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DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

# Modulation of pro-inflammatory response of retina by adenosinergic systems

Neuroinflammation/Neuroprotection

Raquel Sofia Freitas Boia

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Investigadora Doutora Ana Raquel Sarabando Santiago (Faculdade de Medicina da Universidade de Coimbra) e Investigador Doutor Paulo Santos (Faculdade de Ciências e Tecnologia da Universidade de Coimbra).

Raquel Sofia Freitas Boia

2013

“Sê todo em cada coisa. Põe quanto és

No mínimo que fazes.”

Ricardo Reis, in "Odes"
Heterónimo de Fernando Pessoa

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# Abreviations

| | |
|---|---|
| 2-CN-Ado | 2-(6-cyano-1-hexyn-1-yl)adenosine |
| A1R | $A_1$ receptor |
| A2AR | $A_{2A}$ receptor |
| A2BR | $A_{2B}$ receptor |
| A3R | $A_3$ receptor |
| AC | Adenylyl cyclase |
| ADP | Adenosine 5'- diphosphate |
| AMP | Adenosine 5'- monophosphate |
| ARs | Adenosine receptors |
| ATP | Adenosine 5'- triphosphate |
| BRB | Blood-retinal barrier |
| cAMP | Cyclic adenosine 5'-monophosphate |
| CGS 21680 | 4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride |
| CNS | Central Nervous System |
| CREB | cAMP responsive element binding protein |
| CSC | 8-(3-chlorostyryl)caffeine |
| DAPI | 4',6-diamidino-2-phenylindole |
| DMSO | Dimethyl sulfoxide |
| G proteins | GTP-binding proteins |
| GABA | gamma-aminobutyric acid |
| GCL | Ganglion cell layer |
| GFAP | Glial fribrillary acidic protein |
| Gi | Inhibitory G-protein |
| Gs | Stimulatory G-protein |

| | |
|---|---|
| HPLC | High performance liquid chromatography |
| Iba1 | Ionized calcium binding adaptor molecule 1 |
| iBRB | Inner blood-retinal barrier |
| IL | Interleukin |
| INL | Inner nuclear layer |
| IOP | Intraocular pressure |
| IPL | Inner plexiform layer |
| I-R | Ischemia-reperfusion |
| LPS | Lipopolysaccharide |
| MAP | Mitogen-activated protein |
| MHC | Major histocompatibility complex |
| NFL | Nerve fiber layer |
| oBRB | Outer blood-retinal barrier |
| OCT | Optimal cutting temperature |
| OLM | Outer limiting “membrane” |
| ONL | Outer nuclear layer |
| OPL | Outer plexiform layer |
| PI3 | Phosphoinositide 3 |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| Prot | Protein |
| POS | Photoreceptor outer segments |
| REAL | Regulators of Endogenous Adenosine Levels |
| RGCs | Retinal ganglion cells |
| ROS | Reactive oxygen species |
| RPE | Retinal pigment epithelium |

| | |
|---|---|
| SAH | S-adenosyl-homocysteine |
| SCH 58261 | 2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine |
| SSC | Saline-citrate |
| TNF | Tumor necrosis factor |

# Resumo

O glaucoma é uma doença neurodegenerativa e uma das principais causas de cegueira em todo o mundo. O aumento da pressão intraocular é considerado um dos principais factores de risco para o desenvolvimento da doença. Contudo, mesmo pacientes com a pressão intraocular controlada continuam a perder a visão. Deste modo, é imperativo a existência de tratamentos mais eficazes e, a neuroprotecção das células ganglionares da retina mostra ser uma alternativa com potencial terapêutico. As células da microglia desempenham um papel importante no desenvolvimento do glaucoma, uma vez que, quando estas células ficam reactivas libertam mediadores inflamatórios que contribuem para o processo neurodegenerativo.

A adenosina é um neuromodulador do sistema nervoso central e é um dos sistemas de neuroprotecção mais promissores no CNS. A modulação do recetor de adenosina A2A (A2AR) tem sido um assunto intensamente abordado na literatura como um potencial alvo terapêutico, visto que o bloqueio do A2AR confere neuroproteção, após dano no sistema nervoso central.

A cafeína é o psicoestimulante mais usado em todo o mundo e os seus efeitos são maioritariamente mediados pelo bloqueio dos recetores de adenosina. Vários estudos mostram que o consumo de cafeína confere neuroproteção, após dano no sistema nervoso central.

Os principais objetivos deste trabalho foram a) investigar se a modulação da atividade do A2AR controla a neuroinflamação na retina num modelo de isquémia-reperfusão (I-R); e b) investigar os efeitos anti-inflamatórios e neuroprotectores da cafeina no modelo de I-R.

Para tal foram usados dois grupos distintos de animais. O primeiro grupo de ratos Wistar foi previamente tratado através de injeção intravítrea de CGS 21680 (agonista do A2AR) ou SCH 58261 (antagonista do A2AR), 2 horas antes da indução de I-R na retina. Ao segundo grupo de ratos Wistar foi administrado cafeína na água, duas semanas antes da indução de I-R. Com isto, avaliou-se a reatividade das células da microglia, a produção de citocinas pro-inflamatórias e de espécies reativas de oxigénio.

A injeção intravítrea do agonista ou antagonista do A2AR não alterou os níveis de TNF, IL-1β e de IL-6, no entanto, o antagonista diminuiu o número de microglias/macrófagos

reativas induzidas pela I-R da retina. Após 24 h de isquémia, a cafeína aumentou a reatividade das células da microglia, induzida pela I-R e aumentou a morte celular na retina. Por outro lado, após 7 dias de isquémia, a cafeína diminuiu a reatividade da microglia e a produção de citocinas e de espécies reativas de oxigénio. A cafeína também atenuou a morte das células ganglionares da retina, induzida por I-R.

Em suma, os nossos resultados mostraram que a ativação ou o bloqueio do A2AR e o consumo de cafeína foi capaz de controlar a neuroinflamação, controlando a reatividade das células da microglia, conferindo neuroprotecção às células da retina.

Palavras-chave: Glaucoma, neuroinflamação, recetores A2A de adenosina, cafeína, neuroproteção

# Abstract

Glaucoma is a retinal neurodegenerative disease and one of the main causes of blindness in the world. The increase in intraocular pressure (IOP) is considered the main risk factor for the development of glaucoma, but patients continue to lose vision despite adequate IOP control. Therefore, new and more effective treatments are necessary, and neuroprotection of retinal ganglion cells (RGCs) is considered a potential alternative therapy. Neuroinflammation plays an important role in the development of glaucoma. Microglia becomes reactive, leading to increased production of inflammatory mediators that contribute for the neurodegenerative process.

Adenosine is a neuromodulator in the central nervous system (CNS). It has been claimed that adenosine is one of the most promising neuroprotective systems in CNS. Much attention is being given to adenosine A2A receptor (A2AR) modulation as potential therapeutic target since the blockade of A2AR affords robust neuroprotection upon CNS injury.

Caffeine is the most commonly used psychostimulant in the world and its effects are mainly mediated by antagonizing adenosine receptors. Many studies show that caffeine consumption affords neuroprotection upon CNS injury.

The main goals of this work were a) To investigate whether the modulation of A2AR activity controls retinal neuroinflammation in ischemia-reperfusion (I-R) model; and b) To investigate the anti-inflammatory and neuroprotective effects of caffeine in I-R model.

Two groups of animals were used. The first group of Wistar rats was pretreated either with CGS 21680 (A2AR agonist) or SCH 58261 (A2AR antagonist) by intravitreal injection, 2 hours prior to I-R injury. The second group of Wistar rats received with caffeine (1 g/L) in drinking water, 2 weeks prior to injury. Microglia reactivity, levels of pro-inflammatory cytokines, ROS generation, and cell death and RGC loss were assessed.

Intravitreal injection of A2AR agonist or antagonist did not change the levels of TNF, IL-1β and IL-6. However, A2AR antagonist decreased the number of reactive microglia/macrophages induced by I-R injury. After 24h post-ischemia, caffeine exacerbated the reactivity of microglia induced by I-R injury, and increased cell death. On the other hand, 7 days post-ischemia, the treatment with caffeine decreased microglia

reactivity, and the production of cytokines and reactive oxygen species (ROS). Caffeine was also able to attenuate RGC death, induced by I-R injury.

In summary, our results demonstrated that the activation or blockage of A2AR and the caffeine consumption was able to control the neuroinflammatory response by microglia, controlling their reactivity and affording neuroprotection to retinal cells.

Keywords: Glaucoma, neuroinflammation, adenosine A2A receptors, caffeine, neuroprotection

# *Chapter 1*

# 1. Introduction

## 1.1. Vision

"The eyes are our door to the world". Our perception of the world in terms of its colors and its shapes is given to us by the eyes. This is a highly specialized organ of photoreception, in which light energy from the environment produces changes in specialized nerve cells in retina, thereby forming nerve action potentials which are transmitted through optic nerve to the brain, where the information is processed.

## 1.2. Anatomy of eyeball

The eye is divided into three layers (Figure 1). The outermost layer of the eye is composed by the sclera, a conjunctive tissue layer that helps maintaining the eye shape and protects the internal structures. This opaque layer is contiguous to the cornea, in the anterior part of the eye, a specialized transparent tissue that allows light rays to enter the eye.

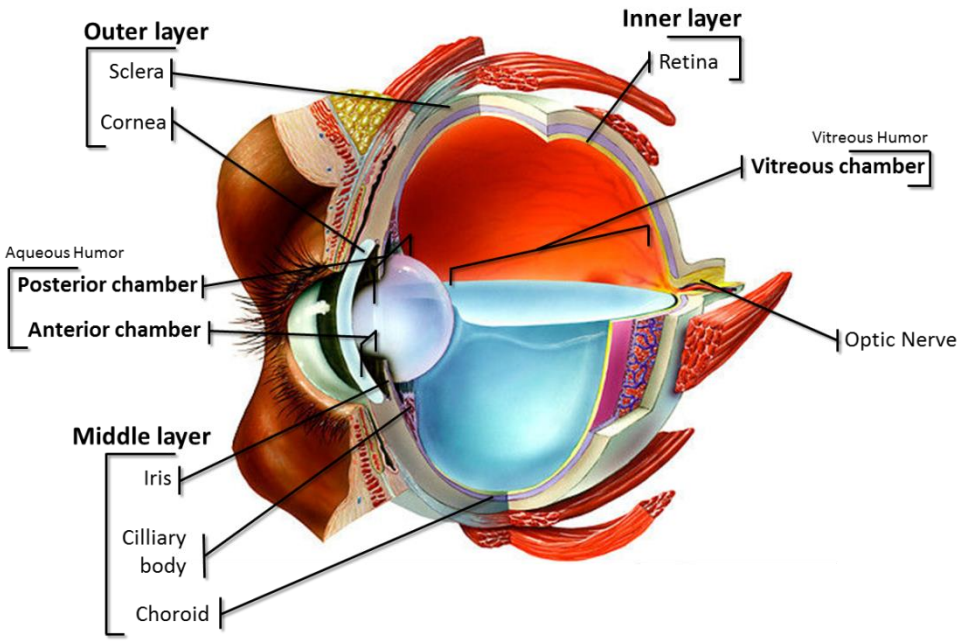


Figure 1 - Anatomy of human eyeball (adapted from http://www.sciencephoto.com/).

The immediately adjacent layer is the uveal tract which includes three distinct structures: iris, ciliary body and choroid. The iris is the colored portion of the eye and can be seen through the cornea. It contains two sets of muscles with opposite actions that can increase or decrease the pupil's diameter to admit more or less light entering. The ciliary body is a muscular component and it is attached to the lens by zonule fibers whose function is to determine the refractive power of lens, allowing image formation. The ciliary body has a vascular component which produces the aqueous humor that fills the anterior and posterior chambers of the eye (Figure 1). Aqueous humor is secreted in the posterior chamber and passes through the pupil into the anterior chamber. Choroid is rich in blood vessels and its main function is to nourish the outermost layers of the retina. The retina is the most inner layer of the eye and it is a light sensitive tissue that has the function of photoreception and transmits impulses to the brain. The portion of the eye located between the lens and the surface of retina is the vitreous chamber and it is filled by a viscous substance called vitreous humor (Purves *et al.*, 2001).

## 1.3. Retina

Retina is part of the central nervous system (CNS) and it is constituted by three main types of cells: neurons, glial cells and blood vessels (Kolb *et al.*, 1995).

### 1.3.1. Neurons

The neuronal component of the retina is composed by five different types of neuronal cells: photoreceptors (rods and cones), bipolar cells, horizontal cells, amacrine cells and retinal ganglion cells (RGCs) (Figure 2).

The retinal pigment epithelium (RPE) is the outermost layer of retina and is composed by a single layer of hexagonal cells. Interactions between retinal pigmented cells and photoreceptors are essential for visual function (Kolb *et al.*, 1995). Neuronal cells bodies are divided into three different layers: the outer nuclear layer (ONL) that contains the cell bodies of rods and cones, the inner nuclear layer (INL) that contains the cell bodies of bipolar, horizontal and amacrine cells, and the ganglion cell layer (GCL) that contains cell bodies of ganglion cells and displaced amacrine cells. The axons of ganglion cells travel towards the optic nerve within the nerve fiber layer (NFL). Moreover, there are two

layers of synapses: the outer plexiform layer (OPL), where photoreceptors make synapses with bipolar and horizontal cells, and the inner plexiform layer (IPL) that connects bipolar cells to RGCs (Kolb *et al.*, 1995).

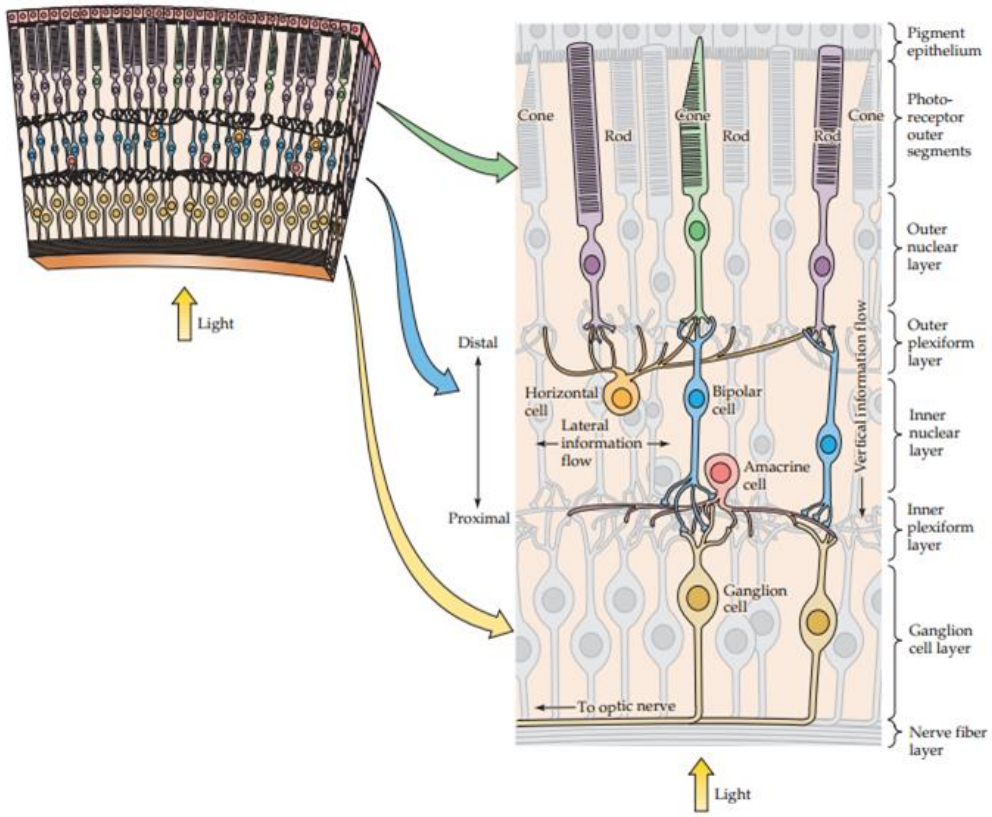


Figure 2 - Structural layers of the retina. Photoreceptors, bipolar cells and ganglion cells provide the most direct route for transmitting visual information to the brain. Horizontal and amacrine cells mediate lateral interactions in the outer and inner plexiform layers (adapted from Purves *et al.*, 2001).

When light reaches the retina it passes across these layers and photoreceptors traduce this light into electrical signals. In the vertical signal pathway, photoreceptors transmit signals to bipolar cells which make synaptic contact with ganglion cells. The horizontal pathway, provided by horizontal cells, enables interactions between photoreceptors and bipolar cells at OPL, and amacrine cells, which affect bipolar and ganglion cells interactions (Cervia and Casini, 2012).

#### *1.3.1.1. Photoreceptors*

Photoreceptors consist of 1) an outer segment containing the visual pigment molecules (rhodopsin); 2) an inner segment that contains mitochondria, ribosomes and membranes where opsin molecules are assembled and passed to be part of the outer

segment discs; 3) a cell body with nucleus of photoreceptor cell; 4) a synaptic terminal where neurotransmission with other neuronal cell types occurs.

In the retina there are two types of photoreceptors: rods and cones. Cones are robust conical-shaped structures with their outer/inner segments protruding the RPE. In darkness there is a high release of glutamate by cones. Rods are slim rod-shaped structure filling the space between cones (Kolb *et al.*, 1995).

#### *1.3.1.2. Bipolar cells*

Bipolar cells receive neurotransmission signals from photoreceptors and transmit them to amacrine and ganglion cells. In the human retina, ten different bipolar cells are present to receive signals from cones and one type to receive inputs from rods (Kolb *et al.*, 1995).

The postsynaptic neurons in OPL express different glutamate receptors which allow the distribution of signal into multiple pathways, based on how cone bipolar cells react to glutamate released by cones. Horizontal and OFF cone bipolar cells express ionotropic glutamate receptors and are hyperpolarized by light. ON cone bipolar cells express metabotropic glutamate receptors and are depolarized by light (Kolb *et al.*, 1995).

#### *1.3.1.3. Ganglion cells*

Ganglion cells collect signals from bipolar and amacrine cells and transmit them through axons to the brain. Ganglion cells are also divided into OFF and ON and have a different response, according to pathways initiated by bipolar cells (Kolb *et al.*, 1995).

#### *1.3.1.4. Horizontal cells*

Horizontal cells are the interneurons in the retina and the second neurons that contact directly with photoreceptors. These cells provide negative signals to cones contributing to the maintenance of visual sensitivity to light (Kolb *et al.*, 1995).

#### *1.3.1.5. Amacrine cells*

Amacrine cells are interneurons that interact with bipolar cells and RGCs at the level of IPL, and different kinds of amacrine cells can be found in the retina. These are inhibitory interneurons which contribute to visual function (Kolb *et al.*, 1995).

