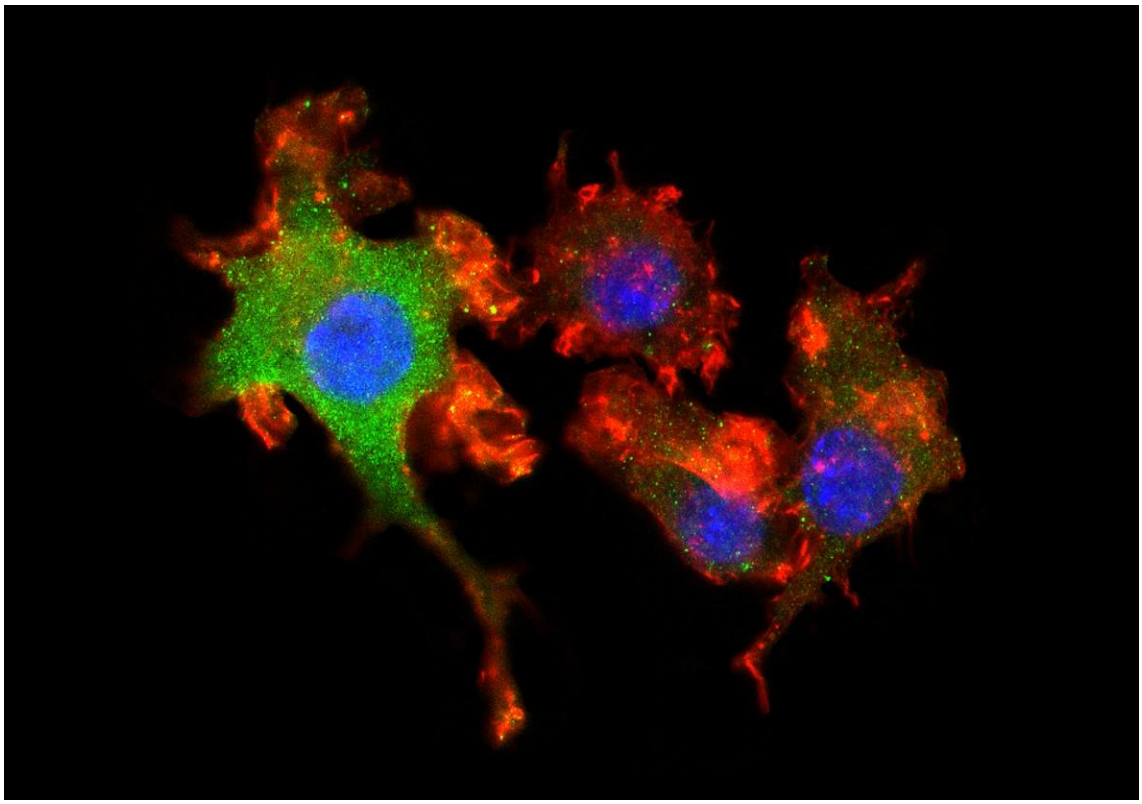

The impact of elevated hydrostatic pressure in microglia: changes in the adenosinergic system and inflammatory responses

Ana Catarina Rodrigues Neves

Dissertação para a obtenção do grau de Mestre em Investigação Biomédica sob orientação científica da Doutora Ana Raquel Sarabando Santiago e co-orientação do Doutor António Francisco Rosa Gomes Ambrósio e apresentada à Faculdade de Medicina da Universidade de Coimbra.

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On the front page:

Microglial cells (BV-2) stained with phalloidin (red) and iNOS (green). Cell nuclei stained with DAPI (blue).

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Dissertação apresentada à Faculdade de Medicina da Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Investigação Biomédica. Este trabalho foi realizado no grupo *Retinal Dysfunction & Neuroinflammation* do Instituto de Imagem Biomédica e Ciências da Vida (IBILI), Faculdade de Medicina da Universidade de Coimbra, sob a orientação científica da Doutora Ana Raquel Sarabando Santiago e co-orientação do Doutor António Francisco Rosa Gomes Ambrósio.

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“A mente que se abre a uma nova ideia,

jamais volta ao seu tamanho inicial”

ALBERT EINSTEIN

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CONTENTS

ABBREVIATIONS

4-AA	4-aminoantipyrine
A_1R	A_1 receptor
$A_{2A}R$	A_{2A} receptor
$A_{2B}R$	A_{2B} receptor
A_3R	A_3 receptor
AC	Adenylate cyclase
ADA	Adenosine deaminase
ADK	Adenosine kinase
ADP	Adenosine 5’-diphosphate
AMD	Age-related macular degeneration
AMP	Adenosine 5’-monophosphate
AP	Alkaline phosphatases
ARs	Adenosine receptors
ATP	Adenosine 5’- triphosphate
BAB	Blood-aqueous barrier
BCA	Bicinchoninic acid
bp	Base pair
BRB	Blood-retinal barrier
BSA	Bovine serum albumin
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
CNT	Concentrative nucleoside transporter
CREB	cAMP responsive element binding protein
CTCF	Correct total cell fluorescence

DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle medium
DPPIV	Dipeptidyl peptidase IV
EHP	Elevated hydrostatic pressure
EHSPT	N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline
ELISA	Enzyme-linked immunosorbent assay
E-NNP	Ectonucleotide pyrophosphatase/phosphodiesterases
ENT	Equilibrative nucleoside transporter
E-NTPDases	Ectonucleoside triphosphate diphosphohydrolases
FBS	Fetal bovine serum
G proteins	GTP-binding proteins
GABA	Gama-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCL	Ganglion cell layer
GFAP	Glial fibrillary acidic protein
G_i	Inhibitory G-protein
G_s	Stimulatory G-protein
HBSS	Hank's balanced salt solution
H_2O_2	Hydrogen peroxide
iBRB	Inner blood-retinal barrier
IL	Interleukin
INL	Inner nuclear layer
I-R	Ischemia-Reperfusion
iNOS	Inducible nitric oxide synthase
IOP	Intraocular pressure
IPL	Inner plexiform layer
KO	Knockout
LPS	Lipopolysaccharide

MAP	Mitogen-activated protein
NFL	Nerve fiber layer
NO	Nitric oxide
oBRB	Outer blood-retinal barrier
ONH	Optic nerve head
ONL	Outer nuclear layer
OPL	Outer plexiform layer
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PI3	Phosphoinositide 3
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PNP	Purine nucleoside phosphorylase
POD	Peroxidase
PVDF	Polyvinyl difluoride
qPCR	Quantitative polymerase chain reaction
RGCs	Retinal ganglion cells
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
RPMI	Roswell Park Memorial Institute medium
SAH	S-adenosylhomocysteine
SCH 58261	2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
TE	Tris EDTA

TLRs	Toll-like receptors
TNF	Tumor necrosis factor
XOD	Xanthine oxidase

RESUMO

O glaucoma é uma doença neurodegenerativa e sem cura, caracterizada pela morte das células ganglionares da retina e pela atrofia do nervo ótico. É a segunda causa de cegueira irreversível em todo o mundo, estimando-se que a sua prevalência aumente para o dobro até 2040. O aumento da pressão intraocular (PIO) é considerado o principal fator de risco para o desenvolvimento de glaucoma e, os atuais tratamentos focam-se no controlo da PIO. Contudo, alguns doentes continuam a perder a visão mesmo com a PIO dentro de valores considerados normais. Assim, torna-se extremamente necessário desenvolver novas estratégias terapêuticas sendo a proteção das células ganglionares da retina uma estratégia com potencial. A neuroinflamação tem um papel crucial no desenvolvimento de glaucoma, e tem sido dada especial atenção ao aumento da reatividade das células da microglia observada em modelos animais e pacientes com glaucoma.

A adenosina é um neuromodulador do sistema nervoso central (SNC), envolvida em respostas inflammatórias, que atua através da activação de recetores acoplados a proteínas G: A_1, A_{2A}, A_{2B} and A_3. Sabe-se atualmente que o bloqueio do recetor A_{2A} de adenosina confere neuroproteção em várias doenças neurodenegerativas através do controlo da neuroinflamação, e diversos estudos focam-se no uso de antagonistas do recetor A_{2A} como potencial estratégia neuroprotetora.

