Maria Helena Bica Madeira

CONTROLLING NEUROINFLAMMATION IN THE RETINA THROUGH A2AR MODULATION: POTENTIAL THERAPEUTIC IMPLICATION IN GLAUCOMA

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UNIVERSIDADE DE COIMBRA

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CONTROLO DA NEUROINFLAMAÇÃO NA RETINA PELA MODULAÇÃO DO RECETOR A$_{2A}$: POTENCIAIS IMPLICAÇÕES TERAPÊUTICAS NO GLAUCOMA

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Contents

Abbreviations

A_1R   Adenosine A_1 receptor

$A_{2A}R$ Adenosine A_{2A} receptor

$A_{2B}R$ Adenosine A_{2B} receptor

A_3R   Adenosine A_3 receptor

AD   Alzheimer's disease

ADA   Adenosine deaminase

ADP   Adenosine di-phosphate

AMP   Adenosine mono-phosphate

AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA   Analysis of variance

ATP   Adenosine triphosphate

BCA   Bicinchoninic acid

BDNF   Brain-derived neurotrophic factor

bp   Base pairs

BRB   Blood-retinal barrier

BSA   Bovine serum albumin

Ca^{2+}   Calcium

cAMP   Cyclic adenosine monophosphate

CD73   Ecto-5'-nucleotidase

CD39   Ectonucleoside triphosphate diphosphohydrolase 1

cDNA   Complementary DNA

CGS21680   3-[4-[2-[[6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid

CI   Circularity index

CNS   Central nervous system

CREB   cAMP responsive binding element

CTCF   Corrected total cell fluorescence

D_2DR   D2 dopamine receptor

DAF-FM   4-amino-5-methylamino-2',7'-difluororescein diacetate

DAPI   4',6-diamidino-2-phenylindole

DMEM   Dulbecco's modified Eagle's medium

DNA   Deoxyribonucleic acid

EDTA   Ethylenediaminetetraacetic acid

EGTA   Ethylene glycol tetraacetic acid

EHP   Elevated hydrostatic pressure

ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signaling kinase
EVC	Episcleral vein cauterization
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GABA	Gamma-Aminobutyric acid
GCL	Ganglion cell layer
GFAP	Glial fribrillary acid protein
HBSS	Hank's balanced salt solution
HD	Huntington's disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
I-R	Ischemia-Reperfusion
IgG	Immunoglobulin G
IL-1β	Interleukin-1β
IL-6	Interleukin-6
INL	Inner nuclear layer
iNOS	Inducible nitric oxide synthase
IOP	Intraocular pressure
IPL	Inner plexiform layer
ipRGCs	Intrinsically photosensitive retinal ganglion cells
K$^+$	Potassium ion
KCl	Potassium chloride
KW6002	8-[(1E)-2-(2-(3,4-Dimethoxyphenyl)ethenyl]-1,3-diethyl-3,7-dihydro-7-methyl-1H-purine-2,6-dione
LGN	Lateral geniculate nucleus
LP	Laser photocoagulation
LPS	Lipopolysaccharide
M-CSF	Macrophage-colony stimulating factor
MAP	Mitogen-activated protein
MgCl$_2$	Magnesium chloride
MHC	Major histocompatibility complex
mRNA	Messenger RNA
NaCl	Sodium chloride
NaF	Sodium fluoride
NFL	Nerve fiber layer
NMDA	N-methyl-D-aspartate

NO	Nitric oxide
NTG	Normal tension glaucoma
OCT	Optimal cutting temperature
OHT	Ocular hypertension
ONH	Optic nerve head
ONL	Outer nuclear layer
OPL	Outer plexiform layer
PACG	Primary angle closure glaucoma
PBS	Phosphate-buffered saline
PCR	Polimerase chain reaction
PD	Parkinson's disease
PFA	Paraformaldehyde
PI3	Phosphoinositide 3
PKA	Protein kinase A
PKC	Protein kinase C
POAG	Primary open angle glaucoma
qPCR	Quantitative PCR
RGC	Retinal ganglion cells
RIN	RNA integrity number
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
s.e.m	Standard error of the mean
SCH 58261	5-Amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo(4,3-e)-1,2,4-triazolo(1,5-c)pyrimidine
SC	Superior colliculus
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase (TdT)- mediated dUTP nick end labelling

Abstract

Glaucoma is the second leading cause of blindness worldwide, being characterized by loss of retinal ganglion cells (RGCs) and optic nerve damage. Early and exarcerbated activation of microglial cells, the immunocompentent cells in the central nervous system (CNS) and strong neuroinflammatory response have been described in glaucoma, which are thought to be involved the processes that lead to RGC death. Advanced age and elevated intraocular pressure (IOP) are considered major risk factors for the development of glaucoma. Currently, the therapeutic approach in glaucoma is lowering the IOP, but many patients continue to lose vision despite the successful control of IOP. Therefore, the development of novel neuroprotective strategies, aimed at preventing RGC loss, might offer potential for the treatment of glaucoma.

Adenosine is a crucial neuromodulator in the CNS, which is up-regulated under harmful conditions. It acts on the purinergic P1 inhibitory receptors, A_1 and A_3, and in the P1 facilitatory A_{2A} and A_{2B} receptors. Adenosine A_{2A} receptor ($A_{2A}R$) antagonists have emerged as potential neuroprotective agents in brain neurodegenerative diseases, namely by controlling the microglia-mediated neuroinflammatory response.

Taking in account the contribution of microglia-mediated neuroinflammation in the pathophysiology of glaucoma, in this work we aimed to evaluate the contribution $A_{2A}R$ blockade in the control of retinal neuroinflammation and its potential neuroprotective effects on RGCs, using *in vitro* and animal models of glaucoma.

In the first part of this work, retinal organotypic cultures were exposed to lipopolysaccharide (LPS; 3 μg/mL), used as an inflammatory stimulus, or elevated hydrostatic pressure (EHP; 70 mmHg above normal atmospheric pressure), to mimic ocular hypertension (OHT), in the presence or absence of the $A_{2A}R$ selective antagonist SCH 58261 (50 nM). An up-regulation of $A_{2A}R$ was detected after exposure to LPS or EHP, and it was paralleled by activation of microglial cells and increased expression of pro-inflammatory markers and reduction in the number of RGCs. The blockade of $A_{2A}R$ prevented the microglia activation and neuroinflammatory response triggered by both conditions. Furthermore, SCH 58261 prevented the loss of RGCs triggered by exposure to LPS and EHP, an effect that was also observed in the presence of antibodies against tumor necrosis factor (TNF) and interleukin-1β (IL-1β). These results suggested that blockade of $A_{2A}R$ prevented the loss of RGCs through the control of the neuroinflammatory response.

In order to further elucidate the direct effects of $A_{2A}R$ blockade on the control of retinal microglial cells reactivity, in the second part of this work, primary retinal microglial cell cultures were challenged with LPS or EHP, after a pre-treatment with SCH 58261 (50 nM). Similar to what we observed in retinal organotypic cultures, the blockade of $A_{2A}R$ prevented the effects of LPS and EHP on microglia activation and on the expression of the inflammatory mediators TNF,

IL-1β and nitric oxide (NO). The A$_{2A}$R antagonist also prevented the phagocytic activity of microglia elicited by LPS.

Notably, in an animal model of high IOP-induced transient ischemia (I-R), the intravitreal administration of SCH 58261 (100 nM; 5 μL) reduced the I-R-induced neuroinflammatory response in the retina and the loss of RGCs. The injection of antibodies against TNF and IL-1β recapitulated the effects of A$_{2A}$R antagonist, further supporting the hypothesis that A$_{2A}$R blockade confers protection to RGCs by reducing the microglia-mediated neuroinflammatory response.

Caffeine is an antagonist of adenosine receptors, and it has been shown to confer neuroprotection to the brain through the antagonism of A$_{2A}$R. Therefore, in the third part of this work, we investigated the effects of caffeine administration (1 g/L, in water) in an animal model of OHT, induced by laser photocoagulation (LP) of the perilimbar and episcleral veins. Caffeine decreased IOP of OHT animals, although this decrease may not be physiologically relevant. Nevertheless, after 7 days of OHT, caffeine administration prevented the OHT-induced microglia activation and neuroinflammatory response and increased the survival of RGCs. Still, caffeine was not able to rescue the functional damage in the retrograde axonal transport in the optic nerve.

In conclusion, our results provide evidence for the ability of A$_{2A}$R antagonists and caffeine to control retinal microglia reactivity and neuroinflammatory response, as well as to confer neuroprotection to RGCs, in both *in vitro* and animal models of glaucoma. Taking in account the contribution of microglia-mediated neuroinflammation to the pathogenesis of glaucoma, our results open the possibility for the use of A$_{2A}$R antagonists as therapeutic options to manage neuroinflammation and RGC loss in glaucoma.

Resumo

O glaucoma é a segunda causa de perda de visão em todo o mundo, sendo caracterizado por danos no nervo ótico e morte de células ganglionares da retina. A ativação precoce e exacerbada das células da microglia, as células imunocompetentes do sistema nervoso central, e a resposta neuroinflamatória foram também descritas em glaucoma, e pensa-se que podem estar envolvidas nos processos que conduzem à morte das células ganglionares da retina. A idade avançada e o aumento da pressão intraocular são considerados os principais fatores de risco para desenvolver esta doença. Atualmente, os tratamentos existentes baseiam-se na diminuição da pressão intraocular. Contudo, em muitos doentes a doença continua a progredir apesar de um controlo efetivo da pressão intraocular. Por isso, o desenvolvimento de novas estratégias, direcionadas à proteção das células ganglionares da retina, pode oferecer um potencial tratamento para o glaucoma.

A adenosina é um neuromodulador essencial no sistema nervoso central, cujos níveis aumentam em condições nocivas. A adenosina atua em recetores purinérgicos do tipo P1: recetores A_1 e A_3 (inibitórios) e recetores A_{2A} e A_{2B} (facilitatórios). Antagonistas do recetor A_{2A} de adenosina ($A_{2A}R$) surgiram como potenciais agentes neuroprotetores em doenças cerebrais neurodegenerativas, nomeadamente através do controlo da resposta neuroinflamatória mediada por células da microglia.

Tendo em conta o contributo da neuroinflamação mediada pelas células da microglia no desenvolvimento de glaucoma, neste trabalho o principal objetivo foi avaliar a contribuição do bloqueio do $A_{2A}R$ no controlo da resposta neuroinflamatória na retina e os seus potenciais efeitos neuroprotetores nas células ganglionares da retina, usando para isso modelos *in vitro* e animais de glaucoma.

Na primeira parte deste trabalho, culturas organotípicas de retina foram expostas a um estimulo inflamatório com lipopolissacarídeo (LPS; 3 μg/mL), ou a pressão hidrostática elevada (70 mmHg acima da pressão atmosférica normal), para mimetizar a hipertensão ocular, na presença ou ausência de um antagonista seletivo do $A_{2A}R$, SCH 58261 (50 nM). Após exposição a LPS ou a pressão hidrostática elevada ocorreu um aumento da expressão do $A_{2A}R$, em paralelo com aumento da reatividade das células da microglia e da expressão de mediadores pró-inflamatórios, assim como uma redução do número de células ganglionares da retina. O bloqueio do $A_{2A}R$ preveniu a resposta neuroinflamatória induzida pelos dois estímulos, assim como a reatividade das células da microglia. Além disso, o tratamento com SCH 58261 preveniu a perda de células ganglionares da retina, um efeito que foi também observado na presença de anticorpos anti-fator de necrose tumoral (TNF) e anti-interleucina-1β (IL-1β). Estes resultados sugerem que o bloqueio do $A_{2A}R$ previne a perda de células ganglionares da retina através do controlo da resposta neuroinflamatória da retina.

De forma a elucidar o efeitos diretos do bloqueio do $A_{2A}R$ no controlo da reatividade das células da microglia da retina, na segunda parte deste trabalho, culturas primárias de microglia de retina, foram expostas a LPS ou pressão hidrostática elevada, após pré-tratamento com SCH 58261 (50 nM). Semelhante ao que observamos nas culturas organotípicas de retina, o bloqueio do $A_{2A}R$ preveniu a ativação destas células induzidas por LPS ou pressão hidrostática elevada, nomeadamente a expressão de marcadores inflamatórios como TNF e IL-1β ou monóxido de azoto (NO). A atividade fagocítica das células microglia induzida por LPS foi também prevenida pelo antagonista do $A_{2A}R$.

Adicionalmente, num modelo animal de pressão intraocular elevada induzida por isquémia-reperfusão (I-R), a injeção intravítrea de SCH 58261 (100 nM; 5 μL) reduziu a resposta neuroinflamatória induzida por I-R e a perda de células ganglionares da retina. De facto, a injeção de anticorpos contra TNF e IL-1β mimetizou os efeitos do antagonista do $A_{2A}R$, suportando assim a hipótese de que o bloqueio do $A_{2A}R$ confere proteção às células ganglionares da retina ao reduzir a resposta neuroinflamatória mediada pelas células da microglia.

A cafeína é um antagonista dos recetores de adenosina que confere neuroproteção no cérebro, através dos efeitos antagonísticos no recetor $A_{2A}R$. Por isso, na terceira parte deste trabalho, investigámos o efeito da administração de cafeína (1 g/L, em água) num modelo animal de hipertensão ocular, induzida por fotocoagulação a laser das veias perilimbares. A administração de cafeína reduziu parcialmente a pressão intraocular em animais com hipertensão ocular, contudo este efeito poderá não ter relevância fisiológica. Todavia, após 7 dias de hipertensão ocular, a administração de cafeína aumentou a sobrevivência das células ganglionares da retina, preveniu a resposta neuroinflamatória e reatividade das células da microglia induzidas por hipertensão ocular. Porém, a cafeína não foi eficaz na prevenção de danos funcionais no transporte axonal retrógrado no nervo ótico.

Concluindo, os nossos resultados forneceram evidências da capacidade dos antagonistas do $A_{2A}R$ no controlo da reatividade das células da microglia da retina e resposta neuroinflamatória, e também de conferir neuroproteção às células ganglionares da retina, quer em modelos *in vitro* quer em modelos animais. Tendo em conta a contribuição da neuroinflamação mediada pelas células da microglia na patogénese do glaucoma, os nossos resultados abrem a possibilidade para o uso de antagonistas do $A_{2A}R$ como opções terapêuticas para controlar a perda de células ganglionares da retina no glaucoma.

CHAPTER I -
General Introduction

1. Introduction

1.1 The visual system

The visual system is the portion of the central nervous system (CNS) that enables the visual process. This system includes the eyes and the sensory cells that project to the cerebral cortex, where action potentials will convey and give origin to visual information (VanPutte, 2014).

The eye is a highly specialized and organized organ, which comprises the ocular globe (also called eyeball) and the optic nerve. The ocular globe comprises three primary tunics (Figure 1). The outermost tunic is a fibrous layer, named sclera that consists of dense collagenous connective tissue with elastic fibers, acting as a protective layer, maintaining the shape of the ocular globe and the intraocular pressure (IOP), and being also an attachment point for the extrinsic eye muscles (Malhotra et al., 2011). In the anterior part, the sclera becomes transparent, forming the cornea, an avascular tissue that allows the input of light into the eye, causing the reflection or refraction of the light that enters (Kolb, 1995a; VanPutte, 2014).
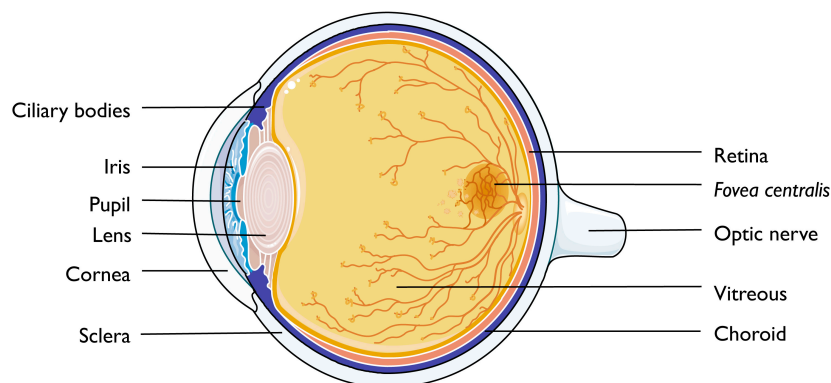
Figure 1 – Sagittal section representing the anatomy of the human eye.

The middle tunic of the ocular globe is the uvea or uveal tract, a vascular layer and nutritive functions. The uvea is composed by the choroid (posteriorly), ciliary bodies (intermediate) and the iris (anteriorly). The choroid is a thin layer that extends from the optic nerve to the *ora serrata* (where the sensory retina ends), providing oxygen and nourishment to the outer layers of the retina (Malhotra et al., 2011; VanPutte, 2014). The iris is a thin, contractile, pigmented structure, with a central aperture called the pupil, which is located between the cornea and the lens (Malhotra et al., 2011). The ciliary body consists of an outer ciliary ring and an inner group of ciliary processes, which are continuous posteriorly with the choroid and anteriorly with the iris. It contains smooth ciliary muscles, which enable the lens to change shape during the accommodation process (focusing near and distant objects). The ciliary

processes are a complex of capillaries and cuboidal epithelium that produces the aqueous humor (Kolb, 1995a; Malhotra et al., 2011; VanPutte, 2014).

The lens is a transparent, biconvex structure, located behind the iris, which consists of multiple layers of cells arranged in concentric pattern. Together with the cornea forms the optical system that focuses impinging light rays into an image in the retina (Malhotra et al., 2011).

The inner layer of the ocular globe is the retina, a light-sensitive tissue, which can be grossly divided in the retinal pigment epithelium (RPE) and the neurosensory retina (Kolb, 1995a). In the human retina, the macula, a small yellow area containing the fovea, is located in the center of the retina. It is this region that provides central vision, being the fovea the portion of the retina with greatest visual acuity. Nevertheless, the commonly used rodent models in research, mouse and rat, do not possess a macula. Still, it was already demonstrated that these models feature a specialized retinal region resembling the key structures of the human macula (Huber et al., 2010). The optic disc is located just medial to the fovea, being the region where the central retinal vessels emerge, allowing the blood supply of the retina. It is also in the optic disc that the axons of retinal ganglion cells (RGCs) pass to form the optic nerve (Malhotra et al., 2011; VanPutte, 2014). Therefore, this region is usually called "blind spot" due to the absence of normal retinal tissue.

The optic nerve, composed by RGC axons and support cells, transmit the visual information from the retina to the brain. It leaves the eye and enters the cranial cavity until the optic chiasm, where the axons of the RGCs can project to the same or opposite side of the brain, until the visual centers in the brain (VanPutte, 2014).

The eye contains three fluid chambers: the anterior chamber, the posterior chamber and the vitreous. The anterior chamber is located between the cornea and the iris, and the posterior chamber between the iris and the lens, being both filled with aqueous humor. The ciliary processes produce the aqueous humor as a blood filtrate, which is then returned to circulation mainly through the trabecular meshwork, located at the base of the cornea. The ratio between the production and the removal of aqueous humor is fundamental for the maintenance of constant IOP and the eye shape. The aqueous humor also refracts light and provides nutrition for the structures of the anterior chamber, such as the avascular cornea. The vitreous chamber occupies the space between the lens and the retina, and in the human represents two thirds of the eye volume. It is filled with vitreous humor, a transparent and viscous substance composed mainly by water bound to soluble proteins, which also helps to maintain the shape of the eyeball (Kolb, 1995a; Mafee et al., 2005; VanPutte, 2014).

1.1.1 The retina

The retina is a thin tissue that covers the inner surface of the eyeball and enables the first step of the visual process.

Located in the outermost part of the retina, the RPE is formed by a single layer of cells that, together with the choroid, nourish retinal cells, being also involved in several other processes, as light absorption, control of ion homeostasis, and secretion of protein required for retinal homeostasis. Furthermore, the RPE contributes for the retinal immune modulation by directly communicating with the immune system (Strauss, 2005).

The neural part of the retina (Figure 2) is responsible for the conversion of the light stimulus into neural impulses. The three main cell types that constitute the retina are: neurons (photoreceptors, bipolar cells, horizontal cells, amacrine cells and RGCs), glial cells (Müller cells, astrocytes and microglial cells) and cells that constitute the retinal vessels (endothelial cells and pericytes) (Fischbarg, 2006; Kolb, 1995c)
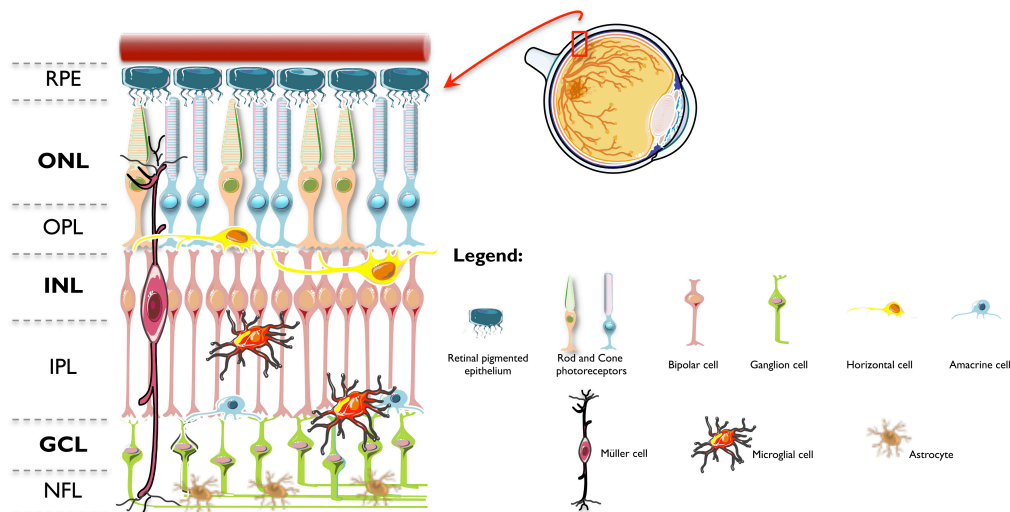
Figure 2 – Schematic representation of the retinal anatomy, depicting the structural layers and major retinal cell types. From top to the bottom: retinal pigment epithelium (RPE) followed by the outer nuclear layer (ONL) containing the nuclei of rod and cone photoreceptors; then it is the outer plexiform layer (OPL), where photoreceptors synapse with bipolar and horizontal cells, which have their nuclei in the inner plexiform layer (INL). Next is the inner plexiform layer (IPL), where retinal ganglion cells (RGCs) synapse with bipolar and amacrine cells. RGC cell bodies are located in the ganglion cell layer (GCL), and their axons give origin to the nerve fiber layer (NFL) and to the optic nerve. Müller cells span all retinal layers, astrocytes are mainly located near the NFL and microglial cells are found in the IPL and GCL (adapted from Madeira et al., 2015).

The cells are organized in three layers of cell bodies and two layers of synapses (Figure 2). The outermost layer of the neural retina, located near to the RPE, is the outer nuclear layer (ONL) that contains the cell bodies of the photoreceptors (rods and cones). The inner nuclear layer (INL) contains the cell bodies of bipolar, horizontal and amacrine cells, and the ganglion cell layer (GCL) is composed by the nuclei of the RGCs and displaced amacrine cells. The synapses occur in the outer plexiform layer (OPL), located between ONL and INL; and in the inner plexiform layer (IPL), between the INL and GCL (Fischbarg, 2006; Kolb, 1995c; VanPutte, 2014).

1.1.1.1 Retinal neuronal cells

The mammalian retina has five main types of neuronal cells, subdivided in more than 60 sub-types of distinct neurons, each playing a specific role in the visual image process (Masland, 2012).

Photoreceptors

Rods and cones are the cells sensitive to light, being located in the outer part of the retina, the more distant region from incoming light (Pascale et al., 2012). These two types of cells differ in numerous ways, being the most important differences their relative sensitivity to the light, their morphology, their number and distribution in the retina (Hubel, 1988). Still, in both cell types it is possible to distinguish three main regions: the outer segment (contiguous to the pigment epithelium), formed by membranous disks light sensitive photopigments, which is responsible by the phototransduction process; inner segment, which contains the nucleus; and the synaptic terminals that contact with bipolar and horizontal cells (Pascale et al., 2012; Purves et al., 2011).

Rods are responsible for vision with low light levels, presenting high sensitivity to capture the few available photons. Their outer segments are cylindrical and the membranous disks contain the visual pigment rhodopsin (Hubel, 1988; VanPutte, 2014). Cones are shorter and have conical morphology, being involved in the visual response in the normal light. The outer segments of cones contain a visual pigment iodopsin, which consists of retinal combined with a photopigment opsin protein, divided in three sub-types, each one sensitive to a different wavelength of light, blue, red, and green (Hubel, 1988; VanPutte, 2014).

The absorption of light by the photopigment in the outer segments gives rise to a cascade of events, which leads to alterations in the membrane potential and consequently modulates the amount of glutamate neurotransmitter released into the synapse (VanPutte, 2014).

Bipolar cells

Bipolar cells are the first "projection neurons" in the visual system. There are at least 12 distinct types of bipolar cells that synapse with rod and cone photoreceptors cells and transmit their synaptic impulse to RGCs (Euler et al., 2014). The interaction between rods and cones with bipolar cells is distinct and can be divided in ON and OFF. ON bipolar cells are depolarized by light, whereas OFF bipolar cells are depolarized in the dark (Euler et al., 2014; Masland, 2012).

One bipolar cell is able to receive input from several rods and one RGC receives input from numerous bipolar cells, occurring spatial summation and signal enhancement, allowing the sensitivity to stimuli from dim sources, but decreases the visual acuity. On the other hand, one cone synapses only with one bipolar cell, reducing the light sensitivity but enhancing the visual acuity (Kolb, 1995a; VanPutte, 2014).

Horizontal and amacrine cells

The cell bodies of horizontal and amacrine cells are located in the INL with the processes limited to the outer and inner nuclear layers, respectively (Purves et al., 2011).

The processes of horizontal cells modulate the interactions between photoreceptors and bipolar cells that are thought to preserve the contrast sensitivity in the visual system. The processes of amacrine cells are post-synaptic to bipolar cells and pre-synaptic to RGCs and modulate the signal transmission between these cells (VanPutte, 2014). The different sub-types of amacrine cells are involved in contrast, color, brightness and movement (Kaneda, 2013).

Retinal ganglion cells

Located in the inner part of the retina, the RGCs represent the output neurons of the vertebrate retina, receiving information from photoreceptors via two intermediary neuronal types: bipolar cells and amacrine cells (Pascale et al., 2012). Synapses between RGCs and bipolar or amacrine cells occur in the IPL and give rise to a division of RGCs according to their response to light: ON, OFF and ON-OFF RGCs (Wong et al., 2012).

In fact, the mammalian retina comprises approximately 20 types of RGCs, based on the morphology, molecular and functional criteria (Erskine and Herrera, 2014; Wong et al., 2012). The axons of RGCs are assembled, forming the optic nerve that transmits the visual information to the main image forming centers in the brain, such as lateral geniculate nucleus (LGN), the visual part of the thalamus, and the superior colliculus (SC) (Erskine and Herrera, 2014). Each RGC type participates in distinct retinal circuits and projects to specific targets in the brain, being responsible for encoding different aspects of the visual scene. The delineation of the

encoding of each RGC is defined by a combination of synaptic inputs, neurotransmitter and the intrinsic physiological properties of the cell (Wong et al., 2012).

In the human retina, the most common types of RGCs are the parasol cells, also called M cells; and the midget cells, known as P cells; the last ones comprising 80% of the total number of RGCs. These two types of cells have different size and dendritic trees: the M cells are larger, and with more complex dendritic trees and larger axons than P cells (Pascale et al., 2012). Additionally, an atypical population of RGCs expresses the photopigment melanopsin and is intrinsically photosensitive (ipRGCs). Accumulating evidence indicates that ipRGCs consist of several subtypes, which are morphologically and physiologically distinct, and project to several brain nuclei that regulate differently image-forming and non-image-forming visual functions, including regulation of the circadian photo-entrainment melatonin secretion cycle, sleep, masking behavior and pupillary reflex (Nadal-Nicolas et al., 2015; Pickard and Sollars, 2012).

1.1.1.2 Retinal and brain visual pathways

Retinal neuronal cells translate the visual information into nervous impulses, which are transmitted to the brain through the optic nerve. Synapses between photoreceptors, bipolar cells and RGCs form the so-called vertical pathway of retinal transmission, which is modulated by horizontal and amacrine cells, the horizontal pathway (Pascale et al., 2012; Willoughby et al., 2010).

The processing of light in the retina involves three main steps, which constitute the vertical pathway: 1) transduction of light input into electrical signal by the photoreceptors; 2) transmission of electrical signal from photoreceptor to bipolar cells; 3) propagation of the electrical signal from bipolar cells to RGCs, which will be carried from the retina to the brain through the optic nerve (Pascale et al., 2012). There are two parallel vertical pathways: cones and rods pathways. In the cone pathway, cones make direct synapses with bipolar cells (designated cone bipolar cells), which synapse with RGCs. This pathway can be further divided at level of bipolar cells, being light responses of photoreceptors transmitted into ON- or OFF-pathways (Kolb, 1995b). In contrast, rod photoreceptors are presynaptic to only a single morphological type of bipolar cell. Rod bipolar cells contact only two distinct types of amacrine cell, presenting higher sensitivity under scotopic conditions (conditions of very little ambient light) (Bloomfield and Dacheux, 2001).

The optic nerve extends from the eye to the optic chiasm, the point where the optic nerve decussates (Figure 3). From the optic chiasm, RGC axons continue through the optic tract synapse in the LGN, where the retinal input is processed, in order to be transmitted to the visual processing centers in the visual cortex (De Moraes, 2013; Nauhaus and Nielsen, 2014).

Some axons project to the SC, instead of the LGN, which is responsible for coordinating eye and head movements in response to visual stimuli (De Moraes, 2013).
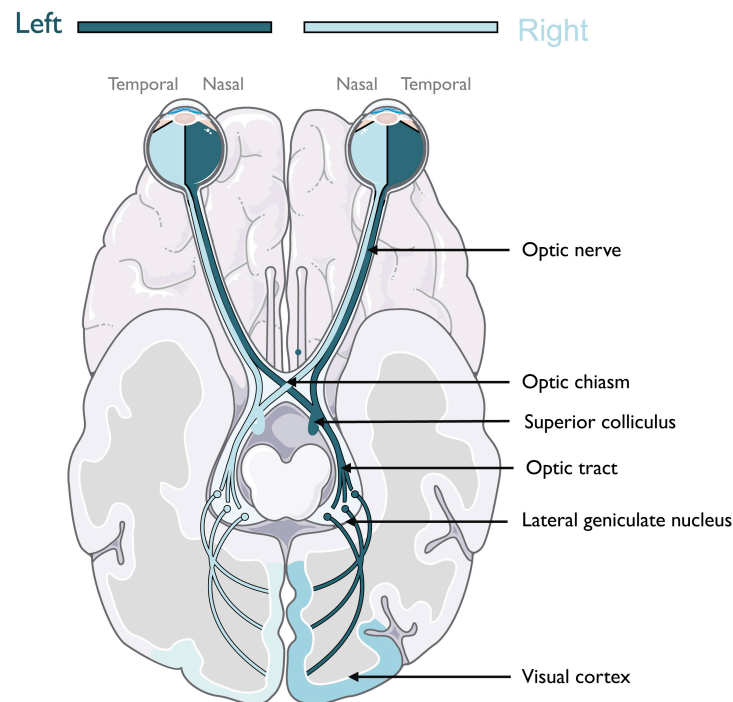
Figure 3 – Schematic representation of the central projections of retinal ganglion cells. Each visual field is divided into a temporal and a nasal half. The axons of RGCs project from the eye to the optic chiasm. Axons from the nasal part of the retina cross and project to the opposite side of the brain. The optic tract consists of axons from both eyes, and will finish in the lateral geniculate nucleus, superior colliculus or visual cortex.

1.1.1.3 Retinal blood vessels

The human retina is the tissue with highest metabolic request, with constant consumption of oxygen and nutrients. This function is fulfilled by the retinal vasculature, which comprises two different sources: the central retinal artery and the choroidal blood vessels (Kur et al., 2012). The central retinal artery enters the retina through the optic and it branches into superior and inferior branches that subdivide into nasal and temporal arteries. These branches of the central retinal artery are responsible for the nourishment of the inner retina. Choroidal blood vessels are responsible to nourish the outer retina (Bek, 2013).

The blood-retinal barrier (BRB) consists of cells that are joined tightly together to prevent certain substances from entering the tissue of the retina. It is crucial in maintaining the so-called eye immune privilege and retinal homeostasis, by limiting the transport of cells and molecules. Generally, the BRB can be divided in two components: the inner BRB and the outer BRB. The inner BRB is formed by tight junctions between endothelial cells from the inner retinal vasculature, pericytes, which are contractile smooth muscle cells that regulate retinal vascular

flow, and glial cells, important in the development and maintenance of the barrier (Cunha-Vaz et al., 2011). The outer BRB is composed of tight junctions between the endothelium of the choriocapillaries, Brunch's membrane and the RPE (Pournaras et al., 2008).

1.1.1.4 Retinal glial cells

The vertebrate retina contains three types of glial cells: Müller cells, astrocytes and microglia. In some species, oligodendrocytes are also present in the retina, associated with myelinated RGC axons (Vecino et al., 2015).

Glial cells are involved in several critical roles in the CNS, such as maintenance of architecture, nutrition and regulation of neuronal communication, as well as regulation of synaptic activity and synaptic pruning (Vecino et al., 2015).

Müller cells

Müller cells constitute the predominant glia in the vertebrate retina, representing 90% of the retinal glia. Müller cells span the entire thickness of the retina and provide architectural support. Retinal sections show an intimate association between Müller cells and retinal neurons, with Müller cells wrapping neuronal cell bodies and processes. This relation promotes the modulatory role of Müller in neuronal activity and homeostasis, by regulation of the ionic concentration and neurotransmitter clearance (Madeira et al., 2015; Vecino et al., 2015). Additionally, these cells are not only involved in the regulation of the synaptic activity in the inner retina but they also contribute to increase photon absorption by cones (Labin et al., 2014; Reichenbach and Bringmann, 2013).

Astrocytes

Astrocytes were named due to its star shaped morphology, with flattened cell bodies and fibrous radiating processes. These cells enter the developing retina from the brain along the developing optic nerve, being confined to the innermost retinal layers, a distribution correlated with retinal blood vessels. Astrocytes actively envelope RGC axons forming axonal and vascular glial sheaths, and are connected to blood vessels of the NFL, being part of the BRB. In fact, as main producers of vascular endothelial growth factor, astrocytes are strongly implicated in the retinal vascularization (Newman, 2015; Vecino et al., 2015). Together with Müller cells, astrocytes have important roles in the maintenance of retinal homeostasis, by providing mechanical and neurotrophic support and contributing to the maintenance of the BRB integrity (Vecino et al., 2015).

Microglia

Microglial cells, the CNS resident immune cells, are constantly surveying the parenchyma and are crucial effectors and regulators of changes in homeostasis during development and in health and disease (Kettenmann et al., 2011).

Microglial cells were first described by Pio del Río-Hortega in 1932, as a unique cell type that differs from other glial and neuronal cells in morphology and constitute approximately 5 to 12% of the cells of the CNS (Ginhoux et al., 2013). These cells are from mesodermal/mesenchymal origin deriving from myeloid progenitors that migrated from the periphery during late embryonic and post-natal life (Chan et al., 2007; Ginhoux and Prinz, 2015). In fact, these cells present many features of circulating monocytes, including immunological signaling cascades, involving chemokines and cytokines (Kettenmann et al., 2011). Taking into account the similarities between microglia and peripheral macrophages, it is reasonable to understand the major challenge that researchers have been facing to distinguish these two cell types. Nevertheless, recent evidence provided by gene expression profile studies suggest that microglia differs considerably from macrophages allowing the identification of unique molecular signature (Butovsky et al., 2014; Gautier et al., 2012).

Although the crucial immune functions of microglial cells have long been recognized, their role in the non-injured brain has now become more apparent and diverse (reviewed in Tremblay et al., 2011). In fact, for decades, it was accepted that microglial cells present a *resting* phenotype, which switches for a reactive state under pathological conditions (Figure 4). In the *resting state* microglial cells are characterized by small cell bodies with elongated ramified processes, presenting a low expression of major histocompatibility complex (MHC) proteins and other antigen-presenting surface receptors (reviewed in Kettenmann et al., 2011; Lull and Block, 2010). Nevertheless, in the last decade it has become clear that microglial cells have an active role in the healthy CNS, being involved in the surveillance of the local environment with their highly motile processes, as well as synaptic homeostasis and synaptic pruning (Paolicelli et al., 2011; Wake et al., 2009). The so-called *resting phenotype* might reflect defined state of an active cell and, therefore, was proposed to be replaced by *surveillance state* (Hanisch and Kettenmann, 2007).

Microglial cells are in intimate contact with other glial and neuronal cells. Neuronal cells express or secret distinct molecules that bind to microglia receptors, which may indicate a threat to the CNS integrity and trigger a transformation of the microglia surveillance state to a reactive state. Neuronal signals can be called OFF and ON, depending on their effect on the immune state of microglial cells. An example of OFF signal is CD200, which expressed by neurons and binds to the CD200 receptor on microglial cells, initiating a cascade that leads to down-regulation of the activation state (Kierdorf and Prinz, 2013; Ransohoff and Perry, 2009).