### 1.3.2. Blood vessels

The retinal tissue has higher levels of oxygen consumption per unit of tissue weight than any other human tissue. For this reason, the retina needs an efficient supply of oxygen and nutrients (Klaassen *et al.*, 2013). There are two distinct vascular beds that provide blood supply to the retina, the choroidal blood vessels and the central retinal artery (Kolb *et al.*, 1995; Pournaras *et al.*, 2008). The first one provides blood supply to photoreceptors in the outer retina, while the central retinal artery gives rise to capillaries that innervate the inner retina (Klaassen *et al.*, 2013).

Cellular barriers are required to maintain an appropriate, tightly regulated environment around cells. Endothelial cells which constitute the blood-retinal barrier (BRB) play a critical role in restricting the nonspecific transport of hydrophilic substances and facilitating the transport of essential molecules between the circulating blood and the retina (Fernandes *et al.*, 2011).

The BRB is divided into two components (Figure 3), the inner blood-retinal barrier (iBRB) and the outer blood-retinal barrier (oBRB) (Pournaras *et al.*, 2008). The iBRB is constituted by the endothelium, that composes intraretinal blood vessels; pericytes which are modified smooth muscle cells of capillaries and may have contractile functions that regulate retinal vascular flow by dilating and contracting (Gardner *et al.*, 2002; Pournaras *et al.*, 2008); and glial cells, which provide signals that have impact on the development and maintenance of the barrier (Pournaras *et al.*, 2008). The oBRB is composed by the endothelium of the choriocapillaris, Bruch's membrane and RPE (Pournaras *et al.*, 2008).

Several mechanisms for BRB loss and leakage have been proposed and some diseases, such as diabetic retinopathy, age-related macular degeneration, retinal vein occlusion and uveitis may be associated with BRB breakdown (Klaassen *et al.*, 2013).

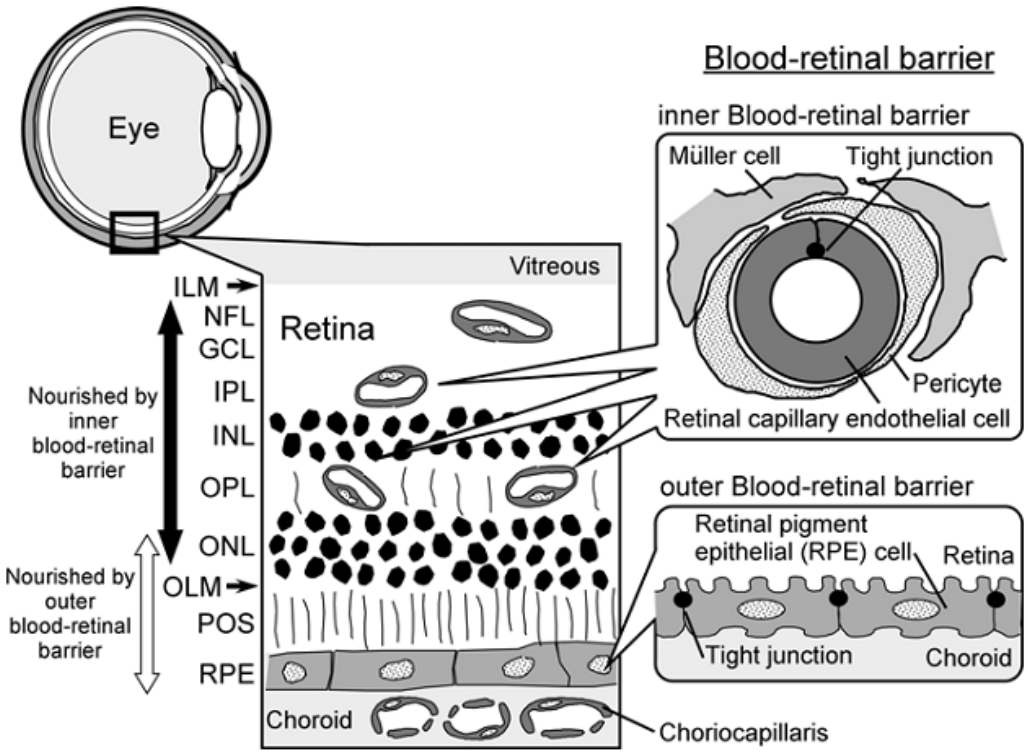


Figure 3 - Schematic diagram of the blood-retinal barrier. RPE: retinal pigment epithelium; POS: photoreceptor outer segments; OLM: outer limiting "membrane"; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; NFL: nerve fiber layer; ILM: inner limiting "membrane" (Hosoya and Tomi, 2008).

### 1.3.3. Glial cells

The third type of cells that compose the retina are glial cells. There are three types of glial cells in retina: Müller cells, astrocytes, and microglia, which have a role of neuro-supporting and immunocompetent defense (Langmann, 2007).

#### *1.3.3.1. Müller cells*

Müller cells are the principal glial cells which span the entire depth of the retina and play an essential role in the normal function of the retina. The main function of Müller cells is the regulation of extracellular concentration of $K^+$, which increases after light stimulation. Müller cells also play an important role in removing neurotransmitters, such as glutamate, from extracellular space following their release in the synaptic terminal (Newman and Reichenbach, 1996; Gardner *et al.*, 2002).

#### *1.3.3.2. Astrocytes*

Astrocytes are mostly located in NFL and they have several important physiological properties that are related to CNS homeostasis (Gardner *et al.*, 2002). In response to stimuli, astrocytes proliferate, change their morphology and increase the expression of glial fribrillary acidic protein (GFAP), a process designated as astrogliosis (Hasko *et al.*, 2005).

Moreover, astrocytes are part of the BBR, having a close association with retinal vessels and regulating its properties. In addition, due to their abundant contents in glycogen, they form a nutritive support in providing glucose to neurons (Kolb *et al.*, 1995).

#### *1.3.3.3.Microglia*

Microglia cells are the resident macrophages of the CNS and their function is the surveillance of the microenvironment. At this stage, microglia has a ramified morphology in which long thin processes extend from the cell body (Kettenmann *et al.*, 2011; Morrison and Filosa, 2013).

When CNS homeostasis is impaired by several factors, such as infection, trauma, ischemia and neurodegenerative disease, microglia adopts a so-called activated state with a globular phagocyte structure (Hasko *et al.*, 2005; Bosco *et al.*, 2011). Despite this morphological transition being clearly described, the different stages of microglial activation are not well understood. A recent study has focused on this issue, proposing that there are six stages of bidirectional microglial activation and deactivation (Jonas *et al.*, 2012).

Upon inflammation and infection, exogenous signals can lead to more microglial activation through the recognition of pro-inflammatory mediators by surface molecules of microglia (cytokines receptors, scavenger receptors, pattern recognition receptors and chemokines receptors). Activation of these receptors leads to a microglial phenotype transition into an activated state, which contributes to the ongoing inflammation process (Kierdorf and Prinz, 2013). However, chronic microglial activation leads to neuronal death, which is closely related with neurodegenerative diseases progression such as Alzheimer's disease, Parkinson's disease, and also glaucoma. More attention has been given to interactions between neurons and glia as being the cause underlying the

degenerative process in these diseases. As recently reviewed, neurotransmitters, such as glutamate, gamma-aminobutyric acid (GABA), adenosine 5'-triphosphate (ATP), catecholamines (adrenaline, noradrenaline and dopamine) and acetylcholine, may modulate microglial-mediated neuroinflammation, since microglia express most of the receptors for those transmitters. ATP is released at high concentrations upon cell death and tissue destruction and later, it is rapidly degraded to adenosine 5'-diphosphate (ADP) and adenosine which then play their physiology functions by activating specific receptors (Lee, 2013).

#### *1.3.3.4. Retinal microglial cells*

There is a lot of research data that focuses in microglia homeostasis in CNS, but less is known about the function of microglial cells of the retina.

In the retina, microglial cells soma are located at the NFL, GCL as well as within IPL or above the INL (Bosco *et al.*, 2011).

When microglial cells become active, their neurosupportive functions are compromised, leading to increased vulnerability of RGCs and driving their axons to injury (Tezel, 2011). The localization of microglia in the GCL and IPL suggests that microglia can signal to RGCs, and in case of exacerbated microglial activation, promote cell death.

In different retinal regions, microglia cells are responsible for the surveillance of the environment with their highly motile protrusions (Figure 4A). Various stimuli lead to degeneration alert microglia (Figure 4B), which migrate to the lesion site and turn into their active state (Figure 4C) (Karlstetter *et al.*, 2010).

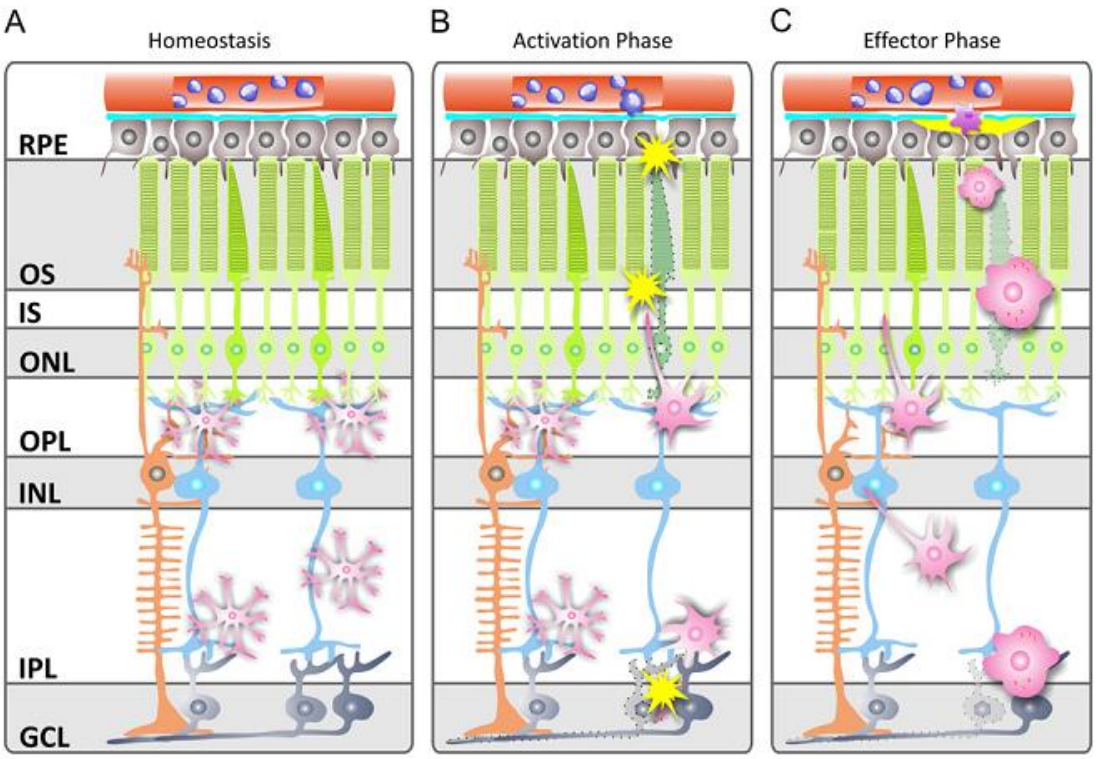


Figure 4 - Schematic representation of three common phases of microglial activity in the retina: A) in normal retina, B) various different insults leading to abnormal cell functions or degeneration in the RPE, the photoreceptor layer, and the ganglion cell layer rapidly alert microglia, C) microglial transformation into amoeboid morphology leading to an inflammatory response (RPE: retinal pigment epithelium; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer) (Karlstetter *et al.*, 2010).

## 1.4. Glaucoma

Glaucoma is one leading cause of blindness in the world. Globally, it was estimated that 60 million people have glaucomatous optic neuropathy, and an estimated 8.4 million people are blind as a result of glaucoma (Cook and Foster, 2012).

In the past, glaucoma was defined by an elevation of intraocular pressure (IOP). Today, glaucoma is considered a neurodegenerative disease, characterized by degeneration of the optic nerve and loss of RGCs (Vohra *et al.*, 2013).

Elevated IOP and age are the most recognized risk factors and the current therapies available are focused in reducing IOP (Johnson and Morrison, 2009). Nevertheless, even patients with normal IOP can be affected by this neuropathy.

Currently, the exact mechanism that initiates and leads to a glaucomatous optic nerve degeneration and RGCs loss is not fully understood. Many studies propose several

mechanisms which play an important role in glaucomatous neurodegeneration. Oxidative stress, due to mitochondrial dysfunction is one of many mechanism proposed (Tezel, 2011). Another proposed mechanism for glaucoma development is related with vascular deregulation (Leske, 2009).

Microenvironment signals, which are triggered by macroglial and microglia cells, are suggested to be important enhancers of glaucomatous neurodegeneration (Johnson and Morrison, 2009; Tezel, 2011).

### 1.4.1. Glaucoma and neuroinflammation

During the last years, microglia has been linked to the development of neurodegenerative disorders in which neuroinflammation is an integral part, in an initial phase of disease development and microglia initiates repair mechanisms. However, an exaggerated microglial reaction may lead to chronic inflammation (Langmann, 2007; Karlstetter *et al.*, 2010).

In an animal model of glaucoma, the DBA/2J mouse, with ocular hypertension, much effort has been made better to understand the role of glia. Microglial changes occur earlier than RGCs pathology, and no correlation between IOP elevation and microglial activation was found, indicating that IOP elevation may not be a contributing factor in the initial changes of this disease development (Bosco *et al.*, 2011). In contrast, Son et al. mentioned that activation of microglia is a process that occurs after large-scale neuronal loss in DBA/2J animal model of chronic glaucoma (Son *et al.*, 2010).

Likewise, in human glaucoma, abnormal activated ameboid cells were found (Neufeld and Liu, 2003). Regarding pro-inflammatory cytokines, it is described that there is an increase in their levels in the retina of glaucoma patients (Tezel *et al.*, 2001). Moreover, aqueous humor analysis of glaucoma patients demonstrate increased levels of pro-inflammatory cytokines (Kuchtey *et al.*, 2010; Sawada *et al.*, 2010; Chua *et al.*, 2012).

It is commonly accepted that glial activation and the prominent stimulation of pro-inflammatory signalling are a hallmark of neuroinflammation in the CNS which, in the presence of accumulating risk factors, can result in dysfunction of regulatory mechanisms.

### 1.4.2. Ischemia-Reperfusion Model

In several eye diseases, like in glaucoma, retinal ischemia is a clinical entity involved in the disease development and is a common cause of visual impairment and blindness. Despite this, the role of retinal ischemia in these diseases is not well understood (Cervia and Casini, 2012; Chen *et al.*, 2013).

Ischemia may be defined as an inefficient blood supply to the tissue, resulting in hypoxia and failure of tissue nutrient demands (Osborne *et al.*, 2004).

In clinical diseases, like glaucoma, it is very difficult to study pathophysiology in human patients. For this reason, the development of animal models of glaucoma is extremely important, in order to understand the underlying mechanism of disease and to develop new therapies. A wide variety of rodent models for glaucoma exist and ischemia-reperfusion (I-R) model is one of the examples. This I-R model is characterized by an elevation of IOP above systolic pressure and allows us to control the magnitude and duration of ischemia, as well as the duration of reperfusion, which are very important parameters concerning RGCs death (Selles-Navarro *et al.*, 1996; Johnson and Tomarev, 2010).

Although it does not directly follow the exact pathophysiology that occurs in human glaucoma, it is important to induce a specific insult to RGCs (Johnson and Tomarev, 2010).

## 1.5. Adenosine

Adenosine is a purine nucleoside present in all tissues and body fluids that behave as a general modulator of biological functions, such as energy metabolism. Under basal conditions, adenosine concentration is kept constant at low levels (20-200 nM) but under metabolic stress or under pathophysiological conditions there is an increase of adenosine levels to 10 μM (Schulte and Fredholm, 2003; Cunha *et al.*, 2007). Also in the retina, adenosine concentration increased after 5 min of ischemia and even during reperfusion period (Li and Roth, 1999).

The first report of adenosine's physiological effects was described in cardiovascular system, in 1929, but adenosine also plays an important role in the immune system, cell growth, proliferation, apoptosis and in the central nervous systems (Schulte and

Fredholm, 2003). Also, in retina, adenosine plays an important role in many physiological mechanisms such as in the regulation of the IOP (Wan *et al.*, 2011).

Adenosine is produced intracellularly as well as extracellularly (Figure 5). Intracellular adenosine production is either by breakdown of adenosine 5'-phosphates (AMP, ADP, ATP) which is mediated by an intracellular 5'-nucleotidase or by hydrolysis of S-adenosyl-homocysteine (SAH). The extracellular adenosine can be originated from intracellular pool through nucleoside transporters present in membrane or by fast nucleotide hydrolysis catalyzed by a cascade of ectoenzymes (ecto-ATPase, ecto-ATP-diphosphohydrolase and ecto-5' nucleotidase). Adenosine can be metabolized by phosphorylation to AMP or degradated to inosine, which is then catalyzed by adenosine kinase and adenosine deaminase, respectively (Schulte and Fredholm, 2003; Hasko *et al.*, 2005; Haskó *et al.*, 2008).

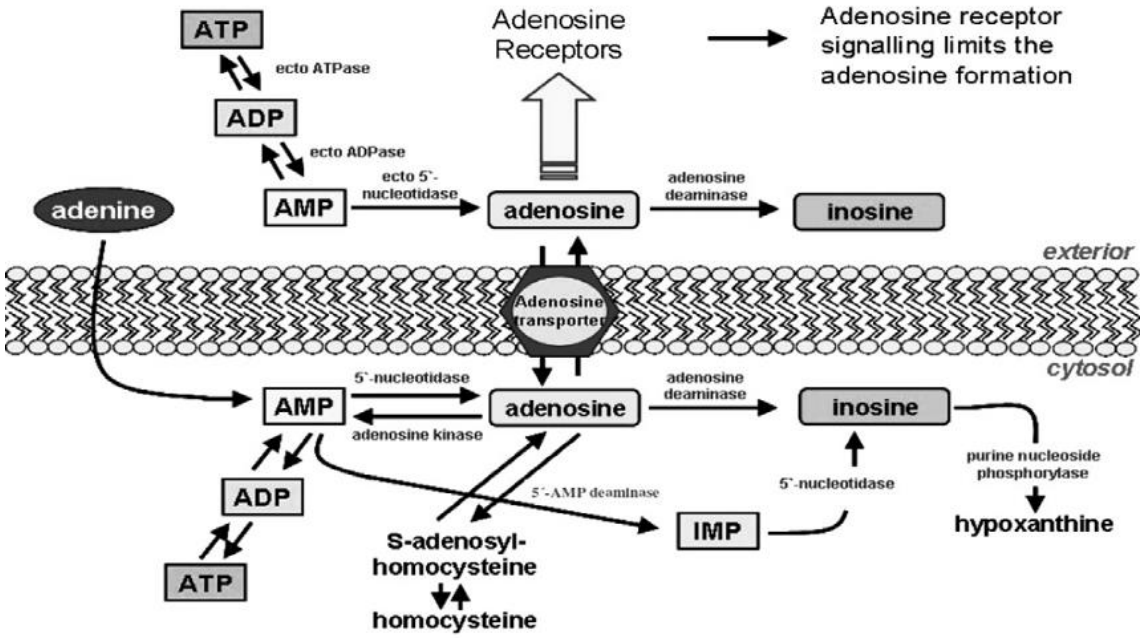


Figure 5 - Mechanism underlying formation of extracellular adenosine (Sachdeva and Gupta, 2012).