Neste estudo pretendemos avaliar o efeito do aumento da pressão hidrostática, estímulo usado para mimitezar a hipertensão ocular, no sistema adenosinérgico das células da microglia. Adicionalmente, pretendemos compreender se o bloqueio do recetor A_{2A} previne a resposta inflamatória resultante do aumento da pressão hidrostática. Foram para isso utilizados dois modelos *in vitro*: as células BV-2 (linha celular de microglia), nas quais o bloqueio do recetor A_{2A} foi efetuado farmacologicamente usando um antagonista seletivo (SCH 58261), e culturas organotípicas de retina de ratinhos *knock-out* (KO) para o recetor A_{2A}.

A exposição das células BV-2 a pressão hidrostática elevada levou à alteração do sistema adenosinérgico, promovendo um aumento dos níveis de adenosina. Este aumento dos níveis de adenosina pode ativar os recetores A_{2A}, cuja expressão aumentou em condições de pressão hidrostática elevada. Adicionalmente, o aumento da pressão hidrostática diminuiu a expressão do recetor A_1 e não intereferiu com a expressão do recetor A_3. O

bloqueio do recetor A_{2A} preveniu a expressão e libertação de iNOS e TNF, respetivamente, nas células da microglia. Nas culturas organotípicas de retina, a exposição a pressão elevada aumentou os níveis de IL-1β e TNF e a morte das células ganglionares. A inativação genética do recetor A_{2A} não preveniu o aumento da libertação de TNF.

Em síntese, os nossos resultados mostraram que a pressão elevada conduz a alterações no sistema adenosinérgico nas células da microglia e que o bloqueio do recetor A_{2A} pode ser uma estratégia promissora na modulação da reatividade da microglia após a exposição a pressão elevada.

Palavras-chave: glaucoma, pressão hidrostática elevada, sistema adenosinérgico, receptor A_{2A} de adenosina, neuroinflamação, células da microglia, culturas organotípicas de retina, neuroproteção.

ABSTRACT

Glaucoma is the second leading cause of irreversible blindness worldwide and it is expected that the prevalence of this disease increases to the double until 2040. It is a neurodegenerative disease, characterized by the progressive loss of retinal ganglion cells (RGCs) and nerve optic atrophy (RGC axons), leading to vision loss. Intraocular pressure (IOP) is the one of the major risk factors for the development of glaucoma and the current therapeutic approaches are focused on lowering IOP. However, some patients continue to loose vision despite the effective control of IOP, highlighting the urgent need to develop new therapeutic strategies, and neuroprotection of RGCs is consider to have great potential. Neuroinflammation has a preponderant role in the pathogenesis of glaucoma. In particular, special attention has been given to the exacerbated microglial cell response in glaucoma experimental models and in human patients.

Adenosine is a neuromodulator in CNS involved in inflammatory responses that acts by the activation of four G protein-coupled receptors, A_1, A_{2A}, A_{2B} and A_3. The blockade of $A_{2A}R$ has been shown to afford robust neuroprotection in several neurodegenerative diseases, probably by the control of neuroinflammation. Therefore, several studies have been focused in $A_{2A}R$ antagonists as a neuroprotective strategy.

In this study, we aimed to investigate the potential alterations induced by elevated hydrostatic pressure (EHP), a stimulus use to mimic increased IOP, in the adenosinergic system in microglial cells. Moreover, we aimed to assess whether the blockade of $A_{2A}R$ could prevent the pro-inflammatory environment prompted by EHP. In order to accomplish our aims, two *in vitro* models were used: the BV-2 cells (microglial cell line), using the selective antagonist SCH 58261 and retinal organotypic cultures obtained from wild-type and knock-out (KO) mice for $A_{2A}R$.

The exposure of BV-2 cells to EHP showed an impairment of the adenosinergic system promoting increased levels of extracellular adenosine. The increase in adenosine might activate $A_{2A}R$, which we found to be upregulated in BV-2 cells exposed to EHP. We also found that EHP decreased the protein levels of A_1R, without interfering with A_3R protein levels. The blockade of $A_{2A}R$ prevented some inflammatory features (iNOS and TNF levels), in microglia. In organotypic cultures, the exposure to EHP increase both IL-1β and TNF levels and RGCs loss. Nevertheless, the genetic blockade of $A_{2A}R$ did not prevent the increased of

TNF levels. In summary, our results demonstrated that EHP impacts the adenosinergic system in microglia and the blockade of $A_{2A}R$ may be promising strategy to modulate microglia reactivity triggered by increased pressure.

Keywords: glaucoma, elevated hydrostatic pressure, adenosinergic system, A_{2A} receptor, neuroinflammation, microglial cells, retinal organotypic cultures, neuroprotection.

CHAPTER 1

Introduction

1. INTRODUCTION

1.1 Vision

The eyes are like windows to the outside of the world that allow the perception of the surroundings. The eyes capture, adjust and transform light into a chemical code that only the brain can decipher.

1.2 Anatomy of the eye

The eye is a highly specialized and organized structure composed by an optical system, responsible for the focus of the external visual image and a neuronal system that converts the visual image into electrical impulses transmitted to the brain through the optic nerve.

The eye is separated into three different layers (Figure 1). The sclera and the cornea form the external layer. The sclera is a conjunctive tissue that helps to maintain the structure of the eye and protects its internal structures. The cornea is a transparent and avascular tissue that allows the entrance of light.

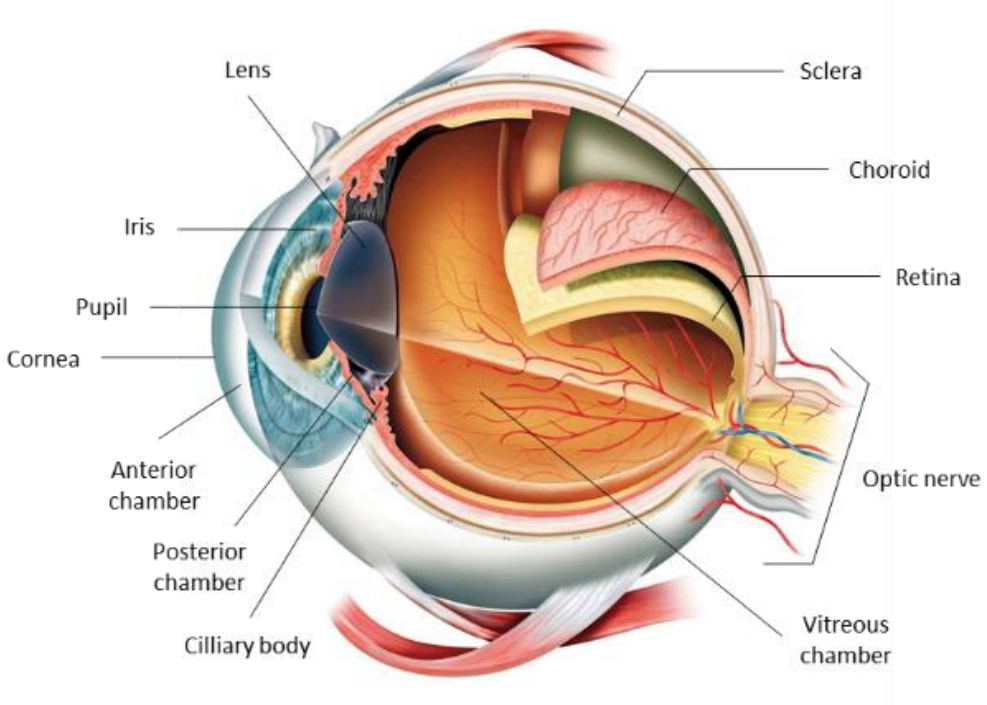

Figure 1: Anatomy of the human eye (Modified from: http://www.iris-pharma.com/c/early-testing/early-pk).