Another factor involved in the OFF control of microglia reactivity is fractalkine, also called CX3CL1 ligand, which is expressed on different neuronal subsets and binds to the CX3CR1 receptor, present in macrophage-like cells, such as microglial cells. The binding of fractalkine to the microglial CX3CR1 is fundamental in the CNS and it occurs under healthy conditions, as demonstrated in microglial cells deficient in CX3CR1 that present an over-activated phenotype (reviewed in Kierdorf and Prinz, 2013). On the other hand, as ON signals, factors such as chemokines and glutamate can act on microglial cells, inducing them to enter in a reactive phenotype (reviewed in Biber et al., 2007).
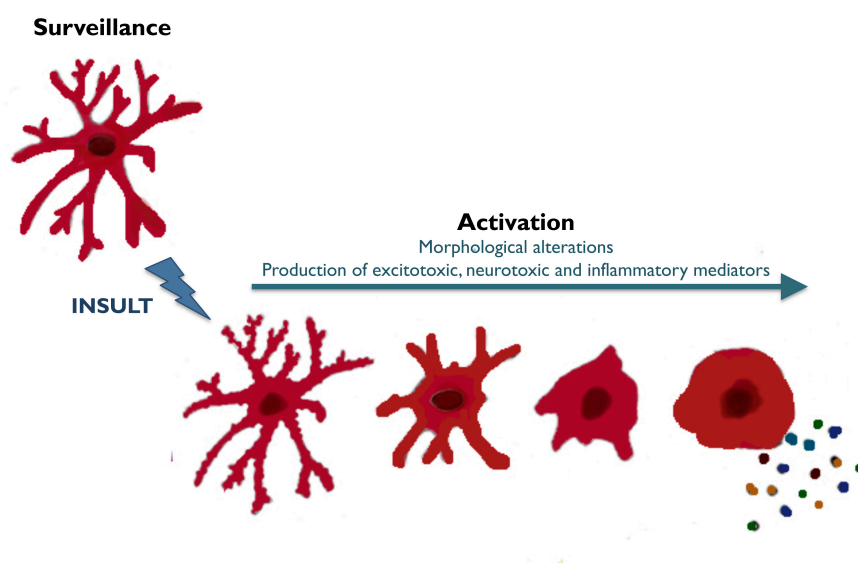
Figure 4 – Microglia activation after injury. Microglial cells are constantly surveying the surrounding environment by extending and retracting their processes. In noxious conditions, microglial cells change their morphology, become more reactive, and increase the production of excitotoxic, neurotoxic and inflammatory mediators.

The microglial reactive state is characterized by an amoeboid morphology, which favors an increase in the phagocytic activity. The morphological alterations are accompanied by changes in the cell signaling and gene expression, with increased expression and release of inflammatory mediators, such as the pro-inflammatory cytokines tumor necrosis factor (TNF) and interkeukin-1β (IL-1β), chemokines, nitric oxide (NO) and reactive oxygen species (ROS) (Lull and Block, 2010).

Similarly to macrophages, the reactive states of microglia were initially classified in two categories: M1 and M2. The M1 is the designation of the classical activation, where oxidative metabolites and proinflammatory cytokines are produced by microglial cells stimulated by a diverse set of ligands (e.g. lipopolysaccharide [LPS]); whereas the M2 alternative activation leads to a reparative, matrix remodeling and anti-inflammatory response (Biber et al., 2014).

Nevertheless, it is now clearly accepted that microglial cell activation is a variable and adaptive process and both M1 and M2 states can convert into different population subsets and develop specific patterns of activity (Jonas et al., 2012).

When studying microglial cell reactivity, one major point is the dichotomy between their contribution to neurodegeneration and neuroprotection. The production of pro-inflammatory mediators, in a properly directed response, is known to be crucial in preventing further damage in the CNS, and promotes the recovery and repair upon damage. Excessive pro-inflammatory microglia activation may lead to neurotoxic effects, which in chronic state could be involved in the onset and progression of neurodegenerative disorders. Therefore, the suppression of the deleterious effects of microglia have emerged as potential targets to prevent neurodegeneration (Czeh et al., 2011; Kawabori and Yenari, 2015; Smith et al., 2012).

1.1.1.5 Retinal microglial cells

Although nowadays there is a vast knowledge about the role of microglial cells in the brain, relatively little is known about their role in retinal homeostasis. In recent years the interest in retinal microglial cells and their contribution to retinal diseases has been boosted (Karlstetter et al., 2010; Madeira et al., 2015). Studies have reported similarities between retinal and brain microglial cells, both presenting immunological functions associated with the so-called *immune privilege* of the CNS (Albini et al., 2005; Chen et al., 2002).

In the retina, precursors of microglial cells emerge during retinal development, prior to vascularization, via the ciliary margin, and differentiate in ramified, quiescent parenchymal microglia in the adult retina (Diaz-Araya et al., 1995). A second population of macrophage-derived precursors invades the retina through the developing vasculature, mainly via optic nerve head (ONH) and differentiates in perivascular microglia, being involved in the constant microglia turnover and replacement (Xu et al., 2007). In the developing retina, microglial cells have been found to be crucial for retinal growth and neurogenesis (Huang et al., 2012). Additionally, undifferentiated microglial cells have also been associated with increased production of NO (Sierra et al., 2014) and promotion of neuronal cell engulfment during retinal development (Ferrer-Martin et al., 2014).

In the adult retina, microglial cells are distributed in the plexiform layers, GCL and nerve fiber layer (NFL), with highly motile protrusions that survey the surrounding environment (Chen et al., 2002; Diaz-Araya et al., 1995; Ellis-Behnke et al., 2013; Hume et al., 1983; Provis et al., 1996). The movement of their processes occurs in all directions, and it is unaccompanied by soma migration (Lee et al., 2008), suggesting that the process dynamics may also serve to exchange signals between neighboring microglia, and may help explaining laminar retinal microglia distribution (Santos et al., 2008). Interestingly, in the adult retina, microglial cells have

different morphologies throughout the different layers. In the NFL, microglial cells are scarce and have a bipolar morphology, with long axis parallel to the course of RGC axons. Multipolar microglial cells, with round or oval cell bodies and some main processes, can be found in the GCL. Microglial cells in the IPL have small round cell bodies with three main branches that are stratified and distributed through the entire retina (Sobrado-Calvo et al., 2007).

The functions of microglia in the physiology of the retina are not fully elucidated yet. Microglial cells are required for normal retinal growth and neurogenesis (Huang et al., 2012) and proper retinal blood vessel formation (Checchin et al., 2006).

Similar to the brain, the activation of retinal microglial cells is commonly accompanied by morphological alterations, from ramified to a more amoeboid shape, with only a few branches (Lee et al., 2008). When activated, microglial cells migrate to the injured site, accumulate in the retinal nuclear layers and sub-retinal space, and increase their phagocytic activity, facilitating the regenerative process (Chen et al., 2002; Karlstetter et al., 2010).

Early microglia activation has been reported in animal models of retinal degeneration (Bosco et al., 2015; Bosco et al., 2011; Rivera et al., 2013; Zeiss and Johnson, 2004), being suggested that these responses are not only bystanders of retinal neurodegeneration, but contribute to the retinal neurodegenerative process (Karlstetter et al., 2015; Madeira et al., 2015). Retinal degenerative diseases, as glaucoma, age-related macular degeneration and diabetic retinopathy, are among the main causes of blindness worldwide (Casson et al., 2012; Ting et al., 2015; Zarbin et al., 2014). These retinal diseases are characterized by chronic neuroinflammation and microglial cells have a key role in the initiation and perpetuation of the inflammatory response. The overactivation of microglia results in excessive production of inflammatory mediators that accumulate to levels, which are harmful to neurons, further contributing to retinal neurodegeneration (reviewed in Madeira et al., 2015).

1.2 Glaucoma

According to the World Health Organization, glaucoma is the second leading cause of blindness in the world, affecting approximately 70 million people worldwide, and nearly 2% of the population over the age of 40. The term glaucoma describes a group of ocular disorders with multifactorial etiology and characterized by clinically visible alterations at the ONH encompassing thinning of the neuroretinal rim and excavation of the optic disc, due to the progressive loss of RGCs and their axons (Casson et al., 2012). Patients with glaucoma may present characteristic changes in the optic nerve and corresponding visual field loss, which progresses slowly and may lead to total vision loss. However, more than 50% of the people affected are unaware of their condition (Mantravadi and Vadhar, 2015).

Several factors are associated with the development and progression of glaucoma, such as family history, systemic hypertension, diabetes and cigarette smoking, but the main risk factors are elevated IOP (above 21.5 mmHg) (Figure 5) and advanced age (Qu et al., 2010). In general, IOP values ranging 30 mmHg lead to a slow glaucomatous damage over several years, while IOP ranging 40 and 50 mmHg may determine a rapid visual loss (Cohen and Pasquale, 2014).
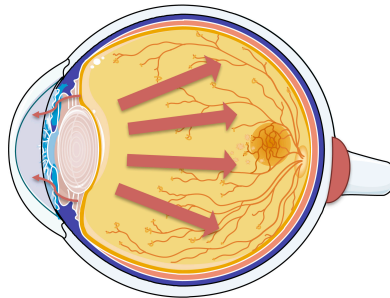
Figure 5 – Schematic representation of increased the effect of elevated IOP in the ocular globe.

Typically, IOP results from a balance between aqueous humor production, by the ciliary bodies, and its drainage by the trabecular meshwork into the Schlemm's canal (and posteriorly through the episcleral veins or the ciliary muscle), or through the uveoscleral pathway (Mantravadi and Vadhar, 2015). Alterations in the aqueous humor outflow result in elevated IOP, which might lead to ONH abnormalities, namely in the lamina cribrosa, affecting the axonal transport in the RGCs or by leading to vascular deficits and ischemic damage (Caprioli et al., 2010).

Until recently, it was considered that elevated IOP played a major role in RGC apoptosis and a relationship between increased IOP and RGC loss in experimental glaucoma was reported, with lowering of the IOP resulting in the slowing down the progression of the degenerative changes (reviewed in Qu et al., 2010). In fact, current therapeutic approach is focused on lowering IOP pharmacologically, surgically or with laser. However, despite efficient IOP control, a vast majority of patients continue to lose vision (Brubaker, 1996). Additionally, only one-third

to half of the glaucomatous patients present elevated IOP in the initial stages of the disease; and 30 to 40% of the patients with glaucomatous visual loss are diagnosed with normal tension glaucoma (NTG). Therefore, the elevation of IOP is recognized as an important risk factor in the pathophysiology of glaucoma, without being the main and unique factor responsible for RGC loss and optic nerve damage (Agarwal et al., 2009).

1.2.1 Classification of glaucoma

Glaucoma is classified according the cause of aqueous humor obstruction is primary or secondary and according the status of the iridocorneal angle (whether open or closed) (Mantravadi and Vadhar, 2015) (Figure 6).

Primary open angle glaucoma (POAG) is the most common form of glaucoma. Clinically, it is possible to detect excavation of the ONH and decreased visual function sensitivity in the mid-peripheral field, which will eventually lead to loss of central and peripheral vision (Quigley, 2011). Due to its huge heterogeneity, at both clinical and molecular levels, the etiology of POAG, still remains to elucidate (Janssen et al., 2013).

Primary angle closure glaucoma (PACG) can be an acute process, with more immediate signs and symptoms than POAG, and may lead to a more destructive subtype (Mantravadi and Vadhar, 2015). It accounts approximately for half the cases of glaucoma worldwide and occurs with acute increase of the IOP due to blockade of the trabecular meshwork, typically by the iris (Yip and Foster, 2006). Vision loss in PACG can be two times faster than with POAG and in acute cases it is considered an ocular emergency, because loss of vision can occur within hours to days (Mantravadi and Vadhar, 2015). The elevation of IOP usually occurs as chronic, asymptomatic disorder, with similar effects to those observed in POAG (Quigley, 1999).

Normal tension glaucoma is a progressive optic neuropathy that mimics POAG, but lacks elevated IOP or other mitigating factors that can lead to optic neuropathy. Patients with NTG have higher incidence of optic disk hemorrhage, earlier decrease of NFL, different shape of the visual field defect, as well as presence of vascular problems or abnormal perfusion (Glaucoma Study Group, 1998). Contrary to the dominant role of IOP in POAG, IOP-independent factors play an important role in the mechanism of NTG. Despite the differences in these two forms of glaucoma, there should be a similar signaling pathway of apoptosis that results in the loss of RGCs (Mi et al., 2014).

Cornea
Anterior chamber
Iris
Trabecular meshwork and Schlemm's canal
Ciliary bodies
Lens

Normal angle

Open-angle glaucoma

Angle-closure glaucoma
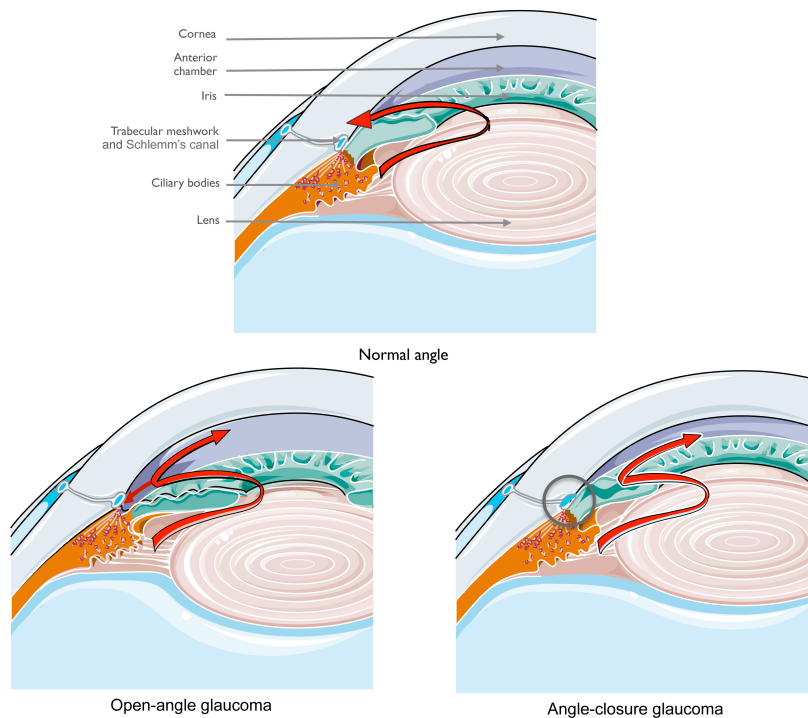
Figure 6 – Aqueous humour flow in healthy and glaucomatous eyes. The aqueous humor that fills the anterior chamber is produced by the ciliary body and flows between the iris and lens, through the pupil and to the drainage angle at the junction of the iris and the cornea. Aqueous fluid exits the eye mainly through trabecular meshwork and into the Schlemm's canal (top image). The secondary route is the uveoscleral drainage. Bottom images represent open angle glaucomatous eye, with reduced drainage of the aqueous humor (left) and angle-closed glaucoma, with structural occlusion of the aqueous humor outflow (right).

1.2.2 Current treatments in glaucoma

Although IOP is no longer part of the definition of glaucoma, it is the only modifiable risk factor, and therefore the primary goal of the currently used therapies is the lowering of IOP. In fact, control of the IOP decreases both the risk of disease onset and its progression, with series of randomized clinical trials showing that lowering IOP can confer protection to the optic nerve and attenuate the visual field loss in glaucomatous patients (reviewed in Chang and Goldberg, 2012). Additionally, lowering of the IOP has been shown to be effective even in patients with NTG, with a 30% reduction in IOP decreasing the long-term risk of progression (Glaucoma Study Group, 1998; Song and Caprioli, 2014).

Five pharmacological classes of drugs are commonly used in glaucomatous patients: cholinergic agents, β-blockers, carbonic anhydrase inhibitors, adrenergic agonists and prostaglandin analogues (Cohen and Pasquale, 2014).

Cholinergic agents facilitate the lowering of IOP by increasing the aqueous humor outflow, while β-blockers and carbonic anhydrase inhibitors decrease the production/secretion of the aqueous humor. Adrenergic agonists are sympathetic drugs that reduce the production of the

aqueous humor and increase the uveoscleral outflow. Still these therapies present several ocular and systemic side effects and limitations (reviewed in Cohen and Pasquale, 2014; Donegan and Lieberman, 2015; Kolko, 2015).

The current gold standard for the treatment of glaucoma is the topical (?) administration of prostaglandin analogues, the single most effective agent in reducing IOP with adequate diurnal control. Prostaglandin analogues have been shown to lower IOP mainly via increasing uveoscleral outflow, along with conventional aqueous humor outflow (reviewed in Donegan and Lieberman, 2015).

Surgical or laser treatments are often required in glaucomatous patients when the pharmacological control of IOP fails. Laser treatments, such as trabeculoplasty, aim to increase the aqueous humor drainage. Other example is laser cyclophotocoagulation, which is intended to decrease aqueous humor inflow, by destroying epithelial cells of the ciliary body (reviewed in Dietlein et al., 2009). IOP lowering therapies present several challenges, such as patient compliance and tolerance with multidrop therapies; as well as the low satisfying rate of success in the surgical approaches (Chang and Goldberg, 2012).

Several studies have reported that despite successful IOP control, in some patients the disease continues to progress, as demonstrated by changes in the optic nerve and visual field (Chang and Goldberg, 2012). Therefore, attention must be given to approaches directed to control RGC loss and degeneration of the optic nerve. Numerous new therapeutic targets are being studied regarding their potential neuroprotective properties; particularly directed to mechanisms involved in the glaucomatous neurodegeneration, such as excitotoxicity, oxidative stress, mitochondrial dysfunction and/or inflammation (Chang and Goldberg, 2012; Kolko, 2015).

1.2.3 Experimental models of glaucoma

A wide variety of *in vitro* and *in vivo* glaucoma models have been developed in order to study cellular and molecular mechanisms involved in the glaucomatous pathology, such as effect of elevated IOP in optic nerve and RGC degeneration, as well as to provide information regarding possible therapeutic approaches. Numerous species have been used as animal models of glaucoma including monkeys, dogs, cats, rodents, and several other species. Though, for various advantages, rodent animals, including mice and rats, have been widely developed as models to study various aspects of glaucoma and to evaluate possible novel therapies (Chen and Zhang, 2015).

1.2.3.1 *In vitro* models of glaucoma

In vitro systems are useful for producing highly controlled experimental conditions, allowing manipulating specific variables contributing for degenerative alterations. *In vitro* and *ex*

vivo glaucoma models have been developed to improve the accuracy and reproducibility of experimental conditions, as well as to investigate pathological mechanisms, especially in the acute phase of the elevation of IOP (Ishikawa et al., 2015; Kretz et al., 2004).

Several *in vitro* models of glaucoma have been described, using cell or tissue cultures, such as RGCs, optic nerve head astrocytes, retinal organotypic cultures. In these models, OHT is mimicked by using pressure loading systems (Beckel et al., 2014; Lei et al., 2011; Ricard et al., 2000; Sappington et al., 2006; Tezel and Wax, 2000; Wax et al., 2000).

Models of elevated hydrostatic pressure have been developed to assess retinal cell response to pressure, using distinct time points and pressure levels, from 15 mmHg to 100 mmHg above atmospheric pressure (Agar et al., 2006; Beckel et al., 2014; Sappington et al., 2006). Elevated hydrostatic pressure (EHP) has been shown to induce remarkable glaucoma-associated alterations, such as RGC apoptosis (Agar et al., 2006; Agar et al., 2000; Beckel et al., 2014; Sappington et al., 2006), increased inflammatory markers (Tezel and Wax, 2000; Wax et al., 2000), mitochondrial dysfunction (Ju et al., 2009) and oxidative stress (Liu et al., 2007). In fact, not only RGCs respond to pressure alterations in vitro; increased hydrostatic pressure has been shown to lead to alterations in the cell structure and migration of ONH astrocytes (Salvador-Silva et al., 2004; Tezel et al., 2001).

1.2.3.2 Animal models of glaucoma

Animal models are one of the most viable tools for researchers to study disease processes, being several times critical in the development of therapeutics and treatments. Namely, in glaucoma, which has a highly complex etiology, animal models that mimic RGC and optic nerve loss are considerably relevant to elucidate the mechanism involved in the disease mechanisms progression and evaluate possible new therapeutic targets (Chen and Zhang, 2015; Struebing and Geisert, 2015).

Generally, animal models of glaucoma are classified in two categories: IOP-dependent or IOP-independent models; being the last ones sub-divided into spontaneous or induced models (Chen and Zhang, 2015; Ishikawa et al., 2015). Owing the complexity the disease, there is not yet an ideal model that completely recapitulates human glaucoma (Chen and Zhang, 2015).

1.2.3.1.1 IOP-dependent glaucoma animal models

1.2.3.1.1.1 Spontaneous IOP-dependent models of glaucoma

A variety of natural-occurring models of glaucoma have been described in different animal species, including dog (beagle) (Gelatt et al., 1977), New Zealand rabbit (Kolker et al., 1963) and the DBA/2J mouse (Anderson et al., 2002; John et al., 1998). Additionally, several transgenic and knockout models have been developed, namely regarding alterations in eye drainage structures (Johnson and Tomarev, 2010).

Regarding rodents, the most well characterized spontaneous model is the DBA/2J inbred line (Chang et al., 1999; John et al., 1997; John et al., 1998; Libby et al., 2005) that develops a pigmentary form of glaucoma, characterized by abnormal iris pigment dispersion in the anterior chamber, which accumulates in the trabecular meshwork with consequent obstruction of aqueous humor outflow and IOP elevation (John et al., 1998). These mice present progressive RGC loss and optic nerve degeneration by the age of 8 to 10 months (Schlamp et al., 2006), retinal function impairment (Perez de Lara et al., 2014) and axonal damage at the ONH, which might be a primary lesion in this model (Jakobs et al., 2005).

Nonetheless, DBA/2J mice show a high degree of individual variability and asymmetry in disease development that limits the application of this mouse model in numerous studies (Schlamp et al., 2006).

1.2.3.1.1.2 Induced IOP-dependent models of glaucoma

The course of spontaneous glaucoma models often presents long periods of experimental manipulation. The development of induced models of glaucoma allows a faster approach and a greater control over the extent of the pathology, offering an ideal model for advancing in the elucidation of the disease pathogenesis and for screening novel therapies (Chen and Zhang, 2015).

Laser photocoagulation of the perilimbar and episcleral veins

One common approach to induce elevated IOP in animal models is by experimentally reducing the aqueous humor outflow by laser photocoagulation (LP) of the limbar tissues, involved in the drainage processes (Agudo-Barriuso et al., 2013). The photocoagulation of the episcleral and perilimbar veins, using diode laser, results in a substantial IOP elevation, which doubles within 12 hours, being maintained significantly elevated for the first week in mice and during 4 weeks in rats (then gradually reducing to the basal IOP levels) (reviewed in Vidal-Sanz et al., 2012). Retrograde axonal transport is impaired in approximately 75% of the RGC population in 8 days, leading to progressive loss of the RGCs (Agudo-Barriuso et al., 2013;

Salinas-Navarro et al., 2010; Vidal-Sanz et al., 2012). These alterations do not progress further between 8 days and 2 months, although it results in damage to the inner and outer nuclear layers of the retina, affecting retinal function and morphology (Salinas-Navarro et al., 2009). In fact, recent work revealed that LP-induced OHT also results in the loss of cone photoreceptors (Ortin-Martinez et al., 2015). Furthermore, increased IOP results in compromised inner retinal blood supply (Flammer et al., 2002).

Unilateral induction of OHT by LP in mice, leads to increased astroglia and microglia reactivity, not only in the eye with OHT, but also in the contralateral eye (de Hoz et al., 2013; Gallego et al., 2012). Additionally, glial reactivity and neuronal loss were also already described in the LGN of monkeys subjected to unilateral chronic OHT induced by LP, suggesting the critical role of glial response in the OHT glaucoma models (Dai et al., 2012; Ito et al., 2011),

LP-induced OHT models, using distinct laser types, such as argon or diode laser, have been used to investigate neuroprotective strategies in glaucoma, as brain-derived neurotrophic factor (BDNF) (Valiente-Soriano et al., 2015), mynocycline (Levkovitch-Verbin et al., 2014) and brimomidine (Lambert et al., 2011). Nevertheless, this model has several limitations, since in some conditions it might require repeated laser treatments, which might induce ocular inflammation and corneal opacity (Ishikawa et al., 2015). Additionally, differences in pigmentation in the trabecular meshwork can result in distinct effects of LP concerning the magnitude of IOP elevation (Ishikawa et al., 2015).

Episcleral vein cauterization (EVC)

Cauterization of three episcleral veins was first described in 1995 (Shareef et al., 1995), and is associated with increased outflow resistance (Ruiz-Ederra and Verkman, 2006; Sawada and Neufeld, 1999). The EVC model was the first model describing the loss of apoptotic RGCs. This model has allowed pharmacological trials of pressure-reducing and neuronal protection drugs (Vecino, 2008). Complications of EVC include thermal damage to the sclera, intraocular inflammation and damage of the ocular surface (Ishikawa et al., 2015; McKinnon et al., 2009), as well as the potential for ocular ischemia and neovascularization (Goldblum and Mittag, 2002).

Hypertonic saline injection

Injection of a hypertonic saline solution to the episcleral veins was described as a method to increase resistance of the aqueous humor outflow (Jia et al., 2000; Morrison et al., 1997). Nevertheless, this procedure is difficult to perform and usually several injections are necessary to accomplish sustained elevated IOP (Ruduzinski and Saragozi, 2005).

Microbead injection model

Obstruction of the trabecular meshwork by intracameral injection of microparticles has a long track record as a method for the generation of experimental glaucoma (Morgan and Tribble, 2015). Weber and Zelenak (2001) have first established the injection of sterile latex microspheres into the anterior chamber of the eye as a simple and cost effective method for inducing chronic elevation of IOP and experimental glaucoma in primates. The "microbead occlusion model" in rats performed by (Sappington et al., 2010), revealed a modest and sustained OHT that triggered axonal loss. Nevertheless, the principal disadvantage of this model is that beads can easily move after injection. To circumvent this problem, more recently, others have described similar procedures using magnetic microbeads, which allow directing the beads specifically to the iridocorneal angle (Bunker et al., 2015; Samsel et al., 2011).

This model is relatively easy to perform and does not require special equipment (Ishikawa et al., 2015). However, a consensus and reproducible protocol has been difficult to achieve. This method has been frequently altered, both in rats and mice, with several groups proposing distinct bead components, different sizes, soluble vehicles and also time and repetition of the injections (Chen et al., 2011; Cone et al., 2010; Matsumoto et al., 2014; Morgan and Tribble, 2015; Rho et al., 2014).

Acute IOP elevation model by ischemia-reperfusion (I-R) injury

Cannulation of the anterior chamber of rat or mouse eyes with a needle allows a precise control of the IOP and suppresses retinal blood supply; the removal of the needle results in the beginning of a reperfusion period (Buchi et al., 1991). Induction of retinal ischemia, for a period between 30 to 120 minutes and followed by reperfusion, causes death of several retinal cells (Buchi et al., 1991; Osborne et al., 2004).

This model involves structural and functional damage in various retinal layers (Grozdanic et al., 2003; Szabo et al., 1991), increased expression of neuroinflammatory markers (Gustavsson et al., 2008) and triggers apoptotic RGC death (Lam et al., 1999). Hence, it can be considered a model of global retinal degeneration rather than a glaucoma model. Still, it has been frequently used to investigate RGC dysfunction and death, and screen potential therapeutic approaches (Abcouwer et al., 2013; Galvao et al., 2015; Li et al., 2014; Martins et al., 2015; Zhang et al., 2015).

1.2.3.1.2 IOP-independent glaucoma animal models

Optic nerve crush and optic nerve transection

When studding RGCs and optic nerve associated pathophysiological alterations in glaucoma it is sometimes desirable to induce a specific insult in the absence of increased IOP. Although the mechanisms in these studies do not completely reflect the human glaucomatous damage, they can contribute to a better comprehension of the RGC neurodegenerative process (Johnson and Tomarev, 2010).

The most commonly models used to study the RGC neurodegenerative process in the absence of IOP elevation are the optic nerve crush and optic nerve transection, both inducing a mechanical injury to the RGC axons and triggering a retrograde Wallerian degenerative response (Schwartz, 2004).

The optic nerve crush model is obtained by optic nerve exposure to a consistent amount of force (using forceps), without interruption of the retinal blood supply. In the case of optic nerve transection, the optic nerve is exposed and transected, also preserving the retinal blood supply (Parrilla-Reverter et al., 2009).

Degeneration of RGCs begins quickly after mechanical damage within the first week (Galindo-Romero et al., 2011; Levkovitch-Verbin et al., 2000), accompanied by disruption of the BRB, and astro- and microgliosis (Frank and Wolburg, 1996).

The advantages of using these models rely on the quick onset of the degenerative process. Although these models are not based on OHT, they play a critical role in the understanding of the mechanisms underlying RGC loss and axonal transport impairment. Still, the procedure is variable, depending mainly on the area and applied force in the local of insult, as well as in the efforts required to maintain the blood supply integrity (Johnson and Tomarev, 2010).

Intraocular injection of excitotoxic agents

Another IOP-independent animal model used to study the pathophysiology of RGC loss is induced by intravitreal administration of excitotoxic agents, which over-activate ionotropic glutamate receptors, leading to an increase in the intracellular calcium (Ca^{2+}) levels and consequent cell dysfunction and death (Johnson and Tomarev, 2010).

Intravitreal injection of N-methyl-D-aspartate (NMDA), glutamate or kainic acid have been frequently used to achieve neuronal loss, namely in the inner layers of the retina (Chidlow and Osborne, 2003; Munemasa et al., 2006; Zhang et al., 2004). The use of this model can provide critical information regarding the roles of different genes and proteins in the RGC death pathways (McKinnon et al., 2009). In addition, over the years, it has been frequently used to investigate therapeutic targets directed to RGC neuroprotection (Galvao et al., 2015; Gomez-

Vicente et al., 2015; Sakamoto et al., 2014; Santos-Carvalho et al., 2013; Schuettauf et al., 2011; Shimazawa et al., 2005).

1.2.4 Neurodegeneration in glaucoma

The mechanisms underlying the initial damage of RGCs in glaucoma are still not completely known. RGCs present distinct functional compartments that can be differently affected by diverse disease stimulus, leading to a "compartmentalized degeneration". This concept of degeneration suggests that whatever the ultimate cause of RGC loss is, the primary event might be the activation of one or more compartmentalized self-destruct programs, being RGC death a secondary event (Whitmore et al., 2005).

Numerous data point to the lamina cribrosa (where the RGC axons exit the eye) as the initial site of damage in glaucoma (Burgoyne et al., 2005; Quigley and Addicks, 1981; Quigley et al., 1980). A main hypothesis has been postulated as the "mechanical damage model", suggesting that increased IOP distorts the laminar region anatomy, resulting in compression or binding of the axons. Notably, in animal models, both anterograde and retrograde transport was shown to be impaired in glaucomatous eyes (reviewed by Burgoyne et al. 2005; Nickells, 2007), leading to decreased transport of several crucial molecules, as neurotrophins (e.g. BDNF) (Pease et al., 2000; Quigley et al., 2000) and motor proteins, as dynein (Martin et al., 2006), which may help explaining the apoptotic death of RGCs (Whitmore et al., 2005).

There are two basic patterns of axonal degeneration, depending on the severity and extent of the lesion. Severely damaged axons, such as after axotomy, undergo in a rapid degeneration along the entire length of the processes, which is called Wallerian degeneration. In less severe insults, the axons undergo a slower degenerative process, named "die-back", which usually begins at the synaptic end and progresses in a retrograde fashion toward the neuronal soma (reviewed in Nickells, 2007; Whitmore et al., 2005).

No clear mechanism is known regarding how IOP elevation causes disruption of the axonal transport or exactly how axon transport defects contribute to axonal damage. However, it is clear that axonal transport deficits precede axonal and optic nerve degeneration in glaucoma, events that occur earlier than RGC loss, as shown in animal models (Buckingham et al., 2008; Galindo-Romero et al., 2011; Jakobs et al., 2005; Salinas-Navarro et al., 2010; Salinas-Navarro et al., 2009; Vidal-Sanz et al., 2012). Importantly, neurotrophic deprivation appears to be a crucial event in the onset of glaucomatous axonal degeneration, affecting signaling cascades in RGCs, with numerous studies showing that replacement of neurotrophins in animal model of RGC loss attenuates the cell death process (Galindo-Romero et al., 2013; Harper et al., 2011; Pease et al., 2009; Sanchez-Migallon et al., 2011).

Pressure-induced RGC axonal insults also include microvascular insufficiency or I-R injury (Osborne et al., 2001). Ischemia, associated with reduced energy stores, can negatively affect axonal ionic balance, leading to increased levels of intracellular Ca^{2+}, which stimulates axonal degeneration (reviewed in Whitmore et al., 2005). Additionally, ischemia leads to an unbalanced metabolic demand and production of reactive oxygen species (ROS), contributing to oxidative stress (Osborne et al., 2004).

Increases in the axonal Ca^{2+} concentration or redistribution of calcium storage are an early event and critical feature of axonal degeneration, inducing membrane depolarization, microtubule disassembly and mitochondrial deregulation, including release of cytochrome c, cell swelling and energetic failure. These features contribute to the activation of the caspase pathway and consequently to the activation of autophagic pathways or the apoptotic cell death process (Nickells et al., 2012; Whitmore et al., 2005). Indeed, several other cell death mechanisms, caspase dependent or independent, have been shown to be involved in the RGCs fate in glaucoma, namely inflammatory pathways, contributing to the pathogenesis of glaucoma (Wax and Tezel, 2009).

The contribution of microglia-mediated neuroinflammatory pathways to the neurodegenerative process of glaucoma has gain increased attention in recent years (Soto and Howell, 2014). Early neuroinflammatory responses by astrocytes, microglia, and other blood-derived immune cells are observed in the ONH (Howell et al., 2013) and in the GCL (Bosco et al., 2015; Bosco et al., 2011; Naskar et al., 2002), suggesting a primary role of inflammation in glaucoma. In fact, previous studies have shown that control of microglia activation is able to prevent the neurodegenerative process, supporting the contribution of microglia reactivity to the onset of glaucoma (Bosco et al., 2012; Bosco et al., 2008).

Moreover, it is important to notice that, in addition to RGCs degeneration, dysfunction of other retinal neuronal cell was already documented in glaucomatous animal models, particularly photoreceptors (Fernandez-Sanchez et al., 2014; Ortin-Martinez et al., 2015; Pelzel et al., 2006).

1.2.5 Neuroprotection in glaucoma

The identification of alternative therapeutic approaches, independent of the IOP lowering therapies, is a highly pursued ambition, due to the limited effects of IOP reduction in the prevention of RGC loss. Glaucoma is a disease with a complex etiology and multiple mechanisms involved in the initiation of the process of RGC loss, such as excitoxicity, neutrophic withdrawal, mitochondrial dysfunction and inflammation. All these mechanisms culminate in the activation of apoptotic pathways, leading to the loss of RGCs, and therefore become interesting targets for neuroprotection (reviewed in Baltmr et al., 2010; Chang and Goldberg, 2012).

Brimonidine is a Food and Drug Administration (FDA, USA) approved drug for glaucoma, targeted for lowering IOP. Interestingly, studies in OHT animals have shown neuroprotective effects of systemic administration of this drug, potential by up-regulating the levels of BDNF, modulation of the release of glutamate or function of the NMDA receptor (Galanopoulos and Goldberg, 2009; Hernandez et al., 2008; WoldeMussie et al., 2001) In fact, blocking glutamate excitotoxicity has been one of the most discussed approached. For instance, memantine, an NMDA receptor antagonist and the first neuroprotective drug approved for AD, has shown protective effects against RGC loss in animal models of glaucoma (Hare and Wheeler, 2009; WoldeMussie et al., 2002). However, it failed in clinical trials since although patients receiving memantine showed lower progression of the disease, there was no significant benefit when compared with patients receiving the placebo (Vasudevan et al., 2011).

New gene and cell therapeutics encoding neurotrophic factors are emerging for both neuroprotection and regenerative treatments for retinal diseases (reviewed in Nafissi and Foldvari, 2015). Replacement of neurotrophic factors has been shown to have the ability to promote the survival of RGCs (Galindo-Romero et al., 2013; Roubeix et al., 2015; Xiao and Zhang, 2010).

Evidence suggested that the adenosinergic system has potential to be targeted in the treatment of glaucoma. Studies have shown that adenosine receptors might be involved in the control of IOP (reviewed in Zhong et al., 2013). In addition, a recent work have shown the potential of the activation of the adenosine A_3 receptor (A_3R) in the protection of RGCs against damage induced by I-R and partial optic nerve transection models (Galvao et al., 2015). In the brain, modulation of the activity of adenosine A_1 and A_{2A} receptors has been shown to exert neuroprotective functions, which might also be transposed to retinal neurodegenerative diseases (Cunha, 2005; Santiago et al., 2014).

1.2.6 Neuroinflammation in glaucoma

Neuroinflammation has been recognized as playing an important role in the pathogenesis of glaucoma. Increased levels of inflammatory mediators, such as TNF (Balaiya et al., 2011; Tezel et al., 2004; Yuan and Neufeld, 2000; Yuan and Neufeld, 2001), IL-6 (Chen et al., 1999b; Chidlow et al., 2012; Cvenkel et al., 2010; Sappington and Calkins, 2008), IL-9, IL-10, IL-12 (Chua et al., 2012) and NO (Cho et al., 2011; Neufeld et al., 2002) are found in the retina and aqueous humor of patients and of experimental models. In fact, recent work, using the DBA/2J mice, has shown an early dysregulation of cytokine signaling, not only in the retina but also in distal retinal targets, such as SC, even prior to elevation of IOP (Wilson et al., 2015).

Although the specific triggers for inflammatory responses in glaucoma remain poorly defined, inflammatory processes, mediated in part by astrocytes and resident microglia, clearly

play a crucial role in glaucoma (Soto and Howell, 2014). Evidence from both human glaucoma and animal models of glaucoma suggests that immune responses are mediated, at least in part, by toll-like receptors (TLRs), as TLR2, TLR3, and TLR4 are up-regulated (Luo et al., 2010). Additionally, proteomic analysis of animal models and donor eyes with glaucoma revealed increased expression of kinases involved in the activation of the NF-κB pathway, resulting in the production of inflammatory cytokines (Yang et al., 2011).

TNF has been implicated as a mediator of RGC death in glaucomatous retina (Tezel et al., 2001; Yuan and Neufeld, 2000). Production and release of TNF increase following elevated IOP or ischemia, suggesting TNF as an attractive therapeutic target. Indeed, the use of a neutralizing antibody against TNF attenuated the apoptotic process of RGC (Tezel and Wax, 2000). Moreover, Etanercept (Enbrel®), a widely used TNF antagonist, attenuates inflammation and RGC loss in a glaucoma animal model (Roh et al., 2012). Recent studies have demonstrated that in an animal model of OHT, TNF stimulates the expression of Ca^{2+}-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, modulating RGC death (Cueva Vargas et al., 2015).

In I-R injury, IL-1β plays an important role in mediating ischemic and excitotoxic damage in the retina (Yoneda et al., 2001). Moreover, studies have shown that polymorphisms in IL-1β gene might contribute to the increased risk of POAG, but not to its progression (Markiewicz et al., 2013; Markiewicz et al., 2015).

Inducible nitric oxide synthase (iNOS) is usually up-regulated by inflammatory mediators producing large amounts of NO (Hannibal, 2015). Up-regulated iNOS and increased NO levels were found in the ONH of glaucomatous patients (Liu and Neufeld, 2000) and in the retina and ONH of glaucoma animal models (Cho et al., 2011; Vidal et al., 2006). Inhibition of iNOS with aminoguanidine confers neuroprotection to RGCs in an animal model of glaucoma (Neufeld et al., 1999), supporting a role of NO in the pathophysiology of glaucoma.