Once present extracellularly, adenosine effects are mediated by four distinct membrane adenosine receptors. All of the adenosine receptors are metabotropic and are composed of seven transmembranar domains with the N-terminus at the extracellular and the C-terminus at the intracellular face of the membrane; and are coupled to intracellular GTP-binding proteins (G proteins). These receptors are called pleiotropic receptors because different types of receptors are coupled to different G proteins with

different transducing systems according to the degree of activation and localization of the receptors (Cunha, 2005). So the effect of adenosine is mediated by four adenosine receptors (ARs) – $A_1$ receptor (A1R), $A_{2A}$ receptor (A2AR), $A_{2B}$ receptor (A2BR) and $A_3$ receptor (A3R). A1R and A3R are coupled to an inhibitory G protein ($G_i$) whereas A2AR and A2BR are coupled to a stimulatory G protein ($G_s$), inhibiting or stimulating adenylyl cyclase (AC) activity, respectively (Jacobson and Gao, 2006).

### 1.5.1. A1 receptors

The A1R is coupled with Gi, and when this receptor is activated, AC activity is inhibited with no increase in levels of cyclic adenosine 5'-monophosphate (cAMP) (Jacobson and Gao, 2006). Additionally, this receptor is linked to various kinase pathways including protein kinase C (PKC), phosphoinositide 3 (PI3) and mitogen-activated protein (MAP). A1R activation can, also, activate $K^+$ channels and inhibit $Ca^{2+}$ channels (Figure 6) (Hasko *et al.*, 2008).

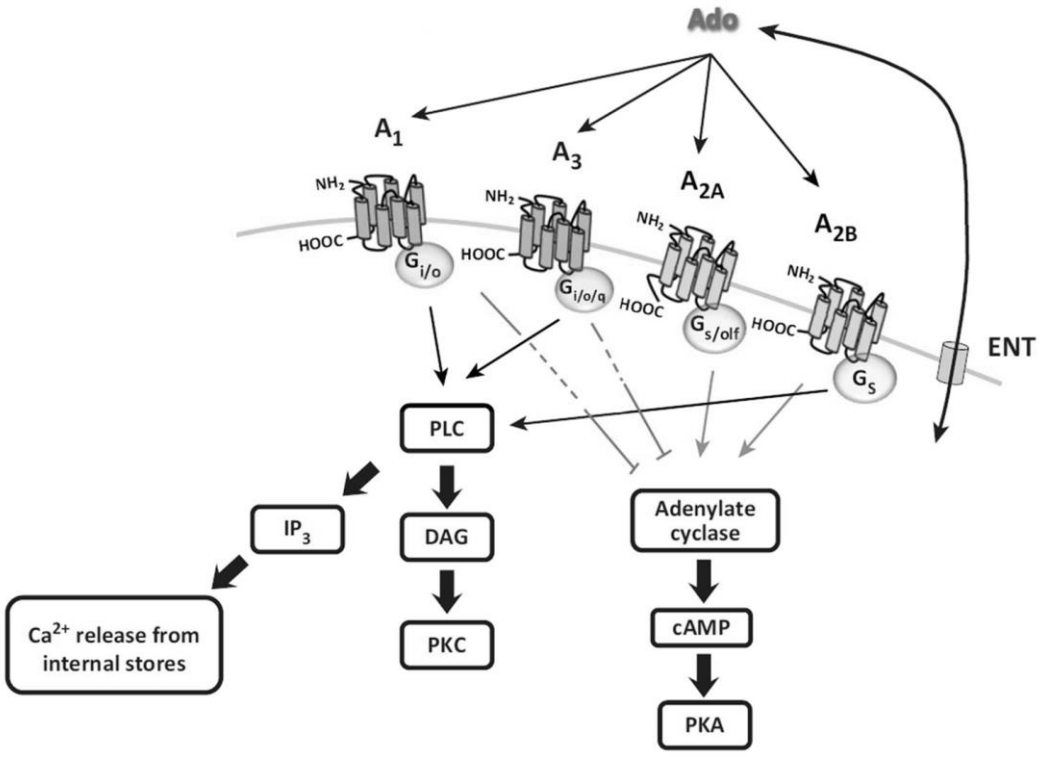


Figure 6 - Schematic diagram depicting the downstream signaling pathways of adenosine receptors (Adapted from (Dias *et al.*, 2013).

A1R are the most abundant and widespread adenosine receptors in the brain. This receptor is most abundant in limbic and neocortical regions, and is mostly concentrated in neurons, especially in synapses (Cunha, 2005; Cunha *et al.*, 2007). A1R are also present in astrocytes and microglia although in less number (Cunha, 2005).

When A1R are activated in presynaptic neurons, the release of excitatory neurotransmitters is inhibited and when they are activated in postsynaptic neurons, the excitatory action potential is inhibited. This action has a profound impact on synaptic transmission, controlling the release of glutamate, acethylcoline and serotonin, causing a depressant, sedative and anticonvulsant effect (Abbracchio and Cattabeni, 1999; Cunha, 2005).

The agonists of this receptor have been used as a therapy for cerebral ischemia and brain trauma. However, the administration of A1R has collateral effects such as profound sedation and hypotension (Abbracchio and Cattabeni, 1999).

The improvement of the neuroprotective effect of A1R resides in the development of partial agonists for these receptors, reducing the extent of desensitization and in the identification of A1R subtypes that can lead to better pharmacology manipulation. Another process that can improve neuroprotective effects of A1R is the development of Regulators of Endogenous Adenosine Levels (REAL) agents which influence adenosine transport, metabolism and/or release, decreasing adenosine levels after trauma or ischemia (Abbracchio and Cattabeni, 1999).

### 1.5.2. A2A receptors

A2AR is coupled to Gs protein, and its activation leads to an increase of cAMP levels due to increased AC activity. These high levels of cAMP lead to activation of protein kinase A (PKA) which proceeds through phosphorylation of the transcription factor of cAMP responsive element binding protein (CREB), resulting in its activation (Figure 6) (Hasko *et al.*, 2008).

A2AR were initially identified in striatum and are implicated in regulation of motor functions. These receptors are also present in the hippocampus and cortex, which are abundantly located in synapses. A2AR are also located in astrocytes and microglia cells, as well as in blood vessels. In striatum, A2AR are most abundant post-synaptically and in

other brain sites in presynaptic neurons (Cunha, 2005). A2AR can also be found in the retina, being highly expressed in retinal microglial cells (Liou *et al.*, 2008).

An increase of expression and density of A2AR was reported as a result of Alzheimer's, Parkinson's and Huntington's disease (Sebastiao and Ribeiro, 2009). Moreover, activated microglia cells also present an increased density of A2AR (Lee, 2013) suggesting that blockade of A2AR has a preponderant effect in latter stages of brain damage and in chronic noxious brain conditions (Cunha, 2005).

Unlike to A1R, the prolonged administration of A2AR antagonists does not provoke desensitization, which makes this receptor an attractive target for modulation of brain injury.

### 1.5.3. A2B receptors

A2BR stimulation can trigger AC activation via Gs protein, which leads to cAMP formation and by activation of PKA also leads to CREB phosphorylation, resulting in its activation (Figure 6) (Schulte and Fredholm, 2003).

A2BR are present in many cell types although in low abundance. Unlike A2AR, this receptor has low affinity for adenosine (Fredholm *et al.*, 2001; Schulte and Fredholm, 2003), thus A2BR activation requires pathophysiological conditions when adenosine levels are high and capable of their activation (Hasko *et al.*, 2008).

### 1.5.4. A3 receptors

Similarly to A1R, activation of A3R is coupled to Gi, inhibiting AC activity and stimulating phospholipase C (PLC) (Figure 6) (Abbracchio and Cattabeni, 1999).

A3R distribution in the brain is diffuse and can be detected in all brain areas although in a much lower density than the other subtypes of receptors (Abbracchio and Cattabeni, 1999).

These receptors are activated under specific conditions characterized by elevated local adenosine concentrations, since A3R have low affinity for adenosine (Mendonca *et al.*, 2000).

## 1.6. Neuroprotective role of A2AR modulation

Activated microglia cells secrete inflammatory mediators which can contribute to the resolution of the inflammatory process or to neuronal damage. Several results show that the blockade of A2AR provides neuroprotection controlling microglia reactivity which is an important target in several brain conditions (Cunha, 2005).

### 1.6.1. A2AR agonist (CGS 21680) or antagonist (SCH 58261)

Several pharmacological tools are used to study ARs. CGS 21680 is an A2AR-selective agonist but it is less potent and selective in humans than in rats. Moreover, in organs with levels of A2AR, CGS 21680 also binds to sites unrelated with A2AR. SCH 58261 is the most selective A2AR antagonist (Fredholm *et al.*, 2001). It is controversial in the literature if activation or blockade of A2AR plays a protective role in CNS injuries. It is proposed that in CNS A2AR exerts a bidirectional effect (protective and deleterious) that depends on several factors: a) different development stages, b) different stages of pathological process after injury and c) different routes and times of A2AR drug administration (Dai and Zhou, 2011).

Recently, it was shown that SCH 58261 has the ability to control neuroinflammation induced by lipopolysaccharide (LPS) which is a constituent of bacteria's membrane and a potent activator of microglia. Interestingly, SCH58261 also attenuated LPS-induced increase in interleukin (IL)-1β levels and activated microglia recruitment (Rebola *et al.*, 2011). Moreover, some studies show that this selective A2AR antagonist ( SCH 58621) can prevent the hippocampal neuronal dysfunction and neurotoxicity triggered by the administration of LPS (Cunha, 2005). In addition, the administration of SCH 58261 significantly reduced, in about 30%, the ischemic injury in the rat model of focal cerebral ischemia (Monopoli *et al.*, 1998).

### 1.6.2. Caffeine

Caffeine (1,3,7–trimethylxanthine), which is present in coffee, tea, soft drinks, and chocolate, is the most commonly used psychostimulant in the world. This molecule has been subjected to extensive research with the objective to determine if it has impact on various biochemical and physiological processes.

In humans, after ingestion, 99% of caffeine is absorbed from the gastrointestinal tract in humans in about 45 min. Due to its hydrophobic properties caffeine is able to cross all biological membranes. Oral ingestion of a single cup of coffee in humans provides a caffeine dose of 0.4 to 2.5 mg/kg, leading to a peak serum concentration of 0.25 to 2 mg/L or approximately 1 to 10 µM (Fredholm *et al.*, 1999).

Caffeine is almost completely metabolized in the liver and only 2% of the quantity ingested can be found in urine unaltered (Xu *et al.*, 2010). For doses lower than 10 mg/kg, the half-life of caffeine varies from 0.7 to 1.2 h in rat and mouse and 2.5 to 4.5 h in humans (Fredholm *et al.*, 1999). The first metabolic step of caffeine metabolism, which represents on average 80% of the total, is via N-3 demethylation to paraxanthine (1,7–dimethylxanthine), primarily by the cytochrome P450 1A2 enzyme. Another two important metabolites are theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine) which represent about 16% of caffeine's metabolism (Xu *et al.*, 2010). In rodents, after caffeine administration, paraxanthine levels are high as well as levels of theophylline.

In animal experiments, it was demonstrated that a treatment with caffeine (1g/L) supplied in drinking water confers prevents memory impairment (Duarte *et al.*, 2009; Cognato *et al.*, 2010), and also prevents neurodegeneration in a chronic Parkinson's disease rat model (Sonsalla *et al.*, 2012). Moreover, epidemiologic studies have shown that caffeine consumption is inversely correlated with the risk of developing neurodegenerative diseases, which are associated with chronic neuroinflammation (Prediger, 2010). Regarding inflammation, caffeine has also the ability to attenuate inflammation induced by LPS (Brothers *et al.*, 2010b).

It seems that prolonged administration of A2AR antagonists does not desensitize the receptor. In particular, long exposure of caffeine leads to a rapid A1R desensitization but maintains the A2AR mediated response (Cunha, 2005).

Despite of extensive research about caffeine effects in CNS, little is known about its effects in the retina and its neuroprotective role in the I-R model.

# Aims

Glaucoma is a neurodegenerative disease characterized by damage in the optic nerve and loss of RGCs. Neuroinflammation is believed to contribute to the pathophysiology of glaucoma, increasing the reactivity of microglia and in the release of pro-inflammatory mediators. The blockade of A2AR confers robust neuroprotection in several brain noxious conditions, possibly through the control of neuroinflammation.

The main aims of this project were to investigate whether the modulation of the adenosinergic system, in particular the role of A2AR, controls retinal pro-inflammatory responses and if it confers neuroprotection to the retina in a model of I-R. The role played by A2AR was assessed by injecting the A2AR agonist or antagonist in the vitreous.

Caffeine is a non-selective antagonist of adenosine receptors and it has been reported that this substance prevents the neurodegenerative process in some diseases. Therefore, the effects of caffeine in the modulation of the retinal pro-inflammatory responses and in neuroprotection were also investigated.

In order to accomplish our goals, we assessed the reactivity of microglia and the production of pro-inflammatory cytokines, as well as the production of reactive oxygen species (ROS). Cell death and loss of RGCs were also evaluated.

# *Chapter 2*

# 2. Materials and Methods

## 2.1. Animals and Drug administration

Wistar rats aged 8 weeks were housed under controlled environment (21.8±0.1 °C of temperature and, 67.6±1.6 % of relative humidity, 12 h-light/dark cycle) with free access to food and water. The animals were treated in agreement with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in vision and ophthalmic research.

### 2.1.1. Intravitreal administration of A2AR agonist or antagonist

Animals were randomly divided for the treatment with saline, A2AR agonist or antagonist. The A2AR agonist CGS 21680 (Tocris Bioscience, Cambridge, UK) and the A2AR antagonist SCH 58261 (Tocris Bioscience, Cambridge, UK) were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 100 mM, and then diluted and stored in Mili-Q water at 30 mM and 5 µM, respectively. Immediately prior to the intravitreal injection, CGS 21680 and SCH 58261 were diluted in sterile saline (0.9% NaCl, Labesfal) to a concentration of 2 mM and 100 nM, respectively. CGS 21680 dose was selected based on previous report (Li and Roth, 1999). Intravitreal injection of CGS 21680 (1 µl + 4 µl saline) and SCH 58261 (5 µl) was performed using a 10 µL Hamilton syringe, in both eyes 2h before the induction of retinal ischemia-reperfusion (I-R), as indicated in Figure 7.

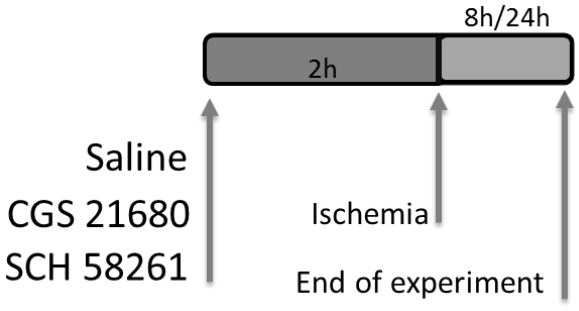


Figure 7 - Schematic representation of A2AR agonist (CGS 21680) or antagonist (SCH 58261) administration experimental procedure.

The animals were randomly divided into three experimental groups:

i) Intravitreal injection of 5 µl of sterile saline (0.9% NaCl) followed by retinal I-R (saline);

ii) Intravitreal injection of 5 µl of 2 mM CGS 21680 followed by retinal I-R (CGS 21680);

iii) Intravitreal injection of 5 µl of 100 nM SCH 58261 followed by retinal I-R (SCH 58261).

### 2.1.2. Caffeine administration

Animals were randomly divided for the treatment with caffeine or water. Caffeine (1 g/L, Sigma-Aldrich) was supplied in the drinking water for two weeks before performing retinal I-R and until the end of the experiment, as indicated in Figure 8. This dose of caffeine was selected based on previously reported ability to affords protection upon CNS injury (Xu *et al.*, 2010).

Caffeine solution was prepared fresh every two days.

The animals were divided into three experimental groups:

i) Retinal ischemia-reperfusion injury, drinking water (water);

ii) Retinal ischemia-reperfusion injury, treatment with caffeine (caffeine);

iii) Non ischemia-reperfusion injury, treatment with caffeine (non I-R caffeine).

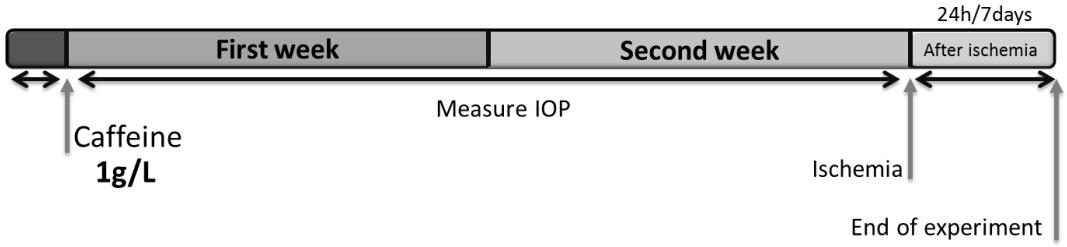


Figure 8 - Schematic representation of caffeine (1 g/L) administration experimental procedure.

## 2.2. Measurement of intraocular pressure

The IOP was measured bilaterally 3 days a week with a rebound tonometer specifically designed for rodents (Tonolab®, Icare, Espoo, Finland), and was conducted between 1:00 p.m. and 4:00 p.m. (Figure 9). Six reliable measurements were made by internal software which generates and displays an average, after elimination of high and low readings. For

the purpose of this study, this machine-generated average was considered as one reading. For each measurement, six machine-generated readings were recorded in each eye. An average of these readings was then calculated and was reported as the IOP for that eye. Basal IOP was measured one week before starting the treatment with caffeine.

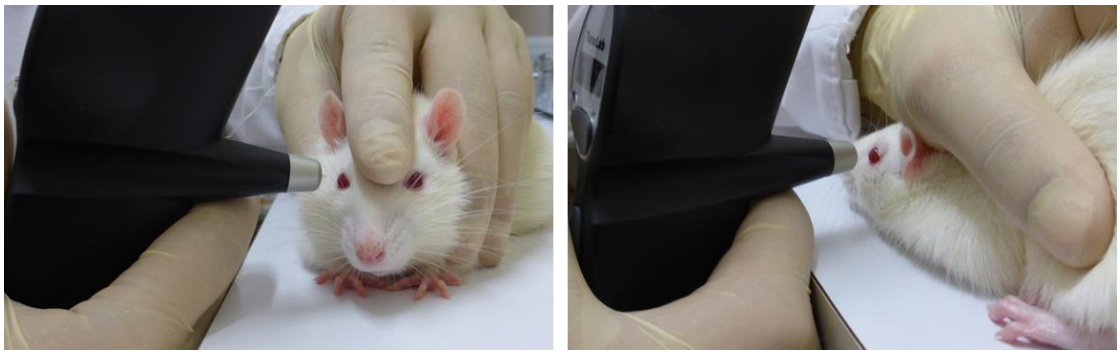

Figure 9 - Experimental procedure for measurement of IOP.