The intermediate layer, the uveal layer, is divided in two parts: the anterior, composed by the iris and the ciliary body; and the posterior, composed by the choroid. The

iris is the colored part of the eye that functions like a diaphragm. This circular structure is composed by two types of muscles that contract and relax, adjusting the size of the pupil, in order to control the amount of light that reaches the eye. The lens is a transparent body localized behind the iris that helps the eye focusing near or distant objects, through a process called accommodation. This structure is suspended by ligaments, called zonule fibers that are attached to the anterior portion of the ciliary body: the ciliary muscles. The ciliary body is also composed by ciliary processes, which are responsible for the aqueous humor formation. The choroid provides oxygen and nourishment to the outer retina and it also plays an important role in the drainage of the aqueous humor from the anterior chamber (via uveoscleral pathway) (Nickla and Wallman, 2010).

The eye possesses three fluid chambers: the anterior chamber (between cornea and iris), the posterior chamber (located between iris and lens) and the vitreous chamber (between lens and retina). The aqueous humor fills both anterior and posterior chambers, nourishing the cornea and the lens, whereas the vitreous chamber is filled with a more viscous fluid: the vitreous humor (Kolb et al., 1995). These fluids are colorless and important to maintain the structure of the eye.

The innermost layer of the eye is the retina, which converts optical images into electric signals that are sent to the brain through the optic nerve (Seeley et al., 2008).

1.3 Retina

The retina is a thin multi-layer of neural tissue located in the back of the eye (Figure 2) and it is part of the central nervous system (CNS). The light travels through all retinal layers to reach the photoreceptors. Indeed, the absorption of photons by the visual pigment of the photoreceptors, rhodopsin, is converted into a biochemical message and then translated in an electrical message that can stimulate the succeeding neurons of the retina until being transmitted to the brain via optic nerve .

The retinal pigment epithelium (RPE) layer is the outermost layer of the retina and is composed by continuous cuboidal epithelial cells. This monolayer of cells reduces the backscattering of light that enters in the eye, supplies trophic factors for the retina, and renews the photopigments by phagocytosing the disks of the photoreceptors (Purves et al., 2004, Rizzolo et al., 2011). Adjacent to the RPE, there is the photoreceptor layer constituted by the outer and inner segments of photoreceptors, and the outer nuclear layer (ONL)

constituted by nuclei of photoreceptors. The axons of the photoreceptors are located in the outer plexiform layer (OPL), where they make synapses with the dendrites of bipolar and horizontal cells. The following layer is the inner nuclear layer (INL) that contains the nuclei of bipolar, horizontal and amacrine cells. The axons of bipolar and amacrine cells are localized in the inner plexiform layer (IPL) and contact with the dendrites of retinal ganglion cells (RGCs). The cell bodies of RGCs and displaced amacrine cells constitute the ganglion cell layer (GCL). The axons of RGCs converge to form the nerve fiber layer (NFL) and constitute the optic nerve, responsible to transmit the impulse to the brain (Litzinger and Del Rio-Tsonis, 2002).

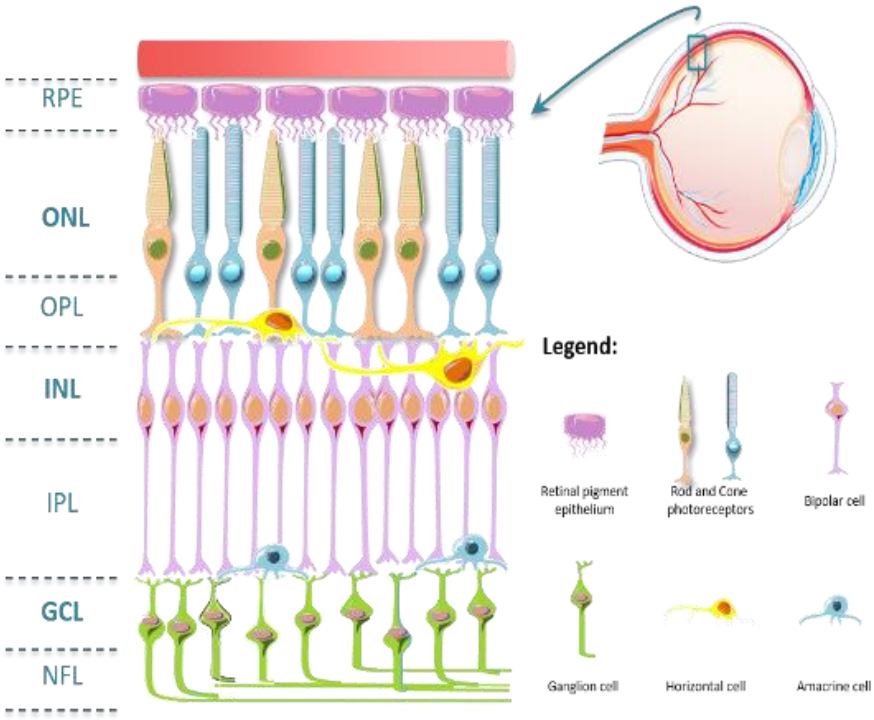

Figure 2: Structural layers of the retina. RPE - retinal pigment epithelium, ONL - outer nuclear layer, OPL - outer plexiform layer, INL - inner plexiform layer, IPL - inner plexiform layer, GCL - ganglion cell layer, NFL - nerve fiber layer. The axons of RGCs constitute the optic nerve and lead the information to the brain (Modified from (Madeira et al., 2015a).

1.3.1 Neurons

There are five types of neurons in the retina: photoreceptors (rods and cones), horizontal cells, bipolar cells, amacrine cells and RGCs.

1.3.1.1 Photoreceptors

Rods and cones are the two types of photoreceptors present in the mammalian retinas. Both types have an outer segment containing the visual pigment, an inner segment containing mitochondria and ribosomes, a cell body containing the nucleus and a synaptic terminal where neurotransmission occurs with second-order neurons (Kolb et al., 1995). The absorption of light by the photopigment initiates a cascade of events that changes the membrane potential, modulating the release of glutamate at the photoreceptor terminals. The process of converting photons into electrochemical signals is called phototransduction (Purves et al., 2004). Rods are extremely sensitive to light being responsible for dark vision that is characterized by lower spatial resolution. On the other hand, cones are responsible for light vision providing chromatic and detail vision (Litzinger and Del Rio-Tsonis, 2002).

1.3.1.2 Bipolar cells

Bipolar cells are the second order neurons that constitute the vertical visual pathway. These cells receive the synaptic input from photoreceptors and transmit them to the RGCs and to the amacrine cells. The human retina possesses ten different types of cone bipolar cells and only one type of rod bipolar cells. Multiple pathways can be activated whether ON- or OFF-center bipolar cells are stimulated. The differences on the response of ON- or OFF-center bipolar cells to light are explained by the fact that they express different types of glutamate receptors. Off-center bipolar cells express ionotropic receptors and ON-center bipolar cells express metabotropic glutamate receptors (Purves et al., 2004).

1.3.1.3 Retinal ganglion cells

The RGCs collect the visual information through their dendrites from the amacrine and bipolar cells and transmit it to the brain by their axons that constitute the optic nerve. RGCs can also be subdivided into two subgroups: ON- and OFF-center, depending on whether the information is passed by an ON- or OFF-center bipolar cell, respectively (Purves et al., 2004). Furthermore, these cells are classified in different types based on the morphology of their dendritic arborization and size of cell bodies and dendrites. The human retina is composed by 18 different morphological types of ganglion cells (Kolb et al., 1995).

1.3.1.4 Horizontal cells

Horizontal cells mediate the lateral interactions between photoreceptor terminals and the dendrites of bipolar cells. Indeed, there are two types of horizontal cells that modulate the neurotransmitter release from cones and rods into bipolar cells by different mechanisms of feedback to maintain the sensitivity of the visual system to the luminance contrast over a wide range of light intensities (Perlman et al., 1995, Purves et al., 2004, Wassle, 2004).