Interleukin-6 (IL-6) has been proposed has a key component of pressure-induced responses by retinal microglia (Sappington et al., 2006). In genetic animal models of glaucoma alterations in the expression of IL-6 and IL-6 receptors have been detected (Sims et al., 2012). Similarly, in the aqueous humor of patients with neovascular glaucoma, the levels of IL-6 increase spatial and temporarily correlated with the grade of neovascularization of the patient (Chen et al., 1999b). Nevertheless, IL-6 increases the survival of RGCs challenged with pressure, suggesting that it may be an attempt to regenerate RGC axons (Chidlow et al., 2012; Sappington et al., 2006).

Specific changes in autoantibody profiles have been described in glaucomatous patients and animal models (Joachim et al., 2008; Reichelt et al., 2008), which are associated with antibody depositions in the serum and aqueous humor of glaucomatous patients, and increased

microglial cell activation in the retina of experimental models (Joachim et al., 2012). These changes have been linked with the inflammatory process that precedes RGC degeneration and clearance of cell debris (Joachim et al., 2014).

1.2.7 Glial cells in glaucoma

Extrinsic signals from the microenvironment are critically important for the neuronal fate during glaucomatous neurodegeneration. The progressive degeneration of the optic nerve axons and RGCs is accompanied by chronic structural and functional alterations in glial cells, associated with inflammatory responses, which initiate aiming to restore tissue homeostasis. Nevertheless, failure in the control of the glial-mediated immune response might help to propagate neuronal injury (reviewed in Tezel, 2013).

Besides the compartmentalized degeneration that occurs in RGCs, also the inflammatory response might differ between compartments, mediating the immune response in unique distinct ways in axons and in the retina (reviewed in Mac Nair and Nickells, 2015).

1.2.7.1 Müller cells in glaucoma

Müller cells are critical for a healthy retinal environment, being involved in the retinal metabolism of ions, glucose and neurotransmitters (Bringmann et al., 2006). Reactive Müller cells are commonly characterized by increased expression of the intermediate filament glial fribrillary acid protein (GFAP), and activation of signaling mechanisms. These mechanisms might be primarily protective for retinal neurons, but in chronic conditions may proceed to uncontrolled neuronal damage (Seitz et al., 2013).

Increased expression of GFAP in Müller cells was observed in post-mortem retinas from human glaucomatous patients (Tezel et al., 2003). Similar observations have been made in spontaneous or induced animal models of glaucoma (Gallego et al., 2012; Inman and Horner, 2007; Wang et al., 2000). In fact, in DBA/2J mice, a relationship between GFAP expression and increased IOP was observed (Inman and Horner, 2007). Nevertheless, in animals with OHT induced by EVC, glial activation lasted even after the IOP normalization, excluding a direct correlation of this process with IOP elevation (Kanamori et al., 2005).

Several evidences suggest that Müller cells react to RGC injury by either increasing the expression of neuroprotective molecules (Honjo et al., 2000; Kirsch et al., 2010; Sarup et al., 2004), either by increasing the expression of detrimental factors, such as NO and TNF (Chen et al., 2013a; Lebrun-Julien et al., 2009).

1.2.7.2 Astrocytes in glaucoma

Astrocytes become reactive with any injury affecting the optic nerve (Mac Nair and Nickells, 2015). Optic nerve axotomy, in mice, has been shown to lead to axonal swelling and astrocyte degeneration (Fitzgerald et al., 2010; Qu and Jakobs, 2013), accompanied by strong and rapid alterations in the expression of inflammatory genes, and a decrease in astrocyte markers (Qu and Jakobs, 2013). Still, it is important to note that the structure of the ONH in mice differs from the humans, as it lacks the lamina cribrosa; which may trigger distinct astrocytic responses between mice and glaucoma patients (Mac Nair and Nickells, 2015).

In both, animal models and glaucomatous patients, astrocytes become reactive as RGC cell bodies begin to deteriorate (Inman and Horner, 2007; Tezel et al., 2003), being involved in the up-regulation of neurotrophic factors and inflammatory mediators (Yang et al., 2015). Nevertheless, the role of astrocytic activation in RGC survival remains poorly understood (Mac Nair and Nickells, 2015).

1.2.7.3 Microglia in glaucoma

Microglial cells are considered to have a key role in the inflammatory environment in glaucomatous conditions. Several studies focusing on the role of microglial cells in glaucoma have shown that these cells have alterations in morphology, gene expression, cell proliferation, cell adhesion and immune response, compatible with a reactive phenotype (Ebneter et al., 2010; Neufeld et al., 1999; Taylor et al., 2011; Tezel and Fourth, 2009). In fact, growing evidence demonstrates that the interactions between RGCs and glia are critically important for glaucomatous neurodegeneration (Pascale et al., 2012; Tezel and Fourth, 2009; Vohra et al., 2013) (Figure 7).

In glaucomatous human eyes, studies have demonstrated that microglia redistributes and becomes reactive in the ONH and retina (Neufeld, 1999; Yuan and Neufeld, 2001). In the eyes from glaucomatous patients, microglial cells are more amoeboid, clustering in the lamina cribrosa and surrounding blood vessels, a process that was suggested to be related with a protective role against damage to the BRB (Neufeld, 1999). This observed microglial activation is accompanied by increased expression of several pro-inflammatory responses, such as TNF (Neufeld, 1999; Yang et al., 2011) and iNOS (Liu and Neufeld, 2000).

Abnormal microglia reactivity and distribution have also been observed in the retina of animal models of RGC degeneration, as OHT (Ebneter et al., 2010), optic nerve axotomy model (Schuetz and Thanos, 2004; Thanos, 1991; Zhang and Tso, 2003) and retinal ischemia (Cho et al., 2011; Zhang et al., 2005), suggesting that microglial cells become reactive secondary to RGC degeneration. Additionally, in glaucomatous animal models, increased expression of MHC-II and CD200 are early detected in the retina, namely adjacent to the optic nerve, suggesting this

process accompanies ongoing axonal degeneration (de Hoz et al., 2013; Ebneter et al., 2010; Gallego et al., 2012; Naskar et al., 2002; Rojas et al., 2014; Taylor et al., 2011).

Activation of death cascades
Inactivation of pro-survival pathways
Impaired axonal transport
Dendritic and axonal retraction

Microglial activation

Glutamate

ROS

NO   Chemokines

IL-1β

IL-6

TNF

Neuronal death

Neurotoxicity
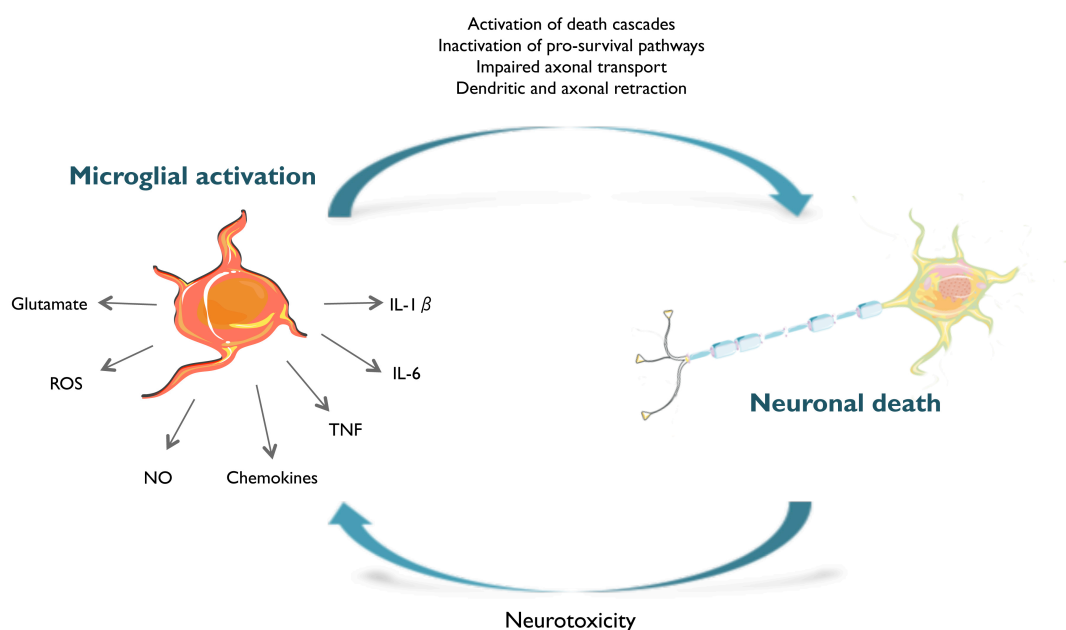
Figure 7 – Relationships between microglia activation and neuronal cell death. In response to changes in the environment, microglia change to a more reactive phenotype, characterized by alterations in cell morphology, gene expression and pro-inflammatory mediators release. The sustained release of inflammatory factors perpetuates the neuroinflammatory process further activating microglia, contributing to neuronal dysfunction and to pathology (adapted from Madeira et al., 2015).

Direct evidence of the contribution of microglia to the loss of RGCs in glaucoma was provided by the observations that microglial cells proliferate in the vicinity of RGCs (Inman and Horner, 2007) and that the recruitment and activation of microglial cells occur before RGC death (Bosco et al., 2015; Bosco et al., 2011). Additionally, minocycline, a tetracycline derivative known to inhibit microglia activation, suppresses RGC degeneration in ischemia and glaucoma models (Abcouwer et al., 2013; Levkovitch-Verbin et al., 2014) and improves the integrity of the optic nerve (Bosco et al., 2008), further supporting a role for microglia in glaucomatous neuropathy. *In vivo* monitorization of microglial cell alterations has been suggested to predict the severity of the neurodegenerative process in DBA/2J mice (Bosco et al., 2015). Moreover, a high-dose of irradiation has been shown to reduce microglia reactivity and proliferation in the central retina and in the ONH region of animal models of glaucoma (Bosco et al., 2012). The reduction of microglia reactivity is associated with a decrease in RGC degeneration and an improvement of the structural and functional integrity of RGC axons (Bosco et al., 2012).

Reactive microglial cells are also observed in all retinal layers of eyes contralateral to experimental glaucoma, although with different morphology, suggesting an attempt for maintenance of tissue homeostasis, protecting axons of the non-injured eye (de Hoz et al., 2013; Gallego et al., 2012; Rojas et al., 2014).

Microglia reactivity in glaucoma is not confined to the retina. Increased microglia reactivity following OHT is also apparent in the optic nerve and optic tract (Ebneter et al., 2010). Activated microglial cells in the LGN, the primary processing center for visual information received from the retina, have also been observed in glaucomatous monkeys (Imamura et al., 2009; Shimazawa et al., 2012), and it can be correlated with neuronal degeneration in the LGN (Gupta et al., 2006; Gupta and Yucel, 2007; Ito et al., 2009; Shimazawa et al., 2012).

1.3 Adenosine

Adenosine is a naturally occurring purine nucleoside, which is ubiquitously distributed through the body as a metabolic intermediary. In the CNS, adenosine is a neuromodulator that acts as a homeostatic factor, controlling the neuronal excitability and neurotransmitter release, as well as modulating synaptic activity and functions (Chen et al., 2014). It is also involved in key pathways of primary metabolism, namely, nucleotide, nucleoside and amino acid metabolism, (Cunha, 2005).
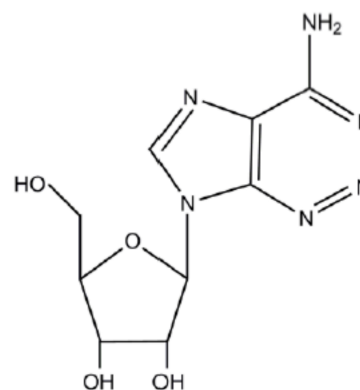
Figure 8 – Molecular structure of adenosine.

In physiological conditions the extracellular levels of adenosine are 30-300 nM, but in noxious conditions, the levels of adenosine rise to 10 μM or higher (Schulte and Fredholm, 2003), being able to modulate the release of excitatory mediators, limit Ca^{2+} influx, hyperpolarize neurons and to exert modulatory effects in glial cells (Rebola et al., 2005).

In fact, adenosine does not act as a classical neurotransmitter, since it is not enriched or stored in vesicles nor released in response to an action potential. Instead, there are two main sources of extracellular adenosine: direct release from the intracellular space and extracellular conversion from adenine nucleotides (such as ATP, ADP and AMP) by a specific cascade of ectonucleotidases, namely CD39 and CD73 (Hasko et al., 2005). Equilibrative nucleoside transporters, which follow the concentration gradient, and concentrative nucleoside transporters that are dependent on the Na^+ gradient, regulate the extracellular concentration of adenosine. Intracellularly, adenosine is formed by an intracellular 5'-nucleotidase (CD73) or through the hydrolysis of S-adenosylhomocysteine (SAH). Additionally, adenosine can also be removed from intra- and extracellular environment, being metabolized into AMP by adenosine kinase, or deaminated into inosine by adenosine deaminase (Boison, 2008; Hasko et al., 2005).

1.3.1 Adenosine receptors

Cellular responses to extracellular adenosine are coordinated by four different G-coupled pleotropic receptors: A_1, A_{2A}, A_{2B} and A_3. These adenosine receptors have unique distribution, pharmacological properties, different G proteins and distinct signaling pathways (Chen et al., 2014) (Figure 9). A_1 and A_3 receptors inhibit the production of cyclic AMP (cAMP), via G_i protein signaling, while A_{2A} and A_{2B} receptors increase intracellular concentrations of cAMP by coupling to G_s proteins (Fredholm et al., 2011). Additionally, these receptors have been implicated in the adenylate cyclase activity, stimulation of phosphoinositide metabolism and modulation of the potassium (K^+) and Ca^{2+} conductance (Abbracchio and Burnstock, 1998). Adenosine is

approximately equipotent on A_1, A_{2A} and A_3 receptors, whereas A_{2B} receptors require higher concentration of adenosine (Fredholm et al., 2011).
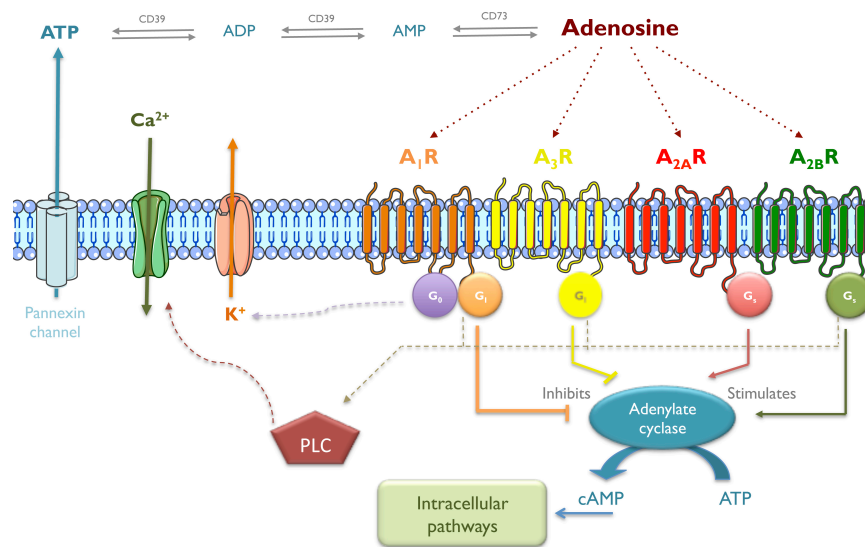
Figure 9 – **Adenosine pathways.** Extracellular degradation of ATP, through a cascade of ectonucleotidades (mainly CD39 and CD37), results in adenosine that will act on four types of G-coupled receptors: the inhibitory A_1R and A_3R and the facilitatory A_{2A}R and A_{2B}R. Acting on these receptors, adenosine can modulate the activity of adenylate cyclase, regulating the levels of cAMP, and consequently several intracellular pathways, being also involved in the regulation of the Ca^{2+} and K^+ transport.

Adenosine receptors have been identified in all CNS regions, including the retina, although with distinct density and distribution (Boison, 2008; Dos Santos-Rodrigues et al., 2015; Vindeirinho et al., 2013). All four receptors were reported to be expressed in macro- and microglial cells (Bjorklund et al., 2008; Boison et al., 2010; Dare et al., 2007; Hammarberg et al., 2003). Adenosine receptors have been implicated in several biological functions, including motor activity, sleep regulation, cognition as well as inflammatory response and in neurodegenerative diseases (reviewed in Boison, 2008).

1.3.1.1 Adenosine A_1 receptors

The adenosine A_1 receptor (A_1R) was the first subtype identified, being widely distributed in the CNS and peripheral tissues, with the highest levels observed in cortical neurons, hippocampus and cerebellum (Dixon et al., 1996; Schenone et al., 2010). In the retina, A_1Rs are mainly localized in the IPL and GCL (Kvanta et al., 1997).

This receptor couples with G_i-protein, leading to the inhibition of adenylate cyclase and activation of K^+ channels. As consequence, it renders the postsynaptic cells less excitable and leads to inhibition of Ca^{2+} channels, decreasing the release of excitatory neurotransmitters such

as glutamate, acethylcoline and dopamine (Cunha, 2005; Schenone et al., 2010). In fact, A_1R activation is linked to various kinase pathways including protein kinase C (PKC), phosphoinositide 3 (PI3) kinase and mitogen-activated protein (MAP) kinases (Hasko et al., 2005).

A_1R activation plays critical functions under pathophysiological environments, such as hypoxia. Under these conditions, the increase in adenosine levels is associated with several sorts of stress or brain injury, and the activation of A_1R appears as an endogenous neuroprotective agent, aimed at limiting the release of excitotoxic neurotransmitters, reducing the damaging effects (reviewed in Schenone et al., 2010). The use of A_1R agonists have been considered in some neurological diseases, such as chronic pain (McGaraughty and Jarvis, 2006) and Huntington's disease (HD) (Ferrante et al., 2014). However, chronic administration of such drugs is ineffective probably because of functional desensitization of the receptors, which might be associated with a reverse effect (Cunha, 2005).

1.3.1.2 Adenosine A_{2A} receptors

Although less expressed than A_1R, in the last decade increased relevance has been given to the adenosine A_{2A} receptor ($A_{2A}R$) (Sebastiao et al., 2012). This excitatory receptor couples to G_s-protein, increasing the levels of cAMP through the stimulation of adenylate cyclase, leading to downstream activation of activation of protein kinase A (PKA), followed by the phosphorylation and consequent activation of cAMP responsive binding protein (CREB), an important transcription mediator of several neuronal functions (reviewed in Greer and Greenberg, 2008; Trincavelli et al., 2010). Activation of $A_{2A}R$ also results in the activation of extracellular signaling kinase (ERK) cascade through a number of different mechanisms, which vary between cell types (reviewed in Milne and Palmer, 2011). In fact, $A_{2A}R$ signaling pathways depend on the cell types and tissue where the receptor is localized, by the specific G protein involved, and the kinases present in the cells (Gomez and Sitkovsky, 2003) (Figure 10).

$A_{2A}R$ has been found highly distributed through the body, with distinct expression levels depending on the tissue and cell type. $A_{2A}R$ can be found in the spleen, thymus, cardiovascular and gastrointestinal tissues, being highly involved in vasodilatiation and protection against ischemic damage (Fredholm et al., 2003).
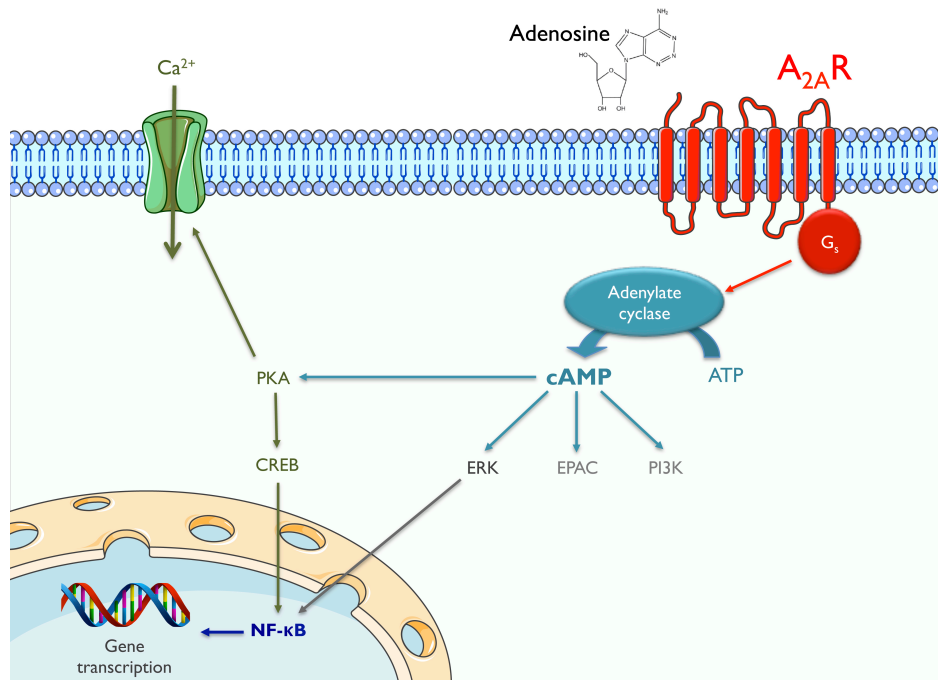
Figure 10 – Simplified overview of the A$_{2A}$R signaling pathway. Activation of A$_{2A}$R results in increased adenylate cyclase activity and cAMP levels, with consequent activation of several transcription factors via ERK pathway and/or CREB, also regulating the intracellular Ca^{2+} concentration.

In the CNS, A$_{2A}$R were initially identified in postsynaptic neurons in the striatum being implicated in the regulation of motor functions (Bruns et al., 1986; Ferre et al., 1991). A$_{2A}$Rs can also be detected in other brain regions as the nucleus accumbens and olfactory bulb, namely in striatopallidal GABAergic neurons and cholinergic interneurons. Additionally, A$_{2A}$R can be detected at lower levels in neurons and microglial cells in other brain regions outside of striatum, including hippocampus and cerebral cortex (Dixon et al., 1996; Svenningsson et al., 1997). In the retina, A$_{2A}$R transcripts were mainly detected in the INL, with a lesser extent in the GCL and ONL, being expressed in retinal microglial cells (Kvanta et al., 1997; Liou et al., 2008).

Evidences indicate that A$_{2A}$R density strongly increases in patients and animal models of several brain neurodegenerative diseases, including Parkinson's disease (PD) and HD (Calon et al., 2004; Popoli et al., 2007; Tarditi et al., 2006). Numerous studies have pointed the potential neuroprotective effects of A$_{2A}$R antagonism (Cunha, 2005; Santiago et al., 2014), with A$_{2A}$R activation being involved in several signaling pathways critical for neuroinflammatory response (Rebola et al., 2011) and neurotrophic modulation (Gomes et al., 2013; Jeronimo-Santos et al., 2014; Vaz et al., 2015).

Notably, A$_{2A}$R activation presents distinct effects in the periphery and CNS. In the periphery, activation of A$_{2A}$R signaling was shown to suppress inflammation (Sitkovsky, 2003), attenuates pulmonary ischemic injury (LaPar et al., 2011; Reece et al., 2005a) and improves

cardiac dysfunction (Reece et al., 2005b). Contrarily, in the CNS it was the blockade of $A_{2A}R$ that has been showing beneficial effects after transient ischemia (Melani et al., 2015), control neurotransmitter release (Gomes et al., 2013) and reduce the neuroinflammatory response (Rebola et al., 2011). This dual role of the modulation of $A_{2A}R$ might reflect the complexity of actions in distinct cell types present in the CNS, such as neuronal and glial cells, which may lead to distinct effects upon CNS injury (Chen et al., 2013b; Cunha, 2005).

1.3.1.3 Adenosine A_{2B} receptors

The adenosine A_{2B} receptor ($A_{2B}R$) is a low affinity receptor able to activate second messenger systems during limited oxygen availability, being mainly activated under pathological conditions. This receptor couples to adenylate cyclase trough G_s-proteins, acting on PLC, the most important pathway responsible for $A_{2B}R$ effects (Ortore and Martinelli, 2010).

The $A_{2B}R$ has been found in several organs, including spleen, lung, colon and kidney, being the vasculature the primary site of expression in all of these tissues (Ortore and Martinelli, 2010; Yang et al., 2006). $A_{2B}R$ was shown to modulate glucose homeostasis and obesity (Johnston-Cox et al., 2012), being involved in cell metabolism in diabetes, which prompts it as a novel promising therapeutic target for the treatment of diabetes (Merighi et al., 2015).

In the brain, studies have shown that $A_{2B}R$ is associated with an instant and tonic increase of glucose transport into neurons and astrocytes (Lemos et al., 2015), being also involved in control of the A_1R-mediated synaptic transmission in the hippocampus (Goncalves et al., 2015).

1.3.1.4 Adenosine A_3 receptors

The adenosine A_3 receptor (A_3R) is the less well-studied receptor, being highly expressed in the lung and liver, with relatively low levels and distributed expression in the brain, namely in the thalamus and hypothalamus (Stone et al., 2009). It is considered that A_3R is expressed in neuronal and glial cells in most species, including humans (Trincavelli et al., 2010), and recent findings have shown their functional presence in microglial cells (Hammarberg et al., 2003). In the retina, A_3R is expressed in RGCs (Zhang et al., 2006).

Classically, A_3R couples to G_i-protein, inhibiting the adenylate cyclase and the G_q-protein that stimulates PLC, inositol triphosphate and the Ca^{2+} uptake (Baraldi et al., 2000). A_3Rs are activated under specific noxious conditions since its activity is mediated by local high adenosine concentrations due to the low affinity of this receptor to adenosine. Furthermore, the A_3R is thought to easily desensitize upon stimulus (Baraldi et al., 2000).

The effects of A_3R activation in several diseases are often controversial, depending on acute and chronic agonist administration. For instance, studies have hypothesized that the initial A_3R activation plays a protective role in ischemia, whereas prolonged A_3R stimulation leads to

deleterious effects, as excitotoxicity (Borea et al., 2015) and oligodendrocyte death (Gonzalez-Fernandez et al., 2014). Nevertheless, it has been reported that models A_3R activation plays a protective role in RGCs (Fishman et al., 2013; Galvao et al., 2015; Zhang et al., 2010).

1.3.2 Neuroprotection mediated by $A_{2A}R$ blockade

Neuromodulatory effects mediated by adenosine rely on a balanced activation of inhibitory A_1R and excitatory $A_{2A}R$, mostly by control of excitatory synapses: A_1Rs impose a tonic pause on excitatory transmission, whereas $A_{2A}Rs$ promote synaptic plasticity (Gomes et al., 2011).

Evidences have supported the hypothesis that the increased release of adenosine during insult activates A_1R and plays a neuroprotective role during hypoxia (Leshem-Lev et al., 2010), transient or global brain ischemia (Miller and Hsu, 1992), excitotoxicity induced by kainate or quinolinic acid (MacGregor et al., 1997) or against dopaminergic neurotoxicity (Delle Donne and Sonsalla, 1994). Therefore acute administration of A_1R agonist or use of strategies aimed to enhance the extracellular levels of adenosine have been seen as a neuroprotective strategies against different types of insults both in *in vivo* and *in vitro* models (Cunha, 2005). Nevertheless, as discussed previously, the A_1R is prone to a rapid desensitization, which limits the time-lapse of action of possible neuroprotective therapies. Hence, A_1R activation is only effective if in the temporal vicinity of the CNS insults. In contrast, blockade of $A_{2A}R$ is able to reduce long-term effects of insults in CNS disorders in different neurodegenerative conditions (reviewed by Gomes et al., 2011; Santiago et al., 2014).

Neuroprotection by an $A_{2A}R$ antagonist was first described in the gerbil brain by reduction of the ischemic injury (Gao and Phillis, 1994). After that, several studies have shown protective effects of the pharmacological blockade of $A_{2A}R$ against excitotoxic insults, such as kainate (Jones et al., 1998), glutamate (Pintor et al., 2004) and quinolinic acid (Scattoni et al., 2007), and also mitochondrial toxins (Blum et al., 2003; Fink et al., 2004).

$A_{2A}R$ antagonism is able to rescue locomotor impairment (Aoyama et al., 2000) and reverse inflammatory processes (Gyoneva et al., 2014) in PD models, being currently proposed as novel therapeutic approach (Jenner, 2014). Moreover, in HD models, inactivation of $A_{2A}R$ has been recently shown to reverse early working memory deficits (Li et al., 2015).

An alternative strategy used to complement pharmacological approached is demonstrating the neuroprotective role of $A_{2A}R$ blockade is the use of mice lacking this receptor (Morelli et al., 2010). In general, $A_{2A}R$ knockout mice are protected from brain damage induced by ischemia or excitotoxins (Chen et al., 1999a; Gui et al., 2009; Li et al., 2009).

The mechanism by which $A_{2A}R$ is able to impact neurodegeneration remains to be defined. Two leading hypotheses have been explored in the past years: control of glutamate

excitotoxicity or the control of neuroinflammatory response (Cunha, 2005). In fact, A_{2A}R-mediated glutamate release has been implicated in excitotoxicity in acute injury in the CNS and in chronic neurodegenerative disorders (Melani et al., 2003), and therefore prevention of glutamate-induced excitotoxicity as been used as neuroprotective strategy (Popoli et al., 2007).

Neuroinflammation, namely mediated by microglial cells, is associated with neurodegenerative diseases, contributing for the disease progression. Blockade of A_{2A}R has emerged as a potential therapeutic strategy, based on its ability to regulate proliferation, chemotaxis and reactivity of glial cells, affording protection in several brain diseases (reviewed by Gomes et al., 2011; Santiago et al., 2014).

Additionally, A_{2A}R can also mediate neuroprotection by acting on the modulation of BDNF synaptic transmission, as shown in the N9 microglial cell line (Gomes et al., 2013), hippocampus (Diogenes et al., 2007) and striatum (Minghetti et al., 2007). In fact, reduced levels and function of BDNF were observed in the brain of A_{2A}R knockout mice (Tebano et al., 2008).

1.3.3 Effects of A_{2A}R modulation in the inflammatory response

The activation of A_{2A}R leads to opposite effects whereas acting on cells of the peripheral nervous system or in the CNS. Moreover, the effects exerted by A_{2A}R are dependent on the type of receptor stimulation, whether the stimulus is acute or chronic seems to influence the outcome of A_{2A}R activation.

Concerning the peripheral nervous system, studies using animal models suggest that A_{2A}R activation on immune cells is beneficial in environments associated with hypoxia (Chouker et al., 2008; Sitkovsky et al., 2004). In fact, recent findings have demonstrated the crucial modulatory effects of adenosine on T cell regulation, through the activation of A_{2A}R (Ohta and Sitkovsky, 2014), being this receptor involved in the development of immunosupression (Ohta et al., 2012).

Contrarily to the beneficial effects of A_{2A}R activation in the peripheral system, the role of A_{2A}R modulation in the control of CNS immune response is considerably less clear. An obvious distinction between peripheral and central inflammatory responses resides in the type of cells involved in these processes, namely the existence of exclusive glial cells in the CNS, which are not present in the periphery, the microglial and macroglial cells (Cunha et al., 2007).

In fact, a bidirectional effect has been attributed to A_{2A}R in the CNS neuroinflammatory response, with activation of A_{2A}R presenting a protective role in acute damaging conditions, whereas chronic activation leads to a deleterious effect, with the blockade of A_{2A}R being shown to confer neuroprotection against a broad spectrum of brain insults (Cunha et al., 2007; Dai and Zhou, 2011; Gomes et al., 2011). This dual role might be a result of the local environment, with higher concentrations of glutamate in chronic conditions being associated with a switch of A_{2A}R

activation from anti-inflammatory to pro-inflammatory, resulting in the aggravation of the CNS injury (Dai and Zhou, 2011).

In neurodegenerative conditions of the CNS, the genetic deletion or pharmacological blockade of $A_{2A}R$ has been shown to confer robust neuroprotection, by reducing microglial-mediated neuroinflammation (Santiago et al., 2014). $A_{2A}R$ blockade has been shown to reduce the release of IL-1β and neuroinflammatory response in the hippocampus and consequent neuronal dysfunction (Rebola et al., 2011; Simoes et al., 2012). Furthermore, $A_{2A}R$ antagonism was recently shown to reverse the retraction of microglial processes in a model of PD (Gyoneva et al., 2014).

1.3.4 Pharmacological applications of $A_{2A}R$ antagonists in the CNS

The therapeutic value of $A_{2A}R$ antagonists has been investigated for several years and medicinal chemistry has indicated a principal role of these compounds in several CNS disorders, namely PD and HD (Preti et al., 2015).

Several lines of evidence support the role of $A_{2A}R$ in HD, with alterations in their function and expression representing susceptibility of the spinal neurons (Varani et al., 2003; Varani et al., 2007). The use of selective antagonists of $A_{2A}R$, such as SCH 58261 and ZM241385, has shown beneficial effects in the glutamatergic neurotransmission in HD (Domenici et al., 2007; Martire et al., 2010; Varani et al., 2007). Still, due to its bidirectional role, $A_{2A}Rs$ can activate both protective and pro-toxic pathways in models of HD, making their role unpredictable, which difficult their therapeutic application (Popoli et al., 2008; Popoli et al., 2007).

Nevertheless, the protective role of the $A_{2A}R$ selective antagonist SCH 58261 has been shown in a rat model of focal cerebral ischemia (Pedata et al., 2005), brain damage and neurological deficits (Melani et al., 2006). Accordingly, $A_{2A}R$ antagonist and $A_{2A}R$ knockout mice present protective effects in striatal and nigral neurons in a variety of ischemic stroke models (Pedata et al., 2005).

Several reports have demonstrated that $A_{2A}R$ are able to form multimers and/or dimers that contribute to the normal brain physiology, and might be seen as a selective drug targets in specific pathologies. The $A_{2A}R$ C-terminus has been shown to interact with distinct proteins, such as D_2 dopamine receptors (D_2DRs), which are highly involved in the onset of PD (Preti et al., 2015). Numerous preclinical studies have demonstrated motor benefits of the antagonistic $A_{2A}R$-D_2DR interaction in rodents and non-human primates (Antonelli et al., 2006; Jorg et al., 2014; Morelli et al., 2007).

Dopamine replacement therapies are the current drugs used to reduce the early motor symptoms of PD; however, these agents are associated with development of motor complications, limiting usefulness in late stages of the disease. Over the past years, several phase

IIb and phase III clinical trials, using the $A_{2A}R$ selective antagonists istradefyline (KW 6002) or preladenant (SCH 420814), in advanced PD patients, have reported modest, but significant, amelioration of the motor symptoms and reduction of the required pharmacological dosage of dopamine replacers (reviewed in Chen et al., 2013b). However, no evidence supporting the efficacy of istradefyline as monotherapy was observed in the phase III clinical trial (Stocchi et al., 2014). Although FDA issued it as non-approvable, istradefyline is approved in Japan as an adjunctive treatment of PD (Dungo and Deeks, 2013).

1.3.5 Neuroprotective role of caffeine

Caffeine is the most consumed psychostimulant drug in the world, known to affect basic and fundamental human processes, such as sleep, arousal, cognition, learning and memory (Glade, 2010). It acts as a non-selective antagonist of adenosine receptors, and has been related to the regulation of the heart rate, smooth muscle relaxation and neural signaling in the CNS (Rivera-Oliver and Diaz-Rios, 2014).

Since the late 1990s, several studies have shown that caffeine, by acting mainly on A_1R or $A_{2A}R$, reduces physical, cellular and molecular damages caused by neurodegenerative diseases such as PD and Alzheimer's diseases (AD) (reviewed by Rivera-Oliver and Diaz-Rios 2014).

Recent studies have shown that caffeine reverses synaptic dysfunction and enhances memory performance in models of chronic stress by blocking $A_{2A}R$ (Kaster et al., 2015). Accordingly, in AD animal models, several beneficial effects of caffeine administration have been identified, including amelioration of cognitive impairment, rescue of neurotrophic factors levels (such as BDNF) and reduced protein accumulation and toxicity (Basurto-Islas et al., 2014; Dall'Igna et al., 2003; Espinosa et al., 2013; Han et al., 2013; Laurent et al., 2014). Moreover, caffeine mitigates several pro-inflammatory and oxidative stress markers found up-regulated in an animal model of AD (Laurent et al., 2014).

Similar to AD, also in progressive models of PD, chronic caffeine consumption prevents disease-related locomotion deficits and neuronal loss (Chen et al., 2001; Sonsalla et al., 2012; Xu et al., 2002). Also in humans, caffeine intake reduces toxin-induced dopaminergic neuron injury (Xu et al., 2010).

These data establish a potential neural basis for the inverse association of caffeine with the development of neurodegenerative diseases, and intensive investigations are under way for more than a decade to dissect the common cellular mechanisms that may underlie the broad spectrum of neuroprotection by caffeine and $A_{2A}R$ inactivation (Kalda et al., 2006).

In fact, similar to $A_{2A}R$ blockade, caffeine administration has been associated with reduced microglial-mediated neuroinflammatory response (Brothers et al., 2010; Frau et al., 2015;

Machado-Filho et al., 2014; Ruiz-Medina et al., 2013; Sonsalla et al., 2012), prompting it as a possible mechanism that leads to the caffeine-mediated neuroprotection.

1.3.6 Adenosine and glaucoma

In the ocular tissue, adenosine has been suggested to regulate several effects, which might be related with glaucoma, such as control of IOP (Crosson, 1995), corneal endothelial ion transport (Riley et al., 1996), retinal and choroidal vasculature (Gidday and Park, 1993; Takagi et al., 1996). Increased adenosine levels have been implicated in the onset in several neurodegenerative diseases, including retinal degenerative diseases, such as glaucoma (Boison, 2008; Dos Santos-Rodrigues et al., 2015).

Particular interest has been given to the role of adenosine in the control of IOP, with several studies showing that the use of adenosine receptor agonists can lead to an acute rise in the IOP, consistent with the activation of A_1R and $A_{2A}R$ (Avila et al., 2001; Crosson, 1995; Crosson and Gray, 1994; Crosson and Gray, 1996; Tian et al., 1997). Furthermore, selective activation of A_3R can increase the aqueous humor secretion, thereby increasing the IOP (Mitchell et al., 1999). These observations supported the critical involvement of adenosine in the regulation of IOP and therefore, several adenosine receptor agonists and antagonists are currently being evaluated as hypotensive drugs for the treatment of glaucoma (Bagnis et al., 2011; Lee and Goldberg, 2011; Zhong et al., 2013).