## 2.3. Retinal ischemia-reperfusion (I-R) injury

Retinal I-R was induced in one eye by elevating the IOP to 80 mmHg for 60 min. Animals were anesthetized with 2.5% isoflurane (IsoFlo, Abbott Laboratories) in 1 L/min $O_2$ with a vaporizer throughout the procedure of ischemia, and were placed on a heating plate to maintain their body temperature. After topical anesthesia with application of oxybuprocaine (4mg/mL, Anestocil, Edol) and pupillary dilation with topical application of tropicamide (10 mg/mL, Tropicil Top, Edol), the anterior chamber of the one eye was cannulated with a 30-gauge needle connected to reservoir infusing sterile saline solution (Figure 10A). The other eye was the contralateral control eye. The IOP was raised by elevating the reservoir to a height of 1.8 m. Retinal ischemia was confirmed by whitening of the iris and loss of the red reflex (Figure 10B). IOP was also measured with the Tonolab and it was increased to 80±1 mmHg. In order to avoid corneal opacity, viscoelastic solution (2% Methocel, Dávi II – Farmacêutica S.A.) was applied to both eyes. After 60 min of ischemia the needle was withdrawn and reperfusion was established. Fusidic acid ointment (10 mg/g, Fucithalmic, Leo Pharmaceutical) was applied in the end of the experiment. Animals were sacrificed 8h, 24h or 7 days after ischemia.

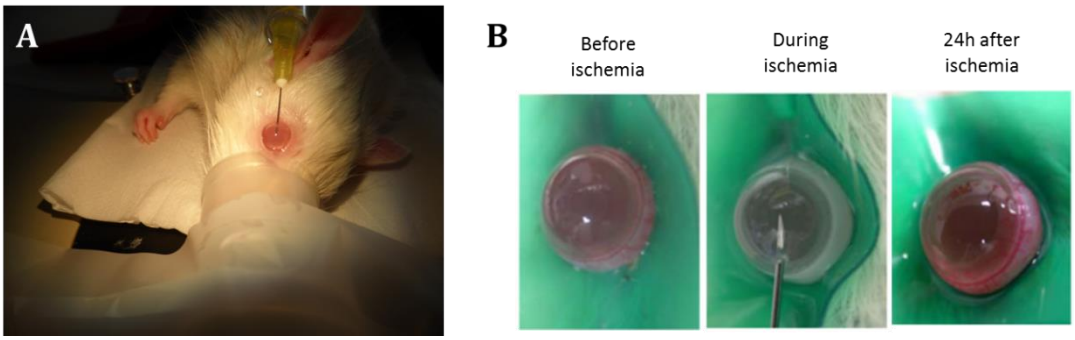


Figure 10 - Retinal ischemia-reperfusion injury. A) Anterior chamber of the eye cannulated with a 30-gauge needle. B) Visual effects of the ischemia in the eyes: whitening of the iris and loss of the red reflex (adapted from Sun et al., 2010). .

## 2.4. Quantification of caffeine levels

At the end of the treatment with caffeine, caffeine concentration was quantified in the serum, liver and brain by high performance liquid chromatography (HPLC) using a reverse-phase column [LiChro-CART 125 x 4 mm LiChrospher 100 RP-18 (5 µm) cartridge fitted into a ManuCART holder (Merck, Darmstadt, Germany)] and a Gilson system equipped with a UV detector set at 274 nm, as previously described (Duarte *et al.*, 2012). Serum samples were obtained after centrifugation of blood at 2000 g, for 15 min. For the quantification in the liver and brain, 10 mg of tissue were homogenized with 100 µL Mili-Q water, and frozen twice in liquid nitrogen. Then, 40% of the sample volume was added to 30% Perchloric Acid and mixed thoroughly, followed by centrifugation at 14000 rpm for 10 min. The supernatant was collected and injected to the column.

The eluent was $KH_2PO_4$ (115 mM) and acetonitrile (89/11, v/v %) at pH 3.10 with the flow rate of 1 mL/min. Chromatograms were recorded at 274 nm with a run time of 12 min. The identification of caffeine was achieved by comparing the peak areas of standard samples, and the concentration of caffeine was calculated by converting the peak areas to concentration values with known standards ranging from 2 to 50 µM.

## 2.5. Preparation of frozen retinal sections

Animals were deeply anesthetized with an intraperitoneal injection of a solution of ketamine (90 mg/kg; Imalgene 1000) and xylazine (10 mg/kg; Ronpum 2%) and then transcardially perfused with phosphate-buffered saline (PBS, in mM: 137 NaCl, 2.7 KCl, 10

$Na_2HPO_4$, 1.8 $KH_2PO_4$; pH 7.4) followed by 4% (w/v) paraformaldehyde (PFA). The eyes were enucleated and post-fixed in 4% PFA for 1 hour. Then, the cornea and the lens were 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) with 30% of sucrose in PBS (1:1), and stored at -80 °C.

The tissue was sectioned on a cryostat (Leica CM3050 S) into 10 µm thickness sections and mounted on Superfrost Plus glass slides (Menzel-Glaser, Thermo Scientific). Glass slides were dried overnight and then stored at -20 °C.

## 2.6. Immunohistochemistry

The glass slides were defrosted at room temperature overnight. The sections were fixed with ice-cold acetone at -20 °C for 10 min, and then rehydrated in PBS twice until OCT was removed. The tissue was surrounded with a hydrophobic pen and it was permeabilized with 0.25% Triton X-100 in PBS for 30 min. The sections were blocked in 10% Normal Goat Serum plus 1% BSA in PBS for 30 min at room temperature in a humidified environment. After washing with PBS, the sections were incubated overnight with primary antibody (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 incubated with the nuclear dye 4',6-diamidino-2-phenylindole (DAPI), diluted 1:2000. The tissue was washed in PBS and mounted with fluorescent mounting medium (Dako, USA).

Table 1 - List of primary and secondary antibodies used in immunohistochemistry.

| | Supplier (cat no) | Host | Dilution |
|---|---|---|---|
| **Primary antibodies** | | | |
| **anti-Iba1** | Wako (019-19741) | Rabbit | 1:1000 |
| **anti-OX-6** | Serotec (MCA46R) | Mouse | 1:200 |
| **anti-IL6** | Abcam (ab6672) | Rabbit | 1:800 |
| **anti-Brn3a** | Chemicon ( MAB1585) | Mouse | 1:200 |
| **Secondary antibodies** | | | |
| **Alexa Fluor 488 anti-rabbit IgG** | Invitrogen (A11008) | Goat | 1:200 |
| **Alexa Fluor 568 anti-mouse IgG** | Invitrogen (A11004) | | |

## 2.7. Assessment of reactive oxygen species

ROS were detected using dihydroethidium (DHE), a probe that in the presence of superoxide anion ($O^{2-}$) is oxidized to ethidium and intercalates with DNA emitting red fluorescence that can be observed with a microscope. The detection of ROS was performed as described previously (Sena *et al.*, 2012), with some modifications as follows. Animals were deeply anesthetized with an intraperitoneal injection of a solution of ketamine (90 mg/kg; Imalgene 1000) and xylazine (10 mg/kg; Ronpum 2%) and then transcardially perfused with sterile saline (0.9% NaCl). The eyes were enucleated and were embedded in OCT with 30% of sucrose in PBS (1:1) and stored at -80 °C. The glass slides were defrosted at room temperature overnight. The sections were rehydrated in PBS twice until OCT was removed. The tissue was surrounded with a hydrophobic pen and it was incubated with 2 µM DHE for 30 min at 37 °C in the dark. After washing with PBS, the sections were fixed with 4% PFA (10 min). Then, the sections were washed with PBS and then incubated DAPI (1:2000). Sections were washed in PBS and mounted with fluorescent mounting medium (Dako, USA).

## 2.8. Assessment of retinal thickness

After defrosting the slides at room temperature overnight, the sections were rehydrated in PBS twice (5 min). Then the sections were incubated with hematoxylin solution for 3 min, followed by a superficial wash (3 seconds) with HCl 0.1% solution. The sections were washed in running tap water for 5 min, and then incubated with 0.5% eosin in 100% ethanol for 3 min. The sections were then washed in running tap water for 30 seconds and then dehydrated in ascending alcohol solutions (95% and 100%, 1 min each). Then, sections were cleared through 2 changes of xylene (5 min). Finally, tissue sections were mounted with DPX mounting medium (FLUKA).

## 2.9. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay

Cell death was detected with a TUNEL assay kit with fluorescein detection following the instructions provided by the manufacture (Promega). Briefly, sections were washed twice in PBS and then permeabilized with proteinase K (20 µg/ml) for 10 min. After washing twice in PBS, the sections were incubated with equilibration buffer (200 mM potassium cacodylate, 25 mM Tris-HCl, 0.2 mM DTT, 0.25 mg/mL BSA and 2.5 mM cobalt chloride) for 10 min. Then, sections were incubated with the recombinant TdT enzyme and with nucleotide mix containing dUTP conjugated to fluorescein at 37 °C for 1 hour. The reaction was stopped by immersing the slides in saline-citrate (SSC) buffer (175 g/L NaCl, 88.1 g/L sodium citrate) for 15 min at room temperature. Then, the samples were washed three times in PBS 5 min, followed by incubation with the nuclear dye DAPI (diluted 1:2000). Sections were washed in PBS and mounted with fluorescent mounting medium (Dako, USA).

## 2.10. Image analysis

The samples were examined in a Zeiss LSM 710 confocal on an Axio Observer Z1 microscope using an EC Plan-Neofluar 40x/1.30 Oil DIC M27 objective. Images (1024x1024 format) were collected using 405, 488 and 561 nm laser lines for excitation. Z-stacks images were acquired and merged using the maximum intensity projection mode of the Zeiss software (Zen 2009, Zeiss).

For the analysis of microglial reactivity, 4 images per section were acquired (4 sections each eye). In each picture, the number of cells immunoreactive to both Iba1 (Ionized calcium binding adaptor molecule 1) and OX-6 ($Iba1^+$ $OX\text{-}6^+$) was counted and it was expressed in percentage of the total number of cells immunoreactive to Iba1 ($Iba1^+$).

The analysis of IL-6 immunoreactivity and DHE staining was performed in the maximum projection images. For each eye, 6 pictures were acquired per section (4 sections). Images were converted to 8-bit pictures and densitometric analysis was performed using the public domain ImageJ program (http://rsb.info.nih.gov/ij/) by quantifying the mean grey value.

The survival of RGC was assessed by counting the number of cells immunoreactive to Brn3a ($Brn3a^+$) in a Leica DM IRE2 fluorescence microscope using the 40x/0.70 HC PL APO CS objective. For each eye, 4 sections were analyzed and the number of cells $Brn3a^+$ was counted in the entire retinal section and normalized to the length of the same section. Representative images were acquired with a Zeiss LSM 710 confocal microscope, as described previously.

The retinal thickness was assessed by hematoxylin and eosin staining visualized in light microscope (Leica DM 4000 B). The average thickness (in µm) of the total retina was measured for each eye, in a total of 4 sections. Measurements, 16 per section, were obtained along the entire retinal section.

The cell death was assessed by counting the number of $TUNEL^+$ cells in a Leica DM IRE2 fluorescence microscope using the 40x/0.70 HC PL APO CS objective. For each eye, 4 sections were analyzed and the number of $TUNEL^+$ cells was counted in the entire retinal section and normalized to the length of the same section. Representative images were acquired with a Zeiss LSM 710 confocal microscope, as described previously.

## 2.11. Quantification of pro-inflammatory cytokines

The concentration of pro-inflammatory cytokines tumor necrosis factor (TNF), IL-1β and IL-6 in the retina was quantified by Enzyme-linked immunosorbent assay (ELISA) following the instructions provided by the manufacturer (Peprotech, UK). The animals were sacrificed, the eyes enucleated and the retinas dissected in ice-cold PBS. The retinas were then homogenized in lysis buffer (20 mM imidazole HCl, 100 mM KCl, 1 mM $MgCl_2$,

1% Triton X-100, 1 mM EGTA,1 mM EDTA, 10 mM NaF, 1 mM $Na_3VO_4$; pH 7.4) supplemented with protease inhibitor (Complete mini, Roche). The tissue was centrifuged at 10.000 g for 5 min at 4 °C, the supernatant was collected and a small aliquot was saved for protein concentration using the BCA Protein assay kit (Pierce, Thermo Scientific). The samples were diluted three times with diluent solution (0.05 % Tween-20, 0.1 % BSA in PBS) and stored at -80 °C until used. The absorbance at 405 nm, with wavelength correction at 650 nm, was measured with a multimode microplate reader (Synergy HT, Biotek, USA). The concentrations of TNF, IL-1β and IL-6 in retinal samples were determined by extrapolation of the standard curve using recombinant cytokines provided by manufacturer and reported as pictograms per milligram protein (pg/mg protein). To normalize the data, a ratio of the pro-inflammatory cytokine levels in the I-R-injured retina to the contralateral eye was calculated and used for statistical analysis.

## 2.12. Statistical analysis

Data that were outside the range mean ± 2 SD were excluded. Results are presented as mean ± SEM for the number of eyes indicated in parentheses above each bar in the graphs. Statistical analysis was performed in Graph Pad Prism 5 software using two-tailed unpaired Student's t-test, and $p<0.05$ was considered to represent a significant difference.

# *Chapter 3*

# 3. Results

In this work, we aimed to investigate whether the modulation of A2AR controls the inflammatory response in the retina in a model of I-R injury. In addition, the potential protective effect of A2AR modulation was investigated. Therefore, A2AR agonists or antagonists were injected intravitreally 2h before ischemia, or caffeine, a non-selective antagonist of A1R and A2AR, was administered orally in the drinking water two weeks before ischemia and until the end of the experiment.

## 3.1. Role of A2AR modulation on retinal I-R injury

### 3.1.1. Levels of pro-inflammatory cytokines

In acute and transient retinal ischemia, potentially toxic mediators are released by activated inflammatory cells, by glial elements, and by injured neurons (Sanchez *et al.*, 2003; Gesslein *et al.*, 2010). In order to investigate the modulatory role of A2AR in retinal I-R injury, selective A2AR antagonist or agonist were injected intravitreally before the injury, and the levels of pro-inflammatory cytokines TNF, IL-1β, and IL-6 were assessed 8h (Figure 11) and 24h (Figure 12) post-ischemia by ELISA.

The levels of TNF, after 8h of reperfusion, in I-R retinas treated with saline were 307.5±103.1 pg/mg prot, which corresponds to a ratio of 1.3±0.3 (Figure 11A). The ratio of TNF levels in the retinas treated with CGS 21680 or SCH 58261 was not significantly different from saline-treated retinas (1.8±0.7 and 1.9±1.1 fold change, respectively).

After 8h of reperfusion, in I-R retinas treated with saline, the levels of IL-1β were 3.2±0.4 fold-increased (Figure 11B) compared to the contralateral retinas (399.2±77.2 pg/mg prot vs 1167.2±191.2 pg/mg prot). The injection of CGS 21680 in the vitreous did not significantly change IL-1β levels ratio of I-R retinas to contralateral retinas (424.9±152.2 pg/mg prot in contralateral retinas vs 570.6±131.0 pg/mg prot in I-R retinas), which corresponds to 1.8±0.7 fold-change. Intravitreal injection of SCH 58261 significantly increased IL-1β ratio by 7.0±2.8 fold (332.3±138.3 pg/mg prot in contralateral retina vs 1564.7±639.5 pg/mg prot in I-R retinas), as compared to the saline-treated retinas.

The levels of IL-6 in the saline-treated retinas contralateral to the injury were 3754.8±1132.9 pg/mg prot, and in I-R retinas were 6691.3±3568.0 pg/mg prot, resulting in a ratio of 1.7±0.7 (Figure 11C). Intravitreal injection of CGS 21680 did not significantly change the IL-6 levels ratio (1.3±0.1 fold-change) as compared to saline-treated retinas. In addition, intravitreal administration of SCH 58261 did not significantly change IL-6 levels ratio (1.9±1.0 fold-change), as compared to saline-treated retinas.

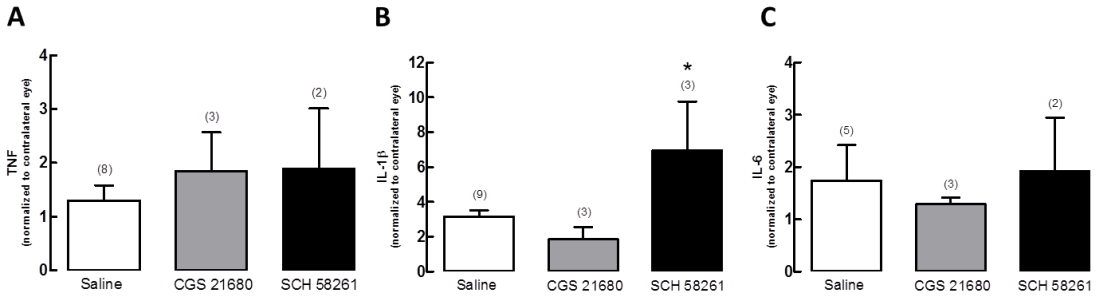


Figure 11 - Effect of CGS 21680 and SCH 58261 in retinal levels of TNF (A), IL-1β (B) and IL-6 (C) induced by I-R injury at 8 hours of reperfusion. Animals were injected in the vitreous with saline, CGS 21680 or SCH 58261 2 h before I-R, and the levels of TNF, IL-1β and IL-6 levels were quantified by ELISA. Both contralateral and I-R eyes were injected. The results represent the mean ± SEM for the number of eyes indicated in parentheses above each bar, and are expressed as the ratio of I-R eye to contralateral eye. *p<0.05, compared with saline, Student's t-test.

After 24h of reperfusion, the levels of TNF after I-R injury in saline-treated animals were 547.1±64.2 pg/mg prot in the contralateral retinas and 620.1±149.9 pg/mg prot in I-R retinas, which corresponds to 1.2±0.3 fold change (Figure 12A). The intravitreal injection of CGS 21680 and SCH 58261 results in 1.9±0.5 and 1.6±0.3 fold change in I-R retinas, respectively.