1.3.1.5 Amacrine cells

Amacrine cells modulate the interactions between bipolar cells and RGCs. Human retinas have around 40 different types of amacrine cells that can be distinguished by their dendritic size, stratification and neurotransmission release. Most amacrine cells in the mammalian retinas are inhibitory, releasing gama-aminobutyric acid (GABA) or glycine (Kolb et al., 1995).

1.3.2 Blood vessels

The retina is a highly differentiated tissue with an elevated rate of oxygen consumption. In the retina, the blood vessels allow to maintain the supply of oxygen and nutrients and the ionic gradients necessary for light transduction and electrical activity (Pournaras et al., 2008). The retina is supplied by two sources of blood: the central retinal artery and the choroidal blood vessels (Kolb et al., 1995). The central retinal artery is responsible for nourishment of the inner retina while choroid blood vessels provide blood supply to photoreceptors (Pournaras et al., 2008).

The blood-ocular system is formed by two barriers: the blood-aqueous barrier (BAB) and blood-retinal barrier (BRB). The first one is important to maintain the aqueous humor conditions, the second one is essential to regulate the content of inner fluids and preserving the internal ocular tissues from variations that constantly occur in the whole circulation. The BRB is divided into two components, the inner blood-retinal barrier (iBRB) and the outer blood-retinal barrier (oBRB) (Tomi and Hosoya, 2010, Cunha-Vaz et al., 2011) (Figure 3). The iBRB is composed by the endothelial cells, which provide structural support to the vasculature, and by pericytes that regulate retinal vascular flow. Besides the vascular cells,

the iBRB is also constituted by glial cells, which sheathe the blood vessels, providing signals that modulate their function. The oBRB is composed by the RPE and the Brunch´s membrane (Pournaras et al., 2008).

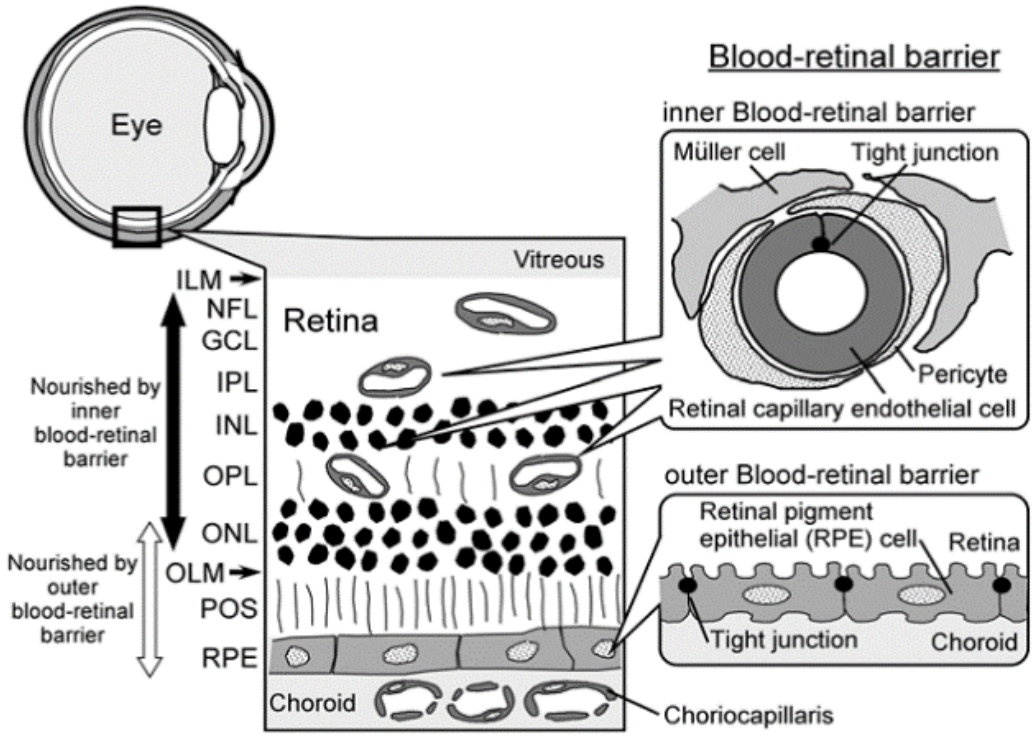

Figure 3: Schematic diagram of the blood-retinal barrier (BRB). Retinal pigment epithelium (RPE); photoreceptor outer segments (POS); outer limiting "membrane" (OLM); outer nuclear layer (ONL); outer plexiform layer (OPL); inner nuclear layer (INL); inner plexiform layer (IPL);ganglion cell layer (GCL);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

1.3.3 Glial cells

1.3.3.1 Astrocytes

Astrocytes seem to be originated from the optic nerve head (ONH) and subsequently migrate to the NFL and GCL. Besides their critical role in the development of retinal vasculature and iBRB maintenance, astrocytes have additional homeostatic functions, including water, ions and glutamate buffering of the neurons (Phatnani and Maniatis, 2015). In response to an insult, astrocytes suffer a process named astrogliosis which consists in morphological changes, proliferation and increase of glial fibrillary acidic protein (GFAP) expression (Hasko et al., 2005).

1.3.3.2. Müller cells

Müller cells are the most abundant glial cell in the retina, being distributed radially across the entire neural retina, and constituting an anatomical and functional connection between retinal neurons and retinal blood vessels (Kolb et al., 1995). Müller cells are involved in the development and maintenance of the BRB. Furthermore, these cells have key functions in maintaining ion and water homeostasis, and they are critically involved in glucose metabolism and neurotransmitter recycling. After an injury, Müller cells become more reactive which leads to proliferation and culminates in a gliotic scar with detrimental effects for the function and structure of retinal neurons (Bringmann et al., 2006, Vohra et al., 2013).

1.3.3.3. Microglial cells

Microglial cells are the resident immune cells of the CNS and constitute the primary response in a defense network. It is well established that these cells are approximately 10 to 15% of all glial cells that constitutes the brain (Lull and Block, 2010). Microglial cells derive from monocytic lineages and invade the brain and the retina during early development, being essential for neuronal homeostasis and innate immune defense.

Under resting conditions, microglial cells present a ramified morphology, surveying the surrounding microenvironment through the projection and retraction of their processes. When a pathological event occurs, such as infection, trauma, ischemia or the development of a neurodegenerative disease, resting microglial cells acquire an activated phenotype, characterized by amoeboid morphology (Langmann, 2007, Kettenmann et al., 2011). Indeed, microglial cells are able to demonstrate a broad range of phenotypes that do not transit only between a ramified/resting or amoeboid/activated phenotype (Karlstetter et al., 2015). In fact, a recent study demonstrated that microglia activation and deactivation occurs through six bi-directional stages (Ellis-Behnke et al., 2013). Microglial cells activation occurs through interaction with immune receptors such as Toll-like receptors (TLRs), scavenger receptors and cytokine and chemokine receptors (Kierdorf and Prinz, 2013, Karlstetter et al., 2015). The interactions between microglia and other retinal cells are essential to control microglia reactivity. In healthy conditions, cells release some microglial regulators, namely, CD200 and CX3CL1 that maintain microglia in a resting state

(Langmann 2007). After a pathological event, some mediators are released from injured cells that activate TLRs, cytokine and chemokine receptors, leading to microglia activation (Langmann, 2007, Kettenmann et al., 2011, Karlstetter et al., 2015, Wang et al., 2015).

Microglial cells are essential for the maintenance of the CNS integrity. They are responsible for the phagocytosis of cellular debris that result from normal cell death. They also provide trophic support to neurons by the release of nerve growth factors and neurotrophins. Besides these functions, microglial cells are also able to release inflammatory mediators (Lull and Block, 2010, Polazzi and Monti, 2010).

During a pathological event, injured cells release several mediators, triggering microglia activation. Once activated, microglial cells migrate to the site of injury and release pro-inflammatory mediators such as tumor necrosis factor (TNF), nitric oxide (NO), reactive oxygen species (ROS), and interleukin (IL)-1β. Moreover, microglia also release chemokines that attract other immune cells towards the injury. An excessive or prolonged microglial activation in the CNS may lead to chronic inflammation, which results in irreversible neuronal loss (Karlstetter et al., 2010).