Also in the retina, adenosine concentration is significantly increased after I-R injury (Ghiardi et al., 1999), which might be due to the extracellular conversion of ATP. In fact, ATP release has been found increased in retinal neuronal and macroglial cells after mechanical strain and short-term increases of IOP (Beckel et al., 2014; Lu et al., 2015). Acute injury to RGC has been show to be mediated by extracellular ATP, with the reduction of this increase preventing the damage to RGCs (Resta et al., 2007).

Pharmacological modulation of adenosine receptors has been proposed to present benefits in the clinical setting concerning glaucoma by attenuation of neuronal cell death (Reichenbach and Bringmann, 2015). Depending on the duration of the insult, both activation of A_1R and blockade of $A_{2A}R$ might afford protection in I-R injury models. Acute response is mediated by A_1R, whereas $A_{2A}R$ antagonists can protect retinal function and structure after prolonged ischemia (Li et al., 1999). Moreover, agonists of A_3R have been shown to confer neuroprotection against I-R-induced retinal degeneration (Galvao et al., 2015), and are also proposed as therapeutic strategies for glaucoma (Fishman et al., 2013).

1.4 Objectives of the study

Glaucoma, the second leading cause of blindness worldwide, is characterized by RGG loss and optic nerve damage. Elevated IOP is the only modifiable risk factor and the treatments are focused in reducing IOP. Still, many patients continue to lose vision despite the control of IOP and neuroprotective strategies have arisen as having potential to prevent the loss of RGCs. In recent years, neuroinflammation and glial reactivity have been associated with the onset of glaucomatous damage, prompting the hypothesis that neuroinflammation control could be a potential strategy to provide protection to RGCs in glaucoma.

Adenosine is crucial neuromodulator in the CNS, involved in key metabolic pathways. In several brain pathological conditions, $A_{2A}R$ antagonists and caffeine have been shown to confer neuroprotection, mainly through the control of the neuroinflammatory response. Considering the neuroprotective properties of $A_{2A}R$ antagonists and the involvement of microglia-mediated neuroinflammation in the pathogenesis of glaucoma, we hypothesized that the modulation of the activity of $A_{2A}R$ could afford protection to RGCs in models of glaucoma through the control of microglia reactivity.

The main aims of this work were:

1)   To investigate whether the blockade of $A_{2A}R$ controls microglia-mediated retinal neuroinflammation;

2)   To investigate the neuroprotective effects of the blockade of $A_{2A}R$ against elevated pressure-induced neuroinflammation;

3)   Evaluate the neuroprotective effects of caffeine in an animal model with ocular hypertension.

In order to achieve these goals, we have used *in vitro* and animal models. In the *in vitro* experiments, retinal microglial cell cultures and retinal organotypic cultures were challenged with LPS in order to induce an inflammatory response, or with EHP, to mimic OHT. As animal models, we used a model of retinal ischemia-reperfusion injury and a model with chronic OHT based on reduced aqueous humor outflow.

In chapter one retinal organotypic cultures were challenged with LPS or EHP in the presence or absence of the $A_{2A}R$ antagonist SCH 58261 to evaluate the potential effects of $A_{2A}R$ in retinal neuroinflammation and survival of RGCs. Moreover, a neutralization experiment with antibodies anti-TNF and anti-IL-1β were used evaluate the direct contribution of neuroinflammation to RGC loss.

In the second chapter we further evaluated the effects of $A_{2A}R$ blockade in the control of retinal microglial cells reactivity and neuroinflammatory response, using primary retinal

microglial cell cultures. Additionally, the protective effects of $A_{2A}R$ blockade were investigated in an animal model of retinal I-R injury.

At last, in the third chapter we have investigated the effects of caffeine administration in an animal model of OHT, namely in the neuroinflammatory response, retrograde axonal transport impairment and survival of RGCs.

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CHAPTER 2 -

Adenosine A$_{2A}$R blockade prevents neuroinflammation-induced death of retinal ganglion cells caused by elevated pressure

Maria H. Madeira, Filipe Elvas, Raquel Boia, Francisco Q. Gonçalves, Rodrigo A. Cunha, António Francisco Ambrósio and Ana Raquel Santiago (2015). Adenosine A$_{2A}$R blockade prevents neuroinflammation-induced death of retinal ganglion cells caused by elevated pressure. Journal of Neuroinflammation **12**(1): 115. doi: 10.1186/s12974-015-0333-5.

NOTE: Work presented as published, with minor modifications.

2.1 Abstract

Background

Elevated intraocular pressure (IOP) is a major risk factor for glaucoma, a degenerative disease characterized by the loss of retinal ganglion cells (RGCs). There is clinical and experimental evidence that neuroinflammation is involved in the pathogenesis of glaucoma. Since the blockade of adenosine A$_{2A}$ receptor (A$_{2A}$R) confers robust neuroprotection and controls microglia reactivity in the brain, we now investigated the ability of A$_{2A}$R blockade to control the reactivity of microglia and neuroinflammation as well as RGC loss in retinal organotypic cultures exposed to elevated hydrostatic pressure (EHP) or lipopolysaccharide (LPS).

Methods

Retinal organotypic cultures were either incubated with LPS (3µg/mL), to elicit a pro-inflammatory response, or exposed to EHP (+70 mmHg), to mimic increased IOP, for 4 or 24 h, in the presence or absence of A$_{2A}$R antagonist SCH 58261 (50 nM). A$_{2A}$R expression, microglial reactivity and neuroinflammatory response were evaluated by immunohistochemistry, quantitative PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA). RGC loss was assessed by immunohistochemistry. In order to investigate the contribution of pro-inflammatory mediators to RGC loss, the organotypic retinal cultures were incubated with rabbit anti-tumour necrosis factor (TNF) (2 µg/mL) and goat anti-IL-interleukin-1β (IL- 1β) (1 µg/mL) antibodies.

Results

We report that the A$_{2A}$R antagonist (SCH 58261) prevented microglia reactivity, increase in pro-inflammatory mediators as well as RGC loss upon exposure to either LPS or EHP. Additionally, neutralization of TNF and IL-1β prevented RGC loss induced by LPS or EHP.

Conclusions

This work demonstrates that A$_{2A}$R blockade confers neuroprotection to RGCs by controlling microglia-mediated retinal neuroinflammation and prompts the hypothesis that A$_{2A}$R antagonists may be a novel therapeutic option to manage glaucomatous disorders.

Keywords: Microglia; adenosine; neuroprotection; glaucoma.

2.2 Background

Glaucoma is the third leading cause of visual impairment and the second cause of blindness worldwide (Resnikoff et al., 2004). It is defined as a group of chronic degenerative optic neuropathies, characterized by the irreversible and progressive loss of retinal ganglion cells (RGCs) and damage of the optic nerve (RGC axons). Although glaucoma is a multifactorial disease, elevated intraocular pressure (IOP) is a major risk factor and the current treatments are mainly focused in reducing on IOP (Caprioli, 2013). However, many patients continue to lose vision despite the control of IOP and neuroprotective strategies aimed to prevent RGC loss are necessary (Cordeiro and Levin, 2011).

Increasing evidence has shown that neuroinflammation has an important role in the pathogenesis of glaucoma (Howell et al., 2011; Joachim et al., 2012; Krizaj et al., 2013). Accordingly, microglial cells display an activated amoeboid-like morphology at the early stages of glaucoma (Bosco et al., 2011; Neufeld, 1999; Taylor et al., 2011; Wang et al., 2002). In parallel, there is an increased expression and release of pro-inflammatory cytokines [e.g. tumour necrosis factor (TNF), interleukin-1β (IL-1β)] and nitric oxide (NO) in the glaucomatous eye (Cho et al., 2011; Gramlich et al., 2013; Tezel and Wax, 2000; Yuan and Neufeld, 2000). The importance of this microglia-associated neuroinflammation in glaucoma is underscored by the observation that the control of microglia activation (Bosco et al., 2008; Wang et al., 2014; Yang et al., 2013) or of pro-inflammatory cytokines expression (Howell et al., 2011; Roh et al., 2012) can prevent the loss of RCG in animal models of glaucoma.

Microglia-associated neuroinflammation is also involved in different brain disorders (Kettenmann et al., 2011). Adenosine is a neuromodulator which can control inflammatory reactions (Hasko et al., 2008; Sitkovsky et al., 2004) and microglia reactivity (Minghetti et al., 2007; Rebola et al., 2011; Saura et al., 2005) mainly through the activation of its G-protein coupled receptor of the A₂ₐ receptor subtype (A₂ₐR) (Gomes et al., 2011). Accordingly, A₂ₐR antagonists afford robust neuroprotection upon ischemia, epilepsy, Alzheimer's or Parkinson's diseases (Gomes et al., 2011).

All these evidence prompt the hypothesis that A₂ₐR antagonists may also control the microglia-associated neuroinflammation and loss of RGC in animal models of glaucoma. Therefore, the main aim of this work was to investigate whether A₂ₐR blockade modulates retinal microglia reactivity, neuroinflammation and loss of RGC triggered by lipopolysaccharide (LPS) or elevated hydrostatic pressure (EHP).

2.3 Materials and methods

2.3.1 Animals

Adult Wistar rats were housed in certified local facilities, in a temperature and humidity controlled environment, and were provided with standard rodent diet and water *ad libitum*, under a 12 h light/12 h dark cycle. All procedures involving animals were approved by the Ethical Committee of the Faculty of Medicine of the University of Coimbra/Center for Neuroscience and Cell Biology and are in agreement with the Association for Research in Vision and Ophthalmology statement for animal use.

2.3.2 Organotypic retinal cultures

Wistar rats (8-10 weeks old) were euthanized and their eyes enucleated. Retinas were dissected in a Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS; in mM: 137 NaCl, 5.4 KCl, 0.45 KH_2PO_4, 0.34 Na_2HPO_4, 4 $NaHCO_3$, 5 glucose; pH 7.4) and placed in tissue culture inserts (Millipore; 0.4-µm pore diameter) with the ganglion cell layer (GCL) facing up. The retinas were cultured for 4 days in DMEM-F12 with GlutaMAX I, supplemented with 10 % heat-inactivated foetal bovine serum and 0.1 % gentamicin (all from Life Technologies) at 37 °C, in 5 % CO_2 humidified atmosphere, as previously described (Kretz et al., 2004). The culture medium was replaced at culture days 1 and 2.

2.3.3 Culture treatments

Organotypic retinal cultures were either incubated with LPS (3 µg/mL, Sigma-Aldrich) or exposed to EHP (70 mmHg above atmospheric pressure) for 4 h or 24 h, before the end of the experiment. For the EHP experiments we used a custom-made humidified pressure chamber equipped with a pressure gauge and a pressure regulator, which allowed maintaining a constant pressure with an air mixture of 95 % air and 5 % CO_2, as described previously (Sappington et al., 2006). The chamber was placed in an oven at 37 °C. The magnitude of pressure elevation (70 mmHg above atmospheric pressure) was chosen in accordance with previous studies (Sappington and Calkins, 2006; Sappington et al., 2006). For ambient pressure experiments, the organotypic retinal cultures were kept in a standard 5 % CO_2 humidified incubator.

The cultures were incubated with a selective A₂ₐR antagonist (50 nM SCH 58261; Tocris Bioscience) 45 min before exposure to LPS or EHP. To test the role of extracellular adenosine, organotypic cultures were treated with 1 U/mL adenosine deaminase (ADA; Roche Applied Science), which catalyzes the irreversible deamination of adenosine to inosine. In order to investigate the contribution of pro-inflammatory mediators to RGC loss, the organotypic retinal cultures were incubated with rabbit anti-TNF (2 µg/mL; Peprotech) and goat anti-IL-1β (1 µg/mL; R&D Systems)

antibodies, or with corresponding immunoglobulin Gs (IgGs), 45 min before exposure to LPS or EHP for 24 h. Organotypic cultures were also incubated with 20 ng/mL TNF and 10 ng/mL IL-1β (ImmunoTools) to evaluate if TNF and IL-1β, by themselves, lead to RGC loss.

2.3.4 Immunohistochemistry

Organotypic cultures were washed with phosphate-buffered saline (PBS; in mM: 137 NaCl, 2.7 KCl, 10 Na₂HPO₄, and 1.8 KH₂PO₄; pH 7.4) and fixed with ice-cold ethanol for 10 min at 4 °C. After washing in PBS, cultures were blocked and permeabilized with 10 % normal goat serum, 3 % bovine serum albumin and 0.1 % Triton X-100 in PBS, for 1 h, and then incubated with the primary antibody (Table 1) for 48 h at 4 °C. After washing, cultures were incubated overnight with the secondary antibody (Table 1), at 4 °C. Retina cultures were then washed and incubated with 4', 6-diamidino-2-phenylindole (DAPI; 1:1000) for 15 min, to stain nuclei. After washing, the preparations were flat-mounted on slides and coverslipped using Glycergel mounting medium.

Table 1: Primary and secondary antibodies used in immunohistochemistry

	Supplier	Host	Dilution
Primary antibodies			
Anti-A₂ₐR	Santa Cruz Biotechnology	Goat	1:100
Anti-CD11b	AbD Serotec	Mouse	1:250
Anti-iNOS	BD Biosciences	Rabbit	1:200
Anti-Brn3a	Chemicon	Mouse	1:500
Secondary antibodies			
Alexa Fluor anti-mouse 568	Life Technologies	Donkey	1:200
Alexa Fluor anti-mouse 488	Life Technologies	Goat	1:200
Alexa Fluor anti-goat 488	Life Technologies	Rabbit	1:200
Alexa Fluor anti-rabbit 488	Life Technologies	Goat	1:200

2.3.5 Image acquisition and densitometric analysis

The preparations were observed with a confocal microscope (LSM 710, Zeiss) on an Axio Observer Z1 microscope using an EC Plan-Neofluar 40x/1.30 Oil DIC M27 objective, and, from each quadrant, at least 3 images of the GCL were randomly acquired (encompassing central and peripheral retina), in a total of 12 images. The settings and exposure times were kept identical for all conditions within each experiment. Densitometric analysis was performed using the public domain ImageJ program (http://rsb.info.nih.gov/ij/). Corrected total cell fluorescence (CTCF) was calculated as previously described (Gavet and Pines, 2010) using the following formula:

CTCF = Integrated density-(area of selected cell x mean fluorescence of background reading)

2.3.6 Circularity index and skeleton analysis

Morphological alterations of microglia were estimated as previously described (Kurpius et al., 2006) using the confocal images of the retinal organotypic cultures labelled with anti-CD11b. Briefly, the particle measurement feature of ImageJ was used to automatically evaluate the circularity index (CI) of microglia, using the formula: $CI = 4\pi(area/perimeter^2)$. A circularity index of 1.0 indicates a perfect circle.

The microglial cell complexity and branch length were assessed by skeleton analysis using ImageJ software, as described previously (Morrison and Filosa, 2013). Briefly, confocal images were converted to 8-bit format, followed by noise de-speckling to eliminate single-pixel background fluorescence. Then, images were converted to binary images, which were analyzed using AnalyzeSkeleton plugin (http://fiji.sc/AnalyzeSkeleton/) to assess the number of microglial cell processes, number of branch endpoints and maximum branch length for each cell. These results were analyzed as average per frame.

2.3.7 ATP quantification

The extracellular levels of adenosine triphosphate (ATP) were quantified with a luciferase ATP bioluminescence assay kit (Sigma-Aldrich) as we previously described (Cunha et al., 2000). Briefly, the supernatants were collected and immediately stored at -80 °C until used. Then, 80 μL of these supernatant were added to a white 96-well plate (designed for bioluminescence) placed in a VICTOR multilabel plate reader (PerkinElmer). The luciferin-luciferase ATP assay mix (40 μL) was automatically loaded in each well, and the luminescence output was converted to ATP concentration by interpolation of a standard curve, which was linear between 2×10^{-12} M and 8×10^{-5} M. ATP concentration was normalized to the total amount of protein of each retina, which was determined by the bicinchoninic acid assay (Pierce Biotechnology).

2.3.8 NO production assay

The production of NO was quantified by the Griess reaction method in the supernatants of the culture medium. The culture medium was centrifuged (10000 g for 10 min) and the supernatant stored at -80 °C until use. Then, the supernatant was incubated (1:1) with Griess reagent mixture (1 % sulfanilamide in 5 % phosphoric acid with 0.1 % N-1-naphthythylenediamine) for 30 min at room temperature, and in the dark. The optical density was measured at 550 nm using a microplate reader (Synergy HT; Biotek). The nitrite concentration was determined from a sodium nitrite standard curve.

2.3.9 Quantitative real-time PCR

Total RNA was extracted using Qiagen RNeasy Mini Kit (Qiagen), according to the instructions provided by the manufacturer. The concentration and purity of total RNA were determined using NanoDrop ND1000 (Thermo Scientifics). Then, 1 µg of total RNA was reversed transcribed using a NZY First-Strand cDNA Synthesis Kit according to the manufacturer instructions (NZYTech, Portugal). The resultant complementary DNA (cDNA) was treated with RNase-H for 20 min at 37 °C, and a 1:2 dilution was prepared for qPCR analysis. All cDNA samples were stored at -20 °C until further analysis.

Genomic DNA contamination was assessed with a conventional PCR for β-actin using intron-spanning primers (Table 2), as described previously (Santiago et al., 2009). SYBR-Green-based real-time quantitative PCR (qPCR) was performed using a StepOnePlus PCR system (Applied Biosystems). The PCR conditions were as follows: iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 200 nM primers (Table 2) and 2 µL of 1:2 dilution of cDNA, in a total volume of 20 µL. Cycling conditions were a melting step at 95 °C for 15 s, annealing-elongation at 60 °C for 45 s, and extension at 72 °C, with 40 cycles. A dissociation curve at the end of the PCR run was performed by ramping the temperature of the sample from 60°C to 95 °C, while continuously collecting fluorescence data. Ct values were converted to "Relative quantification" using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Three candidate housekeeping genes (*hprt*, *Ywhaz*, and *GAPDH*) were evaluated using NormFinder, a Microsoft Excel Add-in (Andersen et al., 2004), and *hprt* was the most stable gene throughout all experimental conditions and samples, and therefore was used as the housekeeping gene.

Table 2: Primers used in qPCR and RT-PCR

Gene	GeneBank number	Forward	Reverse	Amplicon size
Adora2A	NM_053294	5' - GGCTATCTCTGACCAACA - 3'	3' - TGGCTTGACATCTCTAATCT - 5'	106 bp
TNF	NM_012675	5' - CCCAATCTGTGTCCTTCT - 3'	3' - TTCTGAGCATCGTAGTTGT - 5'	90 bp
IL1b	NM_031512	5' - ATAGAAGTCAAGACCAAAGTG - 3'	3' - GACCATTGCTGTTTCCTAG - 5'	109 bp
Nos II	NM_012611	5' - AGAGACAGAAGTGCGATC - 3'	3' - AGAGATTCAGTAGTCCACAATA - 5'	96 bp
hprt	NM_012583	5' - ATGGGAGGCCATCACATTGT- 3'	3' - ATGTAATCCAGCAGGTCAGCAA - 5'	76 bp
actb	NM_031144	5' - GCTCCTCCTGAGCGCAAG - 3'	3' - CATCTGCTGGAAGGTGGACA - 5'	75 bp

2.3.10 Enzyme-Linked Immunosorbant Assay (ELISA)

Culture media was centrifuged (10,000 *g* for 10 min) and the supernatant was collected and stored at -80 °C until use. The levels of TNF and IL-1β in the culture supernatants were quantified by ELISA, according to the instructions provided by the manufacturer (Peprotech).

2.3.11 Retinal ganglion cell counting

Retinal ganglion cells were identified by immunohistochemistry stainning with an antibody anti-Brn3a (RGC marker) and confocal images of the GCL were acquired (as described above). The number of Brn3a-immunoreactive cells per image was counted using ImageJ Cell Counter plugin (http://rsbweb.nih.gov/ij/plugins/cell-counter.html). Results represent the average of Brn3a-immunoreactive cells per image.

2.3.12 Statistical analysis

The results are presented as mean ± standard error of the mean (SEM). The data were analyzed using the non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparison test, as indicated in the figure legends. The statistical analysis was performed using the Prism 6.0 software for Mac OS X (GraphPad Software, Inc).

2.4 Results

The retinal organotypic culture is particularly useful to evaluate molecular and cellular mechanisms in the retina because the retinal structure is maintained (Kretz et al., 2004). Thus, we used this experimental model to investigate the ability of A₂ₐR to control neuroinflammation and RGC death triggered by LPS or EHP (to mimic an increase in IOP).

2.4.1 LPS and EHP increased the expression of A₂ₐR in retinal microglial cells in the GCL

Since the A₂ₐR modulation system undergoes a gain of function upon noxious brain conditions (Gomes et al., 2011), we first assessed if this also occurred in the retina. Therefore, we investigated if LPS or EHP up-regulated the expression of A₂ₐR and bolstered the source of adenosine responsible for the activation of A₂ₐR, i.e. ATP-derived adenosine (Augusto et al., 2013).
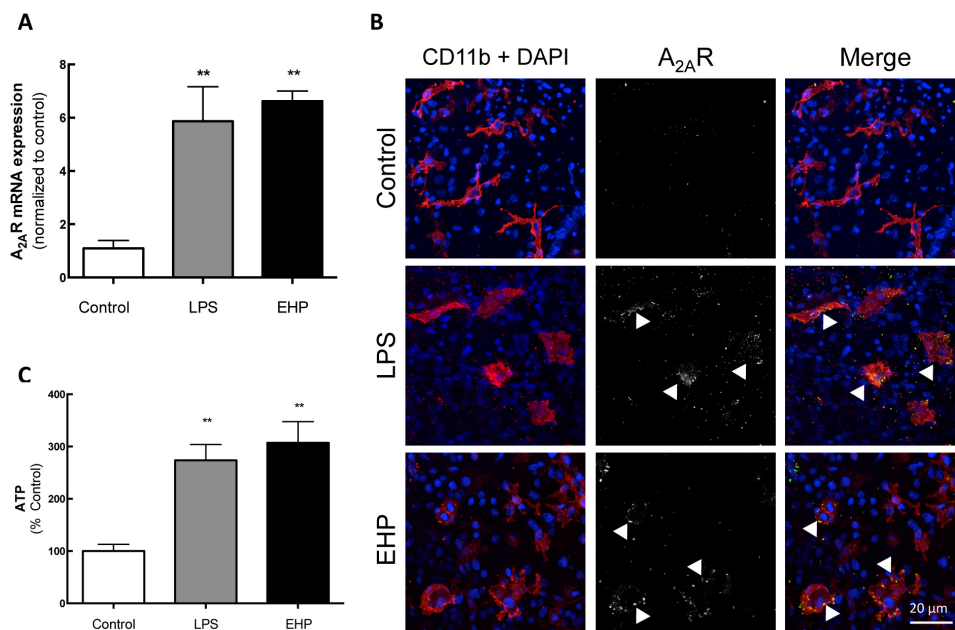

Figure 1 – LPS or EHP increases A₂ₐR expression and density in retinal microglia and increase the extracellular ATP levels. Retinal organotypic cultures were challenged with LPS (3 µg/mL) or EHP (+70 mmHg) for 24 h. **A:** A₂ₐR mRNA expression was assayed by qPCR. Results are presented as fold change of the control, from 6-10 independent experiments. **B:** Organotypic retinal cultures were immunostained for A₂ₐR (*grey/green; arrowheads*) and CD11b (*microglia marker; red*) and imaged in the GCL using a confocal microscope. Nuclei were stained with DAPI (*blue*). Representative images obtained from 4 independent experiments. **C**: The extracellular levels of ATP in the medium were quantified by luciferin-luciferase ATP-dependent reaction. Results are expressed in percentage of control and are mean ± SEM of 6-8 independent experiments. ✱✱$P < 0.01$, different from control; Kruskal -Wallis test, followed by Dunn's multiple comparison test.

LPS or EHP exposure for 4 h significantly increased $A_{2A}R$ messenger RNA (mRNA) expression in the retina by 5.3- and 6.0-fold (n = 6-10), respectively (Figure 1A). Accordingly, 4 h exposure to LPS or EHP, $A_{2A}R$ immunoreactivity increased mainly in CD11b-positive cells in the GCL (Figure 1B), indicating that $A_{2A}R$ in the GCL are mainly present in microglia.

Extracellular ATP levels in control conditions were 0.6 ± 0.3 pmol/μg protein (n = 8), and significantly increased by 173.8 ± 30 % and 215.1 ± 40 % after 24 h of exposure to LPS or EHP (n = 6-8), respectively (Figure 1C).

2.4.2 $A_{2A}R$ blockade prevented the alterations of microglia morphology triggered by LPS or EHP

Modification of cell morphology is one of the hallmarks of microglia activation and has been widely used to categorize different activation states (Kettenmann et al., 2011). As shown in Figure 2A, under control conditions, microglial cells (i.e. CD11b-positive cells) in the GCL typically presented a ramified morphology [circularity index (CI): 0.110 ± 0.02, n = 7; Figure 2B], compatible with a "surveying" phenotype. After 24 h of exposure to LPS or EHP, microglia morphology changed to a more amoeboid-like morphology (CI: 0.242 ± 0.014 and 0.182 ± 0.006, respectively; n = 5-8, p < 0.05 *vs.* control). Incubation with the selective antagonist of $A_{2A}R$ (SCH 58261, 50 nM) prevented the LPS- and EHP-induced alterations of microglia circularity index (n = 5-8) (Figure 2B). In addition, skeleton morphological analysis was used to further document more subtle morphological changes compatible with microglial activation. Retinal microglia from LPS- and EHP-treated cultures presented a decrease in the number of branches (Figure 2C), endpoints (Figure 2D) and maximum branch length (Figure 2E) compared to the control condition. The blockade of $A_{2A}R$ prevented these alterations, indicating that $A_{2A}R$ blockade blunted LPS- and EHP-induced microglia reactivity.
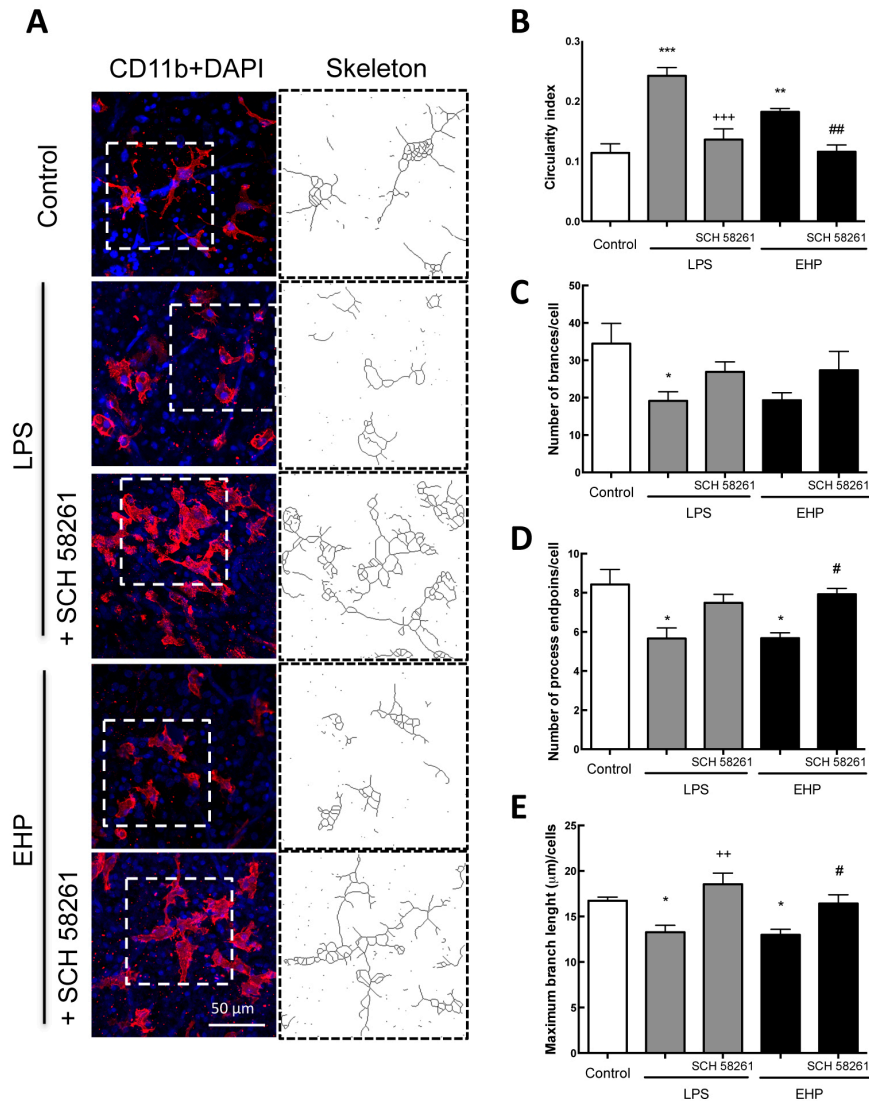
Figure 2 – Blockade of A₂ₐR prevents microglia morphological changes induced by LPS or EHP. Retinal organotypic cultures were pretreated with the A₂ₐR antagonist SCH 58261 (50 nM) and then challenged with LPS (3 µg/mL) or EHP (+70 mmHg) for 4 h. **A:** Organotypic retinal cultures were immunostained for CD11b (*microglia marker; red*) and imaged in the GCL using a confocal microscope. Nuclei were stained with DAPI (*blue*). Representative images obtained from 4-5 independent experiments. **B:** The circularity index; **C**: number of branches per cell; **D:** number of processes endpoints per cell; and **E:** the maximum branch length (µm) per cell were calculated for the different experimental conditions. The bar graphs present data as mean ± SEM of 4-5 independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001, different from control; ++P < 0.01,and +++P < 0.001 different from LPS; #P < 0.05 and ##P < 0.01, different from EHP; Kruskal-Wallis test, followed by Dunn's multiple comparison test.

2.4.3 Blockade of A$_{2A}$R prevented microglia production of nitric oxide (NO)

Since the activation of microglial cells leads to the production of pro-inflammatory and cytotoxic factors like NO, both *in vivo* and *in vitro* (Kraft and Harry, 2011), we tested if A$_{2A}$R could control the up-regulation of inducible nitric oxide synthase (iNOS), which plays a critical role in neuroinflammation by generating high amounts of NO in reactive microglia (Brown, 2007).

As expected, the mRNA expression of iNOS significantly increased by 30.5-fold after 4 h of exposure to LPS (n = 6), and this effect was significantly decreased upon A$_{2A}$R blockade (n = 4) (Figure 3A). The exposure to EHP for 4 h also significantly increased iNOS mRNA expression by 4.6-fold over control (n = 5), and the blockade of A$_{2A}$R also significantly prevented this effect (n = 6) (Figure 3A).

In control conditions, the immunoreactivity of iNOS was barely detected in microglia localized in the CGL (Figures 3B). Exposure to LPS or EHP for 24 h, significantly increased iNOS immunoreactivity mainly in CD11b-immunoreactive cells (Figures 3B and C), confirming that microglial cells are the main producers of NO under these conditions. This effect was abolished by blockade of A$_{2A}$R, since iNOS immunoreactivity was similar to control (Figures 3B and C).

The release of NO was indirectly quantified in the culture medium by Griess reaction 24 h after exposure to LPS or EHP (Figure 3D). In control conditions, nitrite concentration was 5.64 ± 0.17 μM (n = 6). LPS or EHP significantly increased nitrite concentration to 149.5 ± 11 % and 138 ± 3.5 % of the control, respectively (n = 4-5), and these effects were prevented by A$_{2A}$R blockade (n = 3-4) (Figure 3D).
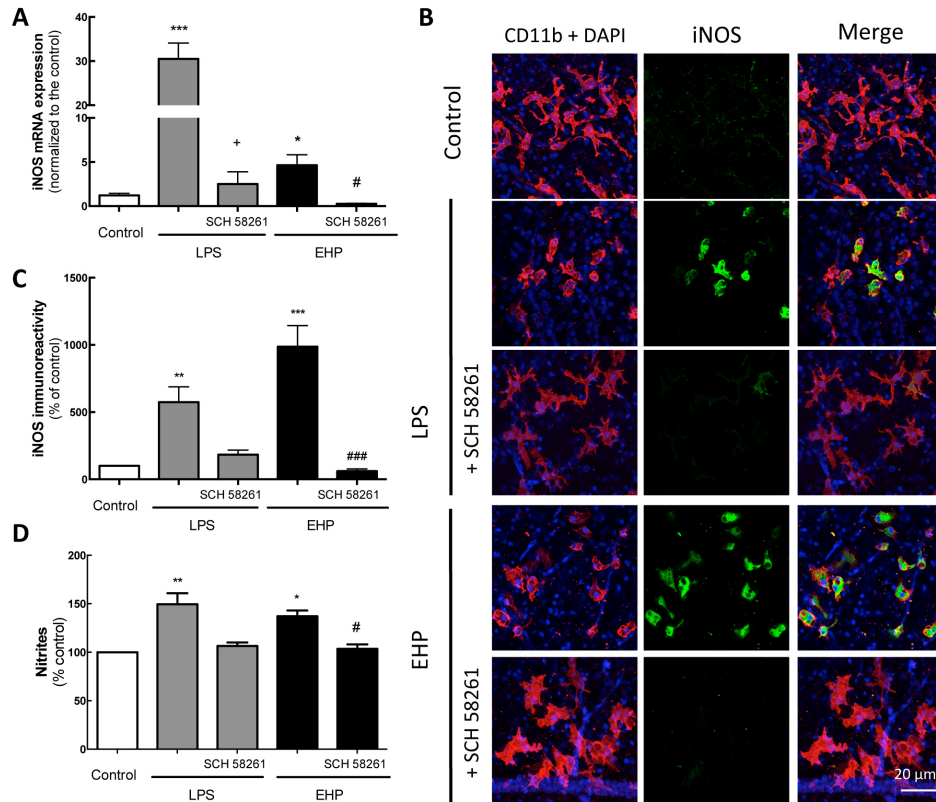
Figure 3 – Blockade of A₂ₐR decreases the expression and immunoreactivity of iNOS and NO production induced by LPS or EHP. Retinal organotypic cultures were pretreated with SCH 58261 (50 nM) and then challenged with LPS (3 µg/mL) or EHP (+70 mmHg) for 4 h. **A:** iNOS mRNA expression was assessed by qPCR. Results are presented as fold change of the control, from 6-12 independent experiments. **B:** Organotypic retinal cultures were immunostained for iNOS (*green*) and CD11b (*microglia marker; red*) and imaged in the GCL using a confocal microscope. Nuclei were stained with DAPI (*blue*). The images are representative of 4-5 independent experiments. **C:** The immunoreactivity of iNOS in microglia localized in the GCL was quantified. Results are expressed in percentage of control from 4-5 independent experiments. **D:** The production of NO was assessed by the Griess reaction in culture supernatants and nitrite formation was quantified. Results are expressed in percentage of control and are mean ± SEM of 4-6 independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, different from control; +$P < 0.05$, different from LPS; #$P < 0.05$ and ### $P < 0.001$, different from EHP; Kruskal-Wallis test, followed by Dunn's multiple comparison test.

2.4.4 A₂ₐR blockade mitigates the inflammatory response induced by LPS or EHP

Activation of microglia leads to an increased expression and release of pro-inflammatory cytokines, such as IL-1β and TNF (Kettenmann et al., 2011). To further test if A₂ₐR blockade prevented the LPS- and EHP-induced inflammatory response, we quantified mRNA levels encoding for IL-1β and TNF by qPCR. As shown in Figure 4A, the exposure of retinal organotypic cultures to LPS or EHP for 4 h significantly increased the transcript levels of IL-1β and TNF (n = 5). Overall, the blockade of A₂ₐR inhibited the LPS- and EHP-induced increase of IL-1β and TNF mRNA levels (n = 5-7) (Figure 4A).

We next quantified the levels of IL-1β and TNF in the culture medium by ELISA (Figure 4B). In control conditions, the concentration of IL-1β in the culture medium was 67.1 ± 5.5 pg/mL and the concentration of TNF was 30.9 ± 53.7 pg/mL (n = 10-15). Incubation with LPS or EHP for 4 h significantly increased IL-1β concentration in the culture medium to 135.3 ± 6.9 pg/mL and 146.7 ± 9 pg/mL, respectively (n = 6-8) and the TNF concentration to 317.6 ± 40.6 pg/mL and 162.8 ± 42.6 pg/mL, respectively (n = 6-9) (Figure 4B). The blockade of A₂ₐR significantly inhibited the LPS- and the EHP-induced increase of IL-1β or TNF levels in the culture medium (n = 5) (Figure 4B).

Additionally we tested if the removal of endogenous extracellular adenosine was equivalent to blocking A₂ₐR in the control of LPS- or EHP-induced neuroinflammation. We found that the pre-treatment of organotypic cultures with ADA (1 U/mL), which removes extracellular adenosine, abrogated the LPS- and EHP-induced increase in the expression (Figure 4A) and extracellular levels (Figure 4B) of both TNF and IL-1 β (n = 3).
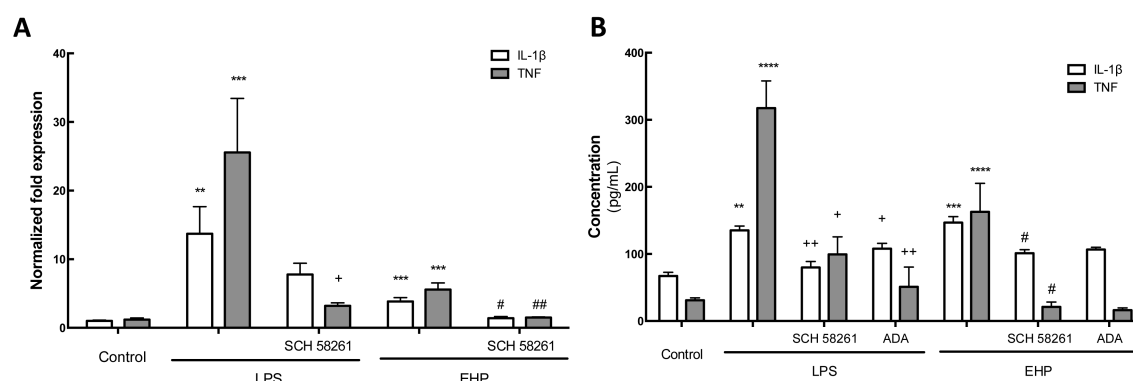

Figure 4 – A₂ₐR blockade partially inhibits the inflammatory response induced by LPS or EHP. Retinal organotypic cultures were pretreated with SCH 58261 (50 nM) and then challenged with LPS (3 μg/mL) or EHP (+70 mmHg) for 4 h. **A:** Effects of A₂ₐR blockade in the LPS- or EHP- induced mRNA expression of pro-inflammatory cytokines IL-1β and TNF were assessed by qPCR. Results are presented as fold change of the control, from 6-13 independent experiments. **B:** The release of IL-1β and TNF to the culture medium was quantified by ELISA. To evaluate the role of endogenous adenosine, the cultures were pretreated with adenosine deaminase (ADA; 1 U/mL). Results are expressed in pg/mL and represent mean ± SEM of 5-10 independent experiments. $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$, different from control; $+P < 0.05$, and $++P < 0.01$ different from LPS; $\#P < 0.05$ $\#\#P < 0.01$, different from EHP; Kruskal-Wallis test, followed by Dunn's multiple comparison test.