IL-1β levels in the saline-treated retinas contralateral to the injury were 334.7±159.7 pg/mg prot vs 653.9±182.1 pg/mg prot in I-R retinas, resulting in 2.5±0.5 fold increase (Figure 12B). In the retinas with intravitreal injection of CGS 21680, the concentration of IL-1β were 845.4±272.0 pg/mg prot and 1029.3±403.8 pg/mg prot, for contralateral and I-R retinas, respectively, corresponding to 2.0±0.7 fold-change. Intravitreal injection of SCH 58261 did not significantly change the IL-1β concentration ratio (392.3±110.9 pg/mg prot and 523.0±177.1 pg/mg prot in contralateral and I-R retinas, respectively, resulting in 1.6±0.4 fold change).

The levels of IL-6 in the saline-treated retinas contralateral to the injury were 2031.1±360.3 pg/mg prot, and in I-R retinas were 2687.7±932.0 pg/mg prot, resulting in a

ratio of 1.4±0.4 (Figure 12C). Intravitreal injection of CGS 21680 and SCH 58261 results in 1.2±0.2 or 1.9±0.5 fold-change in I-R retinas, respectively.

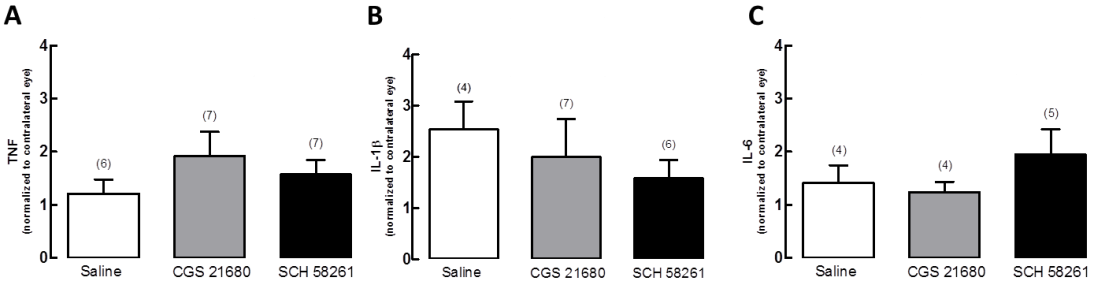


Figure 12 - Effect of CGS 21680 and SCH 58261 in retinal levels of TNF (A), IL-1β (B) and IL-6 (C) induced by I-R injury at 24 hours of reperfusion. Animals were injected in the vitreous with saline, CGS 21680 or SCH 58261 2 h before I-R, and the levels of TNF, IL-1β and IL-6 levels were quantified by ELISA. Both contralateral and I-R eyes were injected. The results represent the mean ± SEM for the number of eyes indicated in parentheses above each bar, and are expressed as the ratio of I-R eye to contralateral eye.

### 3.1.2. Microglial reactivity

Microglia reactivity was assessed by immunohistochemistry using antibodies that recognize the Iba1 which is a microglia/macrophage-specific calcium-binding protein, and can be used as a marker of microglia/macrophages (Ito *et al.*, 2001). OX-6 is an antibody recognizing the major histocompatibility complex (MHC) class II antigen which is only expressed by activated microglia/macrophage (Zhang *et al.*, 2005; Kettenmann *et al.*, 2011). Therefore, microglia reactivity was assessed by immunohistochemistry with antibodies against Iba1 (total number of microglia/macrophages) and OX-6 (active microglia/macrophages). The number of cells immunoreactive to both OX-6 and Iba1 was expressed as a percentage of total microglia/macrophages.

Retinal sections reveal the positioning of cells $Iba1^+$ at the level of internal layers of retina (INL, IPL and GCL). Microglial cells were more ramified (resting) in the saline-treated contralateral retinas than in saline-treated I-R retinas (Figure 13A).

In saline-treated contralateral retinas, most of $Iba1^+$ cells were not immunoreactive to OX-6 (10.6±8.9 % of total; Figure 13B).

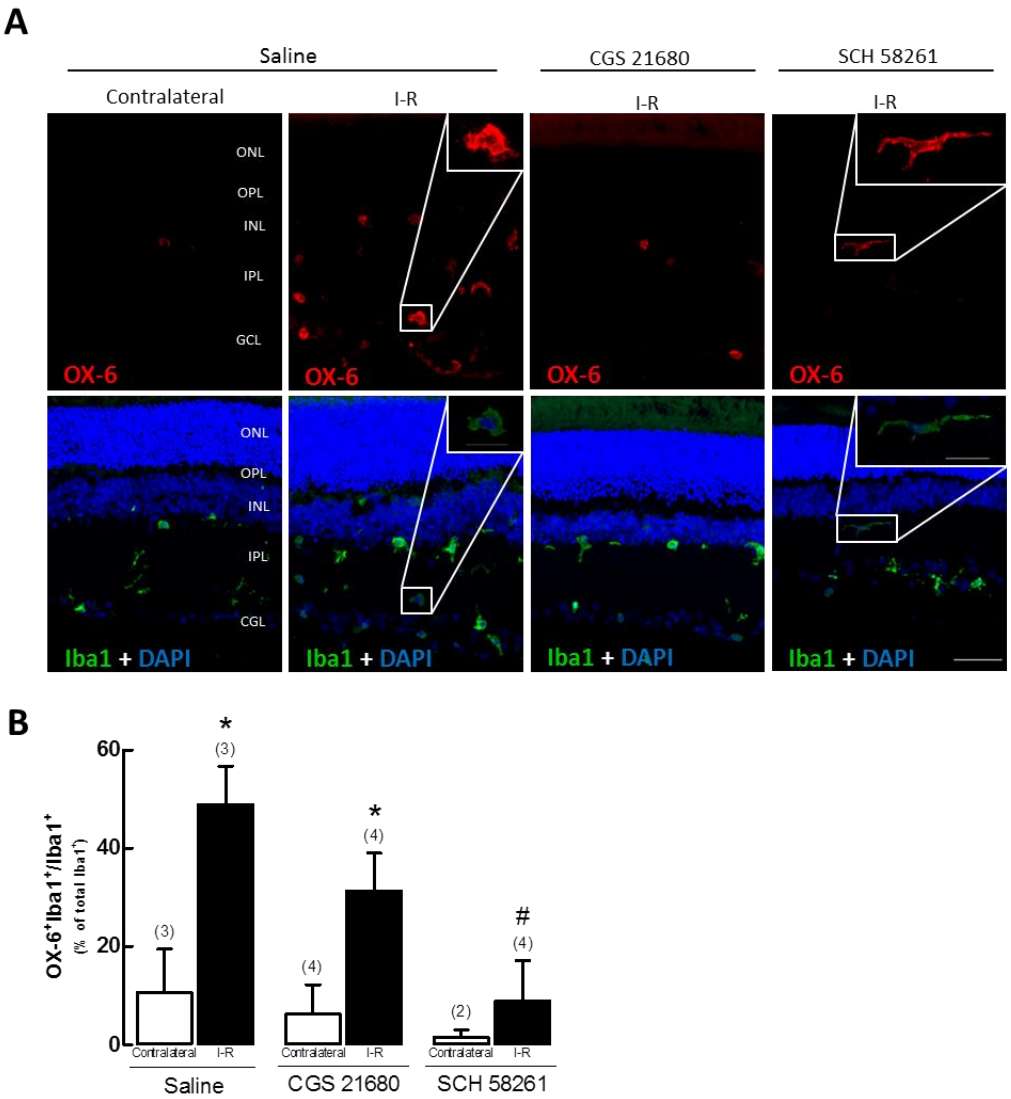


Figure 13 - Effect of CGS 21680 and SCH 58261 in the reactivity of microglia after retinal I-R injury. Animals were injected in the vitreous with saline, CGS 21680 or SCH 58261 2 h before I-R, and were sacrificed 24h post I-R injury. Retinal sections were stained with antibodies against Iba1 (green) and OX-6 (red). Nuclei were stained with DAPI (blue). Representative images are depicted in A. B: Activated microglia/macrophages (OX-6- Iba1-immunoreactive cells) were expressed as the ratio OX-6+Iba1+/Iba1+, and were presented as the percentage of total microglia/macrophages (Iba1-immunoreactive cells). The results represent the mean ± SEM for the number of eyes indicated in parentheses above each bar. From each eye, four retinal sections were analyzed, and from each section six images were randomly acquired. *p<0.05, compared with contralateral eye; #p<0.05, compared with saline-treated I-R retinas, Student's t-test. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar = 20 µm (detail images) or 50 µm.

Retinal I-R injury significantly increased the percentage of OX-6-positive cells (48.9±7.8 % of total, n=3, $p<0.05$). In CGS 21680-treated I-R retinas the percentage of OX-$6^+$ cells induced by I-R injury significantly increased (31.3±7.8% of total, n=4, $p<0.05$) when compared with contralateral eye. In SCH 58261-treated I-R retinas the percentage of OX-$6^+$ cells (8.9±8.2%, n=4) was not significantly different from the contralateral eye, but when compared with saline-treated I-R retinas there is a significantly decrease of OX-$6^+$ cells ($p<0.05$). These results indicate that blockade of A2AR may attenuate microglial reactivity.

## 3.2. Effect of caffeine on retinal I-R injury model

### 3.2.1. Characteristics of animals involved in the study

Caffeine (1 g/L) was administered in the drinking water during 2 weeks prior I-R and until the end of the experiment (24h or 7 days of reperfusion). The animals were housed two per cage. Weight and fluid intake were registered in some animals during the treatment which allowed us to estimate the daily caffeine consumption per animal (Table 2).

Table 2 - Characteristics of animals involved in the study.

| | Water-treated rats | Caffeine-treated rats |
|---|---|---|
| **Fluid intake (mL/day)** | | |
| Basal | | |
| 2 weeks treatment | 36 ± 3 (10) | 30 ± 2 (23) |
| After ischemia | 31 ± 4 (8) | 28 ± 1 (17) |
| **Caffeine consumption (mg/kg/day)** | | |
| Basal | | |
| 2 weeks treatment | - | 125 ± 7 (17) |
| After ischemia | | 110 ± 9 (13) |
| **Serum caffeine (µM)** | | |
| 24 h | nd | 54.6 ± 20.5 (7) |
| 7 days | | 97.5 ± 10.7 (4) |
| **Liver caffeine (ng/mg)** | | |
| 24 h | nd | 9.2 ± 2.8 (7) |
| 7 days | | 14.6 ± 1.9 (4) |
| **Brain caffeine (ng/mg)** | | |
| 24 h | nd | 3.7 ± 1.5 (4) |
| 7 days | | - |
| **IOP (mmHg)** | | |
| Basal | 11 ± 0.2 (5) | 12 ± 0.4 (15) |
| 2 weeks treatment | 12 ± 0.5 (5) | 12 ± 0.3 (15) |
| After ischemia | 12 ± 0.8 (2) | 12 ± 1.0 (3) |

Data are mean ± SEM, the number of animals used for these calculations are given in parenthesis. (nd) Not detected; (-) not measured

Fluid intake was not statistically different throughout the study, and between the two groups of animals.

Caffeine concentration was quantified by HPLC (Figure 14), in the serum, liver and brain samples that were collected immediately after sacrificing the animals 24h and 7 days after I-R injury.

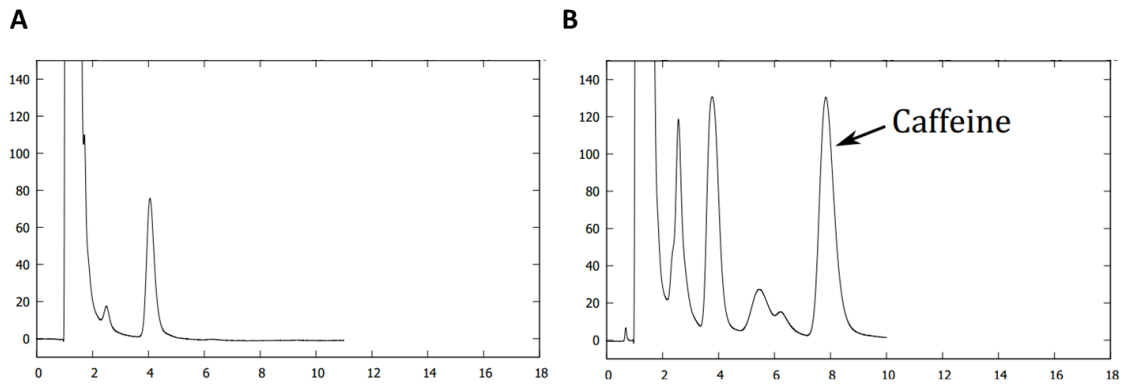


Figure 14 - Representatives HPLC chromatograms of A) rat blank serum and B) rat serum spiked with caffeine.

Some reports have demonstrated that caffeine consumption increases IOP in glaucoma patients (Avisar *et al.*, 2002), while others assume that ingestion of caffeine did not have effect on IOP (Varma and Chandra, 2011). Therefore, IOP was measured regularly with a rebound tonometer. Treatment with caffeine did not significantly change IOP, as compared with animals drinking water (Table 2).

### 3.2.2. Effect of caffeine on retinal I-R injury rat model: 24 h post I-R injury

#### *3.2.2.1. Microglia reactivity*

As already mentioned above, microglia reactivity was assessed by immunohistochemistry with antibodies against Iba1 (total number of microglia/macrophages) and OX-6 (active microglia/macrophages).

Iba1$^{+}$ cells located at the level of internal layers of retina (INL, IPL and GCL) were not changed by caffeine administration (Figure 15A).

The number of cells immunoreactive to both OX-6 and Iba1 was expressed as a percentage of total microglia/macrophages (Figure 15B).

In the animals drinking water, I-R injury increased the number of cells immunoreactive to OX-6 (12.3±5.7% of total, n=5), compared to the corresponding contralateral retinas (2.5±1.6% of total, n=5). In I-R retinas from caffeine-treated animals the number of cells immunoreactive to OX-6 significantly increased (18.2±6.2% of total, n=8, $p<0.05$), when compared with the contralateral retinas from caffeine-treated animals (0.6± 0.6% of total, n=8), indicating an increase in microglia reactivity.

Moreover, no statistical differences were found between contralateral retinas from water-treated group and caffeine-treated group.

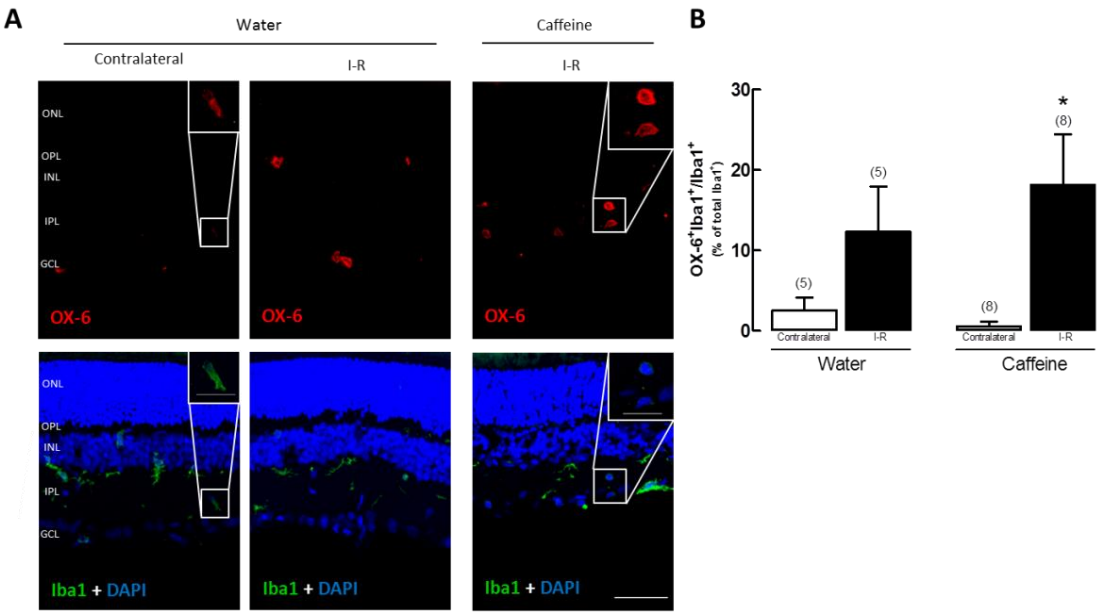


Figure 15 - Caffeine increased the number of activated microglia induced by I-R injury at 24h of reperfusion. Caffeine was administered in the drinking water for 2 weeks prior injury and until the end of the experiment. Retinal sections were stained with antibodies against Iba1 (green) and OX-6 (red). Nuclei were stained with DAPI (blue). Representative images are depicted in A. B: Activated microglia/macrophages (OX-6- Iba1-immunoreactive cells) were expressed as the ratio $OX\text{-}6^{+}Iba1^{+}/Iba1^{+}$, and were presented as the percentage of total microglia/macrophages (Iba1-immunoreactive cells). The results represent the mean ± SEM for the number of eyes indicated in parentheses above each bar. From each eye, four retinal sections were analyzed, and from each section six images were randomly acquired. *$p<0.05$, compared with contralateral eye, Student's t-test. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar = 20 µm (detail images) or 50 µm.

### *3.2.2.2.Levels of pro-inflammatory cytokines*

Caffeine administration did not induce statistically significant changes in the ratio of TNF (Figure 16A), IL-1β (Figure 16B) and IL-6 (Figure 16C) compared to animals drinking water at 24 h of reperfusion.

After 24h of reperfusion, the levels of TNF after I-R injury in water-treated animals were 450.4±137.2 pg/mg prot in the contralateral retinas and 568.7±137.2 pg/mg prot in I-R retinas, which corresponds to 1.3±0.2 fold change (Figure 16A). In caffeine-treated there was a 1.5±0.4 fold change of TNF levels in I-R retinas.

After 24h of reperfusion the levels of IL-1β in the water-treated retinas contralateral to the injury were 532.1±161.4 pg/mg prot vs 1030.2±275.5 pg/mg prot, resulting in 2.0±0.4 fold increase in I-R retinas (Figure 16B). Caffeine administration changed IL-1β concentration by 1.8±0.3 fold (943.3±351.74 pg/mg prot vs 1520.2±437.6 pg/mg prot).

The levels of IL-6 in the water-treated retinas contralateral to the injury were 438.0±79.2 pg/mg prot vs 561.3±258.0 pg/mg prot, resulting in 1.1±0.3 fold changed in I-R retinas (Figure 16C). In caffeine-treated retinas there is an increase of IL-6 concentration by 2.2±0.7 fold (1094.4±264.4 pg/mg prot vs 1779.3±489.5 pg/mg prot).