Although it is very clear that microglia play an essential role protecting the CNS, the overactivation of these cells can lead to an exacerbated and harmful response that is already known to be responsible for the progression of several neurodegenerative diseases, such Alzheimer's and Parkinson's diseases (Madeira et al., 2015a).

1.3.3.4. Retinal microglial cells

Retinal microglial cells are located NFL, GCL, as well within IPL and above INL (Bosco et al., 2011).

Microglial cells response may be protective or deleterious depending on their immunological phenotype and the local milieu. In their resting state, microglia surveil the environment with their long protrusions (Figure 4A). Noxious stimuli lead to cell degeneration triggering microglial cells activation (figure 4B), which migrate toward the lesion site and acquire an amoeboid morphology (Figure 4C) (Karlstetter et al., 2010). Several reports show that deleterious and exacerbated microglial cells responses are implicated in the onset and development of several retinal diseases such as age-related macular degeneration (AMD), diabetic retinopathy and glaucoma (Madeira et al., 2015a).

Moreover, some evidences show that microglia activation occurs early in the development of the diseases, as has been demonstrated in glaucoma experimental models (Bosco et al., 2011, Bosco et al., 2015), indicating the important role of microglial cells for the identification of therapeutic strategies for the treatment of retinal diseases.

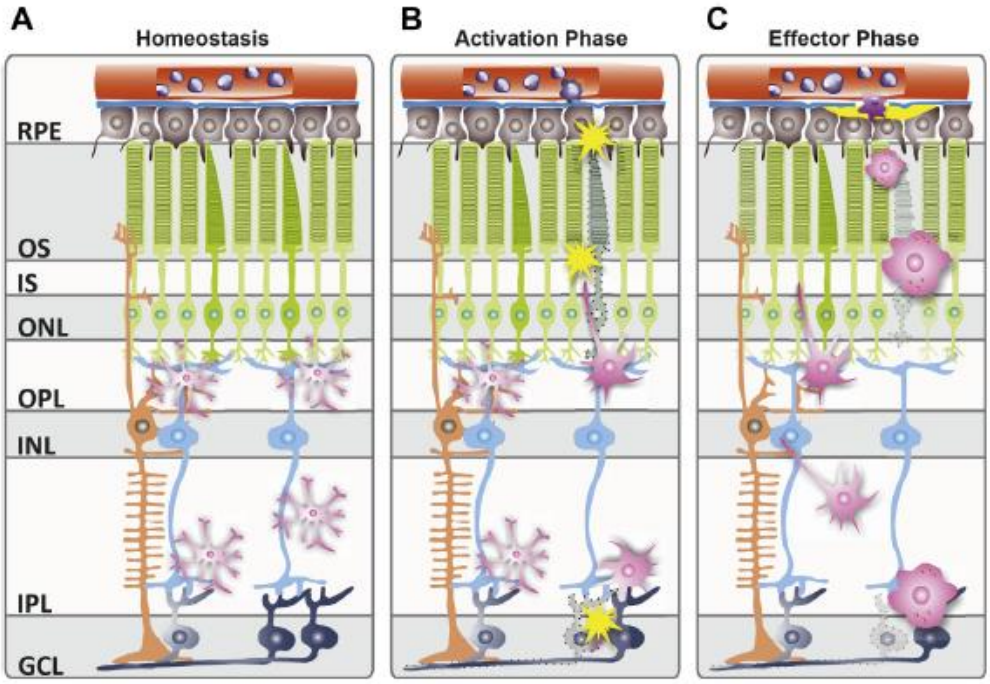

Figure 4: Schematic representation of microglial cells in homeostasis and lesion conditions: A) In resting conditions, microglial cells are mainly located in plexiform layers, scanning the environment with their long protrusions, B) Various noxious stimulus lead to cell degeneration and abnormal function, triggering microglial cells, C) Microglial cells migrate toward the lesion site, acquiring an amoeboid morphology with substantial release of pro-inflammatory mediators (Karlstetter et al., 2010).

1.4 Adenosine

Adenosine is an ubiquitous purine nucleoside that plays a critical role in the maintenance of cellular energetic homeostasis, presenting also an important role in the CNS, acting as a neuromodulator that is able to regulate the neuronal excitability, synaptic plasticity and release of various neurotransmitters including glutamate, GABA, acetylcholine and dopamine (Cunha, 2001, Wei et al., 2011). Adenosine is mainly produced by the adenosine tri-phosphate (ATP) catabolism, being involved in key pathways of primary metabolism, namely, nucleotide and nucleoside metabolism, amino acid metabolism, trans-methylation reaction and handling of ammonia (Cunha, 2005). Due to its modulatory effect, some reports have shown that adenosine is able to prevent or

decrease neuronal damage under different noxious conditions, such as hypoxia/ischemia, excitotoxicity, chemotoxicity or trauma.

Although in physiological conditions, the extracellular adenosine levels are around 30-300 nM, following an insult adenosine levels rise to 10 µM or higher (Schulte and Fredholm, 2003). Increased levels of adenosine has been implicated in the development and/or progression of some retinal diseases such as glaucoma, diabetic retinopathy and ischemic events (Dos Santos-Rodrigues et al., 2015).

1.4.1 Adenosine metabolism

The source of adenosine in the extracellular space can occur through two different mechanisms: direct release of adenosine through adenosine transporters or by the conversion of adenine nucleotides by ectonucleotidases. These proteins are expressed in several cell types, including microglia (Hasko et al., 2005).

There are four types of ectonucleotidases: (i) ectonucleoside triphosphate diphosphohydrolases (E-NTPDases), (ii) ectonucleotide pyrophosphatase/ phosphodiesterase (E-NNP), (iii) ecto-5'nucleotidase (CD73) and (iv) alkaline phosphatase (AP) (Idzko et al., 2014).

(i) Ectonucleoside Triphosphate Diphosphohydrolases (E-NTPDases)

E-NTPases can be recognized in the literature under different names, including ecto-ATPase, apyrase, ATPDase, among others. These ectoenzymes are able to hydrolyze nucleoside tri or/and diphosphates into adenosine monophosphate (AMP). It is recognized the existence of different NTPDases in the E-NTPDase family (NTPDase 1 to 8), being NTPDase 1, corresponding to the lymphoid cell activation antigen CD39, expressed in cells of the immune system, with strong expression in microglial cells (Yegutkin, 2008).

(ii) Ectonucleotide Pyrophosphatase/Phosphodiesterases (E-NNP)

E-NNP family consists in seven structurally related ectoenzymes (E-NNP 1-7), which can dephosphorylate ATP into AMP. The differences between NNP and NTPDase family are poorly understood (Yegutkin, 2008).

(iii) Ecto-5'nucleotidase (CD73)

CD73 is an ectonucleotidase with a broad distribution in the mammalian brain, including synapses, sprouting nerve fibers and microglial cells, being anchored to the plasma membrane by glycosyl-phosphatidylinositol (GPI). Its key role is the conversion of AMP into adenosine. Evidences showed that CD73 expression is upregulated in hypoxia in intestinal epithelium, leading to an increase of extracellular adenosine (Zimmermann, 2006, Yegutkin, 2008).

(iv) Alkaline Phosphatases (AP)

AP are enzymes also anchored to the plasma membrane by GPI, which degrade tri-, di- and monophosphates in adenosine (Zimmermann, 2006, Yegutkin, 2008).

Adenosine can also be generated intracellularly through ATP breakdown by cytosol-5'-nucleotidase that converts AMP into adenosine. Another source of intracellular adenosine is the hydrolysis of S-adenosylhomocysteine (SAH) by SAH hydrolase (Latini and Pedata, 2001).

Adenosine intra- and extracellular levels can be regulated not only by the hydrolysis of ATP, adenosine di-phosphate (ADP) or AMP but also by the nucleoside transporters (Figure 5). Indeed, adenosine can by transported to inside or outside of the cell via equilibrative nucleoside transporters (ENTs) (i), which follow the concentration gradient, or through concentrative transporters (CNTs) (ii), that mediate the influx of adenosine using the sodium gradient (Yegutkin, 2008, Melani et al., 2012).