2.4.5 A$_{2A}$R blockade prevented RGC death through the control of neuroinflammmation

The elevation of the hydrostatic pressure is an experimental strategy to mimic in a retina culture model a situation of IOP increase, which is a major risk factor for glaucoma (Caprioli, 2013). Studies from Sappington (2006) have already provide described RGC death under EHP conditions.

Since A$_{2A}$R blockade prevented microglia activation and the expression and release of pro-inflammatory cytokines, we next tested if A$_{2A}$R blockade also prevented the loss of RGC induced by LPS or EHP in retinal organotypic cultures. Loss of RGCs was evaluated by counting the number of RGC, identified with an antibody against Brn3a (Figure 5A), a marker of RGCs (Nadal-Nicolas et al., 2009; Sanchez-Migallon et al., 2011). The number of Brn3a-immunoreactive cells (Figure 5A and C) significantly decreased when the retinal explants were exposed to LPS or EHP for 24 h, when compared with the control (190.5 ± 12 Brn3a-immunoreactive cells per field in control *vs.* 118.9 ± 11 and 113.9 ± 6 Brn3a-immunoreactive cells per field in LPS and EHP conditions, respectively, n = 6-7), indicating that both insults cause RGC loss. This effect was prevented with the treatment with A$_{2A}$R antagonist (191.2 ± 7.3 and 184.3 ± 9.3 cells per field, respectively; Figure 5A and C; n = 4-5).

Since previous results demonstrated that A$_{2A}$R blockade prevented both inflammatory responses and RGC loss triggered by LPS and EHP, we next investigated if TNF and IL-1β were necessary and sufficient to induce RGC loss under noxious conditions (LPS or EHP). Organotypic retinal cultures were pretreated with antibodies against TNF and IL-1β before incubation with LPS or exposure to EHP, in order to reduce the levels of both pro-inflammatory cytokines. The incubation of organotypic retinal cultures with antibodies against TNF and IL-1β prior incubation with LPS or exposure to EHP fully prevented the loss of RGCs (180.6 ± 8 and 170.2 ± 4 Brn3a-immunoreactive cells, respectively, n = 5; Figure 5B and 5C). As a control, the incubation with rabbit and goat IgGs did not significantly inhibit the decrease in the number of RGC upon exposure to LPS or EHP (n = 4). In addition, incubation with TNF (20 ng/mL) plus IL-1β (10 ng/mL) was sufficient to induce loss of RGC (n = 3) (Figure 5C) to an extent similar to that triggered by LPS or EHP. Moreover, incubation with the neutralizing antibodies in control conditions did not alter the number of RGC cells present in the culture (data not shown). The neutralizing experiments under noxious conditions (LPS or EHP) fully recapitulated the incubation with SCH 58261, further supporting our conclusion that A$_{2A}$R blockade control RGC loss through a control of retinal neuroinflammation.
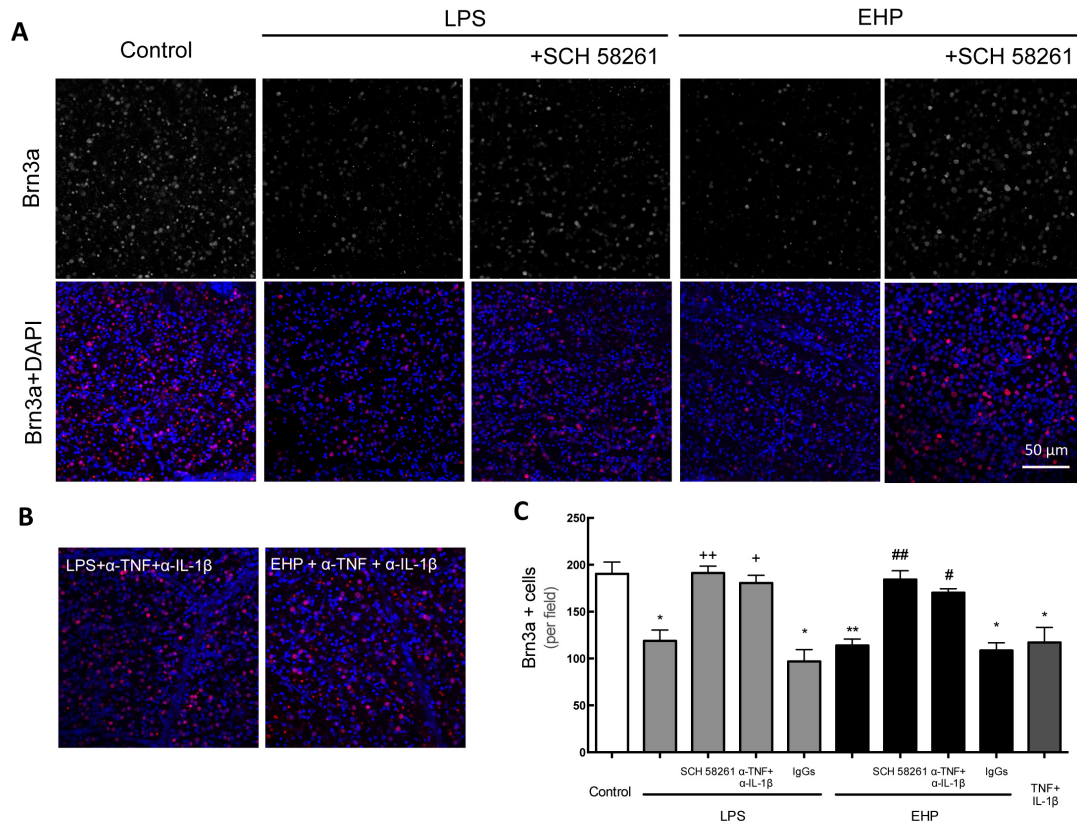
Figure 5 – Blockade of A$_{2A}$R and of TNF and IL-1 β prevents RGC death induced by LPS or EHP. Retinal organotypic cultures were pretreated with SCH 58261 (50 nM) or with anti-TNF and anti-IL-1β neutralizing antibodies and then challenged with LPS (3 µg/mL) or EHP (+70 mmHg) for 24 h. Rabbit and goat IgGs were used as control for the neutralization experiments. **A:** Organotypic retinal cultures were immunostained for Brn3a (*RGC marker, red*) after treatment with SCH 58261 prior to challenge. Nuclei were stained with DAPI (*blue*). **B:** Immunostaining with Brn3a (*red*) after treatment with neutralizing antibodies prior to challenge. Nuclei were stained with DAPI (*blue*). Representative images are depicted. **C:** Surviving RGCs are presented as the number of Brn3a-immunoreactive cells per field and are mean ± SEM of 5-7 independent experiments. *P < 0.05 and **P < 0.01 different from control; +P < 0.01 and ++P < 0.01, different from LPS; #P < 0.05 and ##P < 0.01 different from EHP; Kruskal-Wallis test, followed by Dunn's multiple comparison test.

2.5 Discussion

The present work demonstrates that the blockade of $A_{2A}R$ prevented retinal neuroinflammation and death of RGC in an *ex vivo* model of glaucoma. We exposed retinal organotypic cultures to LPS and EHP, which bolstered microglia reactivity, increased neuroinflammatory response and loss of RGCs. These two noxious conditions up-regulated the $A_{2A}R$ system, as typified by an increase in the extracellular levels of ATP and increased expression and density of $A_{2A}R$ in microglia. Concomitantly, the $A_{2A}R$ system critically contributed to the neuroinflammation and RGC death, since $A_{2A}R$ blockade prevented the activation of microglia, the production of pro-inflammatory cytokines and the death of RGCs.

We took advantage of retinal organotypic cultures, a suitable model to evaluate cellular and molecular signalling mechanisms in which retinal anatomy is maintained (Kretz et al., 2004), and which has been established as a convenient model for screening potential neuroprotective drugs in the retina (Bull et al., 2011). This *in vitro* system enabled us to demonstrate that EHP changed microglia morphology towards an amoeboid-like form, similar to that caused by LPS, which has been extensively used as a microglial activator. Activation of microglial cells is observed as an early event in animals models of glaucoma (Bosco et al., 2011; Naskar et al., 2002), in which increased IOP is a main risk factor (Caprioli, 2013). In retinal organotypic cultures, the observed EHP- and LPS-induced microglia reactivity was paralleled by an increased expression and release of the pro-inflammatory cytokines IL-1β and TNF. Likewise, an increased production of TNF (Tezel et al., 2001; Tezel and Wax, 2000) and IL-1β (Manni et al., 2005; Yoneda et al., 2001) have been observed in glaucomatous animal models and in human glaucoma. Furthermore, the ability of anti-IL1β and anti-TNF antibodies to prevent EHP-induced RGC death provided critical evidence that the death of RGCs upon exposure to EHP or LPS in retinal organotypic cultures actually resulted from the impact of pro-inflammatory cytokines. This is in agreement with previous reports demonstrating that the control of microglia reactivity (Bosco et al., 2012; Bosco et al., 2008; Wang et al., 2014; Yang et al., 2013) or of pro-inflammatory cytokines (Howell et al., 2011; Roh et al., 2012; Sivakumar et al., 2011) prevents the loss of RCG in animal models of glaucoma. Nevertheless, the release of IL-6 by astrocytes and microglia triggered by EHP was reported to protect RGCs (Sappington, 2006), although the authors used purified cultures of microglia, astrocytes and RGCs and did not evaluated the possible interactions between these cells in a more complex *in vitro* model, as the retinal organotypic culture.

The main conclusion of this study was the critical role of $A_{2A}R$ in the control of EHP- or LPS-induced microglia activation, production of pro-inflammatory cytokines and RGC death in retinal organotypic cultures. Indeed, we observed that the blockade of $A_{2A}R$ prevented the EHP- or LPS-induced modification of the production of pro-inflammatory cytokines and of NO as it was previously observed in the rodent hippocampus (Rebola et al., 2011). Accordingly, it was already

demonstrated that activation of $A_{2A}R$ potentiates NO release from reactive microglia in culture, an effect that was associated with microglia neurotoxicity, and $A_{2A}R$ antagonist was suggested as a potential neuroprotective drug (Saura et al., 2005). Moreover, we observed that $A_{2A}R$ blockade prevents EHP- induced microglia morphological alterations, in agreement with recent findings that $A_{2A}R$ antagonism reduces the retraction of processes in LPS-activated microglia (Gyoneva et al., 2014).

Interestingly, these conclusions seems to contradict previous studies reporting that the activation of $A_{2A}R$ reduces microglia reactivity using primary retinal microglia cultures exposed either to LPS, hypoxia or amadori-glycated albumin (Ahmad et al., 2013; Ibrahim et al., 2011a; Ibrahim et al., 2011b). Several factors may explain this discrepancy: 1) while others used cultures of microglial cells we used an organotypic retinal culture in which all retinal cells are present, and thus an additional contribution from other glial cells cannot be excluded (Ferrer-Martin et al., 2014; Johnson and Martin, 2008); this is particularly important given that the control by $A_{2A}R$ of microglia reactivity can be shifted from inhibitory to excitatory by the presence of increased extracellular levels of glutamate (Dai et al., 2010); 2) the insults triggering microglia activation are different and the LPS concentrations and time-points were different; and 3) CGS 21680, the $A_{2A}R$ agonist, at the concentration used in those studies (20 and 40 µM) is no longer selective, being proposed to bind also to A_1Rs (Casado et al., 2010; Halldner et al., 2004). The A_1R is coupled to $G_{i/o}$-proteins and often inhibitory, whereas the $A_{2A}R$ is usually coupled to G_s-proteins, enhancing cAMP accumulation and PKA activity (Fredholm et al., 2011). Nevertheless, the observation of different responses in different models should be taken in account due to the dual role of adenosine receptors and different responses of microglia, which can be elicited with different stimuli and environmental conditions (Blum et al., 2003). In fact, in the brain, it is the blockade rather than the activation of $A_{2A}R$ than reduce microglia activation and neuroinflammation upon different noxious stimuli (Li et al., 2008; Rebola et al., 2011). This probably contributes for the neuroprotection afforded by $A_{2A}R$ antagonists in brain diseases with a neuroinflammatory involvement such as ischemia, epilepsy, traumatic brain injury, multiple sclerosis, Alzheimer's or Parkinson's diseases (reviewed in Gomes et al., 2011). Accordingly, we also observed that $A_{2A}R$ blockade prevented the LPS- and the EHP-induced RGC death in retina organotypic cultures. This might result from the ability of $A_{2A}R$ to control the activation of microglia and the production of pro-inflammatory cytokines that we showed to be sufficient and necessary to trigger RGC death, but it may also involve an ability of $A_{2A}R$ to directly control neuronal viability. In fact, neuronal $A_{2A}R$ can directly affect the degeneration of mature neurons upon exposure to different stimuli (e.g. (Canas et al., 2009; Silva et al., 2007), namely to pro-inflammatory cytokines (Simões et al., 2012), whereas they have an opposite effect in immature neurons (Ferreira and Paes-de-Carvalho, 2001; Rebola et al., 2005) and during neuro-development (Silva et al., 2013).

The relevance of the A$_{2A}$R modulation system in the control of RGC death through a control of neuroinflammation in the retina is further underscored by the observed up-regulation of this system in retinal organotypic cultures exposed either to LPS or to EHP. In fact, LPS and EHP caused an increase of the extracellular levels of ATP. The cellular source of this extracellular ATP is not clear, but it can be released from different cells in the retina, such as RGCs (Xia et al., 2012), microglia (Imura et al., 2013) and Müller cells (Newman, 2003). Moreover, recent work demonstrated that astrocytes present in the optic nerve head can also release ATP through pannexin channels in response to a mechanical strain, suggesting this mechanism as a source of extracellular ATP under chronic mechanical strain, as occurs in glaucoma (Beckel et al., 2014). Actually, elevated levels of extracellular ATP have been reported in the retina as a response to an acute rise in ocular pressure (Reigada et al., 2008; Resta et al., 2007) and the ATP levels are elevated in the aqueous humour of patients with primary acute and chronic angle closure glaucoma, which presents evidence for a contribution of the purinergic signalling in this disease (Li et al., 2011; Zhang et al., 2007). The increased levels of ATP can function as a danger signal, and can either activate P2 receptors, namely P2X7 receptors in the retina (Hu et al., 2010; Niyadurupola et al., 2013; Sugiyama et al., 2013; Xia et al., 2012) or be extracellular catabolized by ecto-nucleotidases into extracellular adenosine that preferentially activates A$_{2A}$R (Augusto et al., 2013; Rebola et al., 2008). Remarkably, EHP and LPS not only bolstered the source of adenosine activating A$_{2A}$R, but also triggered an increased expression of A$_{2A}$R, which was translated into an increased density of A$_{2A}$R in microglia. This is in accordance with the up-regulation of A$_{2A}$R that is observed upon different noxious conditions (reviewed in Cunha, 2005; Gomes et al., 2011), namely in microglia (Gomes et al., 2013; Rebola et al., 2011; Santiago et al., 2014). Thus, noxious stimuli such as LPS or EHP triggered an up-regulation of the A$_{2A}$R system in retinal microglia, which critically contributes to the development of neuroinflammation and RGC death. We cannot rule out the role of A$_{2A}$R present in other cell types of the retinal organotypic culture, but in the GCL A$_{2A}$R was found to be mainly located in microglia.

2.6 Conclusions

The present results demonstrate that EHP can lead to an inflammatory response, similar to LPS, which is associated with the death of RGC. Thus, the organotypic retinal culture exposed to EHP may be an important experimental model to investigate neuroprotective and anti-inflammatory pharmacological strategies against RGC death. Herein we demonstrate for the first time that A$_{2A}$R blockade prevents retinal microglia reactivity and pro-inflammatory responses triggered by LPS or EHP, and confers neuroprotection to RGC by controlling retinal neuroinflammation induced by EHP or LPS. This prompts the hypothesis that A$_{2A}$R antagonists might have therapeutic potential in the treatment of glaucoma.

2.7 Acknowledgments

2.8 References

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CHAPTER 3 -

Selective A$_{2A}$ receptor antagonist prevents microglia-mediated neuroinflammation and protects retinal ganglion cells from high intraocular pressure-induced transient ischemic injury

NOTE: Work presented as published, with minor modifications.

3.1 Abstract

Glaucoma is a leading cause of vision loss and blindness worldwide, characterized by chronic and progressive neuronal loss. Reactive microglial cells have been recognized as a neuropathological feature, contributing to local inflammation and retinal neurodegeneration. In a recent *in vitro* work (organotypic cultures), we demonstrated that blockade of adenosine A$_{2A}$ receptor (A$_{2A}$R) prevents the neuroinflammatory response and affords protection to retinal ganglion cells (RGCs) against exposure to elevated hydrostatic pressure (EHP), to mimic elevated intraocular pressure (IOP), the main risk factor for glaucoma development. Herein, we investigated whether a selective A$_{2A}$R antagonist (SCH 58261) could modulate retinal microglia reactivity and their inflammatory response. Furthermore, we took advantage of the high IOP-induced transient ischemia (ischemia-reperfusion, I-R) animal model to evaluate the protective role of A$_{2A}$R blockade in the control of retinal neuroinflammation and neurodegeneration.

Primary microglial cell cultures were challenged either with lipopolysaccharide (LPS) or with EHP, in the presence or absence of A$_{2A}$R antagonist SCH 58261 (50 nM). Additionally, I-R-injury was induced in adult Wistar rats after intravitreal administration of SCH 58261 (100 nM, 5 µl).

Our results showed that SCH 58261 attenuated microglia reactivity and the increased expression and release of proinflammatory cytokines. Moreover, intravitreal administration of SCH 58261 prevented I-R induced-cell death and retinal ganglion cell (RGC) loss, by controlling microglial-mediated neuroinflammatory response.

These results prompt the proposal that A$_{2A}$R blockade may have great potential in the management of retinal neurodegenerative diseases characterized by microglia reactivity and RGC death, such as glaucoma and ischemic diseases.

3.2 Introduction

Glaucoma is group of ocular disorders with multifactorial etiology characterized by irreversible and progressive loss of retinal ganglion cells (RGCs) and degeneration of the optic nerve (Casson et al., 2012). It is the second leading cause of blindness in the world, affecting approximately 70 million people worldwide, and the main risk factors are advanced age and elevated intraocular pressure (IOP) (Qu et al., 2010). The mainstay in glaucoma treatment is lowering the IOP, but in some patients optic nerve damage and visual field defects progress despite successful IOP control (Chang and Goldberg, 2012). This has prompted the hypothesis that drugs targeting RGC neuroprotection, in addition to IOP-lowering agents, have potential to be used in the treatment of glaucoma.

Chronic neuroinflammation has been documented in glaucoma (Madeira et al., 2015a). Neuroinflammatory processes are orchestrated by reactive microglial cells, the immunocompetent cells of the central nervous system (CNS) (Daré et al., 2007; Kettenmann et al., 2011). Reactive microglial cells have been detected in the retina after high IOP-induced transient ischemia (Abcouwer et al., 2013; Zhang et al., 2005), upon elevated IOP (Bosco et al., 2015; Bosco et al., 2011) and in human glaucoma (Gramlich et al., 2013). A growing body of evidence demonstrates that microglial cells become reactive in the course of retinal degenerative diseases, having a pivotal role in the initiation and propagation of the neurodegenerative process (Karlstetter et al., 2015; Madeira et al., 2015a; Wang et al., 2015b). Interestingly, reactive microglial cells have been found in retinas contralateral to experimental glaucoma, leading the authors to suggest that it may reflect an attempt to maintain tissue homeostasis (de Hoz et al., 2013; Gallego et al., 2012; Ramirez et al., 2010; Rojas et al., 2014).

Activation of microglia is characterized by changes in cell morphology, signaling and gene expression, leading to alterations in the release of pro-inflammatory mediators, such as tumor necrosis factor (TNF), interleukin-1 beta (IL-1β) and nitric oxide (NO) (Bisogno and Di Marzo, 2010; Gyoneva et al., 2009; Karlstetter et al., 2010; Lee et al., 2008). Prolonged and excessive activation of retinal microglial cells has been associated with neuronal degeneration, in particular to the loss of RGCs (Bosco et al., 2012; Bosco et al., 2008; Fischer et al., 2015; Wang et al., 2015a), a feature of glaucoma. This boosts the proposal that therapeutic strategies designed at reducing microglia reactivity may offer beneficial effects for the management of retinal neurodegenerative diseases (Karlstetter et al., 2010; Madeira et al., 2015a).

Adenosine is a neuromodulator that activates four types of adenosine receptors (A$_1$, A$_{2A}$, A$_{2B}$ and A$_3$ receptors) (Fredholm et al., 2011). Adenosine A$_{2A}$ receptor (A$_{2A}$R) has been closely associated to the control of neurodegeneration (Cunha, 2005) since the blockade of A$_{2A}$R affords neuroprotection in several noxious conditions, such as in models of Alzheimer's

disease (Canas et al., 2009), Parkinson's disease (Cerri et al., 2014; Gyoneva et al., 2014), Machado-Joseph's ataxic disease (Goncalves et al., 2013) and ischemia (Chen et al., 1999). One mechanism proposed to explain the neuroprotection afforded by $A_{2A}R$ antagonism is through the control of microglia-mediated neuroinflammation (Blackburn et al., 2009; Santiago et al., 2014).

In a recent work, using organotypic retinal cultures exposed to elevated hydrostatic pressure (EHP), an *in vitro* system to model ocular hypertension, we demonstrated that $A_{2A}R$ blockade plays a critically important role in the protection of RGCs against EHP-induced loss by controlling neuroinflammation (Madeira et al., 2015b). Since microglial cells are endowed with $A_{2A}R$, in this study we aimed to investigate the ability of $A_{2A}R$ blockade in the control of retinal microglia reactivity elicited by lipopolysaccharide (LPS) or EHP. Additionally, we used the high IOP-induced transient ischemia animal model to evaluate the potential anti-inflammatory and neuroprotective properties of the intravitreal administration of a selective $A_{2A}R$ antagonist (SCH 58261).

3.3 Materials and Methods

3.3.1 Animals

Wistar rats were housed in certified local facilities, in a temperature and humidity controlled environment, and were provided with standard rodent diet and water *ad libitum*, under a 12 h light/12 h dark cycle. All procedures involving animals were approved by the Ethical Committee of the Faculty of Medicine of the University of Coimbra and are in agreement with the Association for Research in Vision and Ophthalmology statement for animal use.

3.3.2 Preparation of primary retinal cell cultures

Primary cell cultures were prepared from the retinas of 3-4 days old Wistar rats as described previously (Santiago et al., 2007). Cells were plated at a density of 2×10^6 cells/cm² and cultured for seven days at 37 °C in a humidified atmosphere of 5 % CO_2. The mixed primary cultures contain microglial cells, astrocytes and Müller cells, and retinal neurons (Santiago et al., 2007).

3.3.3 Preparation of primary cultures of retinal microglia

A mixed retinal cell culture was obtained from the retinas of 7-9 days old Wistar rats, as described previously (Liou et al., 2008; Santiago et al., 2007), with some modifications. Cells were plated at 1×10^6 cells/cm² on uncoated 12- or 6-well culture plates and maintained at 37 °C under humidified atmosphere of 5 % CO_2, for three weeks, in Dulbecco's Modified Eagle Medium (DMEM)-F12 with GlutaMAX I, supplemented with 10 % heat-inactivated FBS, 0.1 % gentamicin (all from Life Technologies, Carlsbad, USA) and 2 ng/ml macrophage-colony stimulating factor (M-CSF) (Peprotech, London, UK). Media were replaced every three days.

Retinal microglial cell cultures were obtained by mild trypsinization, as previously described (Saura et al., 2003). Briefly, after three weeks in culture, mixed cell cultures were incubated with Trypsin-EDTA solution (Sigma-Aldrich, St. Louis, USA) diluted 1:3 in DMEM-F12 for 45-60 min. The trypsinization resulted in the detachment of an upper layer, containing astrocytes, whereas the microglial cells kept attached to the culture plate. Microglial cells were cultured in DMEM-F12 with GlutaMAX I supplemented with 2 % heat-inactivated FBS and 0.1 % gentamicin for 3 days. The purity of the culture (93 %) was assessed by immunocytochemistry with anti-CD11b antibody. Each culture preparation was considered one independent experiment.

3.3.4 Retinal transient ischemic injury (ischemia-reperfusion, I-R)

Retinal I-R was performed in anesthetized animals (2.5 % isoflurane; IsoFlo, Abbott Laboratories, USA) following the procedure previously described (Martins et al., 2015). The IOP was increased in only one eye to 80 mmHg for 60 min and the contralateral eye was considered the control eye. In the end, the needle was withdrawn and reperfusion was established. In a different group of animals, eyes were cannulated and the pressure was maintained at normal IOP to serve as a normotensive controls (sham-operated). Animals were sacrificed 24 hours after ischemia. An independent experiment was defined as being the eye, taking into consideration that transient ischemia was induced in only one eye (equivalent to the number of animals per group).

3.3.5 Drug treatment

Cell cultures

Cells were challenged with LPS (100 ng/mL) or exposed to EHP (70 mmHg above atmospheric pressure) for 4 hours or 24 hours, as indicated in the figure legends. For the EHP experiments we used a custom-made humidified pressure chamber equipped with a pressure gauge and a regulator, which maintained a constant pressure, with an air mixture of 95 % air and 5 % CO_2, as described previously (Madeira et al., 2015b; Sappington and Calkins, 2006). The chamber was placed in a 37 °C oven. The magnitude of pressure elevation (70 mmHg above atmospheric pressure) was chosen in accordance with previous studies (Madeira et al., 2015b; Sappington and Calkins, 2006). For ambient pressure experiments, cell cultures were kept in a standard 5 % CO_2 cell incubator.

Prior LPS or EHP exposure (45 minutes), primary retinal microglial cells were incubated with the A2AR selective antagonist (50 nM SCH 58261, Tocris Bioscience, Bristol, UK).

Intravitreal drug administration

Intravitreal injections were performed 2 hours before injury under anesthesia (2.5 % isoflurane; IsoFlo, Abbott Laboratories, Chicago, USA) in Wistar rats (8 weeks old). Under the operating microscope a sclerotomy was created approximately 2 mm posterior to the limbus with a 30-gauge needle, taking special caution to avoid damaging the lens. Intravitreal administration of 5 µL saline (0.9 % NaCl) or SCH 58261 (100 nM) was performed in both I-R-injured and contralateral eyes with a 10 µL Hamilton syringe.

In neutralizing experiments, 5 µL of a solution containing rabbit anti-TNF (0.02 µg/mL; Peprotech, London, UK) and goat anti-IL-1β (0.1 µg/mL; R&D Systems, Minneapolis, USA) or of the isotype-matched controls were delivered to both eyes 1 hour before induction of ischemia.

3.3.6 Retinal cryosections

Animals were deeply anesthetized with an intraperitoneal injection of ketamine (90 mg/kg; Imalgene 1000; Merial, Los Condes, Chile) and xylazine (10 mg/kg; Ronpum 2%; Bayer, Leverkusen, Germany) and then transcardially perfused with phosphate buffered saline (PBS) followed by 4% (w/v) paraformaldehyde (PFA). The eyes were enucleated and post-fixed in 4% PFA for 1 hour. Then, the cornea was carefully dissected out and the eyecup was fixed for an additional 1 hour in 4 % PFA. After washing in PBS, the tissue was cryopreserved in 15 % sucrose in PBS for 1 hour followed by 30 % sucrose in PBS for 1 hour. The eyecups were embedded in tissue-freezing medium (Optimal Cutting Temperature, OCT; Shandon Cryomatrix, Thermo Scientific, Massachusetts, USA) with 30 % of sucrose in PBS (1:1), and stored at -80 °C. The tissue was sectioned on a cryostat (Leica CM3050 S, Solms, Germany) into 10 μm-thick sections and mounted on Superfrost Plus glass slides (Menzel-Glaser, Thermo Scientific, Massachusetts, USA).

3.3.7 Immunolabeling

Primary retinal cell cultures and retinal microglial cells were washed with PBS and fixed with 4 % PFA with 4 % sucrose for 10 minutes. After washing in PBS, cells were permeabilized in 1% Triton X-100 in PBS for 5 minutes. Blocking was performed with 3 % bovine serum albumin (BSA) and 0.2 % Tween 20 in PBS, for 1 hour, and then incubated with the primary antibody (Table 1), prepared in blocking solution, for 90 minutes at room temperature. Following washing, cells were incubated with the secondary antibody (Table 1), prepared in blocking solution, for 1 hour. The cells were then washed in PBS and incubated with the nuclear dye 4',6-diamidino-2-phenylindole (DAPI; 1:2000; Life Technologies, Carlsbad, USA) for 10 minutes. After washing the cells, the coverslips were mounted with Glycergel (DAKO, Glostrup, Denmark) mounting medium.

Retinal cryosections were fixed with ice-cold acetone at -20 °C for 10 minutes, and then rehydrated in PBS twice until OCT was removed. The tissue was permeabilized with 0.25 % Triton X-100 in PBS for 30 minutes. The sections were blocked in 10 % normal goat serum plus 1 % BSA in PBS for 30 minutes at room temperature in a humidified environment. After washing with PBS, the sections were incubated overnight with primary antibodies (Table 1), prepared in 1 % BSA in PBS at 4 °C, in a humidified environment. Then, the sections were rinsed in PBS followed by incubation with the corresponding secondary antibodies (Table 1), prepared in 1 % BSA in PBS, for 1 hour at room temperature, in the dark. The sections were washed with PBS and then the nuclei were stained with DAPI (1:2000). The tissue was washed in PBS and mounted with Glycergel mounting medium.

Table 1: List of primary and secondary antibodies used in immunolabeling

	Supplier	Host	Dilution
Primary antibodies			
Anti-A_{2A}R	Santa Cruz Biotechnology	Goat	1:50
Anti-CD11b	AbD Serotec	Mouse	1:100
Anti-iNOS	BD Biosciences	Rabbit	1:100
Anti- IL-1β	R&D Systems	Goat	1:100
Anti- TNF	Peprotech	Rabbit	1:100
Anti-Iba1	Wako	Rabbit	1:1000
anti-MHC class II	AbD Serotec	Mouse	1:200
Anti-Brn3a	Chemicon	Mouse	1:200
Secondary antibodies			
Alexa Fluor anti-mouse 568	Life Technologies	Donkey	1:200
Alexa Fluor anti-mouse 488	Life Technologies	Goat	1:200
Alexa Fluor anti-goat 488	Life Technologies	Rabbit	1:200
Alexa Fluor anti-rabbit 488	Life Technologies	Goat	1:200

3.3.8 Nitric oxide quantification by DAF-FM staining

The NO indicator DAF-FM diacetate (Molecular Probes, Invitrogen, Life Technologies, Carslbad, USA) was used to detect NO production, as we described previously (Socodato et al., 2015). Briefly, culture medium was collected and stored, and microglial cells were incubated with 5 µM DAF-FM diacetate in Krebs-Henseleit Ringer solution (in mM: 140 NaCl, 1 EDTA, 10 HEPES, 3 KCl, 5 glucose; pH 7.4) for 60 minutes. Then, the solution was replaced by the previously collected medium, and the cells were placed in the cell incubator until the end of the experiment. In order to define cell limits, cells were immunolabeled with an antibody anti-CD11b, as described above. Cells were observed with a confocal microscope (LSM 710, Zeiss, Jena, Germany) and densitometric analysis was performed as described below.

3.3.9 Nitrite quantification assay

The release of NO was indirectly assessed by quantifying nitrite concentration in the culture supernatants using the Griess reaction method. Culture media was centrifuged (10000 g for 10 minutes) and the supernatant stored at -80 °C, until use. The supernatant was incubated (1:1) with Griess reagent mixture (1 % sulfanilamide in 5 % phosphoric acid with 0.1 % N-1-naphtylenediamine) for 30 minutes, in dark conditions. Optical density was measured at 550 nm using a microplate reader (Synergy HT; Biotek, Winooski, USA). The nitrite concentration was determined from a sodium nitrite standard curve.

3.3.10 Phagocytosis assay

Retinal microglial cells were incubated with 0.0025% fluorescent latex beads (1 μm diameter) for 75 minutes at 37 °C, in the cell incubator. After incubation, an immunocytochemistry was performed in these cells with an anti-CD11b antibody, as described above. The preparations were observed with an inverted fluorescence microscope (Leica DMIRE2, Solms, Germany), and from each experimental condition, at least seven fields were randomly acquired.

Phagocytic efficiency was determined using the formula previously described (Boche et al., 2013; Pan et al., 2011):

$$Phagocytic\ efficiency\ (\%) = \left(\frac{(1 \times X1 + 2 \times X2 + 3 \times X3 .... + n \times Xn)}{total\ number\ of\ cells} \right) * 100$$

Xn represents the number of cells containing n beads (n = 1, 2, 3, ..., up to a maximum of 6 points for more than 5 beads engulfed per cell).

3.3.11 Real-time quantitative PCR

Total RNA was extracted from microglial cells using Qiagen RNeasy Mini Kit (Qiagen, Limburg, Netherlands), according to the instructions provided by the manufacturer. Total RNA was isolated from the rat retinas using Trizol reagent (Invitrogen, Life Technologies, Carslbad, USA). RNA samples were dissolved in 16 μL of Mili-Q water. Total RNA concentration was determined using NanoDrop ND1000 (Thermo Scientific, Waltham, MA, USA). The quality of total RNA was determined (2100 Bioanalyser, Agilent Technologies, Santa Clara, CA, USA) and the integrity of RNA, expressed as RNA Integrity Number (RIN) was between 7.7-9.6, indicating, a high quality, non-degraded RNA.

Amplification of cDNA was performed according to the instructions provided by the manufacturer, using 1 μg of total RNA. The resulting cDNA was treated with RNAse-H for 20 minutes at 37 °C, and a 1:2 dilution was prepared for qPCR analysis. All samples were stored at -20 °C until analysis. Genomic DNA contamination was assessed with a conventional PCR for β-

actin using intron-spanning primers (Table 2), as described previously (Santiago et al., 2009). SYBR-Green-based real-time quantitative PCR (qPCR) was performed using StepOnePlus (Applied Biosystems, Carlsbad, CA, USA). The PCR conditions were as follows: iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), 200 nM primers (Table 2), and 2 µL of 1:2 dilution cDNA, in a total volume of 20 µL. Cycling conditions were a melting step at 95 °C for 15 seconds and annealing-elongation at 60 °C for 45 seconds, and extension at 72 °C, with 40 cycles. A dissociation curve at the end of the PCR run was performed by ramping the temperature of the sample from 60 °C to 95 °C, while continuously collecting fluorescence data. Ct values were converted to "Relative quantification" using the $2^{-\Delta\Delta Ct}$ method previously described (Livak and Schmittgen, 2001). Four candidate housekeeping genes (*Gapdh*, *Hprt*, *Ywhaz* and *Rhodopsin*) were evaluated using NormFinder (a Microsoft Excel Add-in) (Andersen et al., 2004). *Yhwaz* and *Hprt* were the most stable genes for purified retinal microglial cells and rat retinas, respectively.

Table 2: Primers for qPCR analysis.

Gene	GeneBank number	Forward	Reverse
Adora2A	NM_053294	5' - GGCTATCTCTGACCAACA - 3'	3' - TGGCTTGACATCTCTAATCT - 5'
Tnf	NM_012675	5' - CCCAATCTGTGTCCTTCT - 3'	3' - TTCTGAGCATCGTAGTTGT - 5'
il-1β	NM_031512	5' - ATAGAAGTCAAGACCAAAGTG - 3'	3' - GACCATTGCTGTTTCCTAG - 5'
Nos II	NM_012611	5' - AGAGACAGAAGTGCGATC - 3'	3' - AGAGATTCAGTAGTCCACAATA - 5'
ywhaz	NM_013011.3	5' - CAAGCATACCAAGAAGCATTTGA - 3'	3' - GGGCCAGACCCAGTCTGA - 5'
hprt	XM_003752155	5' - ATGGGAGGCCATCACATTGT - 3'	3' - ATGTAATCCAGCAGGTCAGCAA - 5'
actin, beta	NM_031144.2	5' - GCTCCTCCTGAGCGCAAG - 3'	3' - CATCTGCTGGAAGGTGGACA - 5'

3.3.12 TNF and IL-1β quantification by Enzyme-Linked Immunosorbant Assay (ELISA)

Protein levels of IL-1β and TNF were quantified using ELISA, according to the instructions provided by the manufacturer (Peprotech, London, UK).

Culture media was collected and centrifuged (10000 *g* for 10 minutes) and the supernatant was stored at -80 °C until used for extracellular quantification. Retinal microglial cells and total retinas were lysed in 20 mM imidazole-HCl, 100 mM KCl, 1 mM MgCl$_2$, 1 mM

EGTA, 1 mM EDTA, 10 mM NaF, 1% Triton X-100, supplemented with protease and phosphatase inhibitors. Then, lysates were sonicated and centrifuged at 16000 g for 10 minutes at 4 °C and at 10000 g for 5 minutes at 4 °C, respectively. The supernatant was collected and stored at -80 °C until use. The cytokine concentration of each sample was normalized to the total protein concentration, which was determined by the bicinchoninic acid (BCA) protein assay according to the instructions provided by the manufacturer (Pierce Biotechnology, Waltham, MA, USA).

In animals with ocular hypertension, microglia activation has been described in the contralateral eye (Gallego et al., 2012). Therefore in order to take into account the possible contribution of cytokine production in contralateral eyes, the results obtained in I-R retinas were normalized to the contralateral eye.