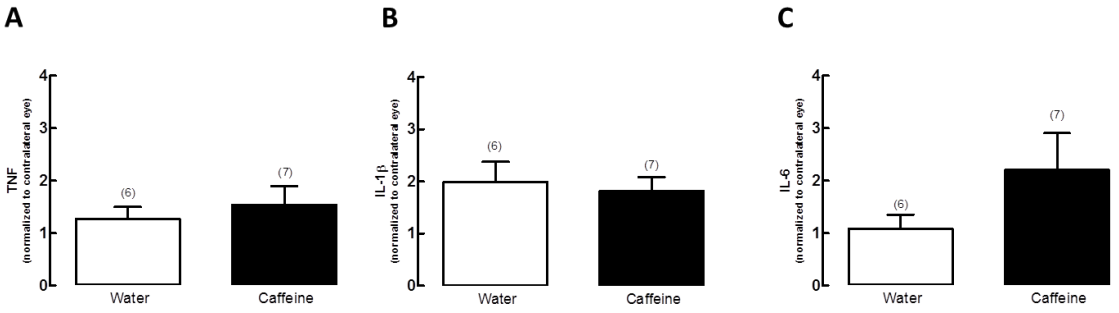


Figure 16 - Caffeine did not change retinal levels of TNF (A), IL-1β (B) and IL-6 (C) induced by I-R injury at 24h of reperfusion. Caffeine was administered in the drinking water for 2 weeks prior I-R injury and until the end of the experiment. TNF, IL-1β and IL-6 levels were quantified by ELISA. The results represent the mean ± SEM for the number of eyes indicated in parentheses above each bar, and are expressed as the ratio of I-R eye to contralateral eye.

### *3.2.2.3. Production of reactive oxygen species*

DHE imaging allows the assessment of superoxide formation, since in the presence of superoxide is oxidized and emits red fluorescence. DHE staining was observed in the entire retina (Figure 17A) and was quantified in the inner retinal layers (INL, IPL and GCL). No statistically differences were detected in DHE staining between the groups and between I-R and contralateral retinas (Figure 17B).

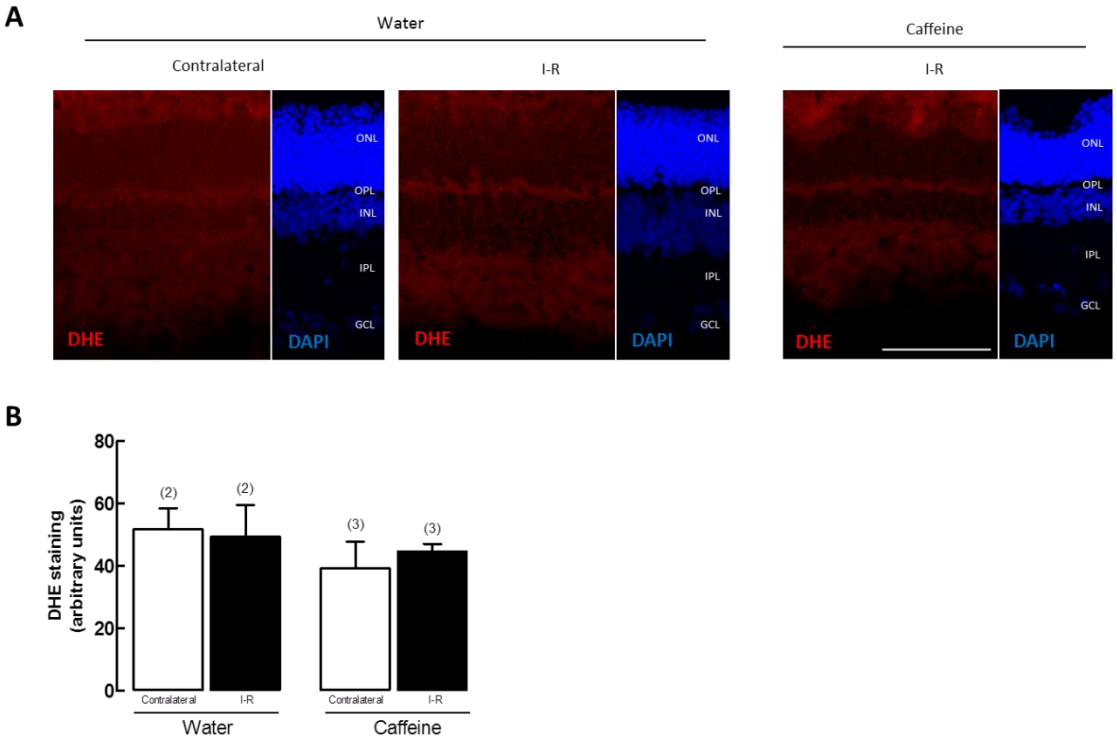


Figure 17 - Effect of caffeine on the production of reactive oxygen species induced by I-R injury at 24h of reperfusion. Caffeine was administered in the drinking water for 2 weeks prior I-R injury and until the end of the experiment. Reactive oxygen species were assayed with DHE fluorescence. Representative images are depicted in A. B: The intensity of DHE staining was quantified using ImageJ software. The results represent the mean ± SEM for the number of eyes indicated in parentheses above each bar. From each eye, four sections were stained and from each section six images were randomly acquired. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar = 50 µm.

### *3.2.2.4. Retinal thickness*

Retinal thickness was assessed after staining retinal sections with hematoxylin and eosin (Figure 18A). Hematoxylin has a deep blue-purple color and stains nucleic acids and eosin is pink and stains proteins nonspecifically (Fischer *et al.*, 2008).

Overall, no major changes were found concerning the thickness of the retina between the five experimental conditions (Figure 18B).

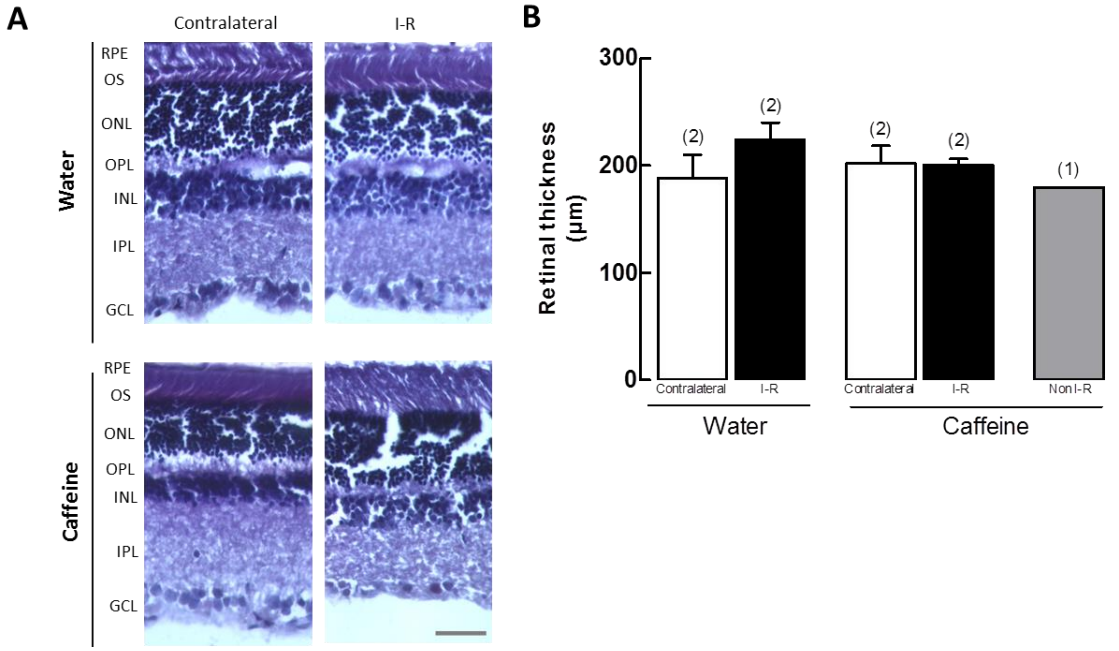


Figure 18 - Effect of caffeine in retinal thickness 24h after I-R. Caffeine was administered in the drinking water for 2 weeks prior I-R and until the end of the experiment. Retinal sections were stained with hematoxylin (blue-purple color) and eosin (pink). Representative images are depicted in A. B: Retinal thickness was quantified using ImageJ software. The results represent the mean ± SEM for the number of eyes indicated in parentheses above each bar. From each eye, four sections were stained and from each section 16 measurements were made. RPE: retinal pigment epithelium; OS: outer segments; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar = 50 µm.

### *3.2.2.5. Cell death*

In the present study we aimed to evaluate if caffeine could prevent I-R injury-induced cell death at 24 h of reperfusion. Cell death was assessed using the TUNEL assay (Figure 19A).

We observed that in water-treated retinas I-R injury significantly increased the number of TUNEL$^+$ cells (15.4±6.8 cells/mm, n=4, $p<0.05$) comparing with contralateral retinas (1.2±0.6 cells/mm). Also, in caffeine-treated subjected to I-R injury retinas there was a significantly increase in the number of TUNEL$^+$ cells (44.5±5.5 cells/mm, n=4, $p<0.001$) comparing with contralateral retinas (0.6±0.3 cells/mm). Moreover, caffeine significantly increased the number of TUNEL$^+$ cells induced by I-R injury ($p<0.05$) compared to I-R retinas of animals drinking water. Caffeine administration in non-I-R retinas did not cause changes compared to the contralateral eyes (Figure 19B).

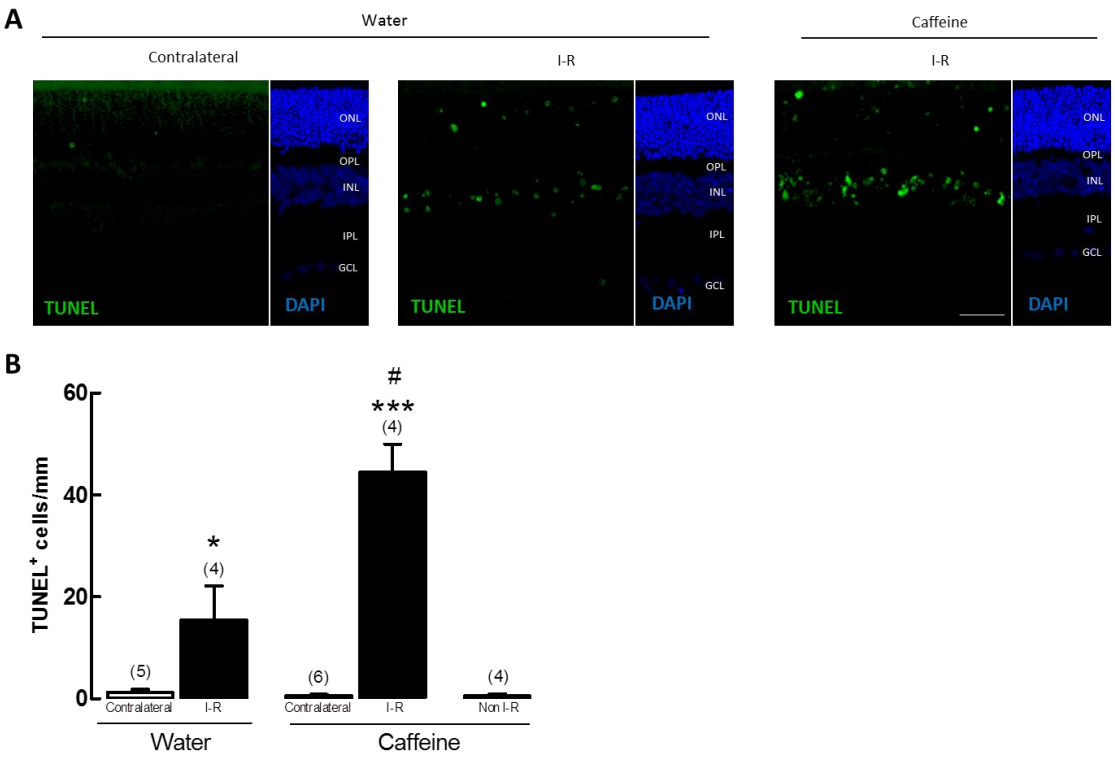


Figure 19 - Caffeine administration increased cell death induced by I-R injury at 24 h of reperfusion. Caffeine was administered in the drinking water for 2 weeks prior injury and until the end of the experiment. Cell death was assayed with TUNEL assay. Nuclei were stained with DAPI (blue). Representative images are depicted in A. B: TUNEL$^+$ cells (green) were counted and were expressed per mm of retina. The results represent the mean ± SEM for the number of eyes indicated in parentheses above each bar. From each eye, four retinal sections were analyzed, and from each section six images were randomly acquired. *p<0.05, ***p<0.0001, compared with contralateral eye, #p<0.05, compared with water-treated I-R retinas, Student's t-test. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar = 50 µm.

### *3.2.2.6. RGC loss*

The loss of RGCs was assessed by immunohistochemistry with an antibody that recognizes the transcription factor Brn3a that in the retina is expressed only in RGCs and can be used to identify these cells (Figure 20A) (Nadal-Nicolas *et al.*, 2009).

As can be observed in Figure 20B, there is a tendency to decrease the number of Brn3a$^+$ cells induced by I-R injury (16.4±1.5 cells/mm, n=4, p=0.0844) compared with contralateral retina (21.3±1.8 cells/mm). In caffeine-treated I-R retinas Brn3a$^+$ cells were changed compared with contralateral retina (17.3±2.6 cells/mm vs 12.0±3.4 cells/mm) (Figure 20B). Moreover, caffeine did not affect the percentage of RGC survival. Caffeine per se did not induce Brn3a$^+$ cells loss, as can be seen in caffeine-treated group non I-R retinas.

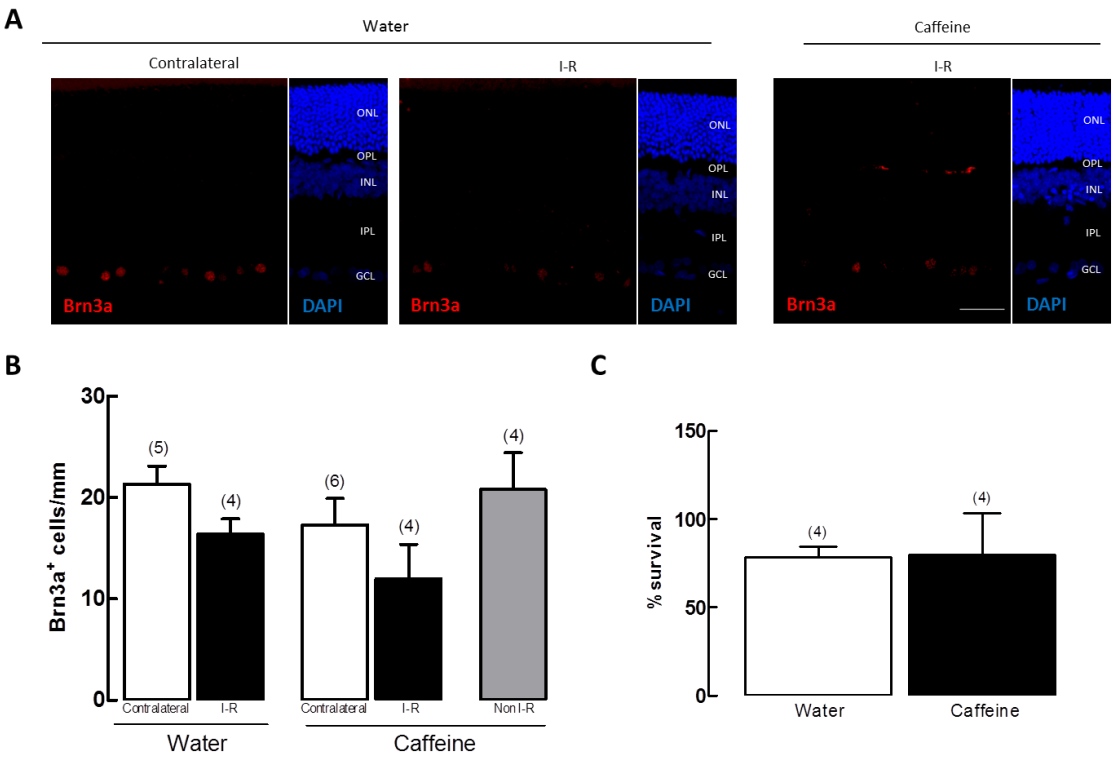


Figure 20 - Effect of caffeine on RGC number after 24h post-injury. Caffeine was administered in the drinking water for 2 weeks prior injury and until the end of the experiment. Retinal sections were stained with an antibody against Brn3a (red). Nuclei were stained with DAPI (blue). Representative images are depicted in A. B: Brn3a-immunoreactive cells were counted and were expressed per mm of retina. C) Percentage of surviving RGCs is expressed as the ratio of I-R eye to contralateral eye. The results represent the mean ± SEM for the number of eyes indicated in parentheses above each bar. From each eye, four retinal sections were analyzed, and from each section six images were randomly acquired. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar = 50 µm.

### 3.2.3. Effect of caffeine on retinal I-R injury rat model: 7 d post I-R injury

Next, we aimed to evaluate if, in a later stage of I-R injury, caffeine could be protective against ischemic damage. For this propose we evaluated damage caused 7 days after ischemia.

#### *3.2.3.1. Microglia reactivity*

As already mentioned, microglia reactivity was assessed by immunohistochemistry with antibodies against Iba1 (total number of microglia/macrophages) and OX-6 (active microglia/macrophages). The number of cells immunoreactive to both OX-6 and Iba1 was expressed as a percentage of total microglia/macrophages.

In the water-treated group, I-R injury significantly increased the number of cells immunoreactive to OX-6 (22.2±1.3% of total, n=2, p<0.01), compared to the corresponding contralateral retinas (0.5±0.5% of total, n=2), indicating an increase in microglia reactivity (Figure 21B).

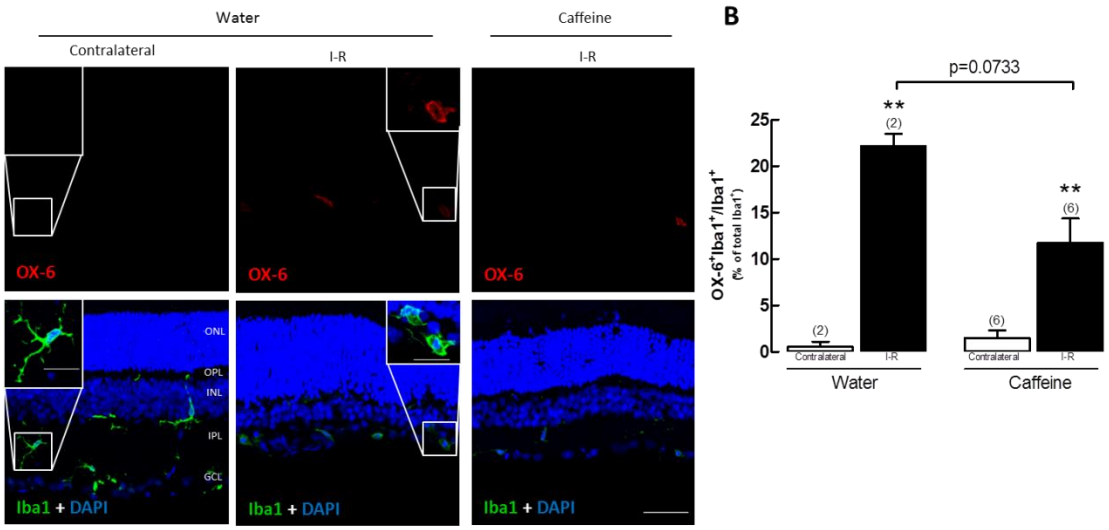


Figure 21 - Caffeine decreased the number of activated microglia induced by I-R injury at 7 days of reperfusion. Caffeine was administered in the drinking water for 2 weeks prior injury and until the end of the experiment. Retinal sections were stained with antibodies against Iba1 (green) and OX-6 (red). Nuclei were stained with DAPI (blue). Representative images are depicted in A. B: Activated microglia/macrophages (OX-6- Iba1-immunoreactive cells) were expressed as the ratio $OX\text{-}6^{+}Iba1^{+}/Iba1^{+}$, and were presented as the percentage of total microglia/macrophages (Iba1-immunoreactive cells). The results represent the mean ± SEM for the number of eyes indicated in parentheses above each bar. From each eye, four retinal sections were analyzed, and from each section six images were randomly acquired. **p<0.01, compared with contralateral eye, Student's t-test. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar = 20 µm (detail images) or 50 µm.