(i) Equilibrative Nucleoside Transporters (ENTs)

There are four isoforms of ENTs: ENT1, ENT2, ENT3 and ENT4. ENT1 is widely expressed, mainly found at the plasma membrane, and is considered the main regulator of homeostatic maintenance of adenosine levels. In mice, it was

described two isoforms known as mENT1a and mENT2b (Dos Santos-Rodrigues et al., 2015).

(ii) Concentrative Nucleoside Transporters (CNTs)

CNTs are classified into three isoforms, known as CNT1, CNT2 and CNT3. CNT1 is a pyrimidine-preferring transporter and does not transport adenosine; CNT2 transports purines although it can also transport uridine and CNT3 has a broad of substrates being able to transport both purine and pyrimidine nucleosides (Dos Santos-Rodrigues et al., 2015).

Adenosine can be removed from intra- and extracellular environment, being metabolized into AMP by adenosine kinase (ADK) or deaminated into inosine by adenosine deaminase (ADA). Although it has a cytosolic localization, ADA can also be expressed on the surface of the cell often associated with CD26/dipeptidyl peptidase IV (DPPIV).

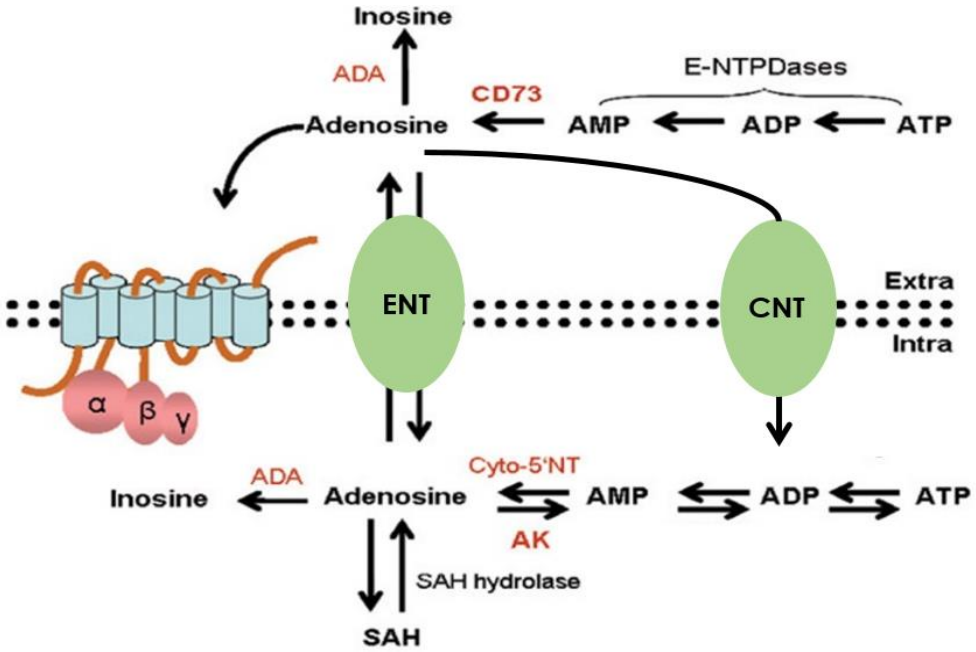

Figure 5: Adenosine metabolism: mechanisms underlying adenosine formation and transport (Modified from (Ham and Evans, 2012).

1.4.2 Adenosine receptors

Adenosine can modulate a variety of physiological responses including inflammation by activating adenosine receptors (ARs): A_1, A_{2A}, A_{2B} and A_3. All ARs are

pleiotropic receptors, i.e., receptors with potential to couple different G-proteins. Although A_1 and A_3 receptors preferentially couple to G_i proteins inhibiting adenylate cyclase and, consequently, the production of cyclic adenosine 5'-monophosphate (cAMP), A_{2A} and A_{2B} receptors stimulate cAMP production by coupling to G_s proteins (Figure 6). ARs are expressed in different layers of the retina (Zhong et al., 2013).

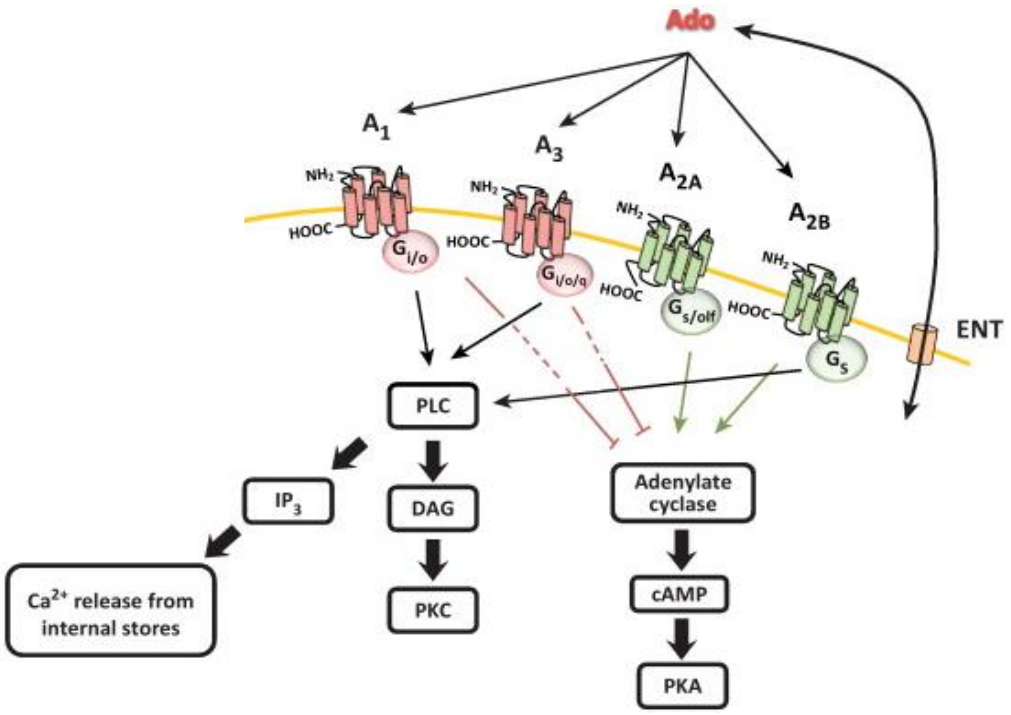

Figure 6: Schematic representation of the downstream signaling pathways of adenosine receptors (Adapted from (Dias et al., 2013)).

1.4.2.1 A_1 receptors

A_1R is linked to G_i-proteins that inhibits the adenylate cyclase (AC), leading to a diminished production of cAMP (Jacobson and Gao, 2006). It is also well established that A_1R activation is linked to various kinase pathways including protein kinase C (PKC), phosphoinositide 3 (PI3) kinase and mitogen-activated protein (MAP) kinases. Additionally, A_1R activation can activate K^+ channels and inhibit specific Ca^{2+} channels (Hasko et al., 2008). Moreover, A_1R play an essential role mainly in the control of neuronal excitability by the regulation of the neurotransmitters release, namely, glutamate, acetylcholine and serotonin (Cunha, 2005).

A protective role for adenosine in epilepsy and in trauma- and ischemia-associated brain injury has been proposed to occur mainly by A_1R activation (Abbracchio and Cattabeni, 1999). Indeed, A_1R activation diminishes the extent of brain damage, by the control of calcium influx, glutamate release and membrane potential. Furthermore, it has been shown that the activation of A_1Rs reduces intraocular pressure (IOP) (Zhong et al., 2013).

However, the therapeutic interest of A_1R agonists has several limitations, hampering their usefulness as therapeutic drugs. Beyond the poor brain permeability, A_1R agonists have a short "window of opportunity", which is limited to a few hours, and the chronic administration of these agonists causes a reverse effect, i.e., chronic A_1R activation exacerbates neuronal loss (Cunha, 2005, Gomes et al., 2011).