3.3.13 Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay

Cell death in retinal slices was detected with a TUNEL assay kit with fluorescein detection following the instructions provided by the manufacturer (Promega, Madison, WI, USA). Nuclei were counterstained with DAPI (diluted 1:2000). Sections were washed in PBS and mounted with fluorescent mounting medium (Glycergel; Dako, Glostrup, Denmark).

3.3.14 Image acquisition and analysis

The preparations were observed with a confocal microscope (LSM 710, Zeiss, Jena, Germany) on an Axio Observer Z1 microscope an EC Plan-Neofluar 40x/1.30 Oil DIC M27 objective or Plan ApoChromat 20x/0.8 objective for cell cultures or retinal cryosections, respectively. Densitometric analysis of retinal microglia immunolabeling was performed using the public domain ImageJ program (http://imagej.nih.gov/ij/). The settings were kept identical for all the conditions and at least eight images per coverslip were randomly acquired. Corrected total cell fluorescence (CTCF) was calculated as previously described (Gavet and Pines, 2010) with the following formula:

CTCF=Integrated density - (area of selected cell x mean fluorescence of background)

For the analysis of microglia reactivity in retinal cryosections, we used four sections from each eye (n), and from each section we acquired 6 images. In each image, the number of cells immunoreactive to both Iba1 and MHC-II (Iba1^{+}MHC-II[+]) was counted and it was expressed in percentage of the total number of microglia (Iba1^{+}).

Cell death and the survival of RGCs were assessed by counting the number of TUNEL^{+} cells and the number of cells immunoreactive to Brn3a (Brn3a^{+}), respectively. From each eye,

four sections were used. The TUNEL[+] and Brn3a^{+} cells were counted in the entire length of each section and the results were expressed as the number of cells per mm.

3.3.15 Statistical analysis

The results are presented as mean ± standard error of the mean (s.e.m). The normality of the data was assessed with Shapiro-Wilk normality test. The data were analyzed using Kruskall-Wallis test, followed by Dunn's multiple comparison test, Mann-Whitney test, or Tukey's Multiple Comparison Test, as indicated in the figure legends. A level of confidence of 0.05 was considered. The statistical analysis was performed in Prism 6.0 Software for Mac OS X (GraphPad Software, Inc).

3.4 Results

We first tested in an *in vitro* model if A$_{2A}$R blockade controlled retinal microglia reactivity and neuroinflammation elicited by either LPS or elevated pressure, since neuroinflammation is a feature of glaucoma and elevated IOP is a major risk factor for the development of glaucoma. We then tested whether A$_{2A}$R antagonist could modulate microglia reactivity in an animal model of high IOP-induced transient ischemia (ischemia-reperfusion, I-R). In fact, acute IOP elevation leads to transient ischemia and triggers microglia reactivity and retinal cell death, including RGCs, and these models have been used to study neuroprotective strategies for retinal degenerative diseases, such as glaucoma (Abcouwer et al., 2013; Husain et al., 2011; Neufeld et al., 2002).

3.4.1 An inflammatory stimulus up-regulates A$_{2A}$R in retinal microglia

In primary retinal neural cultures, which we previously characterized to contain neurons, astrocytes, Müller cells and microglia (Gaspar et al., 2013; Santiago et al., 2009; Santiago et al., 2006), the immunoreactivity of A$_{2A}$R was mainly observed in microglia (labeled with an antibody anti-CD11b) (Fig. 1A) in control conditions and A$_{2A}$R immunoreactivity seems to be selectively increased in microglia in LPS-challenged cultures.
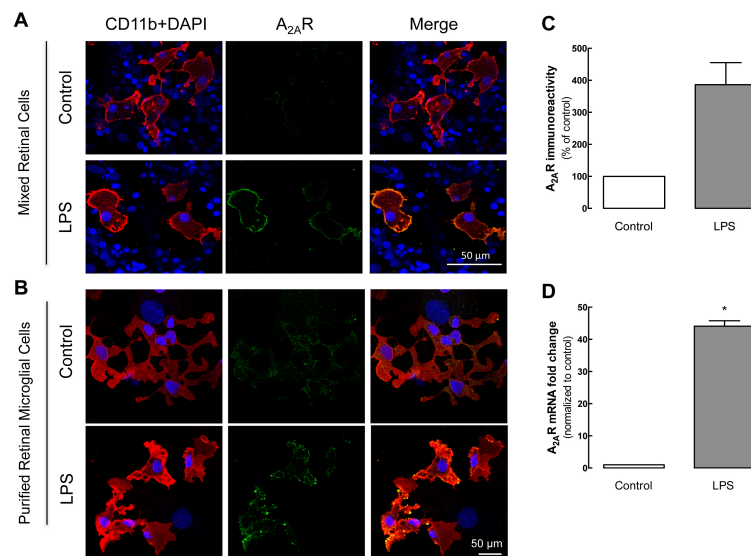

Fig. 1: An inflammatory stimulus up-regulates A$_{2A}$R in retinal microglia. Primary retinal cell cultures and purified retinal microglial cell cultures were challenged with LPS (100 ng/mL) for 24 hours. A: Representative images of primary mixed retinal cell cultures immunostained for A$_{2A}$R (green) and microglia (CD11b; red). Nuclei were stained with DAPI (blue). B: Representative images of each experimental condition in purified retinal microglial cells obtained from 4 independent experiments. Cultures were immunostained for A$_{2A}$R (green) and microglia (CD11b; red). Nuclei were stained with DAPI (blue). C: The immunoreactivity of A$_{2A}$R was quantified in retinal microglial cultures and are expressed as percentage of control from 3-4 independent experiments. D: A$_{2A}$R mRNA expression was assessed in purified retinal microglial cell cultures by qPCR and is presented as fold change of the control from 4 independent experiments. *$P < 0.05$ *vs.* control; Mann-Whitney test.

As observed in the neural retinal cultures, retinal microglial cells were immunoreactive to $A_{2A}R$, and an inflammatory condition elicited by exposure to LPS (100 ng/mL for 4 hours) increased $A_{2A}R$ immunoreactivity (Fig. 1B and 1C). Accordingly, $A_{2A}R$ mRNA expression was also significantly increased upon LPS incubation as compared with the control condition (44.1 ± 1.66 fold-increase) (Fig. 1D), as previously reported (Liou et al., 2008).

3.4.2 $A_{2A}R$ blockade decreases LPS-induced NO production and iNOS expression

Previous studies have shown that microglia produce and release high amounts of NO when exposed to LPS (Nakamura et al., 1999; Saura et al., 2005). Intracellular NO production was assessed by DAF-FM staining (Fig. 2A). LPS increased DAF-FM fluorescence (Fig. 2A and 2B) in retinal microglia cells (149.8 ± 18.23 % of the control). SCH 58261 prevented the increase in DAF-FM fluorescence induced by LPS (91.9 ± 2.24 % of the control), suggesting that $A_{2A}R$ blockade prevents NO production.
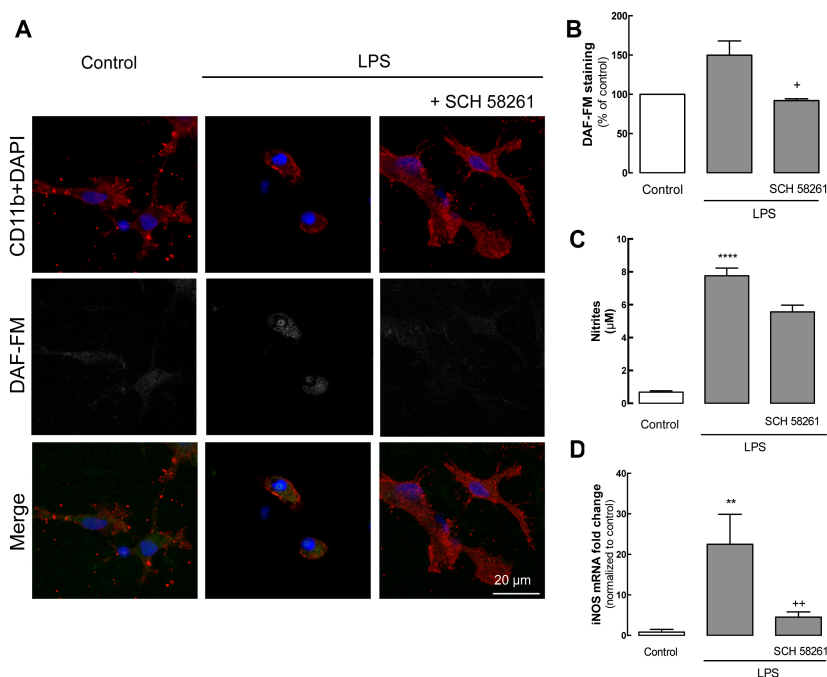

Fig. 2: $A_{2A}R$ blockade decreases LPS-induced NO production and iNOS expression in purified retinal microglial cell cultures. Retinal microglial cells were incubated with SCH 58261 (50 nM, $A_{2A}R$ antagonist) before challenge with LPS (100 ng/mL) for 24 hours. A: The production of NO was assessed by quantifying DAF-FM staining (green) in microglial cells (stained by CD11b; red). Nuclei were stained with DAPI (blue). B: Densitometric analysis of DAF-FM staining from 4 independent experiments. C: The release of NO was assessed by Griess reaction from 5-8 independent experiments. D: iNOS mRNA expression was assessed by qPCR from 4-5 independent experiments and is presented as fold change of the control. **$P < 0.01$ and ****$P < 0.0001$ vs. control; [+] $P < 0.05$ and [++] $P < 0.01$ vs. LPS; Kruskall-Wallis test, followed by Dunn's multiple comparison test.

The release of NO to the culture medium was assessed using the Griess reaction (Fig. 2C). In control conditions, the concentration of nitrites presented in the culture medium was 0.7 ± 0.08 μM. LPS significantly increased the extracellular nitrite concentration to 7.7 ± 0.46 μM, which was decreased by the presence of the $A_{2A}R$ antagonist to 5.6 ± 0.41 μM. The treatment of retinal microglial cells with SCH 58261, without LPS, did not alter the levels of NO (data not shown).

Additionally, we investigated the expression of iNOS, known to be the main enzyme involved in the production of NO by inflammatory stimuli (Saura et al., 2005; Sierra et al., 2014). LPS significantly increased iNOS expression by 22.5 ± 7.3 fold (Fig. 2D) and SCH 58261 significantly decreased iNOS expression, in agreement with the ability of SCH 58261 in decreasing NO production by microglia.

3.4.3 A₂ₐR blockade prevents LPS-induced expression and release of inflammatory mediators

Microglial cells detect and respond to inflammatory triggers by changing to a reactive phenotype, typified by the release of inflammatory factors, such as IL-1β or TNF (Block and Hong, 2005). In control conditions, the immunoreactivity of IL-1β or TNF was barely detected in cultured retinal microglia (Fig. 3A). The exposure to LPS significantly increased the immunoreactivity of IL-1β by 247 ± 7.8 % and TNF by 2162.7 ± 412.6 % of the control (Fig. 3A and 3B); SCH 58261 significantly decreased the LPS-induced increase in TNF immunoreactivity, without altering IL-1β immunoreactivity. LPS also increased the transcript levels of IL-1β and TNF and SCH 58261 prevented the increase elicited by LPS of both IL-1β and TNF mRNA (Fig. 3C).

Protein levels of IL-1β and TNF were quantified by ELISA in cell extracts (intracellular, Fig. 3D) and in the culture medium (extracellular, Fig. 3E). In control conditions, the intracellular levels of IL-1β and TNF were 0.32 ± 0.11 pg/μg of protein and 1.48 ± 0.57 pg/μg of protein, respectively. LPS increased the intracellular levels of IL-1β to 2.26 ± 0.25 pg/μg of protein and TNF to 374.21 ± 78.1 pg/μg of protein. In the presence of SCH 58261 and LPS, the protein levels of IL-1β and TNF were 239.5 ± 9.6 and 0.6 ± 0.1 pg/μg of protein. The extracellular levels of IL-1β and TNF in control conditions were 0.18 ± 0.12 and 0.13 ± 0.08 pg/μg of protein, respectively, which increased to 1.33 ± 0.3 pg/μg of protein and 2.95 ± 0.78 pg/μg of protein, following incubation with LPS; IL-1β levels were significantly reduced in the presence of SCH 58261 and LPS of 1.03 ± 0.12 pg/μg of protein, while TNF levels were 0.53 ± 14 pg/μg of protein.
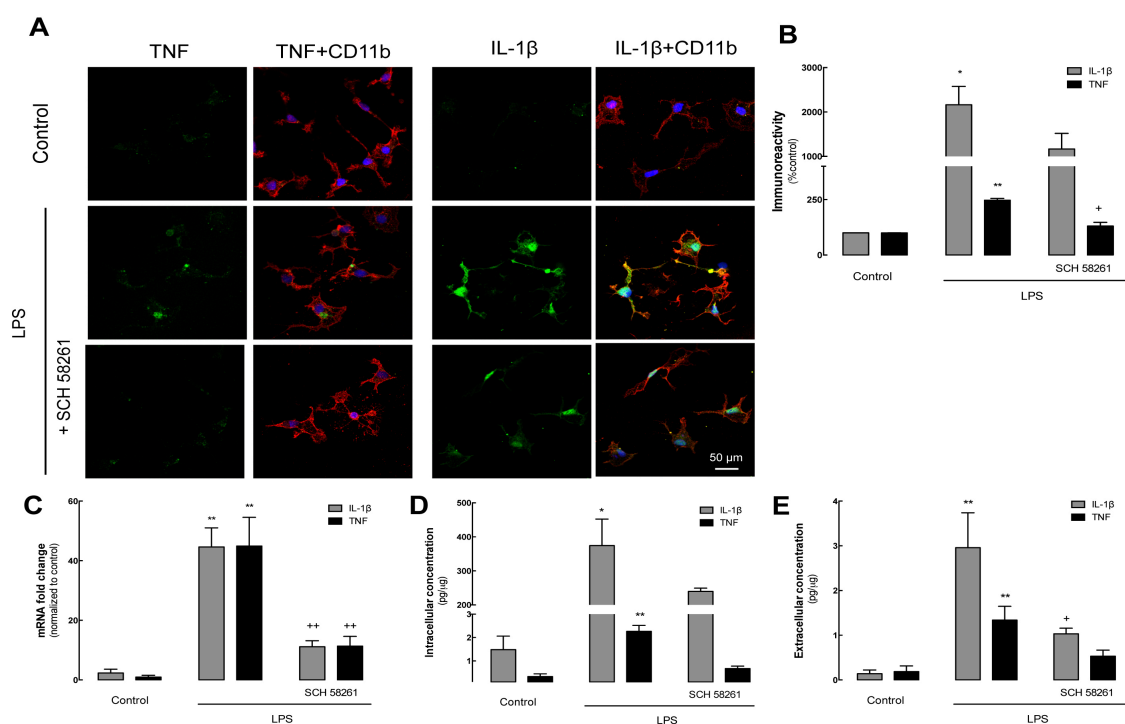
Fig. 3: A$_{2A}$R blockade of prevents the expression and release of TNF and IL-1β. Retinal microglial cells were challenged with LPS (100 ng/mL) for 4 hours with or without pretreatment with the A$_{2A}$R antagonist SCH 58261 (50 nM). A: Cells were immunostained for TNF or IL-1β (green) and microglia (CD11b; red). Nuclei were stained with DAPI (blue). Representative images obtained from 3 independent experiments. B: Densitometric analysis of TNF and IL-1β staining from 4-5 independent experiments. C: TNF and IL-1β mRNA expression was assessed by qPCR and is presented as fold change of the control from 4-5 independent experiments. D and E: Intra- and extracellular protein levels, respectively, of IL-1β and TNF were assessed by ELISA and are expressed in pg/μg of protein from 3-6 independent experiments. * $P < 0.05$ and ** $P < 0.01$ *vs.* control; + $P < 0.05$ and ++ $P < 0.01$ *vs.* LPS; Kruskall-Wallis test, followed by Dunn's multiple comparison test.

3.4.4 A$_{2A}$R blockade prevents LPS-induced phagocytosis by microglial cells

The functional behaviour of retinal microglial cells was evaluated by assessing phagocytosis of fluorescent latex beads (Figs. 4A and 4B). In control conditions, few microglia incorporated beads. LPS increased the number of beads per cell and the number of cells with beads, reflected by a significant increase in phagocytic efficiency (1.85-fold increase). SCH 58261 significantly decreased microglia phagocytosis induced by LPS (Fig. 4).
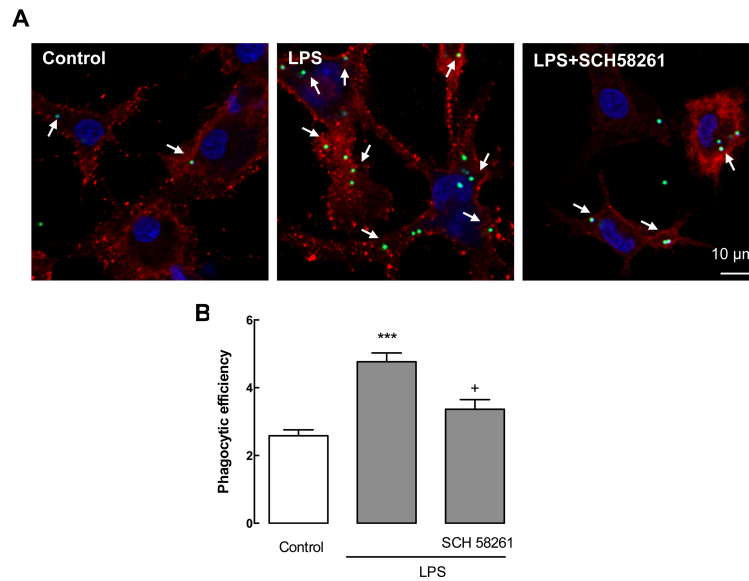
Fig. 4: A$_{2A}$R blockade attenuates the increase in phagocytic activity induced by LPS in microglial cells. Retinal microglial cells were challenged with 1 μg/mL LPS for 24 hours, in the absence or presence of the A$_{2A}$R antagonist SCH 58261 (50 nM). Before the end of the incubation (75 min), cells were incubated with green fluorescent latex beads (1 μm diameter). A: Cultures were immunostained for microglia (CD11b; red). Nuclei were stained with DAPI (blue). Arrows show some beads engulfed by microglia. B: Quantification of phagocytic efficiency from 5 independent experiments. *** $P < 0.001$ *vs.* control; + $P < 0.05$ *vs.* LPS; Kruskall-Wallis test, followed by Dunn's multiple comparison test.

3.4.5 A$_{2A}$R blockade prevents EHP-induced retinal microglial cell activation

Elevated IOP is a major risk factor for glaucoma development (Agarwal et al., 2008), and recent studies have demonstrated that microglial cells have an important role in the pathogenesis of glaucoma (Bosco et al., 2011; Johnson and Morrison, 2009). Since our previous results with LPS showed that A$_{2A}$R blockade decreased microglia reactivity induced by inflammatory conditions, we tested if this also occurred with EHP (to mimic elevated IOP).

We first evaluated if the exposure to EHP altered A$_{2A}$R density and expression by immunocytochemistry (Fig. 5A and 5B) and qPCR (Fig. 5C). As observed with LPS, the exposure of retinal microglia to EHP also up-regulated A$_{2A}$R. Thus, EHP increased A$_{2A}$R immunoreactivity by 231.23 ± 37.8 % (Fig. 5A and 5B) and A$_{2A}$R mRNA expression by 6.65 ± 2.4 fold (Fig. 5C) compared with control cells. Moreover, the exposure of retinal microglia to EHP increased the transcript levels for TNF, IL-1β and iNOS, as determined by qPCR, which was attenuated by SCH 58261 (Fig. 5D). This indicates that A$_{2A}$R blockade controls retinal microglia reactivity triggered by EHP.
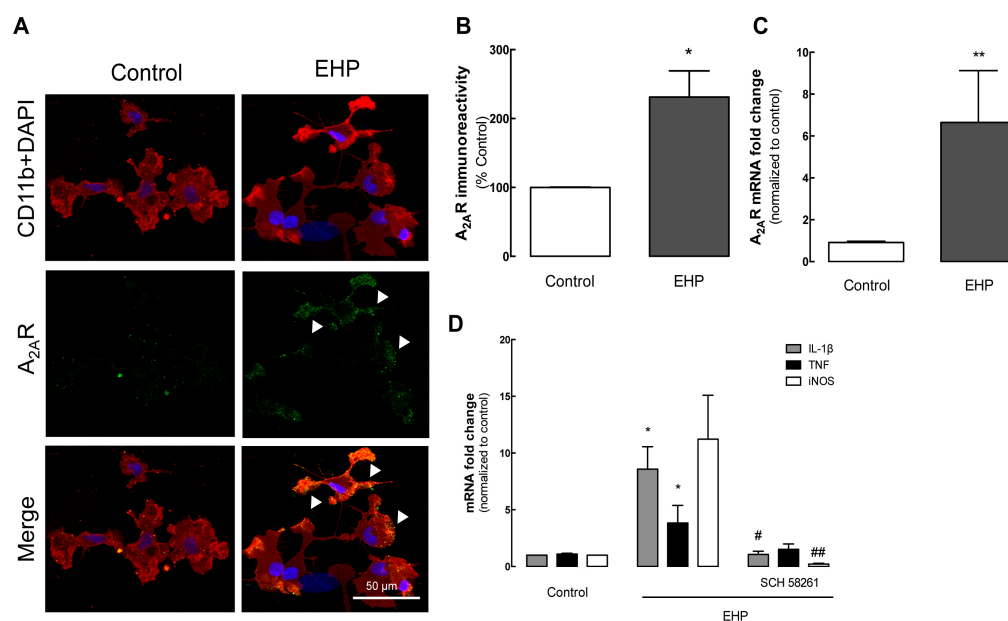
Fig. 5: A$_{2A}$R blockade prevents EHP-induced retinal microglial cell activation. Retinal microglial cells challenged with EHP (+70 mmHg) for 4 hours. A: Cultures were immunostained for A$_{2A}$R (green) and microglia (CD11b; red). Nuclei were stained with DAPI (blue). Arrowheads show A$_{2A}$R immunoreactivity. Representative images obtained from 3 independent experiments. B: Densitometric analysis of A$_{2A}$R staining from 3 independent experiments. C: A$_{2A}$R mRNA expression was assessed in the purified retinal microglial cells by qPCR and is presented as fold change of the control from 4 independent experiments. * $P < 0.05$ and ** $P < 0.01$, different from control; Mann-Whitney test. D: Effect of EHP on the transcript levels of inflammatory mediators in purified retinal microglial cells, evaluated in the absence or presence of the A$_{2A}$R antagonist (SCH 58261, 50 nM). mRNA expression of pro-inflammatory cytokines was assessed by qPCR and is presented as fold change of the control from 4-5 independent experiments. * $P < 0.05$ vs. control; # $P < 0.05$ and ## $P < 0.01$ vs. EHP; Kruskall-Wallis test, followed by Dunn's multiple comparison test.

3.4.6 A$_{2A}$R blockade prevents retinal microglial activation and inflammatory response induced by high IOP-induced transient ischemia[1]

Using the purified retinal microglial cell culture we have demonstrated that blockade of A$_{2A}$R can prevent retinal microglial cell reactivity in response to inflammatory conditions and to EHP. These observations boost the potential neuroprotective effects of the antagonist of A$_{2A}$R in an animal model of glaucoma, by reducing the neuroinflammatory response.

In order to study whether the intravitreal administration of the tested A$_{2A}$R antagonist controlled the inflammatory response in the retina, we took advantage of the high IOP-induced transient ischemia animal model, characterized by increased microglia reactivity, release of neurotoxic mediators and cell death (Davies et al., 2006; Dorfman et al., 2013; Gesslein et al., 2010). As expected, transient ischemia increased microglia reactivity, as gauged by the increase in the number of cells immunoreactive to MHC-II (Supplementary Fig. 1A and 1B), a marker of

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[1] These results are part of the Master Thesis: Boia, R. "Modulation of pro-inflammatory response of retina by adenosinergic systems" (2013), University of Coimbra, and therefore are presented as supplementary figures.

microglia activation (Roy, 2006). In the retinas treated with the $A_{2A}R$ antagonist and subjected to I-R, the number of reactive microglia ($Iba1^+MHC-II^+$ cells) was significantly decreased, when compared with the saline-treated I-R retinas (Supplementary Fig. 1A and 1B). The administration of SCH 58261 to the contralateral non-ischemic retinas did not change the number of reactive microglia.

Since $A_{2A}R$ blockade was able to decrease microglia activation induced by high IOP, we then assessed the effects of $A_{2A}R$ blockade in the mRNA (Supplementary Fig. 1C) and protein (Supplementary Fig. 1D) levels of TNF and IL-1β. When comparing with the contralateral retinas (non-ischemic), IL-1β mRNA was significantly increased by 25.6 ± 6.9-fold and TNF mRNA was also significantly increased by 7.4 ± 1.52-fold in the saline-treated I-R retinas. SCH 58261 attenuated the I-R-induced increase in IL-1β mRNA expression, but it did not prevent the increase in TNF mRNA expression induced by I-R. No significant alterations were found in the transcript levels for IL-1β and TNF in sham-operated retinas (not shown).

Retinal protein levels of IL-1β and TNF were quantified in the retina by ELISA (Supplementary Fig. 1D). IL-1β protein levels were markedly increased in the I-R injured retinas (corresponding to a ratio of 3.4 ± 0.9 comparing with contralateral non-ischemic retinas), and SCH 58261 significantly decreased IL-1β levels. TNF levels were also increased in the retinas subjected to I-R (ratio of 1.8 ± 0.3) in saline-treated group, but the treatment with $A_{2A}R$ antagonist did not significantly attenuate the increased TNF levels.

3.4.7 $A_{2A}R$ blockade prevents cell death and RGC loss through control of neuroinflammation [2]

The potential neuroprotective properties of $A_{2A}R$ blockade in the I-R injured retinas were assessed by TUNEL assay (Supplementary Fig. 2A and 2D). Saline-treated I-R retinas presented a significant increase in the number of $TUNEL^+$ cells (26.8 ± 4.6 $TUNEL^+$ cells/mm), compared with non-ischemic contralateral eye (0.9 ± 0.7 $TUNEL^+$ cells/mm,). The intravitreal injection with SCH 58261 significantly decreased the I-R-induced cell death (3.0 ± 1.3 $TUNEL^+$ cells/mm). Intravitreal injection of $A_{2A}R$ antagonist in the contralateral retinas (non-ischemic) did not modify cell death (0.3 ± 0.3 $TUNEL^+$ cells/mm).

The loss of RGCs triggered by I-R injury was evaluated by staining with an antibody raised against Brn3a, specifically expressed by RGCs and well established to assess RGC loss (Nadal-Nicolas et al., 2012; Nadal-Nicolas et al., 2009; Nadal-Nicolas et al., 2015) (Supplementary Fig. 2B and 2E). I-R injury significantly decreased the number of RGCs (4.9 ± 1.1 $Brn3a^+$ cells/mm) comparing with the non-ischemic contralateral saline-treated retinas (22.3 ± 2.4 $Brn3a^+$

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[2] These results are part of the Master Thesis: Boia, R. "Modulation of pro-inflammatory response of retina by adenosinergic systems" (2013), University of Coimbra; and therefore presented as supplementary figure.

cells/mm). In SCH 58261-treated I-R retinas, the number of Brn3a$^+$ cells was significantly higher (16.7 ± 3.2 Brn3a$^+$ cells/mm), indicating that SCH 58261 afforded protection to RGCs. Treatment with SCH 58261 alone did not alter the number of RGCs (25.9 ± 1.6 Brn3a$^+$ cells/mm).

To grasp if the control of neuroinflammation might mediate the neuroprotective effects of A$_{2A}$R blockade on RGC survival, TNF and IL-1β neutralizing antibodies were injected intravitreally prior I-R (Supplementary Fig. 2C and 2E). TNF and IL-1β neutralizing antibodies attenuated RGC loss induced by I-R, whereas they were devoid of effects in saline-treated contralateral retinas. Moreover, the injection with IgG isotype control did not significantly alter the number of RGCs after I-R injury (data not shown).

3.5 Discussion

In the present work, we showed that a selective $A_{2A}R$ antagonist prevented retinal microglia reactivity and neuroinflammation triggered by exposure to LPS and EHP (*in vitro*) and by I-R injury (animal model) and attenuated RGCs death after I-R injury.

Previous studies reported that $A_{2A}R$ is mainly detected in the GCL (Kvanta et al., 1997), and more recently we, and others, identified the expression of $A_{2A}R$ in retinal microglia (Liou et al., 2008; Madeira et al., 2015b). We now observed that $A_{2A}R$ immunoreactivity was mainly confined to microglia in primary mixed retinal neural cell cultures, where several cell types are present (Santos-Carvalho et al., 2013). Moreover, $A_{2A}Rs$ are up-regulated in microglia upon noxious stimuli (George et al., 2015; Rebola et al., 2011; Yu et al., 2008), including LPS in retinal microglia (Liou et al., 2008; Madeira et al., 2015b). Similarly to what we have previously described in retinal organotypic cultures (Madeira et al., 2015b), we now observed that EHP, used to mimic elevated IOP, also up-regulated $A_{2A}R$ in retinal microglial cell cultures.

Elevated IOP triggers retinal microglia activation and a concomitant increase in inflammatory mediators in the retina (Abcouwer et al., 2013; Bosco et al., 2011; Zhang et al., 2005). Abnormal accumulation and reactivity of microglial cells in the retina could result in exacerbated inflammatory responses that can be deleterious to RGCs (Roh et al., 2012; Sivakumar et al., 2011; Thanos, 1991). Indeed, when we neutralized the actions of TNF and IL-1β (by intravitreal injection of anti-TNF and anti-IL-1β antibodies) the loss of RGCs was attenuated, indicating that controlling inflammation contributes to RGC survival. Furthermore, here we observed that $A_{2A}R$ blockade inhibited retinal microglia reactivity (both in the *in vitro* and animal model) and conferred protection to the retina against I-R injury, supporting the hypothesis that controlling microglia reactivity contributes to the ability of $A_{2A}R$ antagonists of acting as neuroprotectors in different neurodegenerative diseases known to involve neuroinflammation (reviewed previously Gomes et al., 2011; Santiago et al., 2014). This might involve the particular ability of $A_{2A}R$ to control the formation and release of cytokines such as IL-1β and/or TNF, as previously observed in different brain preparations (Dai et al., 2010; Orr et al., 2009; Rebola et al., 2011; Simões et al., 2012; Yu et al., 2008). Interestingly, we observed that $A_{2A}R$ blockade decreased the release of TNF and IL-1β from cultured microglia, whereas $A_{2A}R$ blockade only prevented the I-R injury-induced increased expression and production of IL-1β, without altering the levels of TNF. These findings may be in line with previous reports demonstrating that $A_{2A}R$ blockade prevented the neurotoxicity of IL-1β and quinolinic acid, but not TNF and quinolinic acid (Stone and Behan, 2007). Despite microglial cells being the main producers of cytokines, in the I-R model we did not determine the cell source of cytokines. Activated astroglial cells also produce and release inflammatory mediators upon transient ischemia (Dvoriantchikova et al.,

2009) and may respond to mechanical stress (Beckel et al., 2014). Nevertheless, $A_{2A}R$ may not modulate the activation of astroglial cells upon retinal I-R injury, as described in different models of neurodegeneration (Matos et al., 2012a; Matos et al., 2012b).

Contrary to our findings, previous studies have reported that activation of $A_{2A}R$ confers protection to the retina by attenuating the inflammatory response (Ahmad et al., 2013; Konno et al., 2006; Roh et al., 2012; Sivakumar et al., 2011). However, it has been described that the actions mediated by $A_{2A}R$ in the CNS could be bidirectional (protective and deleterious) (reviewed previously Dai and Zhou, 2011). One possible explanation for this discrepancy is the different routes of administration, doses and exposure times of $A_{2A}R$ agonists and antagonists. In the hippocampus, peripheral administration of the $A_{2A}R$ agonist CGS21680 afforded protection against kainate-induced excitotoxicity, while the direct injection of CGS21680 failed to confer protection (Jones et al., 1998). In the current work, we administered the $A_{2A}R$ antagonist intravitreally, to circumvent peripheral effects, while others have administered the drug intraperitoneally. Also, different injuries and different stages of the pathological processes after injury may explain the different outcome of the actions mediated by either the activation or blockade of $A_{2A}R$.

The increase in the inflammatory response induced by EHP suggests a mechanosensitive role for retinal microglial cells, which become activated under mechanical stress, releasing inflammatory mediators. Similar to LPS, $A_{2A}R$ blockade prevented the enhanced inflammatory response elicited by EHP. Thus, we hypothesized that $A_{2A}R$ activation is directly involved in retinal microglia reactivity triggered by inflammatory and mechanical stimuli and, consequently, plays a pivotal role in microglia mediated-inflammatory responses.
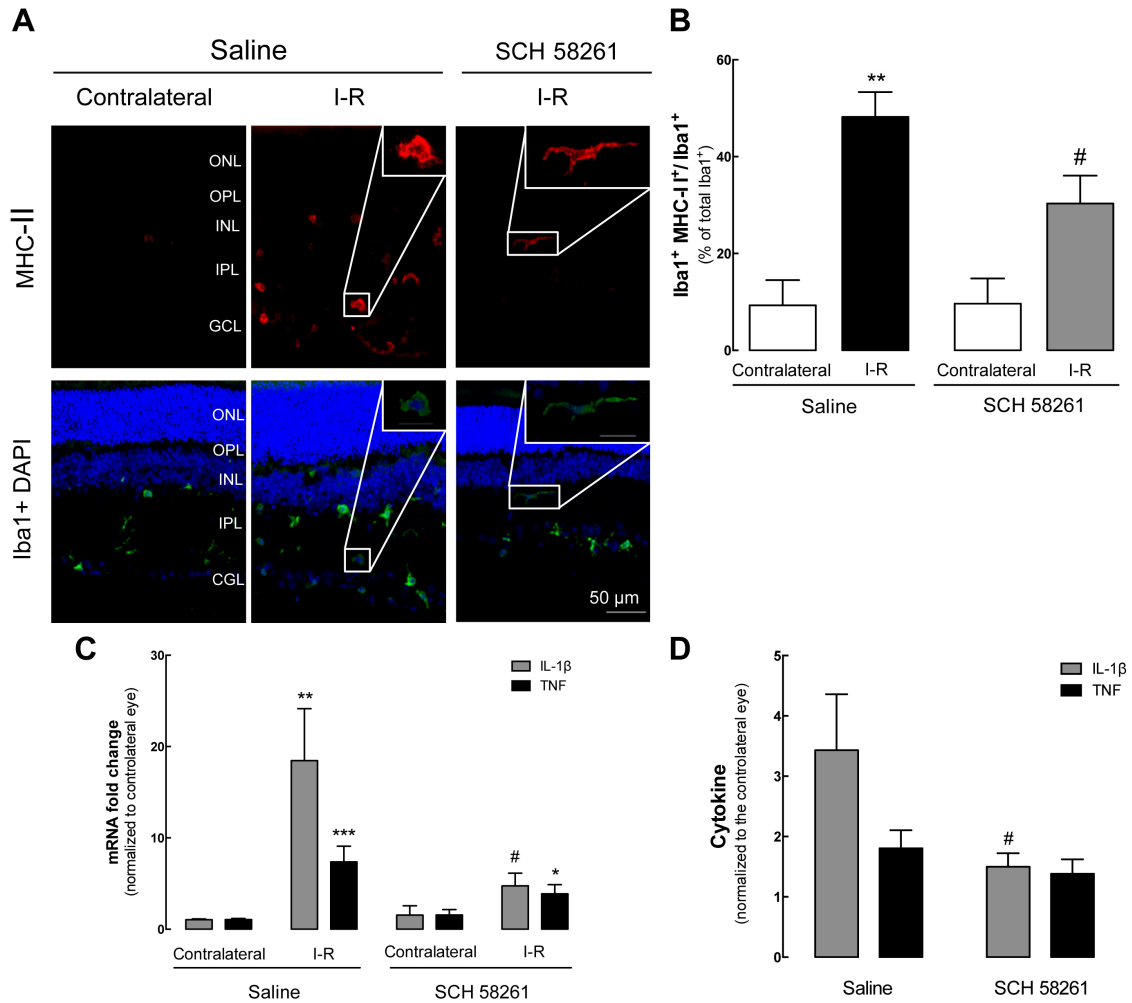
In summary, we now provide evidence that $A_{2A}R$ blockade dampens microglia reactivity and neuroinflammatory response in the retina following high IOP-induced transient ischemia. Furthermore, $A_{2A}R$ blockade prevented retinal cell death and RGC loss through the control of neuroinflammatory response. Therefore, this work demonstrates that $A_{2A}R$ blockade may be envisaged as a potential therapeutic strategy for the treatment retinal diseases involving microglia-mediated neuroinflammation, as is the case of glaucoma.