In caffeine-treated I-R retinas the number of cells immunoreactive to OX-6 significantly increased (11.7±2.6% of total, n=6, p<0.01), when compared with the contralateral retinas from caffeine-treated animals (1.5±2.6% of total, n=6). Caffeine administration tend to decrease the number of cells immunoreactive to OX-6 induced by I-R injury (11.7±2.6% of total, n=6, p=0.0733) comparing with water-treated I-R retinas.

Moreover, no statistical significant differences were found between contralateral retinas of the two groups.

### *3.2.3.2. Levels of pro-inflammatory cytokines*

The levels of TNF in the retina of animals drinking water seven days after ischemia were 1074.5±386.8 pg/mg prot and 2059.7±626.1 pg/mg prot in contralateral and I-R retinas, respectively, which corresponds to 2.2±0.4 fold change (Figure 22A). The TNF levels ratio of I-R retinas to contralateral retinas of caffeine-treated animals significantly decreased (1.2±0.1 fold change, n=6, p<0.05), as compared to animals drinking water.

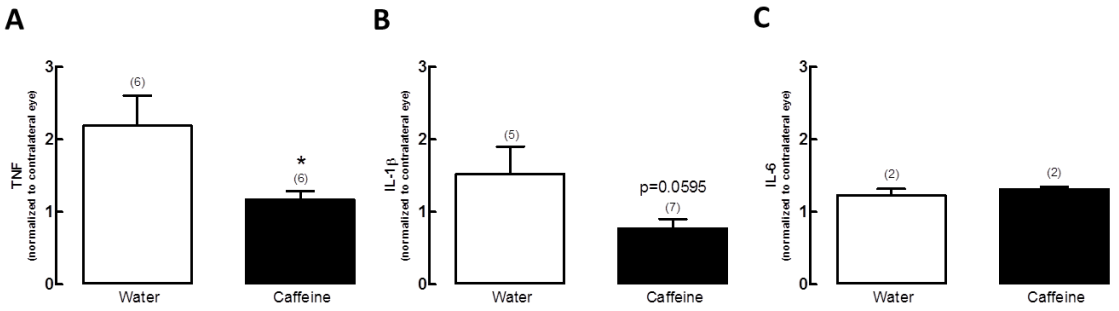


Figure 22 - Caffeine decreased the levels of TNF (A), IL-1β (B) and IL-6 (C) induced by I-R injury at 7 days of reperfusion. Caffeine was administered in the drinking water for 2 weeks prior injury and until the end of the experiment. TNF, IL-1β and IL-6 levels were quantified by ELISA. The results represent the mean ± SEM for the number of eyes indicated in parentheses above each bar, and are expressed as the ratio of I-R eye to contralateral eye. *p<0.05, compared to water-treated group, Student's t-test.

After 7 days of reperfusion the levels of IL-1β (Figure 22B) in the water-treated retinas contralateral to the injury were 1612.4±537.1 pg/mg prot, resulting in 2.2±0.4 fold increase in I-R retinas. Caffeine administration increased IL-1β concentration by 0.8±0.1 fold (3725.8±1491.1 pg/mg vs 2411.1±603.4 pg/mg). IL-1β levels increasing induced by I-R injury in caffeine-treated retinas tend to be lower than in water-treated retinas (0.8±0.1, n=7, p=0.0595).

The levels of IL-6 in the water-treated retinas contralateral to the injury were 2458.0±81.0 pg/mg, resulting in 1.2±0.1 fold increase in I-R retinas (Figure 22C). In caffeine-treated retinas there is an increase of IL-6 concentration by 1.3±0.03 fold (2557.7±135.1 pg/mg vs 3362.3±259.1 pg/mg).

In addition, immunoreactivity of IL-6 in the retina was also assessed (Figure 23). As can be observed, IL-6 immunoreactivity was mainly found in the OPL, IPL, INL and GCL. The immunoreactivity in I-R retinas of animals drinking caffeine is increased compared to the contralateral eye. The treatment with caffeine may decrease IL-6 immunoreactivity of I-R retinas, compared with retinas that were submitted to I-R from animals drinking water.

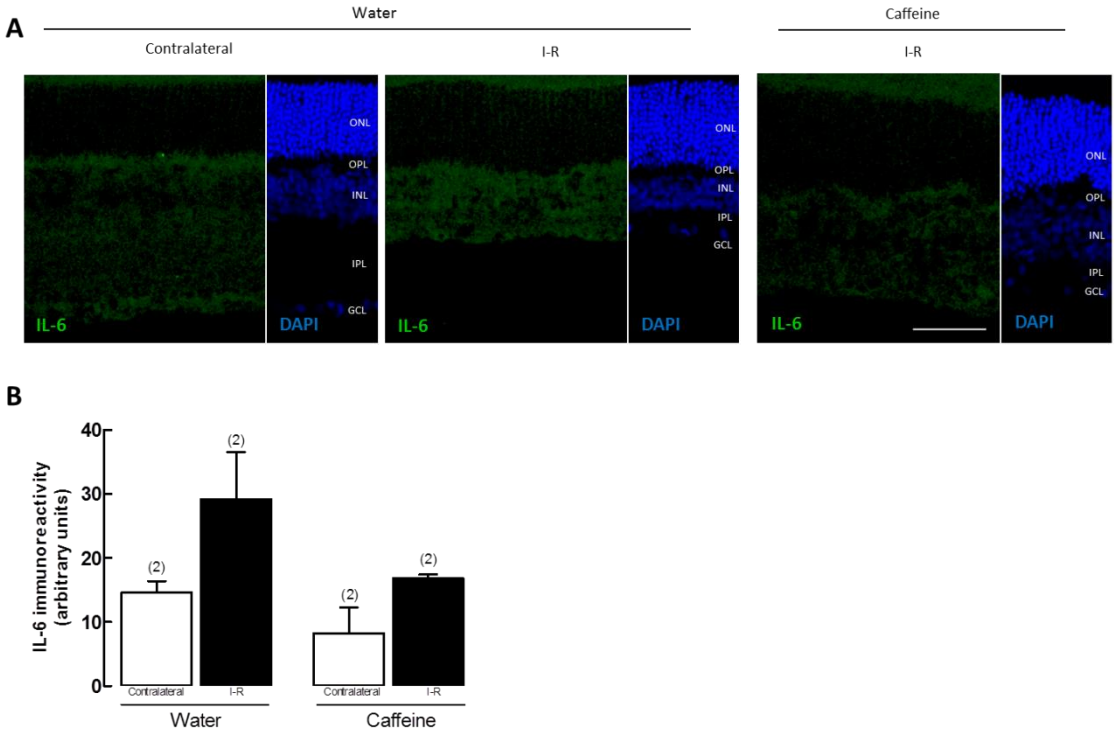


Figure 23 - Caffeine decreased IL-6 immunoreactivity 7 days after I-R. Caffeine was administered in the drinking water for 2 weeks prior injury and until the end of the experiment. Retinal sections were stained with antibodies against IL-6 (green). Nuclei were stained with DAPI (blue). Representative images are depicted in A. B: The intensity of IL-6 staining was quantified using ImageJ software. The results represent the mean ± SEM for the number of eyes indicated in parentheses above each bar. From each eye, four sections were stained and from each section six images were acquired. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar = 50 µm.

### *3.2.3.3. Production of reactive oxygen species*

DHE staining was used to assess the production of ROS 7 days after I-R injury (Figure 24). At 7 days of reperfusion, DHE staining significantly increased in I-R retinas from animals drinking water (n=3, $p<0.05$) compared with contralateral retina.

In caffeine-treated I-R retinas DHE staining is significantly decreased when compared to I-R retinas from animals drinking water (n=3, $p<0.05$).

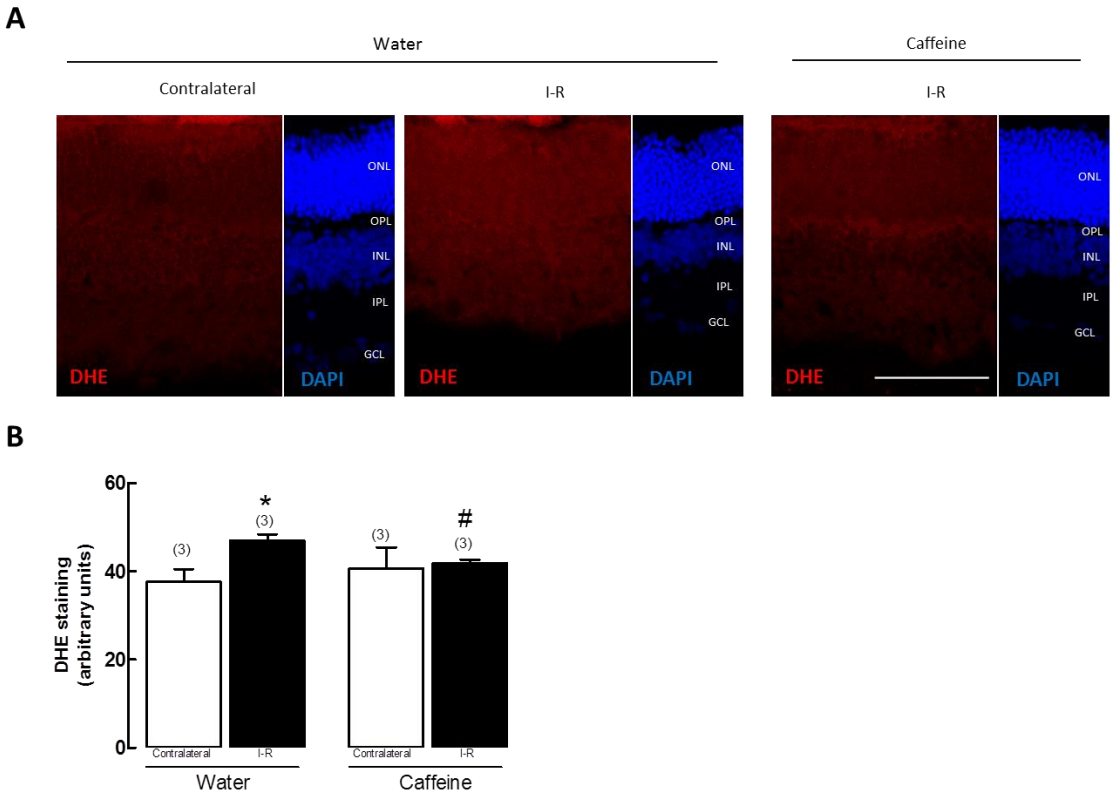


Figure 24 - Caffeine administration decreased the production of reactive oxygen species induced by I-R injury at 7 days of reperfusion. Caffeine was administered in the drinking water for 2 weeks prior injury and until the end of the experiment. Reactive oxygen species was assayed with DHE fluorescence. Representative images are depicted in A. B: The intensity of DHE staining was quantified using ImageJ software. The results represent the mean ± SEM for the number of eyes indicated in parentheses above each bar. From each eye, four sections were stained and from each section six images were acquired. *p<0.05, compared with contralateral eye, #p<0.05, compared with water-treated I-R retinas, Student's t-test. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar = 50 µm.

### *3.2.3.4. Retinal thickness*

After 7 days of reperfusion, retinal thickness significantly decreased in water-treated I-R retinas (147.9±7.8 µm, n=2, p<0.05) comparing with contralateral retinas (Figure 25B). The treatment with caffeine did not affect the thickness of the retina for both contralateral and I-R conditions (234.1±8.1 µm and 181.8±17.3 µm, respectively). The treatment of caffeine in non-I-R retinas does not affect retinal thickness.

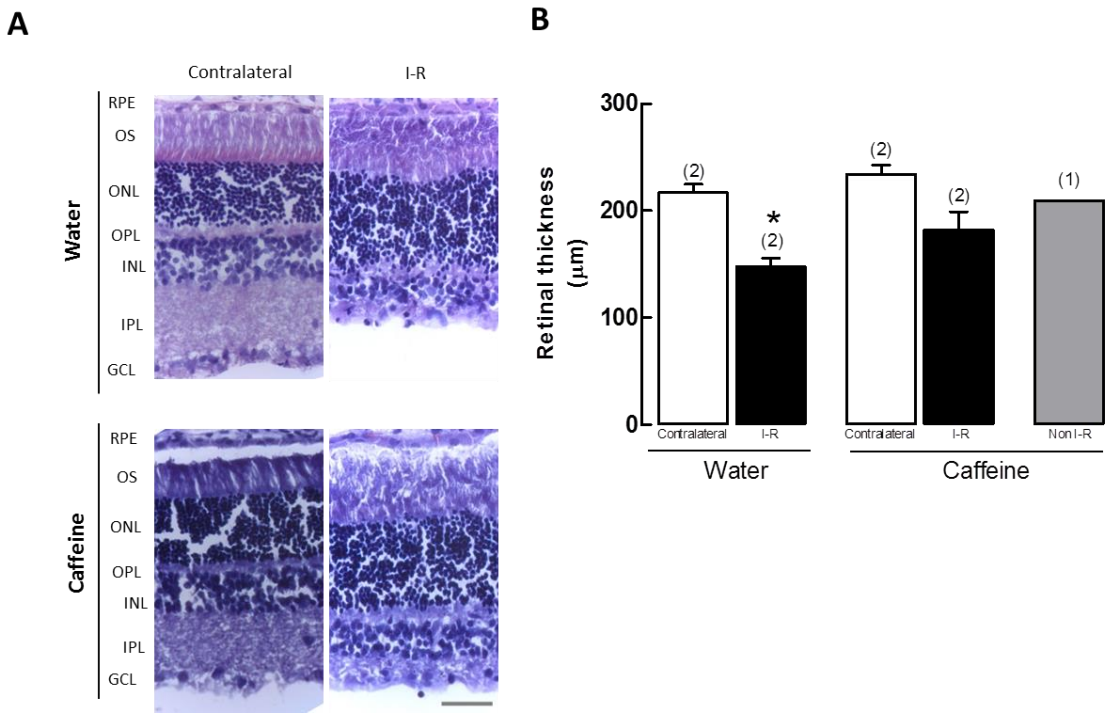


Figure 25 - Caffeine attenuated the decrease of retinal thickness induced by I-R injury 7 days after ischemia. Caffeine was administered in the drinking water for 2 weeks prior injury and until the end of the experiment. Retinal sections were stained with hematoxylin (blue-purple color) and eosin (pink). Representative images are depicted in A. B: Retinal thickness was quantified using ImageJ software. The results represent the mean ± SEM for the number of eyes indicated in parentheses above each bar. From each eye, four sections were stained and from each section 16 measurements were made. *p<0.05, compared with contralateral eye, Student's t-test. RPE: retinal pigment epithelium; OS: outer segments; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar = 50 µm.

### *3.2.3.5. Cell death*

Cell death by apoptosis was assessed by TUNEL assay. We observed that I-R injury in water-treated retinas significantly increased the number of $TUNEL^+$ cells (6.4±1.2 cells/mm, n=3, p<0.01) comparing with contralateral retinas (0.5±0.2 cells/mm) (Figure 26). In caffeine-treated I-R retinas there was an increase in the number of $TUNEL^+$ cells (3.0±0.8 cells/mm, n=5, p<0.01) comparing with contralateral retinas (0.2±0.1 cells/mm), but it was significantly decreased compared to I-R retinas of animals drinking water (p<0.05). Caffeine per se did not cause cell death.

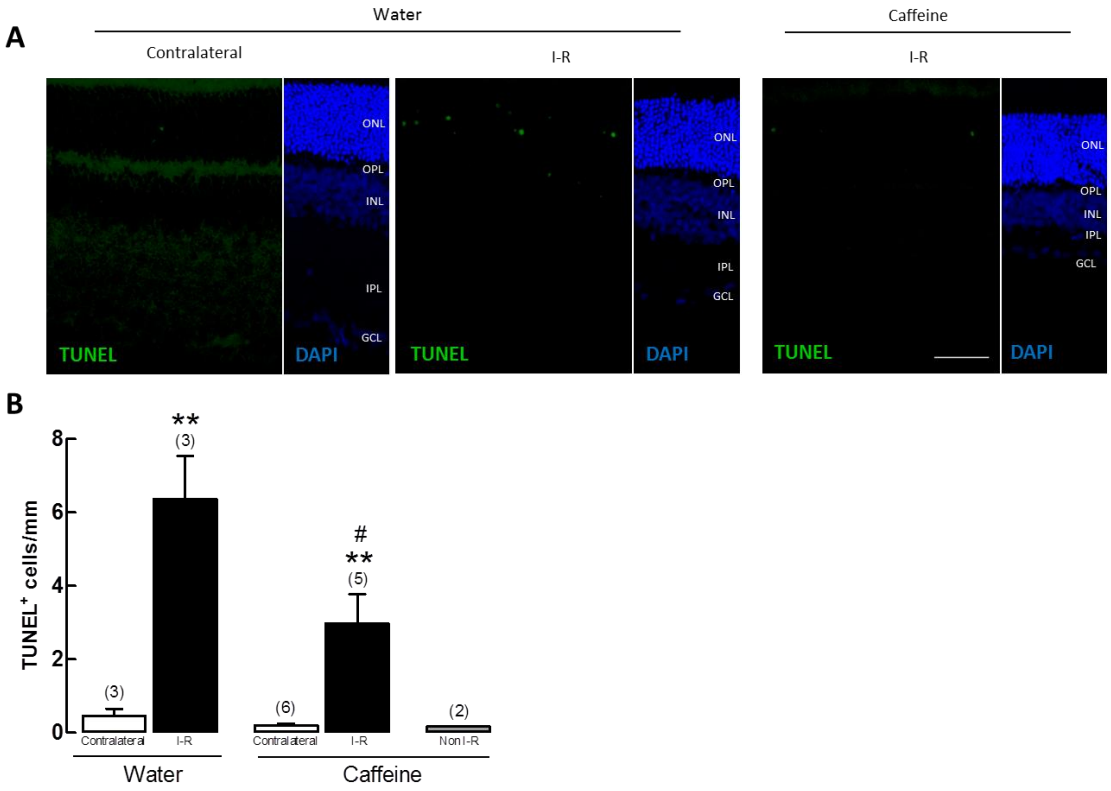


Figure 26 - Caffeine administration decreased cell death induced by I-R injury at 7 days of reperfusion. Caffeine was administered in the drinking water for 2 weeks prior injury and until the end of the experiment. Cell death was assayed by TUNEL assay. Nuclei were stained with DAPI (blue). Representative images are depicted in A. B: TUNEL-positive cells (green) were counted and were expressed per mm of retina. The results represent the mean ± SEM for the number of eyes indicated in parentheses above each bar. From each eye, four retinal sections were analyzed, and from each section six images were randomly acquired. **p<0.01, compared with contralateral eye, #p<0.05, compared with water-treated I-R retinas, Student's t-test. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar = 50 µm.