A_1Rs are widespread in the brain but their localization is not homogeneous. Indeed, A_1Rs are considerably more abundant in neurons, but they are also present in astrocytes, microglia and oligodendrocytes (Cunha, 2005). In the retina, A_1Rs are mainly localized in the IPL and GCL (Hartwick et al., 2004).

1.4.2.2 A_{2A} receptors

$A_{2A}Rs$ are facilitatory receptors that act through G_s-mediated activation of AC pathway, leading to increased levels of cAMP. Moreover, the increased levels of cAMP lead to the activation of PKA that can phosphorylate the transcript factor cAMP responsive element binding protein (CREB), promoting its activation. Activated CREB can mediate gene expression indirectly through others transcript factors or directly by the interaction with gene promoters (Hasko et al., 2008).

$A_{2A}Rs$ are highly concentrated in the striatum, being their density about 20 times greater than elsewhere in the brain. In striatum, these receptors are located in post-synaptic neurons. Moreover, $A_{2A}Rs$ are also expressed in cortex and hippocampus, being predominantly located in pre-synaptic zones. The $A_{2A}R$ can also be located in glial cells (astrocytes and microglia) and in blood vessels (Cunha, 2005). In the retina $A_{2A}R$ expression is detected in GCL, INL and ONL (Kvanta et al., 1997, Zhong et al., 2013), being expressed also in retinal microglial cells (Liou et al., 2008).

Several evidences indicate that the density of $A_{2A}R$ strongly increases in patients and animal models of several neurodegenerative diseases, such Parkinson's, Alzheimer's

and Huntington's disease (Calon et al., 2004, Tarditi et al., 2006, Boison, 2008). Moreover, several studies clearly demonstrate that noxious stimuli lead to an increase of microglia reactivity, which also up-regulate $A_{2A}R$ (Cunha, 2005, Liou et al., 2008, Vindeirinho et al., 2013).

1.4.2.3 A_{2B} receptors

A_{2B} receptors are coupled to G_s proteins and its stimulation triggers AC activation. This leads to an increase in cAMP production that activates PKA and, consequently, the phosphorylation and activation of CREB. Moreover, $A_{2B}R$ have also the ability to activate the PLC pathway through Gq-coupled proteins (Hasko et al., 2008).

A_{2B} receptors are mainly localized in astrocytes in neurons and there is no evidence that this receptor is present in microglia (Cunha, 2005).

$A_{2B}R$ has a lower affinity for adenosine than A_1, A_{2A} and A_3, which means that the activation of this receptor occurs more frequently in pathological conditions, where the levels of adenosine are increased (Jacobson and Gao, 2006).

1.4.2.4 A_3 receptors

The A_3R activation encompasses G_i-mediated inhibition of AC, decreasing the production of cAMP. Additionally, A_3R can activate the PLC pathway through G_q-coupled proteins (Zhang et al., 2006, Hasko et al., 2008).

A_3R can be found in neurons but their function seems more evident in astrocytes and eventually in microglia (Cunha, 2005). In the retina, A_3R are mainly localized in RGCs (Zhang et al., 2006, Zhong et al., 2013).

The role of A_3R in several diseases is often controversial, depending on acute and chronic agonist administration. It has been hypothesized that the initial A_3R activation plays a protective role in ischemia. However, the prolonged A_3R stimulation leads to deleterious effects, as excitotoxicity (Borea et al., 2015). Furthermore, it has been reported an up-regulation of this receptor in hippocampus of transgenic mouse of Alzheimer's disease, whereas no change has been observed in the brains of Parkinson's patients (Borea et al., 2015), supporting the idea that the expression pattern of A_3R is not consistent in different neurodegenerative diseases. Despite its controversial neuroprotective role, studies have

been reported that A_3R activation play a protective role in RGCs (Hu et al., 2010, Zhang et al., 2010).

1.4.3 Neuroinflammation mediated by A2AR

It has been demonstrated that the modulation of ARs may have a strongly impact in the development and progression of several disorders. Much attention has been paid to the $A_{2A}R$, since its modulation plays a preponderant role in the control of inflammation. It is well documented that $A_{2A}R$ activation is beneficial in peripheral inflammation, namely, sepsis and pulmonary disorders, whereas in CNS is the blockade of $A_{2A}R$ that exerts protective effects (Jacobson and Gao, 2006). Several studies reported that $A_{2A}R$ blockade confers neuroprotection in various neurodegenerative diseases, as Parkinson's and Alzheimer's diseases (Santiago et al., 2014). $A_{2A}R$ blockade is able to control microglia reactivity, controlling the toxic milieu that surrounds the damaged cells (Gomes et al., 2013, Santiago et al., 2014). In an initial phase (acute inflammation), the release of inflammatory mediators has a beneficial effect, however, when the response of microglia become dysregulated leads to noxious conditions (chronic inflammation), leading to a neural loss (Karlstetter et al., 2010).

1.5 Glaucoma

Glaucoma is the second leading cause of irreversible blindness worldwide and the prevalence of this disease has been increasing during the last decades. In fact, the current number of people with glaucoma worldwide is estimated to be 64.3 million and this number is expected to increase to 76.0 and 111.8 million in 2020 and 2040, respectively (Tham et al., 2014). Glaucoma is a multifactorial disease mainly characterized by gradual loss of RGCs and optic nerve damage (RGCs axons) (Casson et al., 2012). IOP is one of the major risk factors for the development of this disease and the current therapeutic approach is focused on lowering IOP by pharmacological drugs, surgically or with laser treatment (Cohen and Pasquale, 2014). However, some patients still progress to vision loss despite the effective control of IOP, which clearly demonstrates IOP-independent mechanisms are involved in the pathogenesis of this disease (Zhong et al., 2013), and an urgent need to develop new therapeutic strategies to manage glaucoma (Cordeiro and Levin, 2011).

1.5.1 Glaucoma and neuroinflammation

Neuroinflammation plays a preponderant role in the progression of glaucoma. In the retinas and aqueous humor of patients with glaucoma and animal models it has been reported increased levels of inflammatory mediators, namely, TNF (Tezel and Wax, 2000, Yuan and Neufeld, 2000, Tezel et al., 2004, Balaiya et al., 2011), interleukin (IL)-6 (Chen et al., 1999, Johnson et al., 2011, Chidlow et al., 2012, Sims et al., 2012), IL-9, IL-10 and IL-12 (Chua et al., 2012). Moreover, TNF levels also increase in co-cultures of RGCs that interact with glial cells exposed to elevated pressure, triggering RGCs death (Tezel and Wax, 2000). Studies using an antibody that neutralizes TNF (Tezel and Wax, 2000) or an inhibitor of TNF (Etanercept) (Roh et al., 2012) showed decreased RGC apoptosis, revealing the critical role of this cytokine in glaucoma. Additionally, inducible nitric oxide synthase (iNOS) and NO have been also found in ONH of glaucomatous patients (Neufeld et al., 1997) and in the retinas and ONH of glaucoma animal models (Cho et al., 2011).

Several studies have been studying the role of microglial cells in this disease. In fact, alterations in microglia morphology, proliferation, cell adhesion and immune response, corresponding to a reactive phenotype have been reported in experimental glaucoma and in glaucoma patients (reviewed in (Madeira et al., 2015a)) (Figure 7). Evidences indicate an early and exacerbated microglia activation in several glaucoma animal models (Bosco et al., 2011, Bosco et al., 2015) and in glaucomatous human eyes (Neufeld, 1999a). In fact, the reduction of microglia reactivity leads to a decrease of RGCs loss and a consequent improve of the functional and structural integrity of the RGCs axons. Indeed, a study of our group demonstrated that the blockade of $A_{2A}R$ prevents RGCs loss elicited by elevated hydrostatic pressure (EHP) in rat organotypic cultures by the control of microglia reactivity (Madeira et al., 2015b). Moreover, minocycline reduces the reactivity of retinal microglial cells and improved the integrity of the optic nerve in the DBA/2J mouse model of glaucoma (Wang et al., 2015).