3.6 Acknowledgments

3.7 Supplementary figures

Supplementary Fig. 1: A$_{2A}$R blockde prevents microglia activation and neuroinflammatory response triggered by retinal I-R injury. Animals were injected in the vitreous with saline or with the A$_{2A}$R antagonist SCH 58261 (100 nM, 5 μL) 2 hours before I-R and were sacrificed 24 hours post ischemia. A: Microglial cell reactivity was assessed in retinal sections immunostained for microglia (Iba1; green) and activated microglia (MHC-II; red). Nuclei were stained with DAPI (blue). Representative images show different experimental conditions of 5-7 independent experiments. B: Activated microglia/macrophages (Iba1$^+$MHC-II$^+$-immunoreactive cells) were expressed as the ratio Iba1$^+$MHC-II$^+$/Iba1$^+$. Results are mean ± s.e.m. of 5-7 animals. C: The mRNA expression of IL-1β and TNF was assessed by qPCR are presented as fold change of the contralateral eye from 8-9 independent experiments. D: The protein levels of IL-1β and TNF were quantified by ELISA and are expressed as the ratio of the I-R eye relatively to the contralateral eye from 5-13 independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ *vs.* contralateral eye; # $P < 0.05$ *vs.* saline-treated I-R retinas; Mann Whitney test; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer.
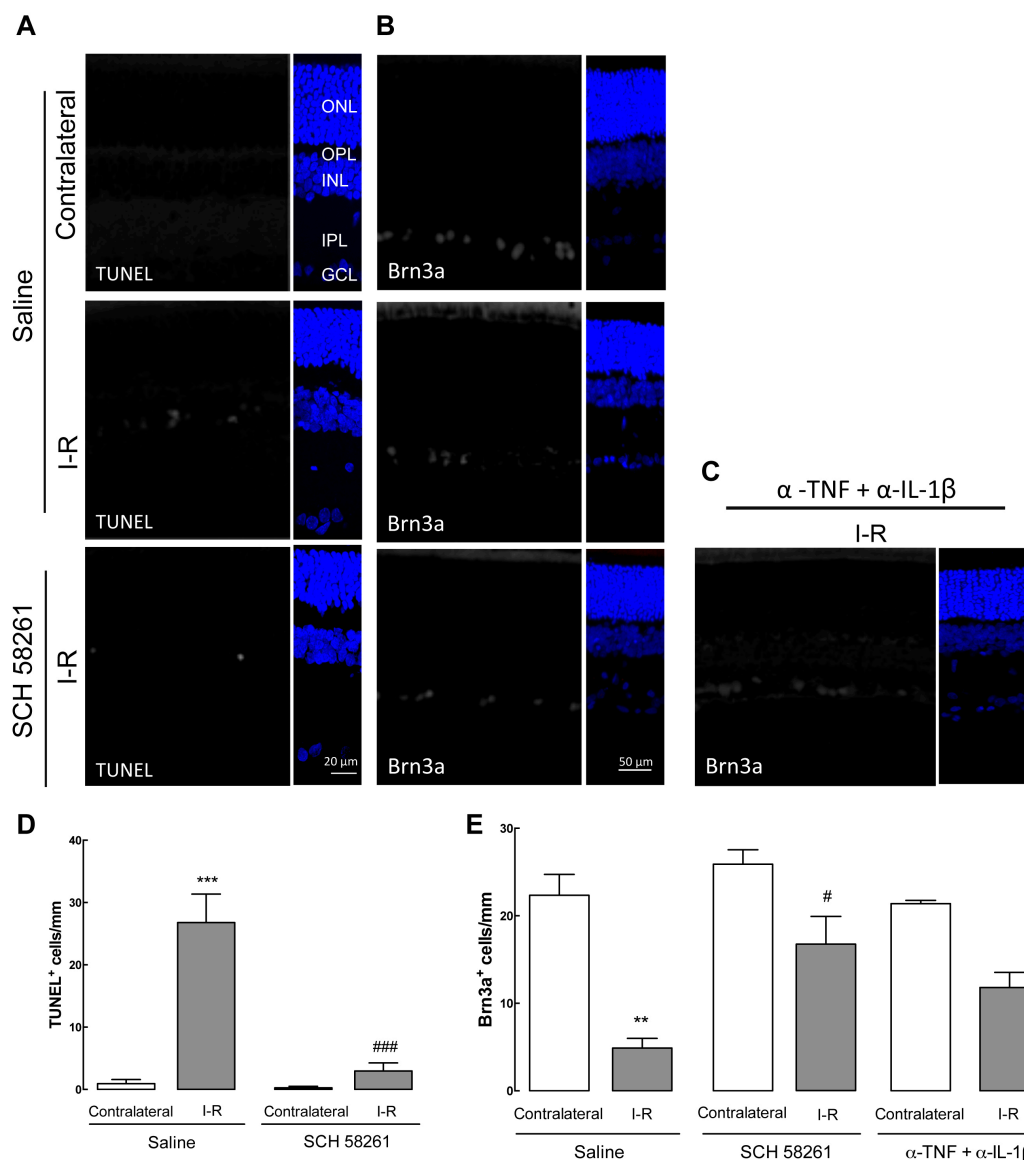
Supplementary Fig. 2: A$_{2A}$R blockade attenuates retinal neuronal cell loss and RGC loss through a control of neuroinflammation. Vehicle or the A$_{2A}$R antagonist (SCH 58261, 100 nM, 5 μL) were intravitreally injected 2 hours before I-R, and the animals were sacrificed 24 hours post ischemia. Retinal section were analyzed with a TUNEL assay (grey) to quantify cell death (A) or with an antibody against Brn3a (grey) (B, C) without or with SCH 58261 (A, B) or after treatment with neutralizing antibodies prior to I-R injury (A, C). Nuclei were stained with DAPI (blue). D: TUNEL$^+$ cells (grey) were counted and were expressed per mm of retina. E: Brn3a$^+$ cells (grey) were counted and were expressed per mm of retina. The results are mean ± s.e.m. of 2-4 independent experiments. ** $P < 0.01$ and *** $P < 0.001$ *vs.* contralateral eye; # $P < 0.05$ and ### $P < 0.001$ *vs.* saline-treated I-R retinas, Tukey's Multiple Comparison Test. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer.

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CHAPTER 4 -

Caffeine administration prevents retinal neuroinflammation and loss of retinal ganglion cells in an animal model of glaucoma

Maria H. Madeira, Arturo Ortin-Martinez, Francisco M. Nadal-Nícolas, António F. Ambrósio, Manuel Vidal-Sanz, Marta Agudo-Barriuso, Ana R. Santiago (2016). Caffeine administration prevents retinal neuroinflammation and loss of retinal ganglion cells in an animal model of ocular hypertension. Submitted to Scientific Reports.

4.1 Abstract

Glaucoma is the second leading cause of blindness worldwide, being characterized by progressive optic nerve damage and loss of retinal ganglion cells (RGCs), accompanied by increased inflammatory response involving retinal microglial cells. The etiology of glaucoma is still unknown, and despite elevated intraocular pressure (IOP) being the main risk factor, the exact mechanisms responsible for RGC degeneration remain unknown.

Caffeine, which is an antagonist of adenosine receptors, is the most widely consumed psychoactive drug in the world. Several evidences suggest that caffeine can attenuate the neuroinflammatory responses and afford protection upon central nervous system (CNS) injury.

We took advantage of a well characterized animal model of glaucoma to investigate whether caffeine administration controls neuroinflammation and elicits neuroprotection.

Herein, we show that caffeine is able to partially decrease the IOP in ocular hypertensive animals. More importantly, we found that drinking caffeine prevented retinal microglia-mediated neuroinflammatory response and attenuated the loss of RGCs in animals with ocular hypertension (OHT).

This study opens the possibility that caffeine or adenosine receptor antagonists might be a therapeutic option to manage RGC loss in glaucoma.

4.2 Introduction

Glaucoma is a group of progressive neurodegenerative multifactorial diseases, characterized by the loss of retinal ganglion cells (RGCs), optic nerve excavation, and axonal degeneration leading to irreversible vision loss (Casson et al., 2012). Although the etiology of glaucoma is still not completely elucidated, elevation of the intraocular pressure (IOP) is considered the main risk factor for the disease onset. Current available treatments for glaucoma are focused on the reduction of IOP (Caprioli, 2013). However, in several patients the disease still progresses, despite the effective control of IOP. Therefore, it is urgent to develop novel therapeutic strategies focused on the neuroprotection of RGCs (Cordeiro and Levin, 2011).

It is currently recognized that degeneration of RGCs in human and experimental glaucoma is accompanied by a neuroinflammatory response, involving retinal microglial cells and increased production of inflammatory mediators, such as tumor necrosis factor (TNF) and interleukin-1β (IL-1β) (Cho et al., 2011; Tezel et al., 2001; Yoneda et al., 2001; Yuan and Neufeld, 2000). In addition, early and exacerbated activation of retinal microglial cells has been described and proposed to contribute to the degenerative process (Bosco et al., 2011; Naskar et al., 2002; Wang et al., 2015), suggesting that the control of microglia reactivity can prevent the glaucomatous loss of RGCs (Bosco et al., 2008; Roh et al., 2012; Wang et al., 2014).

Recently, we showed that the blockade of the adenosine A_{2A} receptor (A_{2A}R) affords protection to RGCs against damage induced by elevated hydrostatic pressure in retinal organotypic cultures (Madeira et al., 2015c) as well as in the high IOP-induced transient ischemic injury animal model (Madeira et al., 2015a). We also demonstrated that A_{2A}R blockade prevents retinal microglia reactivity and the associated neuroinflammatory response, suggesting the control of microglia-mediated neuroinflammation as the mechanism operated by A_{2A}R antagonist to provide retinal protection (Madeira et al., 2015a).

Caffeine is the most widely consumed psychoactive drug in the world. In the center nervous system (CNS), the effects exerted by caffeine, at non-toxic doses, are mediated through the antagonism of adenosine receptors (Fredholm et al., 2005). Caffeine, by blocking A_{2A}R, is able to prevent synaptotoxicity, excitotoxicity and neuronal loss (Cunha and Agostinho, 2010; Espinosa et al., 2013; Matos et al., 2012a; Prediger, 2010). In addition, it has also been reported that caffeine has anti-inflammatory properties in the CNS (Lee et al., 2013), namely by attenuating microglia-mediated neuroinflammation (Brothers et al., 2010).

Taking in consideration the neuroprotective properties of caffeine in the brain mediated by A_{2A}R blockade, together with our previous studies, we now hypothesize that caffeine may confer neuroprotection to RGCs in models of glaucoma by controlling the neuroinflammatory response.

Therefore, the main aim of this work was to investigate whether caffeine administration modulates retinal neuroinflammation and prevents the loss of RGCs in an animal model of ocular hypertension (OHT), obtained by laser photocoagulation (LP) of the trabecular meshwork and perilimbar and limbar veins. Although this model does not completely mimic human glaucomatous optic neuropathy, it has been extensively used to evaluate anatomical and functional alterations associated with glaucomatous damage, such as loss of RGCs and impairment of the retrograde axonal transport in the optic nerve (Agudo-Barriuso et al., 2013; Ortin-Martinez et al., 2015; Salinas-Navarro et al., 2010; Vidal-Sanz et al., 2012).

4.3 Materials and Methods

4.3.1 Animals

All procedures involving animals were approved by the Ethical and Animal Studies Committee of the University of Murcia and were in accordance with the ARVO and European Union guidelines for the use of animals in research. Adult female albino Sprague-Dawley rats (Charles River Laboratories, L'Arbresle, France) were housed in the animal facilities of the University of Murcia, Spain, and were provided with standard rodent diet and water ad libitum, under a 12 h light/12 h dark cycle. Recent studies suggest that injury to one eye may produce significant molecular and structural changes in the intact contralateral eye (de Hoz et al., 2013; Gallego et al., 2012; Ramirez et al., 2010; Rojas et al., 2014). Therefore, comparisons were performed using a group of control animals.

4.3.2 Caffeine administration

Animals were randomly assigned to receive caffeine or normal drinking water. Caffeine (1 g/L, Sigma-Aldrich, St. Louis, MO, USA) was supplied in the drinking water for two weeks before the induction of OHT and was maintained until the end of the experiment. The chosen dose of caffeine was based in a previous a work that reported protective effects of caffeine intake (Goncalves et al., 2013).

The animals were divided into 6 experimental groups: 1) control (intact and water drinking); 2) caffeine (normal IOP and caffeine drinking); 3) water drinking + 3 days OHT; 4) caffeine drinking + 3 days OHT; 5) water drinking + 7 days OHT; 6) caffeine drinking + 7 days OHT.

4.3.3 Induction of OHT and IOP measurements

To avoid the IOP-lowering effect of anesthetic agents, IOP measurements were performed in non-sedated rats that were trained as previously described (Sappington et al., 2010). The IOP was measured in both eyes before surgery (basal) and at days 1, 2, 3, 5 and 7 post-surgery with a rebound tonometer specifically designed for rodents (Tonolab®, Icare, Espoo, Finland).

OHT was induced in the left eyes of anesthetized [intraperitoneal injection of xylazine (10 mg/kg) and ketamine (60 mg/kg)] rats in a single session of diode laser burns (Viridis Ophthalmic Photocoagulator-532 nm, Quantel Medical, Clermont-Ferrand, France), as we described previously (Ortin-Martinez et al., 2015; Salinas-Navarro et al., 2010; Salinas-Navarro et al., 2009a). During recovery, topical ointment containing Tobramycin was applied to the eyes to prevent corneal desiccation.

4.3.4 Retrograde tracing of retinal ganglion cells

One day after laser photocoagulation, 3% fluorogold (FG; Fluorochrome Inc., Engelwood, CO, USA) diluted in 10% in DMSO saline was applied onto the surface of both superior colliculi (SC), as previously described (Nadal-Nicolas et al., 2015; Salinas-Navarro et al., 2010; Salinas-Navarro et al., 2009a). Retinas were analyzed 6 days after the tracing, 7 days after the induction of the OHT.

4.3.5 Immunodetection

All animals were euthanized with an intraperitoneal overdose of 20% sodium pentobarbital and then transcardially perfused with saline followed by 4% (w/v) paraformaldehyde (PFA).

Flat-mounted retinas

Retinas from both eyes were dissected and then permeabilized with 0.5% Triton X-100 in PBS, followed by freezing at -70°C for 15 min, and then a new rinse in 0.5% Triton X-100. Brn3a was detected by incubation with a goat anti-Brn3a antibody (1:750; C-20; Santa Cruz Biotechnology, Heidelberg, Germany), prepared in PBS with 2% Tween-20 and 2% normal goat serum (NGS), overnight, at 4°C. Secondary detection was performed with Alexa Fluor 568 donkey anti-goat conjugated secondary antibody (1:500, Life Technologies, Thermo-Fisher, Madrid, Spain). Finally, retinas were thoroughly washed in PBS and mounted with the RGC layer facing up and covered with anti-fading solution.

Retinal cryosections

The eyes were enucleated and post-fixed in 4% PFA for 1h. Then, the cornea was carefully removed and the eyecup was fixed for an additional 1h in 4% PFA. After washing in PBS, the tissue was cryopreserved in 15% sucrose in PBS for 1h, followed by 30% sucrose in PBS for 1h. The eyecups were embedded in tissue-freezing medium with 30% of sucrose in PBS (1:1), and stored at -80°C. The tissue was sectioned on a cryostat (18 µm thickness) and the sections were mounted on Superfrost Plus glass slides.

Retinal slices were permeabilized with 0.1% Triton X-100 in PBS, followed by blockade with 10% NGS and 1% bovine serum albumin (BSA) for 1h. The slices were then incubated with primary antibodies, as follows: rabbit anti-Iba1 (1:1000; Wako, Osaka, Japan), rabbit anti-MHC-II (1:200; AbDSerotec, Oxford, UK), or mouse anti-GFAP (1:500; Merck Millipore, Billerica, MA, USA), in PBS with 1% BSA, overnight, at 4°C. Secondary detection was performed with Alexa Fluor 568 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit (all 1:500; Life Technologies,

Carlsbad, USA). Nuclei were counterstained with DAPI (Life Technologies, Carlsbad, USA) and the slices were mounted with Glycergel (DAKO, Glostrup, Denmark).

The preparations were observed with a confocal microscope (LSM 710, Zeiss, Oberkochen, Germany) on an Axio Observer Z1 microscope using an EC Plan-Neofluar 40x/1.30 Oil DIC M27 objective.

4.3.6 Transmission electron microscopy

Optic nerve samples were collected at approximately 1 mm from the optic chiasm and fixed with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), supplemented with 1 mM calcium chloride for 2 h. Following rinsing in the same buffer, post-fixation was performed using 1% osmium tetroxide for 1h. Samples were then washed in buffer and dehydrated in a graded ethanol series (30-100 %), impregnated and embedded in Epoxy resin (Fluka Analytical, Sigma-Aldrich, St. Louis, MO, USA). For the evaluation of whole nerve and individual axons, ultrathin sections (70 nm) were mounted on copper grids (300 mesh) and stained with 2% uranyl acetate (15 min) and 0.2% lead citrate (10 min). Observations were carried out using a Tecnai G2 Spirit BioTWIN electron microscope (FEI) at 100 kV.

4.3.7 Image analysis

Retinal whole-mounts were examined and photographed with a microscope (Axioscop 2 Plus; Zeis, Oberkochen, Germany) equipped with a digital-high-resolution camera (ProgRes™ c10; Jenoptic, Jena, Germany) and a computer driven motorized stage (ProScan™ H128; Prior Scientific Instruments Ltd., Cambridge, UK) connected with an image analysis system (Image-Pro Plus 5.1 for Windows®; Media Cybernetics, Silver Spring, MD). Photomontages of the whole-mounts were constructed from 154 consecutive frames captured on the microscope side by side with no gap or overlap between them.

The individual images taken for each retinal photomontage were processed to assess the total number of FG$^+$ cells and Brn3a$^+$ cells in each retina with a specific cell counting subroutine to automatically count labeled cells in each frame, as previously described (Nadal-Nicolas et al., 2009; Salinas-Navarro et al., 2009b).

Density of FG$^+$ cells and Brn3a$^+$ RGCs (cells/mm^2) over the entire retinas were calculated and represented in isodensity maps, as described (Nadal-Nicolas et al., 2009).

4.3.8 Real-time quantitative PCR

Total RNA was extracted from rat retinas using Trizol reagent. RNA samples were dissolved in 16 µL of Mili-Q water and total RNA concentration was determined using NanoDrop ND1000. Amplification of cDNA was performed according to the instructions provided by the manufacturer, using 1 µg of total RNA (NZYTech, Lisbon, Portugal). The resultant cDNA was treated with RNAse-H for 20 min, at 37°C, and a 1:2 dilution was prepared for qPCR analysis. All samples were stored at -20°C until analysis.

Genomic DNA contamination was assessed with a conventional PCR for β-actin using intron-spanning primers (Table 1), as described previously (Santiago et al., 2009). SYBR Green-based real-time quantitative PCR (qPCR) was performed using StepOnePlus, as previously described (Madeira et al., 2015c). Ct values were converted to "Relative quantification" using the $2^{-\Delta\Delta Ct}$ method previously described (Livak and Schmittgen, 2001). Four candidate housekeeping genes (*Tbp*, *Hprt*, *Ywhaz* and *Rhodopsin*) were evaluated using NormFinder (a Microsoft Excel Add-in) (Andersen et al., 2004) and *Yhwaz* was identified as the most stable gene.

Table 1 - Primers used in qPCR and RT-PCR

Gene	GenBank number	Forward	Reverse
Adora1	NM_017155.2	5' - TGAGTGTGGTAGAGCAAGAC - 3'	3' - CAGACGAAGAAGTTGAAGTAGAC - 3'
Adora2a	NM_053294	5' - GGCTATCTCTGACCAACA - 3'	3' - TGGCTTGACATCTCTAATCT - 5'
Tnf	NM_012675	5' - CCCAATCTGTGTCCTTCT - 3'	3' - TTCTGAGCATCGTAGTTGT - 5'
il-1β	NM_031512	5' - ATAGAAGTCAAGACCAAAGTG - 3'	3' - GACCATTGCTGTTTCCTAG - 5'
mhc-ii	NM_013069.2	5' - CCACCTAAAGAGCCACTGGA - 3'	3' - AGAGCTGGCTTCTGTCTTCAC - 5'
Tspo	NM_012515.2	5' - TGTATTCGGCCATGGGGTATG - 3'	3' - GAGCCAGCTGACCAGTGTAG - 5'
trem2	NM_001106884.1	5' - AACTTCAGATCCTCACTGGACC - 3'	3' - CCTGGCTGGACTTAAGCTGT - 5'
Yhwaz	NM_013011.3	5' - CAAGCATACCAAGAAGCATTTGA - 3'	3' - GGGCCAGACCCAGTCTGA - 5'
Actb	NM_031144	5' - GCTCCTCCTGAGCGCAAG - 3'	3' - CATCTGCTGGAAGGTGGACA - 5'

4.3.9 TNF and IL-1β protein levels quantification by Enzyme-Linked Immunosorbent Assay (ELISA)

Protein levels of IL-1β and TNF were quantified by ELISA, according to the instructions provided by the manufacturer (Peprotech, London, UK). Briefly, total retinas were lysed in 20 mM imidazole-HCl, 100 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 10 mM NaF, 1% Triton X-100, supplemented with protease and phosphatase inhibitors (Roche, Basel, Switzerland). Then, lysates were sonicated and centrifuged at 16,000 g for 10 min at 4°C and at 10,000 g for 5 min at 4°C, respectively. The supernatant was collected and stored at -80°C until use.

The cytokine concentration of each sample was normalized to the total protein concentration (determined by the bicinchoninic acid protein assay).

4.3.10 Statistical analysis

The results are presented as mean ± standard error of the mean (s.e.m.). The normality of the data was assessed with Shapiro-Wilk normality test. The data were analyzed using Kruskall-Wallis test, followed by Dunn's multiple comparison test, or Two-Way ANOVA followed by Tukey's Multiple Comparison Test, as indicated in the figure legends. The statistical analysis was performed in Prism 6.0 Software for Mac OS X (GraphPad Software, Inc).

4.4 Results

Ocular hypertension induced by LP of the perilimbar and episcleral vessels of adult rats triggers anatomical and functional alterations associated with glaucoma, such as loss of RGCs and impaired retrograde axonal transport of the optic nerve (Agudo-Barriuso et al., 2013; Salinas-Navarro et al., 2010). We took advantage of this animal model of glaucoma to investigate the ability of caffeine to modulate retinal neuroinflammatory response and evaluate its neuroprotective role.

4.4.1 Effect of caffeine consumption in animal weight, fluid intake and IOP

Caffeine (1 g/L) was administered in the drinking water, starting 2 weeks prior the induction of OHT and until the end of the study. Animal weight and fluid intake were registered in all animals during treatment (Table 2). No significant alterations were observed in the fluid intake or weight between animals drinking water or caffeine.

Table 2– Animal fluid intake and weight

	Animals drinking water (n = 48)	Animals drinking caffeine (n = 49)
Fluid intake (mL/day)	23.7 ± 0.9	22.0 ± 0.7
Animal Weight (g)		
Day 0	200.1 ± 1.1	201.5 ± 1.7
Week 1	200.1 ± 1.1	201.5 ± 1.7
Week 2	234.4 ± 2 .4	236.0 ± 1.8
Week 3	253.4 ± 1.9	250.8 ± 5.7

Since caffeine consumption may change IOP (Avisar et al., 2002), IOP was measured in all animals prior inducing OHT (basal) and at days 1, 2, 3, 5 and 7 post-OHT induction with a rebound tonometer (Figure 1). The basal IOP was similar in all groups (as reference, IOP in control animals was 10.8±0.2 mmHg, n=22). As expected, 1 day post-OHT induction IOP significantly increased in both water- and caffeine-drinking animals (49.5±1.8 and 51.2±1.5 mmHg, respectively, n=37; p<0.0001, when compared with basal IOP). In these animals, IOP

maintained elevated throughout the experiment. Nevertheless, 3 days post-OHT induction, the IOP in caffeine-drinking animals with OHT was statistically lower, when compared with the water-drinking animals subjected to OHT (43.9±1.5 mmHg and 51.2±1.8 mmHg, respectively; $p<0.001$). This effect was maintained until the end of the study, and at day 7 post-OHT induction, IOP of water-drinking animals with OHT was 54.1±2.5 mmHg and in caffeine-drinking animals with OHT the IOP was 40.3±2.8 mmHg ($p<0.001$). Caffeine administration per se did not alter the IOP.
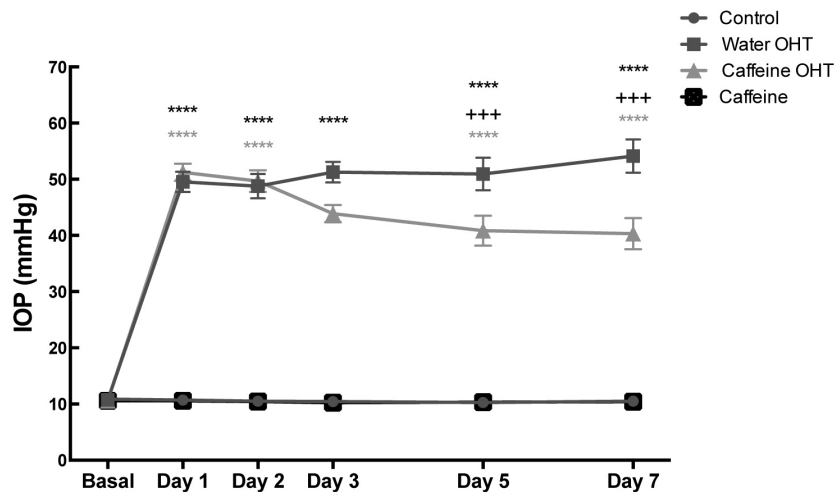

Figure 1 - Caffeine administration reduces IOP in OHT animals. Water or caffeine (1 g/L) was administrated *ad libitum* to Sprague-Dawley rats, during 2 weeks prior induction of OHT, and until the end of the experiment. IOP was measured with a rebound tonometer. Results are expressed in mmHg and represent the mean ± s.e.m of 22 to 37 independent experiments. ****$P<0.0001$, significantly different from control animals; +++$P<0.001$, significantly different from OHT control animals; Two-way ANOVA, followed by Tukey's multiple comparison test.

4.4.2 OHT increases the expression of $A_{2A}R$ and does not affect A_1R expression

In the CNS, including the retina, the pharmacological actions of caffeine are exerted mainly by antagonizing adenosine receptors (Rivera-Oliver and Diaz-Rios, 2014). It has been documented that brain noxious conditions downregulate A_1Rs and up-regulate $A_{2A}Rs$. Therefore, we evaluated whether OHT could alter the expression of A_1Rs and $A_{2A}Rs$. No significant alterations were detected in the mRNA expression of A_1R (Figure 2) at the 2 time-points analyzed (3 and 7 days after inducing OHT). Nevertheless, at 3 days post-OHT we detected a significant increase in the expression of $A_{2A}R$ compared with control animals (2.3-fold change; n=5, $p<0.05$), which was maintained until 7 days post-OHT (2.4-fold change; n=7, $p<0.05$).

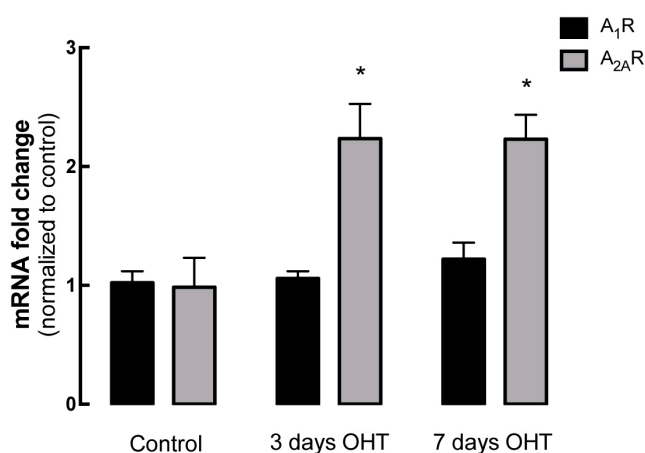**Figure 2 - OHT induces up-regulation of A$_{2A}$R without altering the expression of A$_1$R.** The expression of A$_1$R and A$_{2A}$R mRNA was assessed by qPCR in the retinas of control animals and animals with 3 or 7 days of OHT. Results are presented as fold change comparing with the control animals, and represent the mean ± s.e.m of 5 to 7 independent experiments. [#]$P < 0.05$, significantly different from control animals; Kruskall-Wallis test, followed by Dunn's multiple comparison test.

4.4.3 Caffeine inhibits the inflammatory response triggered by OHT

Since chronic inflammation plays an important role in the pathophysiology of glaucoma (Madeira et al., 2015b), we evaluated mRNA and protein levels of the pro-inflammatory markers TNF and IL-1β. As shown in Figure 3A, 3 days with OHT significantly increased the mRNA levels of TNF and IL-1β (4.3±0.6 and 6.8±0.9 fold-change of the control, respectively; n=7, p<0.01). Similarly, at 7 days with OHT the mRNA levels of these cytokines were still significantly elevated, when compared with the control (2.8±0.6 and 3.2±0.3 fold change for TNF and IL-1β, respectively; n=7, p<0.05). The administration of caffeine to OHT animals significantly inhibited the OHT-induced up-regulation of TNF and IL-1β mRNA levels (n=7, p<0.05) in both time points, without altering the expression of both TNF and IL-1β in the retinas of control animals.

We then quantified the protein levels of TNF and IL-1β, by ELISA, in retinal extracts (Figure 3B). In the retinas of control animals, the expression of TNF and IL-1β was 74.6±8.6 and 150.5±12.6 pg/µg of total protein, respectively. At 3 and 7 days after inducing OHT, IL-1β protein levels were significantly increased (314.7±44.3 and 424.1±51.2 pg/µg of total protein, respectively; n=8-9, p<0.01 and p<0.001), when compared with control animals. In animals drinking caffeine, after 3 days of OHT, the levels of IL-1β in the retina slightly decreased to 194.9±8.1 pg/µg of total protein, comparing with animals drinking water. Moreover, at 7 days of OHT the administration of caffeine significantly decreased IL-1β to 140.9±7.1 pg/µg of total protein (n=7, p<0.001). After 3 days of OHT, the protein levels of TNF were not altered in retinal extracts both in water and caffeine drinking animals, comparing with control animals. Nevertheless, after 7 days of OHT the protein levels of TNF significantly increased to

127.4±13.7 pg/µg of total protein (n=9, p<0.05), which was prevented by caffeine administration (29.9±7.98 pg/µg protein; n=8, p<0.0001). Caffeine administration, per se, did not alter the protein levels of these two cytokines.
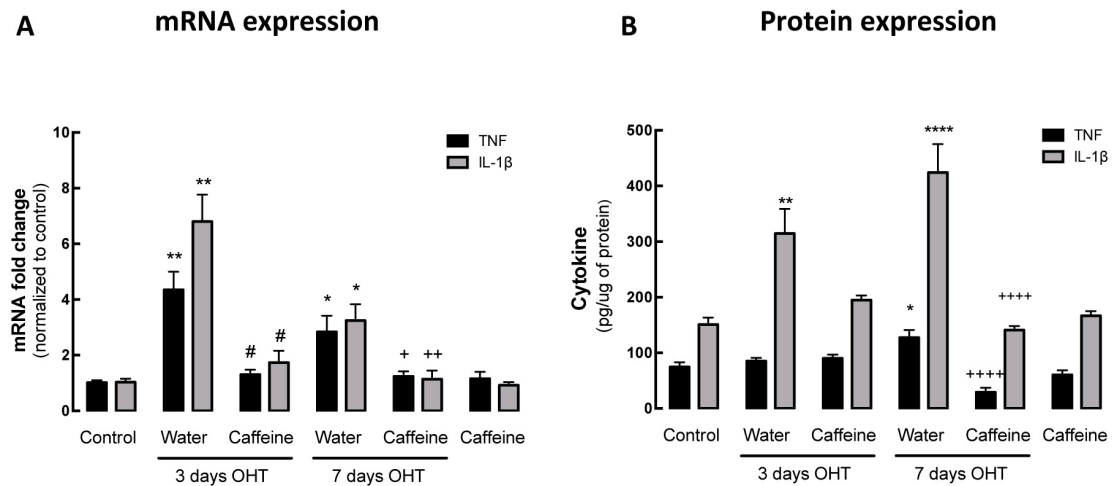

Figure 3 - Caffeine administration prevents the inflammatory response triggered by OHT. The effects of caffeine administration on the retinal neuroinflammatory response in eyes subjected to OHT were evaluated by qPCR and ELISA. **(A)** mRNA expression of pro-inflammatory cytokines IL-1β and TNF were assessed by qPCR. Results are presented as fold change comparing with the control animals, and represent the mean ± s.e.m from 5-7 independent experiments. **(B)** The retinal protein levels of IL-1β and TNF were quantified by ELISA. Results are expressed in pg/µg of protein, and represent the mean ± s.e.m from 5-10 independent experiments. *P<0.05, **P<0.01 and ****P<0.0001, significantly different from control animals; #P<0.05, significantly different from water drinking animals with 3 days OHT; +P<0.05, ++P<0.01 and ++++P<0.0001, significantly different from water drinking animals with 7 days OHT; Kruskall-Wallis test, followed by Dunn's multiple comparison test.

4.4.4 Caffeine prevents OHT-induced microglia activation

Microglia activation appears early in the retina of animal models of glaucoma (Bosco et al., 2011), implicating a role for these cells in the early stages of the disease (Karlstetter et al., 2015; Madeira et al., 2015b). Therefore, we evaluated whether caffeine administration could modulate microglia reactivity elicited by OHT.

By qPCR, we evaluated the mRNA levels of two markers of microglia reactivity, MHC-II (Figure 4A) and TSPO (Figure 4B), as well as CD11b, a general marker of microglia (Figure 4C) and TREM2, which is associated with microglia phagocytic capacity (Figure 4D). After 3 and 7 days of OHT, there was a significant increase in the mRNA levels of MHC-II (3.5±0.8 and 19.8±2.2 fold change, respectively; n=7, p<0.05 and p<0.001). Caffeine administration significantly prevented the OHT-induced increase in MHC-II expression (1.2±0.8 and 4.4±0.3 fold change for 3 and 7 days of OHT, respectively; n=7, p<0.05 and p<0.01). The immunoreactivity for MHC-II was mainly detected in the ganglion cell layer in OHT animals and co-localized with Iba1, a general marker of microglia (Figure 4E). In accordance with the qPCR

results, MHC-II immunoreactivity in the retina was increased in animals with OHT for 7 days; an effect that was not observed in the caffeine-drinking OHT animals.

The TSPO mRNA expression (Figure 4B) was also up-regulated after 3 and 7 days of OHT (11.6±1.5- and 19.8±1.2-fold change, respectively; n=7, p<0.001). The administration of caffeine slightly decreased the levels of the mRNA coding for TSPO (6.7±0.8-fold change) at 3 days in OHT animals, and the decrease reached statistical significance at 7 days in animals with OHT drinking caffeine (4.3±0.8-fold change; n=7, p<0.05), compared with water drinking animals.
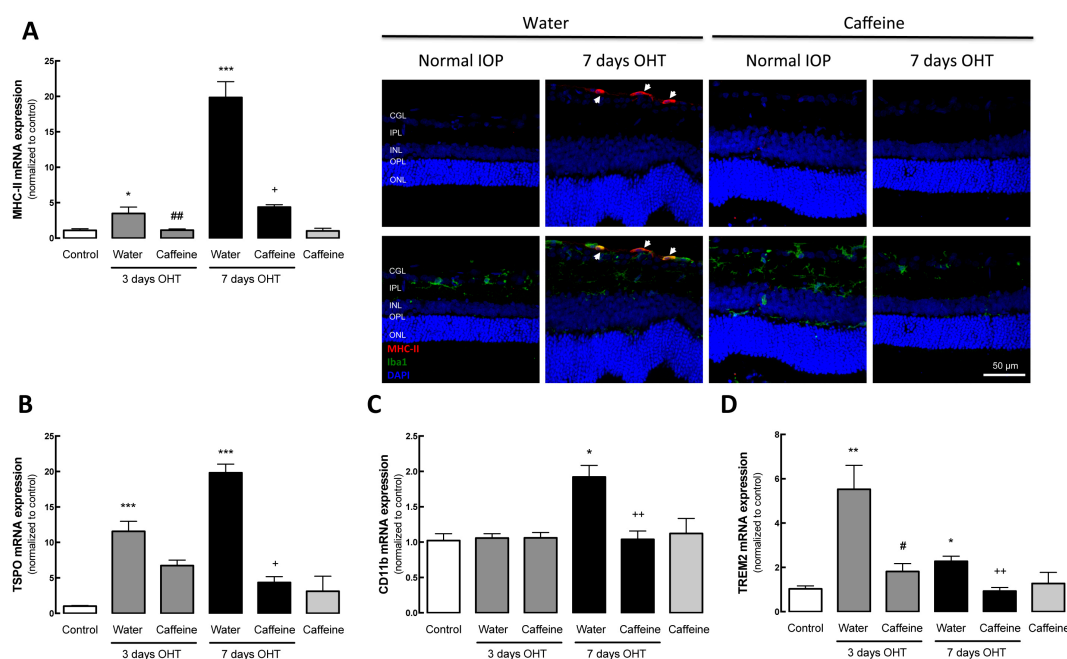

Figure 4 - Caffeine prevents OHT-induced microglia reactivity. Effects of caffeine administration on the mRNA expression of microglial cell markers. **(A)** MHC-II, **(B)** TSPO, **(C)** CD11b and **(D)** TREM2 mRNA levels were assessed by qPCR. Results are presented as fold change comparing with the control, from 5-7 independent experiments. **(E)** Retinal sections were immunostained for Iba1 (general microglia marker; green) and MHC-II (activated microglia marker; red) and then were imaged in a confocal microscope. Nuclei were stained with DAPI (blue). Representative images obtained from 5 independent experiments. Results are presented as fold change comparing with the control, and represent the mean ± s.e.m from 5-7 independent experiments. *P<0.05, **P<0.01 and ***P<0.001, significantly different from control; #P<0.01, significantly different from water drinking animals with 3 days OHT; +P<0.05, ++P<0.01 and ++++P<0.0001, significantly different from water drinking animals with 7 days OHT; Kruskall-Wallis test, followed by Dunn's multiple comparison test.

We then assessed the expression levels of CD11b, and detected a significant up-regulation of the mRNA levels after 7 days of OHT (1.9±0.2-fold change; n=7, p<0.05). This up-regulation was prevented by caffeine administration (1.1±0.2-fold change; n=7, p<0.01). The expression of TREM2 was also assessed in order to estimate microglia phagocytic activity. OHT for 3 and 7 days significantly increased the expression of TREM2 mRNA by 5.5±1.0 and 2.3±0.32 fold-change, respectively (n=7, p<0.05 and p<0.01), and caffeine prevented the OHT-induced

increase in TSPO expression (1.8 ± 0.4- and 0.9 ± 0.2-fold change, for 3 and 7 days, respectively; n=7, $p<0.05$ and $p<0.01$). Administration of caffeine per se did not alter the expression of pro-inflammatory markers.