### *3.2.3.6. RGC loss*

I-R injury significantly decreased the number of Brn3a$^+$ cells in water-treated I-R retinas (8.0±3.6 cells/mm, n=5, p<0.01), comparing with contralateral retinas (22.8±1.4 cells/mm). In caffeine-treated I-R retinas the number of Brn3a$^+$ cells decreased compared with contralateral retinas (16.3±2.1 vs 9.3±3.7 cells/mm) but are not statistically significant. However, caffeine increased the percentage of RGCs in 74.1±27.6 %, comparing with water-treated retinas in which only 34.9±15.7 % of RGCs survived after I-R injury. Caffeine per se did not induce Brn3a$^+$ cells loss (Figure 27B).

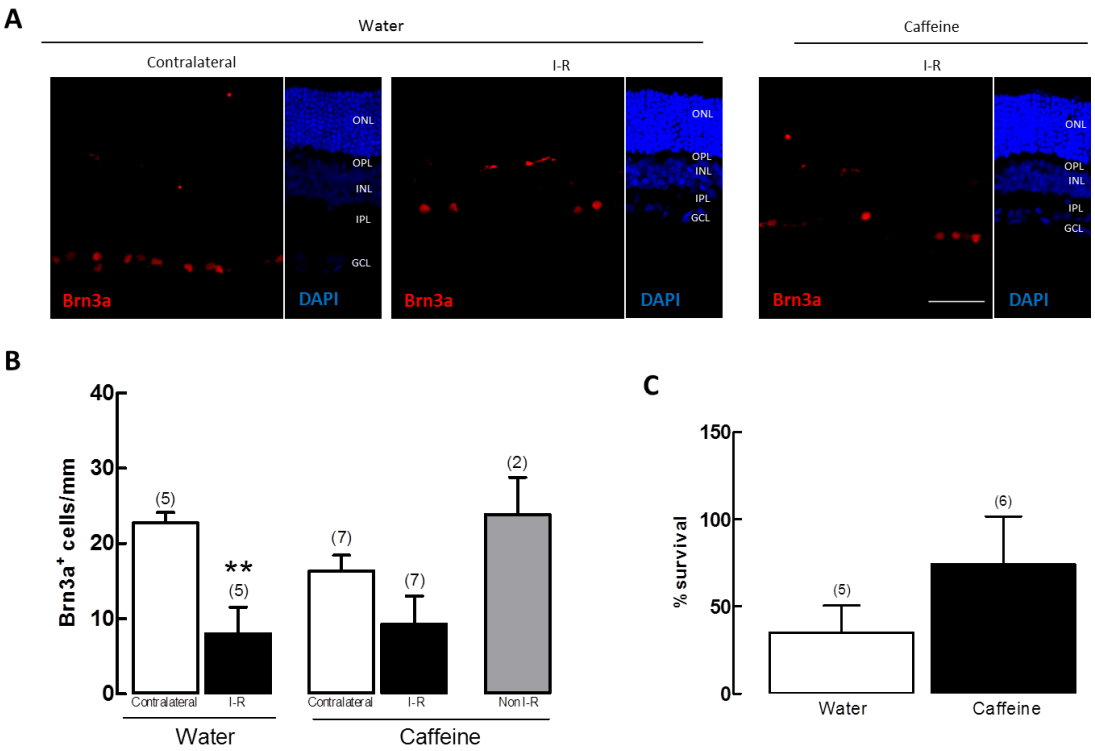


Figure 27 - Caffeine administration decreased RGC loss induced by I-R injury at 7 days of reperfusion. Caffeine was administered in the drinking water for 2 weeks prior injury and until the end of the experiment. Retinal sections were stained with antibodies against Brn3a (red). Nuclei were stained with DAPI (blue). Representative images are depicted in A. B: Brn3a-immunoreactive cells were counted and were expressed per mm of retina. The results represent the mean ± SEM for the number of eyes indicated in parentheses above each bar. From each eye, four retinal sections were analyzed, and from each section six images were randomly acquired. **p<0.01, compared with contralateral eye, Student's t test. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar = 50 µm.

# *Chapter 4*

# 4. Discussion

Glaucoma is one leading cause of blindness in the world and it is characterized by progressive degeneration and death of RGCs (Vohra *et al.*, 2013). Elevated IOP and age are important risk factors for the progression of the disease. The treatments available are directed towards lowering IOP, but the patients continue to lose vision. Therefore, neuroprotection of RGCs is thought as an important therapy in addition to therapies aimed to decrease IOP.

Retinal ischemia is closely associated with pathophysiology of many retinal diseases, like glaucoma. In fact, retinal ischemia followed by reperfusion triggers RGC loss (Dorfman *et al.*, 2012), and damage in various retinal layers (Johnson and Tomarev, 2010), as well as microglia activation (Halder *et al.*, 2013). In the initial phases of the disease, microglia has the role to initiate the repair mechanism. However, an exaggerated microglia reactivity may lead to chronic inflammation, perpetuating the loss of RGCs (Langmann, 2007; Karlstetter *et al.*, 2010). Increased microglia reactivity was observed in the retina of glaucoma patients and experimental animal models (Neufeld and Liu, 2003; Bosco *et al.*, 2011).

Activation of retinal microglial cell through preconditioning with LPS prevented cell damage in retina caused by I-R injury (Halder *et al.*, 2013). Moreover, preconditioning with 5 min of ischemia followed 24h or 72 h later by 60 min also protected retina against the effects of ischemia (Ghiardi *et al.*, 1999). These studies demonstrated that preconditioning initiated a compensatory or adaptive response to excitotoxic insults by microglia.

Moreover, adenosine concentration have large changes after I-R injury which could be injurious to retina, but small adenosine concentration changes may induced a tolerant state ischemic injury (Ghiardi *et al.*, 1999).

Microglia are thought to be the principal source of pro-inflammatory mediators, such IL-1β, TNF, and nitric oxide (NO), which are elevated in the retina after I-R injury (Lee, 2013). Increased production of cytokines and ROS contribute to the perpetuation of the neurodegenerative process (Sanchez *et al.*, 2003; Ozerol *et al.*, 2009; Gesslein *et al.*, 2010).

In I-R model there is a possibility to control the magnitude and the duration time of the elevation of the IOP. Both the duration of ischemia and reperfusion periods influence the proportion of RGC death. An ischemic period between 45 and 60 minutes induces significant RGCs loss, without inducing irreversible damage, and the reperfusion period also influences the proportion of RGCs loss. Cell death begins as soon as 3 to 24 h and after 5 days of reperfusion, a high percentage of cell loss is presently observed (Selles-Navarro *et al.*, 1996). For an ischemic time of 60 minutes, we found that I-R injury induced retinal cell death (assessed with TUNEL assay), loss of RGCs (counting the number of cells immunoreactive to Brn3a), and retinal thinning, which is in agreement with previous reports (Dorfman *et al.*, 2012; Halder *et al.*, 2013).

In the retina, microglial cells are predominantly located in IPL but can be also observed in the GCL (Zhang *et al.*, 2005), in accordance with our results. In addition, intravitreally injection of saline or A2AR agonist or antagonist did not change microglia localization.

In this work, the contralateral eye was considered the control condition, since all eyes were injected with the drugs (also in saline-injected animals). In contralateral eyes (saline-injected or animals drinking water) we did not observe substantial microglia reactivity (cells immunoreactive to OX-6). Interestingly, more OX-$6^{+}$ cells were observed in eyes injected with saline than in animals drinking caffeine. One possible explanation could be the fact that intravitreal injection, even if of saline, can somehow induce changes in the retinal environment and induce microglia reactivity. Recently, it was reported ocular hypertension induces upregulation of GFAP and OX-6 and microglia reactivity in the contralateral retinas (Gallego *et al.*, 2012). Although the experimental models are different, we should be aware that the contralateral eye may not be the best control, and naïve retinas should have been used also.

Several studies reported that blockade of A2AR prevents injury in a number of brain conditions (Cunha, 2005; Cunha *et al.*, 2008; Rebola *et al.*, 2011). Moreover, blockade of A2AR can control neuroinflammation and also the recruitment of microglia (Orr *et al.*, 2009; Simoes *et al.*, 2012). In the retina, microglia cells express A2AR (Liou *et al.*, 2008), therefore we hypothesize that A2AR modulation could control retinal neuroinflammation. However, in general, neither the activation nor the blocking of A2AR altered the levels of pro-inflammatory cytokines, except for 8h reperfusion in which SCH 58261 increased IL-1$\beta$.

Microglia cells became reactive early after I-R injury. In fact, OX-6 (MHC II antigen presenting cells) was reported not to be expressed 6 hours after ischemia, but after 1, 3 and 7 days (Zhang *et al.*, 2005). Intravitreal administration of SCH 58261 decreased the number of cells expressing OX-6 (microglia/machrophages), suggesting that A2AR blockade can, in a certain level, decrease microglia reactivity.

Previously it was reported that activation of A2AR attenuated retinal thinning induced by I-R injury (Konno *et al.*, 2006), suggesting that A2AR activation prevents retinal cell death. In this work, A2AR agonists, 2-(6-cyano-1-hexyn-1-yl)adenosine (2-CN-Ado) and CGS 21680, were administered systemically (30 min before ischemia), intravenously and intraperitoneally, respectively, or topically (2-CN-Ado). Although we did not assess retinal thickness in these experiments, we can speculate that we would not observe prevention in retinal thickness with either CGS 21680 or SCH 58261. The different routes of administration and the time frame of drug administration may help explaining the potential contradictory results. A2AR is known to have bidirectional effects, depending on the route of administration (Dai and Zhou, 2011). For instance, peripheral administration of A2AR agonist protected the hippocampus against kainate-induced excitotoxicity, but intrahippocampal administration did not protect the region to any degree (Jones *et al.*, 1998).

It is important to note that the source of these cytokines was not determined. In the present study, reactive astrocytes also secrete many of the same cytokines. As a consequence of I-R injury, astrocytes become activated, proliferate, produces pro-inflammatory cytokines, chemokines and ROS (Uno *et al.*, 1997; Tezel and Wax, 2000; Reinehr *et al.*, 2007). Astrocyte activation initiates both protective and neurotoxic pathways, and is increasingly associated with worsened outcomes in the injured CNS (Faulkner *et al.*, 2004; Abramov and Duchen, 2005; Fernandez *et al.*, 2007; Toft-Hansen *et al.*, 2011). Neurons also contribute to the inflammatory process and blockade of A2AR also control the deleterious effects of IL-1β on neuronal viability (Rebola *et al.*, 2011). Since we did not detect changes in the expression of pro-inflammatory cytokines after I-R injury, even after SCH 58261 administration causing decreased microglia reactivity, we have to consider that other cells in retina are contributing to inflammation.

Caffeine is the most commonly used psychostimulant in the world and its effects are partly mediated by antagonizing adenosine receptors (Cognato *et al.*, 2010).

In humans, a single cup of coffee provides a peak plasma caffeine concentration of 1-10 µM (Fredholm *et al.*, 1999). In rats, we found that serum caffeine concentration was 55 µM (2 weeks consumption) and 98 µM (3 weeks consumption), which is similar with other studies (Duarte *et al.*, 2009; Duarte *et al.*, 2012). It demonstrates that the caffeine dose selected (1 g/L) represents a high intake of caffeine, corresponding to 5-8 cups of coffee per day in humans.

Caffeine consumption may influence IOP (Avisar *et al.*, 2002). The mechanism of how caffeine contributes to this increased in IOP is not fully understood. By inhibiting phosphodiesterase, caffeine leads to an increase in cAMP, and increased intraocular levels of cAMP leads to a hypotensive effect (Kurata *et al.*, 1998). Moreover, caffeine intake increases blood pressure (Nurminen *et al.*, 1999) which may influences aqueous humor outflow (Xu *et al.*, 2007). Taking this into account, IOP was measured regularly during caffeine administration period and we found that its ingestion did not change IOP in rats.

Caffeine is a non-selective antagonist of A1R and A2AR. These receptors have similar affinity for caffeine, slightly higher for A2AR in brain preparations (Costenla *et al.*, 2010; Orru *et al.*, 2013). Previous reports suggest that chronic daily caffeine administration protects against brain injury in different animal models of neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases, ischemic and traumatic brain injury, and allergic encephalitis (Phillis, 1995; Chen *et al.*, 2001; Popoli *et al.*, 2002; Arendash *et al.*, 2006; Deleu *et al.*, 2006; Riksen *et al.*, 2006; Li *et al.*, 2008; Sonsalla *et al.*, 2012). Caffeine administration also prevents memory impairment triggered by a convulsive episode in early life and in streptozotocin diabetic rats (Duarte *et al.*, 2009; Cognato *et al.*, 2010). More recently, also in an animal model of Machado-Joseph disease caffeine delayed striatal pathology (Goncalves *et al.*, 2013). Regarding these effects, we aimed to evaluate whether long-term caffeine intake prevents retinal neuroinflammation and cell death induced by retinal I-R injury.

Evidence exists regarding the effect of caffeine in controlling neuroinflammation. Using the murine BV2 microglial cell line, it was shown that caffeine suppressed the LPS-induced pro-inflammatory mediators NO, prostaglandin $E_2$ and TNF by inhibiting Akt-

dependent NF-κB activation and the ERK signaling pathway (Kang *et al.*, 2012). Moreover, caffeine administration was reported to reduce LPS- and age-induced microglia activation in the rat hippocampus (Brothers *et al.*, 2010a).

In an animal model of Parkinson's disease caffeine was found to reduce the levels of NO, microglial activation and thereby protect dopaminergic neurodegeneration (Yadav *et al.*, 2012).

We analyzed the effect of caffeine at 24h and 7 days post-ischemia. We found that caffeine exacerbated microglia reactivity at 24 h after ischemia, without significant changes in the levels of cytokines. However, when we analyzed the effect of caffeine 7 days after ischemia we found that caffeine prevented the increase in microglia reactivity and decreased the levels of pro-inflammatory cytokines. The effects of caffeine regarding neuroprotection were mainly due to the prevention of cell death and retinal thickness. One possible explanation for the apparent contradictory results could be the fact that immediately after injury microglia cells become activated to initiate the repair mechanisms. Caffeine may "prime" microglia to respond to injury more effectively, culminating in less cell death and an increase in RGC survival, as observed 7 days after ischemia.

Microglia neuroinflammation is commonly associated with the production of ROS, which are proposed to be involved in RGC death. Production of ROS targets innumerous retinal functions. It was reported that there is an overproduction of ROS after ischemia which mainly occurs during tissue reoxygenation in the reperfusion period (Kuriyama *et al.*, 2001). Controlling the production of ROS, RGC rescue is improved (Yokota *et al.*, 2011; Liu *et al.*, 2012). Our results corroborate these findings demonstrating that caffeine decreases the production of ROS. Caffeine was demonstrated to have properties of scavenging various ROS, namely superoxide and hydroxyl radical (Shi *et al.*, 1991; Devasagayam *et al.*, 1996). Caffeine was also found to be physiologically effective in protecting the lens against ROS-induced damage caused by incubating the lens exposed to UV in kynurenine-containing medium (Varma and Hegde, 2010). The levels of GSH and ATP in the lens were maintained better in the presence of caffeine than in its absence.

The exact molecular mechanism and what cells are contributing to this inflammatory response remains unknown. In line with what has been said so far at 24h after ischemia, the inflammation and microglial reactivity induced by I-R injury are increased in result of

caffeine administration, and in later stages post-injury this procedures is controlled. As cell death can be the result of inflammatory process the exaggerated microglial activation and inflammation leads to large increase of cell death. It seems that caffeine by controlling inflammatory process induced by I-R injury, decrease cell death in retina.

Retinal thinning may be explained by loss of cells and synapse arborization. Retinas in average are 170 µm thick (Dorfman *et al.*, 2012). The most affected layer by I-R injury is IPL, as its thickness start to decrease after 72 h of a 60 min period ischemia (Liu *et al.*, 2012). We did not detect changes in the thickness of the retina after 24h of ischemia, which is in agreement with the lack of statistical significance in the number of RGCs present.

One important aspect to consider is that the markers used to identify microglia are also present in macrophages. As there is no known cell-surface marker to distinguish CNS-resident from blood-borne macrophages, it is difficult to discern the resident microglia from monocytes that enter the CNS from the bloodstream and subsequently adopt microglial-cell morphology (Flugel *et al.*, 2001). The neuroinflammatory response, either resident microglia or infiltrating monocytes, becomes a critical issue in determining, not only the nature of the response and characteristics of the injury, but also the effectiveness of modulating their response. In our model, it is established that the BBB is compromised (Tong *et al.*, 2013), leading to leakage to the retinal parenchyma, suggesting that monocytes infiltrate contributing to the inflammatory response. Nevertheless, it was reported that caffeine protects against high cholesterol diet-induced disruption of the BBB (Chen *et al.*, 2008), suggesting that it may also influence the permeability of the BRB.

In conclusion, intravitreal administration of A2AR agonist or antagonist does not seem to modulate the pro-inflammatory response in the retina induced by I-R injury. Nevertheless, long-term caffeine administration prior I-R may attenuate retinal neuroinflammation and prevent cell death, namely of RGCs.

# *Chapter 5*

# 5. Conclusions and Future directions

The results presented in this thesis allowed us to conclude that:

- Retinal I-R injury increased microglia reactivity, which was prevented by A2AR antagonist;
- The A2AR agonist or antagonist did not change the levels of pro-inflammatory cytokines in the retina after I-R injury;
- Caffeine induced a high inflammatory response 24 h post-injury, exacerbating the effect of I-R;
- Caffeine decreased the number of reactive microglia, pro-inflammatory cytokines and the production of ROS at 7 days post-injury;
- Caffeine prevented the increase in cell death and retinal thinning induced by I-R injury.

Further research should be performed to investigate the effects of systemic administration of A2AR agonist and antagonist. For this, we aim to evaluate the effect of CGS 21680 and SCH 58261 injected intraperitoneally in the I-R injury model.

Considering previous data on the effect of caffeine in the hemato-retinal barrier, we intend to assess its influence on the I-R injury model.

Moreover, in order to complete these results the study may be conducted in a glaucoma model.

# *Chapter 6*

# 6. References

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