summing up, in the light of the reported results, we consider the adequate modulation of the MGC responses to be a promising therapy for DR. Our next challenge will be to design a proper therapy that returns trophic factors to their normal concentrations, decreases chronic inflammation, prevents neovascularization and activates protective pathways, in order to guarantee neuronal and vascular preservation.

Conflict of interest

The authors declare no conflict of interest.

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Abbreviations

α_2M: α_2Macroglobulin
AGEs: advanced glycation end
ALEs: advanced lipoxidation end products
ATP: adenosine triphosphate
BDNF: Brain-derived neurotrophic factor
BRB: blood retinal barrier
CRALBP: cellular retinaldehyde-binding protein
DR: diabetic Retinopathy
ECs: endothelial cells
GFAP: glial fibrillary acid protein
GLAST: glutamate-aspartate transporter
HIF-1α: hypoxia-inducible factor 1α
ICAM: intercellular adhesion molecule
IGF-1: insulin like growth factor-1
IL: interleukin
INFγ: interferon gamma
LRP1: membrane receptor low-density lipoprotein-related protein 1
MAPK: mitogen-activated protein kinase
MGCs: Müller Glial Cells
MMPs: matrix metalloproteinases
NGF: nerve growth factor
NPDR: Non- proliferative DR
Nrf2: nuclear factor eritroyd -2-related factor 2
NT: neurotrophin-3
OIR: oxygen-induced retinopathy
PDR: Proliferative DR
PEDF: pigment epithelium-derived factor
PEDF-R: pigment epithelium-derived factor receptor
ROS: reactive oxygen species
STZ: streptozotocin
TNFα: tumour necrosis factor alpha
Trk: Tropomyosin receptor kinase

VEGF: vascular endothelial growth factor
VEGFR: vascular endothelial growth factor receptor
XIAP: X-linked inhibitor for apoptosis protein

Figure Legend

Figure 1: Müller glial cells in DR. The main insults in DR are hyperglycaemia, dyslipidemia, hypoxia, AGEs and ROS. MGCs detect changes in the retinal environment from the early stages in the onset of the disease and set up a gliotic response. MGCs also synthesize and secrete trophic factors to avoid neuronal damage and activate antioxidant pathways. Together with microglial cells, they modulate inflammation, known as a key factor in chronic metabolic diseases. During the proliferative stage, MGCs contribute to the regulation of neovascularization by the secretion of VEGF and MMPs. In addition, due to trophic factor imbalance and an increased inflammatory response, some MGCs could die by piroptosis. Abbreviations: M: Müller glial cells; m: microglial cells; a: astrocytes; P: photoreceptors; H: horizontal cells; B: bipolar cells; A: amacrine cells; G: retinal ganglion cells; ECs: endothelial cells.

Authors Contribution

M.C.S. designed and reviewed the text and the images and edited the review. P.V.S. wrote the review, designed images and edited the review. M.C.P. contributed to the description of MGCs metabolic alterations and added unpublished results obtained in a mouse model of DR. M.E.R participated in Trophic Factors section, mainly in VEGF unbalance and ROP mouse model. V.E.L contributed to the description of IGF-1 and MMPs participation in the proliferative stage of DR. M.V.V. participated in Antioxidant Pathways section. P.F.B. collaborated in the description of BRB dysfunction and α_2M participation in DR. J.D.L general overview and clinical aspects of the pathology. M.C.P., M.E.R., V.E.L, M.V.V., J.D.L. reviewed the review.

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