4.4.5 Caffeine administration prevents microglia reactivity in contralateral retina induced by OHT

Microglia reactivity in the contralateral eye (without OHT) has been reported (Gallego et al., 2012; Rojas et al., 2014). Therefore, we evaluated the ability of caffeine to modulate the microglia reactivity in contralateral eyes.
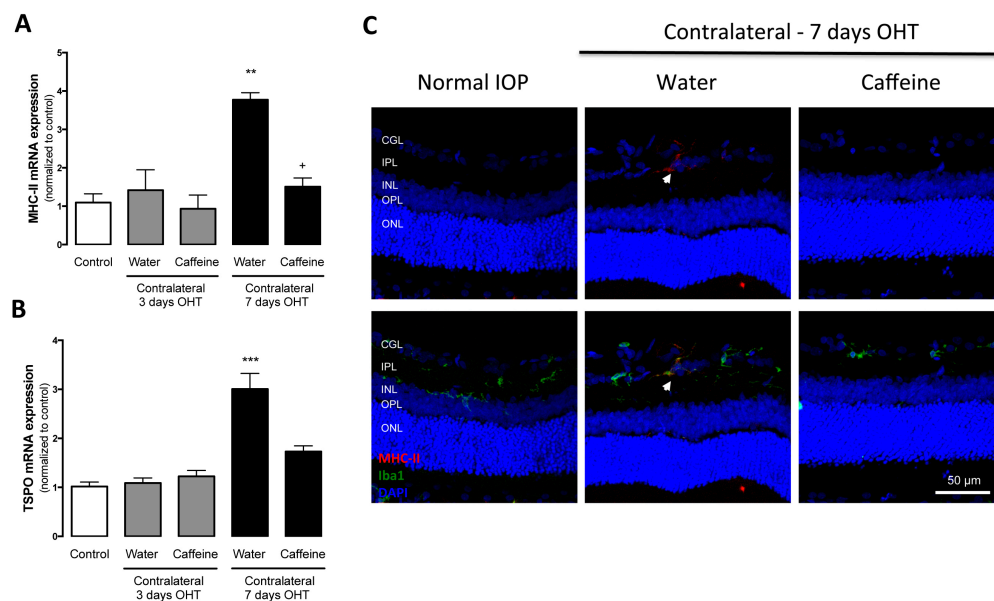

Figure 5 - Caffeine prevents OHT-induced microglia reactivity in the contralateral eye. Effect of caffeine administration on OHT-induced microglia activation in the contralateral eye was evaluated by qPCR to assess mRNA expression of microglial cell activation markers MHC-II **(A)** and TSPO **(B)**. Results are presented as fold change comparing with the control, from 5-7 independent experiments. **(C)** Retinal sections were immunostained for Iba1 (general microglial marker; green) and MHC-II (activated microglia marker; red) and then were imaged in a confocal microscope. Nuclei were stained with DAPI (blue). Representative image obtained from 5 independent experiments. Results are presented as fold change comparing with the control, and represent the mean ± s.e.m from 5-7 independent experiments. $**P<0.01$ and $***P<0.001$, significantly different from control; $^{+}P<0.05$, significantly different from the contralateral eye of water drinking animals with 7 days OHT; Kruskall-Wallis test, followed by Dunn's multiple comparison test.

The expression of mRNA coding for MHC-II and TSPO (Figures 5A and 5B) in the contralateral eye was significantly up-regulated after 7 days of OHT (3.8 ± 0.2- and 3.0 ± 0.3-fold change, $p<0.01$ and $p<0.001$, respectively; n=7). Caffeine administration reduced the expression of MHC-II and TSPO in the contralateral eyes, significantly for MHC-II (1.5 ± 0.2-fold change, $p<0.05$; n=6). By immunohistochemistry, similarly to the OHT eyes, MHC-II immunoreactivity is detected in the microglia (Iba1-positive cells) within the GCL (Figure 5C). Caffeine

administration inhibited the OHT-induced increase in MHC-II immunoreactivity in the contralateral eyes.

4.4.6 Caffeine administration does not ameliorate the OHT-induced impairment in the RGC retrograde transport

Alterations in the structure of the optic nerve and in the retrograde axonal transport of RGCs have been described in glaucomatous animal models (Mabuchi et al., 2003, Salinas-Navarro et al., 2010). Therefore, in OHT animals, we assessed the effect of caffeine administration in both the integrity of the optic nerve and the axonal transport in RGCs. The structural integrity of the optic nerve was assessed by transmission electron microscopy (Figure 6A). We observed that OHT increased the incidence of axon degenerative profiles, with disorganized and abnormal myelin wrapping. Interestingly, in animals drinking caffeine this effect appears to be partially attenuated, with caffeine-drinking OHT animals presenting a reduced number of disorganized myelin structures.

Retrograde axonal transport was assessed after Fluorogold (FG) application in both superior colliculi, the targets of 98% of RGCs (Salinas-Navarro et al., 2009a). Application of FG after induction of OHT is an established method to evaluate the impairment of the retrograde axonal transport of RGC, by counting the total number of FG-positive cells in whole-mounted retinas (FG$^+$-labeled RGCs) (Vidal-Sanz et al., 2012). Isodensity maps allowed us to visualize the distribution of FG$^+$-RGCs in the retina (Figure 6B). In control animals, drinking water or caffeine, the total number of FG$^+$ cells was 73,698±1,611 and 78,125±1,096 cells, respectively (n=5) (Figure 6C), similar to previous reports (Salinas-Navarro et al., 2010). This number was significantly reduced to 19,619±3,990 cells (n=4; p<0.05) in animals with OHT for 7 days. In animals with OHT, caffeine administration did not prevent the OHT-induced reduction in the number of FG$^+$-labeled RGCs (17,630±2,102 cells; n=4, p<0.05).
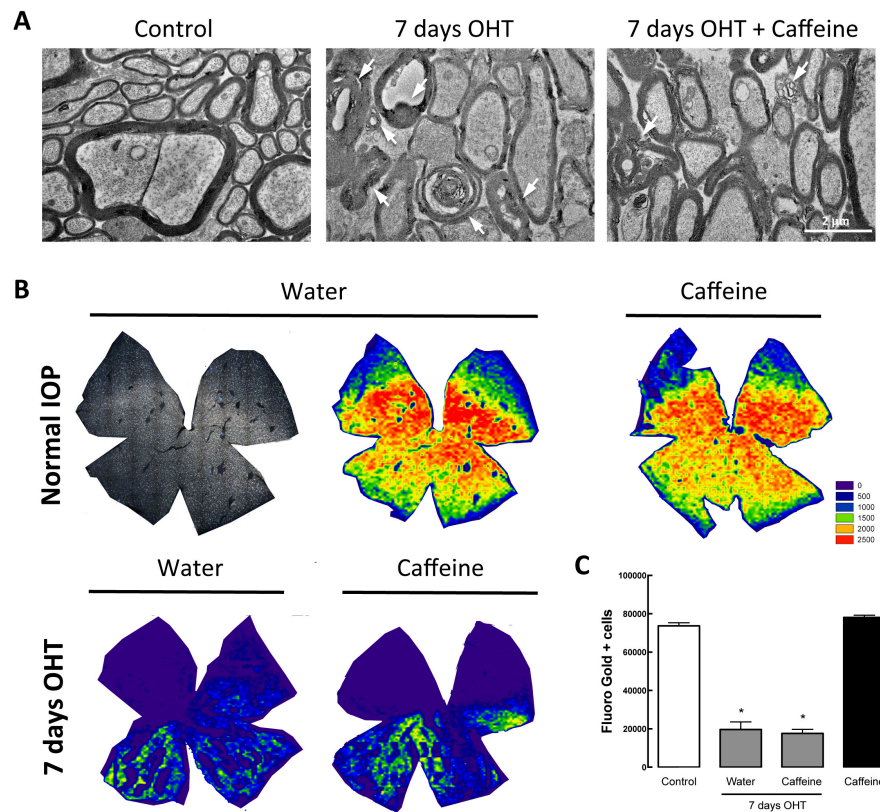
Figure 6 - Caffeine partially prevents OHT-induced optic nerve structural alterations but does not improve axonal transport impairment. (A) Optic nerve structural alterations were observed by transmission electron microscopy. Representative images of semi-thin cross-sections of control, and water drinking and caffeine drinking animals subjected to 7 days of OHT. Alterations in axons structural, including degenerating axons and myelin disarrangement (arrows) can be observed in OHT animals. Scale bar: 2 μm. **(B)** Retrograde axonal transport was assessed by FG application in the superior colliculus 1 day after induction of OHT, and whole-mounted FG-labelled retinas were imaged. Representative isodensity maps showing the topological distribution of FG-positive RGCs, using a color code, according to cell density value within a 28-step color scale range from 0 (dark blue) to 2500 or higher RGCs/mm^2 (red). **(C)** Quantification of FG-positive cells. Graph represents mean ± s.e.m. of the number of FG-positive cells, from 5 to 7 independent experiments. *$P<0.05$, significantly different from control; Kruskall-Wallis test, followed by Dunn's multiple comparison test.

4.4.7 Caffeine increases RGC survival in OHT animals

Taking into consideration the protective properties of caffeine administration (Kalda et al., 2006), we evaluated the potential protective effect of caffeine against the degeneration of RGCs triggered by OHT. In whole-mounted retinas, RGCs were labeled with an antibody that recognizes Brn3a, a marker of RGCs (Nadal-Nicolas et al., 2009) (Figure 7A), and the total number was counted automatically (Figure 7B). In control animals, the total number of RGCs per retina was 70,861±1,258 (n=5), similarly to previous works (Salinas-Navarro et al., 2010). The occurrence of OHT for 3 days triggered a reduction in the number of Brn3a-positive cells to 44,746±6,151 cells (n=4) and 50,021±6,151 cells (n=4) in animals drinking water and caffeine, respectively. Extension of OHT to 7 days resulted in a further significant loss of RGCs in animals

drinking water (24,621±3,443 Brn3a-positive cells, n=7, p<0.01). However, administration of caffeine to animals with OHT for 7 days significantly prevented the loss of RGCs induced by OHT (44,027±5,841 Brn3a-positive cells, n=7, p<0.05). Caffeine administration to animals with normal IOP did not significantly alter the number of RGCs (68,169±1,840 Brn3a-positive cells, n=6).
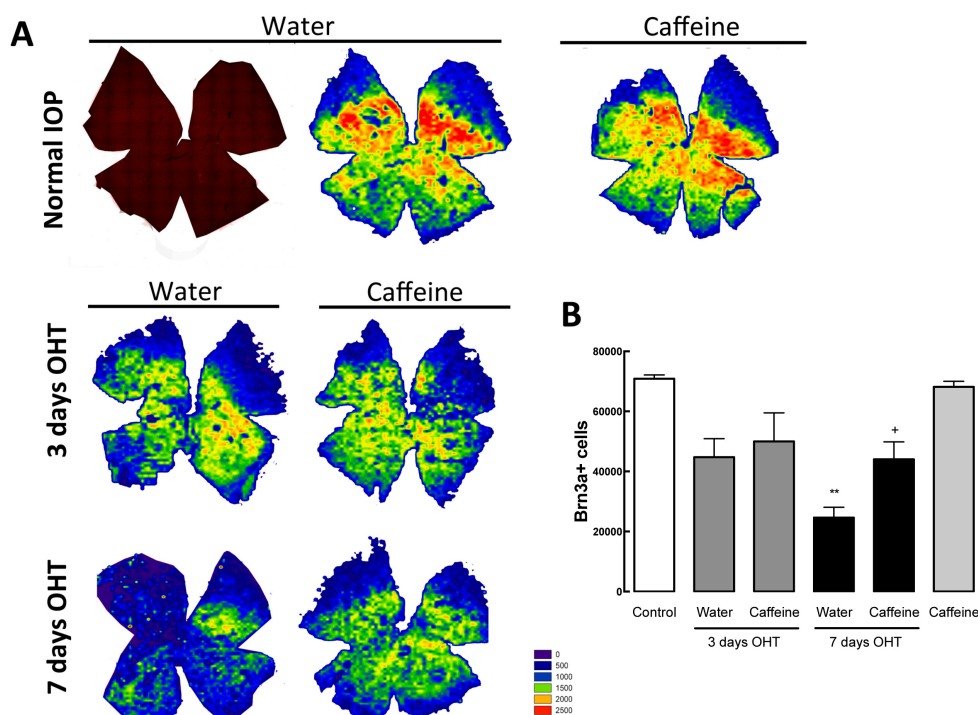

Figure 7 - Caffeine administration inhibits Brn3a-positive cell loss triggered by OHT. Retinal whole-mounts were immunostained for Brn3a (red; RGC marker) and isodensity maps were generated to evaluate RGC survival. **(A)** Representative isodensity maps demonstrating the topological distribution of Brn3a-labelled RGCs, using a color code according to cell density value within a 28-step color scale range from 0 (dark blue) to 2500 or higher RGCs/mm^2 (red). **(B)** The graph represents mean ± s.e.m. of the number of Brn3a-positive cells, from 5 to 7 independent experiments. **P<0.01, significantly different from control; +P<0.05, significantly different from water drinking animals with 7 days OHT; Kruskall-Wallis test, followed by Dunn's multiple comparison test.

4.5 Discussion

The present work demonstrates that caffeine administration prevents retinal neuroinflammation, microglia reactivity and affords protection to RGCs in an animal model of glaucoma.

Similarly to what happens in chronic noxious brain conditions (Rebola et al., 2011; Yu et al., 2008), OHT triggered $A_{2A}R$ upregulation, prompting the hypothesis that the manipulation of this receptor may control neurodegeneration. We previously reported that elevated hydrostatic pressure, to mimic OHT *in vitro*, increases $A_{2A}R$ expression, mainly in retinal microglia located within the ganglion cell layer (Madeira et al., 2015c). Since the actions of caffeine are exerted mainly by blocking adenosine receptors, including the high-affinity A_1R and $A_{2A}R$ (Fredholm et al., 2005), the up-regulation of $A_{2A}R$ by OHT suggested that the effects of caffeine in OHT animals were mediated by $A_{2A}R$ antagonism.

The effects of consumption of caffeine in IOP are not yet clarified. While some authors suggest that caffeine consumption may increase IOP in patients with normotensive glaucoma or ocular hypertension (Avisar et al., 2002), others have shown that caffeine does not significantly alter IOP in patients with glaucoma (Chandra et al., 2011). We regularly measured IOP in animals and we found that administration of caffeine was able to reduce the IOP of OHT animals, without interfering with animals with normal IOP. Nevertheless, the IOP lowering effect of caffeine may not be enough to explain the effects exerted by caffeine, since IOP in caffeine-drinking OHT animals is still elevated (four times higher than in control animals).

Glaucomatous damage is accompanied by early activation of microglia and increased expression of inflammatory mediators (Bosco et al., 2011; Chidlow et al., 2012; Naskar et al., 2002; Roh et al., 2012; Tezel et al., 2001; Tezel and Wax, 2000; Yuan and Neufeld, 2001). It has been suggested that the control of microglia reactivity may represent a therapeutic strategy to manage glaucoma. Reduction of microglia reactivity by irradiation or pharmacological treatment, or the reduction of TNF expression, confers protection in an animal model of glaucoma (Bosco et al., 2012; Bosco et al., 2008; Roh et al., 2012). Several studies demonstrate that caffeine affords protection to the brain in models of neurodegenerative diseases (Prediger, 2010; Rivera-Oliver and Diaz-Rios, 2014) and prevents microglia-mediated neuroinflammatory responses (Brothers et al., 2010). Indeed, OHT animals treated with caffeine presented reduced microglia activation and lower levels of inflammatory mediators, demonstrating that caffeine prevents microglia-mediated neuroinflammation induced by OHT. Increased expression of MHC-II has been also detected in mice contralateral eyes (Gallego et al., 2012; Rojas et al., 2014). Remarkably, caffeine also reduced microglia reactivity in contralateral eyes (without OHT).

Although we did not assess the contribution of other cell types responsible for the inflammatory environment in the retina, we cannot discard the contribution of macroglial cells, which can also release inflammatory mediators (Shin et al., 2014). Nevertheless, caffeine administration did not prevent OHT-induced GFAP up-regulation, a marker of astroglial and Müller cell reactivity (supplementary data; Figure 1), suggesting that caffeine appears to be preferentially modulating microglia responses. This is in line with previous studies reporting the inability of $A_{2A}R$ to modulate the activation of astroglial cells (Matos et al., 2012a; Matos et al., 2012b).

Several studies have shown that axonal transport in the optic nerve is impaired in human glaucoma and in the OHT rat model, preceding the loss of RGCs (Fahy et al., 2015; Salinas-Navarro et al., 2010; Salinas-Navarro et al., 2009a; Vidal-Sanz et al., 2012). In this model, OHT results in Wallerian-like degeneration (Chidlow et al., 2011), which culminates in RGC death (Fahy et al., 2015; Vidal-Sanz et al., 2012). In fact, previous studies have shown that part of the surviving RGC population after one week of OHT exhibits impaired retrograde axonal transport, supporting evidence that not all RGCs die immediately upon impaired axonal transport (Agudo-Barriuso et al., 2013; Vidal-Sanz et al., 2012).

In this work, we found that caffeine is not able to prevent the deficit in the axonal transport induced by OHT. Although caffeine-drinking animals with OHT presented a more preserved optic nerve structure, this was not sufficient to overcome the damage induced by OHT and it was not able to improve axonal transport. Nevertheless, there is a significant attenuation in the loss of RGCs induced by seven days of OHT in animals drinking caffeine. Glaucomatous injury to the optic nerve and optic nerve head appears to be related with the values of IOP, but the detailed mechanism remains to be elucidated (Vidal-Sanz et al., 2012). In fact, previous reports using distinct models of IOP elevation have demonstrated that axonal damage in the optic nerve correlates with the magnitude and duration of IOP elevation (Joos et al., 2010; Mabuchi et al., 2003).

Although most of cell death occurs subsequently to the axonal degeneration, glial-mediated inflammatory response also contributes to the progress of the damage (Munemasa and Kitaoka, 2012). In fact, activation of microglia in a glaucoma animal model occurs prior the loss of RGCs (Bosco et al., 2011). In a model of LP-induced OHT, the presence of markers of microglia reactivity in the retina after 3 days of OHT is paralleled by a decrease in the number of RGCs (Ebneter et al., 2010). Also, markers of reactive microglia are present in the optic nerve and optic tract after 7 days of induction of OHT (Ebneter et al., 2010). Hence, caffeine, by blocking $A_{2A}R$, might be attenuating microglia reactivity, thus protecting the soma of RGCs. Being the response of microglial cells in the optic nerve delayed, it seems plausible to speculate that the effects of caffeine might not be observed at the 7 days time-point. Also, adenosine

receptors might not be directly related with the integrity of the retrograde axonal transport, and therefore caffeine might not be able to alter the functional damage induced by OHT. Indeed, as discussed above, functional impairment of retrograde axonal transport is directly correlated with the elevation of IOP (Joos et al., 2010; Mabuchi et al., 2003).

Notably, we have not observed any detrimental effect related with caffeine administration neither in animals with normal IOP nor with OHT. In fact, caffeine is able to reduce the neuroinflammatory response and increase the survival of RGCs in animals with OHT, in a mechanism independent of lowering IOP.

Taking in account the results obtained in this work, together with our previous works (Madeira et al., 2015a; Madeira et al., 2015c), $A_{2A}R$ antagonists, in combination with IOP lowering agents, might be envisaged as a potential therapeutic strategy to treat glaucoma.

4.6 Funding

4.7 Supplementary figures
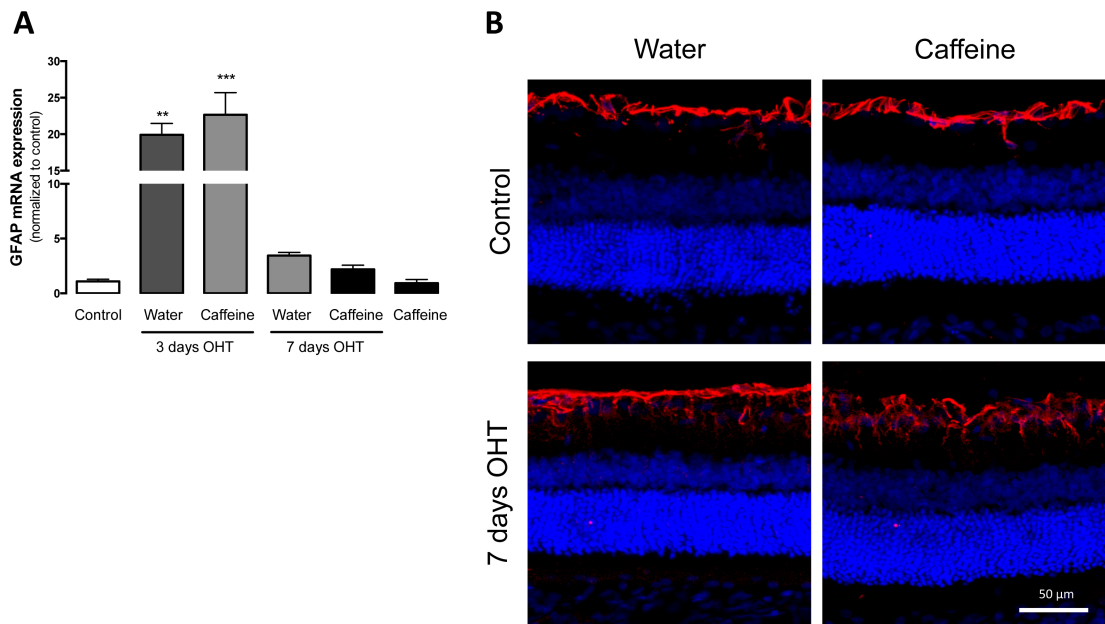
Supplementary figure 1 – Caffeine did not alter the effect of OHT in the expression of GFAP. Effects of caffeine administration on the mRNA expression of GFAP. Results are presented as fold change comparing with the control, from 5-7 independent experiments **(A).** Retinal sections were immunostained for GFAP (marker of astroglial and Muller reactivity; red) and then imaged in a confocal microscope. Nuclei were stained with DAPI (blue). Representative images obtained from 3 independent experiments **(B)..** **P<0.01 and ***P<0.001, significantly different from control; Kruskall-Wallis test, followed by Dunn's multiple comparison test.

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CHAPTER 5 –

General Discussion

5.1 General discussion

Glaucoma, the second leading cause of blindness worldwide, is a retinal degenerative disease mainly characterized by the loss of RGCs and optic nerve damage (Casson et al., 2012). The major risk factors for the development of glaucomatous RGC death and optic nerve damage include elevated IOP, advanced age, positive finding for the condition in the family history, and thin central corneal thickness. However, IOP is the only modifiable risk factor, and therefore, all currently used strategies for the treatment of glaucoma are aimed at lowering or preventing a rise in IOP. Nevertheless, many patients continue to lose vision despite the successful control of IOP. Thus, neuroprotective strategies aimed at preventing the loss of RGCs are imperative for the treatment of glaucoma (Agarwal et al., 2009; Chang and Goldberg, 2012).

In recent years, relevance has been given to the role of neuroinflammation in the pathogenesis of glaucoma (Soto and Howell, 2014). Several groups have reported early activation of microglial cells and increased neuroinflammatory response, which might contribute to the progression of the disease (reviewed in Karlstetter et al., 2015; Madeira et al., 2015; Wang et al., 2015). Hence, therapeutic strategies designed at reducing inflammation may offer therapeutic benefits to manage glaucomatous RGC loss.

Numerous studies demonstrate that selective antagonists of $A_{2A}R$ confer protection against neurodegenerative processes (reviewed in Gomes et al., 2011; Rivera-Oliver and Diaz-Rios, 2014). The blockade of this receptor reduces neuronal damage and neurological deficits in several models of brain damage such as ischemia (Melani et al., 2015; Melani et al., 2006; Pedata et al., 2005) and in neurodegenerative diseases as PD and AD (Aoyama et al., 2000; Bove et al., 2005; Canas et al., 2009; Chen et al., 2001; Dall'Igna et al., 2003; Golembiowska et al., 2013; Gyoneva et al., 2014; Kondo et al., 2015). Although the mechanisms by which $A_{2A}R$ blockade affords neuroprotection remain to be fully clarified, several studies suggest the modulation of the neuroinflammatory process as one of the main hypothesis (Golembiowska et al., 2013; Gyoneva et al., 2014; Rebola et al., 2011; Simoes et al., 2012). Therefore, $A_{2A}R$ antagonists have emerged as potential neuroprotective agents in brain neurodegenerative diseases involving neuroinflammation (Cristalli et al., 2009; Cunha, 2005; Santiago et al., 2014).

In this work, using *in vitro* and animal models of glaucoma, we have shown that blockade of $A_{2A}R$ confered neuroprotection in the retina, by controlling the microglia-mediated neuroinflammatory response.

Using two distinct *in vitro* models, adult rat retinal organotypic cultures and rat retinal microglial cell cultures we have investigated the effect of the exposure to EHP, used to mimic elevated IOP in glaucoma, in the neuroinflammatory response. Retinal organotypic cultures have been considered an appropriate model to study retinal cellular and molecular mechanisms (Kretz et al., 2004). In fact, previous studies have already shown that this model can be a useful

tool to study microglial cell response (Mertsch et al., 2001) and to perform screening of potential RGC neuroprotective therapies (Bull et al., 2011). Nevertheless, one cannot exclude the limitations intrinsic to the preparation, such as the requirement to perform an optic nerve axotomy, which in neonatal organotypic cultures has been associated with microglia reactivity and activation of apoptotic pathways (Engelsberg et al., 2004). Moreover, explantation of rat retina, at postnatal days 11 to 13, triggers activation of stress signaling pathways that might lead to photoreceptor loss and alterations in retinal structure. Although adult rat retinal organotypic cultures do not exactly recapitulate *in vivo* homeostasis, this experimental model allows for direct retinal manipulation, and controlling retinal environment (Bull et al., 2011).

Herein, we described that retinal microglial cells become reactive after exposure to EHP, similarly to what occurs with LPS. Early and exacerbated activation of microglial cells has emerged as a hallmark of the degenerative process in models of glaucoma (Bosco et al., 2015; Bosco et al., 2011; Naskar et al., 2002), suggesting the contribution of the neuroinflammatory response to the pathophysiology of glaucoma. In animal models and glaucoma patients, increased levels of iNOS and NO (Cho et al., 2011; Schneemann et al., 2003), TNF (Tezel et al., 2001; Tezel et al., 2004; Yang et al., 2011), IL-1β (Yoneda et al., 2001) were reported. Concomitantly, after exposure to EHP, in both cell and organotypic cultures, we detected increased expression levels of these pro-inflammatory markers. These results indicate that EHP, triggered by a custom-made pressure chamber, might be seen as an interesting system to investigate molecular and cellular mechanisms involved in glaucoma. Still, with the retinal organotypic culture model, we cannot directly determine the cell source involved in neither the neuroinflammatory response (namely TNF and IL-1β) nor the cells in which A$_{2A}$R antagonist is acting. Nevertheless, when we used retinal microglial cell cultures, we showed that EHP impacted microglial cells and that A$_{2A}$R blockade was able to control microglia reactivity and pro-inflammatory response. These results suggested that even in more complex experimental models, such as the organotypic cultures, A$_{2A}$R blockade attenuates microglia response to EHP.

Increased levels of ATP have been demonstrated in experimental glaucoma (Beckel et al., 2014; Lu et al., 2015; Reigada et al., 2008) and glaucoma patients (Li et al., 2011; Zhang et al., 2007). The exposure of retinal organotypic cultures to EHP (or LPS) increased the extracellular levels of ATP, suggesting ATP as the source of adenosine that would act on A$_{2A}$Rs. Up-regulation of A$_{2A}$R has been described in several noxious conditions (George et al., 2015; Rebola et al., 2011; Yu et al., 2008), highlighting the role of A$_{2A}$R signaling under noxious conditions. We found that both LPS and EHP trigger up-regulation of the A$_{2A}$R expression in cultured retinal microglia and retinal organotypic cultures. Notably, in retinal organotypic cultures, the up-regulation of A$_{2A}$R in the GCL was mainly observed in microglia. Moreover, we also detected the up-regulation of the A$_{2A}$R in the retinas of animals with OHT. Although the cell types up-

regulating $A_{2A}R$ in these animals were not determined, we hypothesized that it might be occurring mainly in microglia. Interestingly, treatment of retinal organotypic cultures with ADA, to remove the extracellular adenosine, prevented the increase in the extracellular accumulation of inflammatory markers, suggesting that adenosine present in the culture medium might be modulating the inflammatory response trough $A_{2A}R$. Taking into account that adenosine formed from ATP degradation, through CD73, preferentially activates $A_{2A}R$ (Augusto et al., 2013), these results demonstrate the important role of $A_{2A}R$ in modulating retinal inflammation as well as in the response to elevated IOP.

Additionally, we demonstrated that both LPS and EHP decreased the number of RGCs in the retinal organotypic cultures. Notably, blockade of $A_{2A}R$ prevented the loss of RGCs, suggesting a neuroprotective action of $A_{2A}R$ antagonists in the retina. Interestingly, the incubation with antibodies anti-TNF and anti-IL-1β depicted similar effects to those obtained with SCH 58261, highlighting the critical contribution of neuroinflammatory processes to the loss of RGCs, and suggesting that $A_{2A}R$ antagonists confer neuroprotection to RGCs through the control of microglia-mediated neuroinflammation. In accordance, in the context of glaucoma, previous studies demonstrated efficacy in controlling microglial cell activation (Bosco et al., 2012; Bosco et al., 2008) or pro-inflammatory cytokines (Sivakumar et al., 2011).

We then used the retinal I-R injury animal model, to assess the potential protective effects of $A_{2A}R$ antagonist against ischemic injury. Although this animal model does not completely mimic the clinical situation of POAG, it has been a widely studied model of retinal degeneration (Abcouwer et al., 2013; Neufeld et al., 2002). In fact, I-R has been widely used in molecular, cellular and pharmacological studies in glaucoma, presenting microglial cell activation, and damage in various retinal layers, including GCL (Johnson and Tomarev, 2010). Similarly to the results obtained with the *in vitro* models, we found that blockade of $A_{2A}R$ prevented microglial cell reactivity and the increase in neuroinflammatory response triggered by I-R. In addition, intravitreal administration of $A_{2A}R$ antagonist prevented the I-R-induced neuronal cell death and RGC loss. Although we did not assess the cell source of the neuroinflammatory molecules, this response appears to play a crucial role in RGC loss, since similarly to what was observed in retinal organotypic cultures, intravitreal injection with antibodies against TNF and IL-1β was also able to reduce RGC loss, further supporting the possible mechanism by which $A_{2A}R$ antagonist is affording protection being the control of retinal neuroinflammation.

Caffeine, the most consumed psichostimulant drug, is an antagonist of adenosine receptors, namely A_1R and $A_{2A}R$ (Fredholm et al., 2005). In several models of brain degenerative conditions, caffeine administration exerts beneficial effects, in particular in the control of neurodegeneration (Espinosa et al., 2013; Kaster et al., 2015; Prediger, 2010) by acting on $A_{2A}R$, regulates rapid microglia responses to injury and consequent neuroinflammatory response

(Brothers et al., 2010; Kang et al., 2012; Lee et al., 2013; Ruiz-Medina et al., 2013). Indeed, several authors have shown that chronic caffeine consumption could exert anti-inflammatory effects in mice models of PD, culminating in neuroprotective effects mediated by $A_{2A}R$ (Kalda et al., 2006; Lee et al., 2013; Ruiz-Medina et al., 2013). Using an animal model of OHT, where the retrograde axonal transport deficits and loss of RGC have been characterized (Agudo-Barriuso et al., 2013; Salinas-Navarro et al., 2010; Salinas-Navarro et al., 2009; Vidal-Sanz et al., 2012), we found that OHT elicited up-regulation of $A_{2A}R$ without alterations in the expression of A_1R, suggesting that the effects of caffeine might be mediated by $A_{2A}R$ blockade. Interestingly, in OHT animals, we demonstrated that, similar to what we previously observed in the *in vitro* models and I-R animal model treated with SCH 58261, caffeine reduced the increased retinal microglia reactivity and neuroinflammatory response and increased the survival of RGCs. Taking together, these observations enrich the hypothesis that caffeine, by blocking $A_{2A}R$, is involved in the control of retinal neuroinflammation, therefore affording neuroprotection and increasing the survival of RGCs in an IOP-independent mechanism.

The effects of consumption of caffeine in IOP are not yet clarified. In fact, a study has previously suggested that caffeine consumption might increase IOP in patients with NTG or OHT (Avisar et al., 2002), whereas, more recently, others have shown that caffeine does not significantly alter IOP in patients with glaucoma (Chandra et al., 2011). Nonetheless, in our work, we showed that caffeine administration reduced IOP only in OHT animals. This effect may not have physiological relevance since the IOP values are still very elevated, and the animals are considered as having OHT, suggesting a protective effect of caffeine in an IOP-independent mechanism (Boia et al., 2016). The effects of adenosine in the control of IOP have been studied in animals with normal IOP (Avila et al., 2001; Crosson and Gray, 1996) and OHT animals (Razali et al., 2015). This modulation occurs mainly by the actions of adenosine in adenosine receptors present in the ciliary tissues, thus regulating aqueous humor production (Donegan and Lieberman, 2015). In our study, we did not evaluate the effect of selective $A_{2A}R$ blockade on IOP, due to the I-R model limitations. Remarkably, topical administration of $A_{2A}R$ antagonists have been shown to reduce IOP (Razali et al., 2015) and might be seen as promising IOP lowering agents (Donegan and Lieberman, 2015).

Nevertheless, despite the caffeine-induced benefits observed in the control of retinal neuroinflammation and in the survival of RGCs in OHT animals, caffeine administration did not prevent functional OHT-induced damage in optic nerve, namely the alterations in the retrograde axonal transport. Since ours and previous results demonstrate the ability of caffeine in preventing microglia reactivity, and taking into consideration that microglial cells located near the RGC soma express $A_{2A}R$, it is tempting to speculate that caffeine increased RGC survival through the control of the OHT-induced retinal microglia reactivity. However, since caffeine did

not improve OHT-impaired axonal transport, the protective properties of caffeine may be lost when assessing other time points.

Contrasting to our results, other authors have reported that it is the activation of $A_{2A}R$ that controls retinal neuroinflammation and confers neuroprotection (Ahmad et al., 2013; Konno et al., 2006). This bidirectional role of $A_{2A}R$ has been described, to be associated with the developmental stages of the animals or distinct stages of the pathological process (Dai and Zhou, 2011). In fact, distinct functions have been attributed to $A_{2A}R$ in peripheral inflammation and CNS neuroinflammation (reviewed in Cunha et al., 2007). While in the CNS, blockade of $A_{2A}R$ has been widely shown to confer neuroprotective effects and control neuroinflammation (reviewed in Gomes et al., 2011; Santiago et al., 2014), in the periphery, it is the activation of $A_{2A}R$ that controls the inflammatory response (Hasko and Pacher, 2008). Therefore, not only the doses and exposure times of $A_{2A}R$ antagonist should be taken into consideration when planning studies in the CNS and preparing therapeutic protocols, but also the administration routes are particularly important. For instance, it was previously reported that local injection of an $A_{2A}R$ agonist in the CNS failed to afford protection, whereas peripheral administration of the same compound presented beneficial effects against CNS excitotoxicity (Jones et al., 1998); this might be related with the opposite modulation of $A_{2A}R$.

Still, more studies are required to complety clarify the potential benefits of $A_{2A}R$ blockade in this pathology, as well as doses and administration routes. Although intravitreal injections of $A_{2A}R$ antagonists, as used in the I-R model, circumvent the limitations of peripheral administration, it still presents some restrictions, as the need to repeat the injections. Therefore, sustained-release drug delivery systems might be seen as a possible administration strategy to administer $A_{2A}R$ antagonists in models of glaucoma and possibly, in the future, in glaucoma patients. Furthermore, other selective pharmacological antagonists of $A_{2A}R$ should be evaluated in glaucomatous animal models. A good candidate to be tested is KW-6002, an $A_{2A}R$ antagonist with excellent bioavailability via oral administration, long half-life and good brain penetration (Yang et al, 2007). Currently, KW-6002 is one of the most advanced $A_{2A}R$ antagonists in drug development. In fact, it has been extensively investigated as therapeutic adjuvant for the treatment of PD and it was found that it exerts beneficial effects in the control of neuroinflammation and neuroprotection in animal models of the disease (Kalda et al., 2006; Lee et al., 2013) and human patients (Kondo et al., 2015; Pinna, 2014). In Japan it has been approved in the adjunctive treatment of PD, yet FDA issued it as non-approvable (Yu et al., 2008). The results presented in this work were obtained by incubating/administrating $A_{2A}R$ antagonist prior the noxious stimulus, albeit important as a proof-of-concept on the beneficial properties of $A_{2A}R$ antagonists do not mirror the clinical situation. Therefore, other studies

administering KW-6002 (or other A$_{2A}$R antagonist) after inducing OHT would definitely elucidate on the potential of A$_{2A}$R blockade as a strategy to treat glaucoma.

Likewise, the involvement of A$_{2A}$R modulation on microglia-mediated neuroinflammatory response in the context of glaucoma should be further explored. Future strategies should aim the depletion of retinal microglial cells in animal models of glaucoma, for instance by using clodronate-containing liposomes (Arroba et al., 2014), to evaluate the direct contribution of microglial cells to RGC loss. Strategies aiming the retinal microglial cell-targeted A$_{2A}$R silencing in animal models of glaucoma might further elucidate the effects of A$_{2A}$R in glaucomatous neuroinflammation and RGC loss.

In summary, we provide strong evidences that selective antagonists of A$_{2A}$R and caffeine administration are able to modulate retinal microglial cell reactivity and the consequent neuroinflammatory response. These effects lead to an increased survival of RGCs, which, in an animal model of OHT, occurred in an IOP-independent way. These results support the already described crucial contribution of microglia-mediated neuroinflammation to the development of glaucoma.

The results obtained in this work prompt the use of A$_{2A}$R antagonists (or caffeine), as a potential therapeutic strategy to manage the glaucomatous damage, and might even be envisaged as a combination with IOP-lowering agents in order to circumvent other glaucomatous damage. Moreover, blockade of A$_{2A}$R can also be seen as a neuroprotective strategy in other retinal diseases involving microglia-mediated neuroinflammation.

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CHAPTER 6 –

Main Conclusions

6.1 Main conclusions

The results obtained in this work allowed us to draw the following main conclusions:

- The exposure of retinal purified microglial cell cultures and organotypic cultures to LPS or EHP leads to up-regulation of $A_{2A}R$ and increased inflammatory response. Moreover, OHT elicits an up-regulation of $A_{2A}R$ in the retina, prompting the important role of this receptor in the retinal responses to elevation of IOP.

- The blockade of $A_{2A}R$ prevents microglia reactivity and pro-inflammatory responses triggered by LPS or EHP in both retinal purified microglial cell cultures and retinal organotypic cultures as well as triggered by transient retinal ischemia.

- The blockade of $A_{2A}R$ confers protection to RGCs through the control of neuroinflammation elicited by EHP (organotypic retinal cultures) or by transient retinal ischemia or OHT.

- Caffeine administration prevents microglia activation, neuroinflammatory response and RGC death elicited by OHT, probably by blocking the $A_{2A}R$.

Taking together, our results demonstrate that the antagonism of $A_{2A}R$ may be envisaged as a potential therapeutic strategy for the treatment of glaucoma, and caffeine consumption may exert prophylactic effects on glaucomatous damage.