Since microglia and inflammatory mediators have a preponderant role in the pathogenesis of glaucoma, the control of microglia reactivity may be an effective approach for the neuroprotection of RGCs.

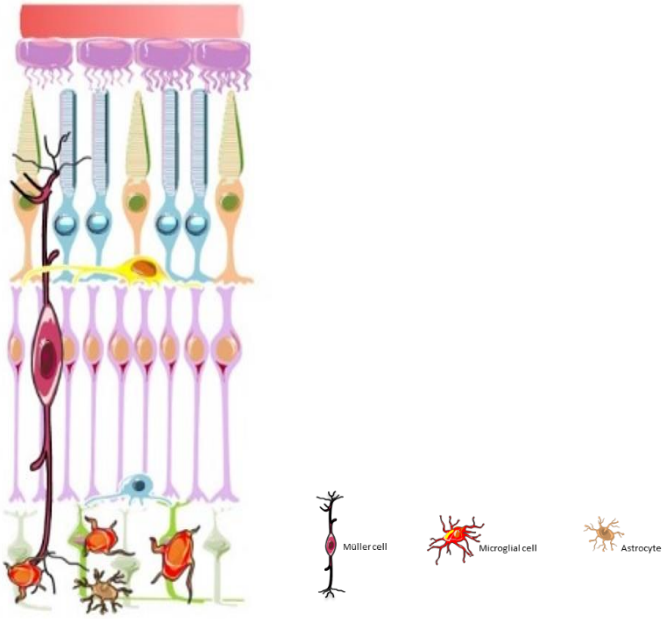

Figure 7: Microglial cells activated in ganglion cell layer. (Modified from (Madeira et al., 2015a)

1.6 Elevated hydrostatic pressure model

The mechanisms behind RGCs death in glaucoma remain unclear. Since IOP is the main risk factor of this disease, the majority of animal models developed are based on ocular hypertension. Moreover, several i*n vitro* models have been developed to assess the cell response after exposure to elevated hydrostatic pressure (EHP) (Agar et al., 2000, Agar et al., 2006, Sappington et al., 2006, Ju et al., 2009). Several studies have been using cell cultures exposed to 30-100 mmHg above atmospheric pressure during different time points in a range (Agar et al., 2000, Agar et al., 2006, Ju et al., 2009), mimicking chronic or acute ocular hypertension. A previous study demonstrated that 70 mmHg of EHP triggers loss of RGCs in a relative short time (24-48 hours) (Sappington et al., 2006). In addition, EHP increases the extracellular levels of ATP in bovine eyecup preparations (Reigada et al., 2008) and impairs glutamate metabolism leading to axonal swelling (Ishikawa et al., 2010). Furthermore, EHP induces mitochondria impairment with an increase in cytochrome C release, mediating apoptotic cell death (Ju et al., 2009), and it can also trigger TNF production by glial cells contributing to RGC death (Tezel and Wax, 2000).

AIMS

It is well established that neuroinflammation contributes for the pathophysiology of glaucoma, the second leading cause of blindness worldwide.

Adenosine is a neuromodulator in the CNS acting through the activation of four adenosine receptors. Under noxious conditions, the extracellular levels of adenosine increase and the expression of adenosine receptors alters. In addition, the blockade of $A_{2A}R$ has been shown to confer protection against neurodegeneration possibly by controlling neuroinflammation.

Taking this into account, the aim of this work was to evaluate whether EHP, which mimics ocular hypertension, (increased IOP is the main risk factor) affects the adenosinergic system in microglial cells, by assessing adenosine transporters, enzymes, and receptors. In addition, we also aimed to investigate whether the blockade of $A_{2A}R$ was able to prevent the inflammatory response triggered by EHP.

In order to accomplish our aims, we used a microglial cell line (BV-2 cells) to dissect the impact of elevated pressure in microglial cells, and mice retinal organotypic cultures to assess the effect of pressure in a more complex system. The effect of the $A_{2A}R$ was evaluated pharmacologically in microglial cells using a selective antagonist (SCH 58261) or by genetic inactivation ($A_{2A}R$-KO mice) in retinal organotypic cultures.

CHAPTER 2

Materials and methods

2. MATERIALS AND METHODS

2.1 Animals

Wild type and A_{2A}R-KO C57BL/6 mice were housed under controlled conditions (21.8±0.1°C of temperature and 67.6±1.6% of relative humidity, 12h light/ dark cycle), with free access to food and water. All procedures involving animals were approved by the Ethical Committee of the Faculty of Medicine of the University of Coimbra and are in agreement with the Association for Research in Vision and Ophthalmology statement for animal use.

2.2 Mice genotyping

Mice were genotyped through DNA extraction from tail biopsy. Briefly, tails were digested during 4 h at 55°C with lysis buffer (5 mM EDTA, 200 mM NaCl, 10 mM Tris, 0.3% sodium dodecyl sulfate (SDS), pH 8.0) and 20 mg/ml proteinase K (Izasa). The solution was centrifuged at 13400 x g for 15 min at 4°C. Ice-cold 100% ethanol was added to the supernatant. Then, the samples were centrifuged at 1500 x g for 15 min at 4°C and the supernatant was discarded. The pellet was washed with ice-cold 75% ethanol and centrifuged at 1500 x g for 15 min at 4°C. The supernatant was discarded and the pellet was left to dry overnight. Then, the pellet was dissolved in Tris-EDTA (TE) buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) at 70°C during 10 min. The DNA concentration and purity was determined with NanoDrop ND-1000. Samples were amplified using a mix of DNA polymerase (Invitrogen Life Technologies), dNTP's (Nzytech) and specific design primers (Invitrogen, Life technologies) (Table 1).

Table 1: Primers for mice genotyping.

Gene	Primer sequence
A_{2A}R	**F:** AGCCAGGGGTTACATCTGTG
	R: TACAGACAGCCTCGACATGTG
NEO	**F:** TCGGCCATTGAACAAGATGG
	R: GAGCAAGGTGAGATGACAGG

The PCR consisted of 94°C for 4 min, then 35 cycles with denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 50 sec and a final cycle at 72°C for 7 min (Figure 8).

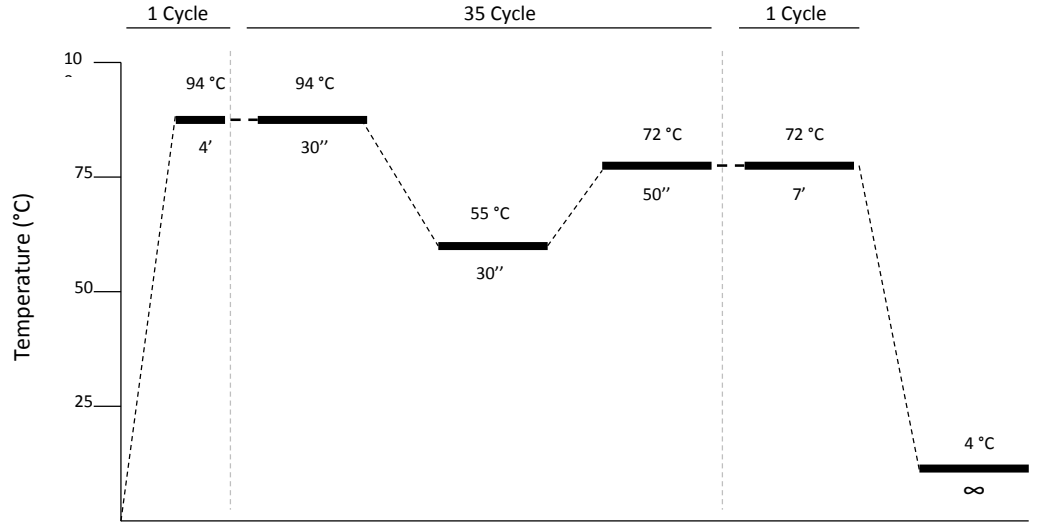

Figure 8: Schematic representation of the PCR cycles profile.

The samples were kept at 4°C until use. Then, the PCR products were separated by electrophoresis in 2% agarose gel with 5% ethidium bromide during 40 min at 120 V using a standard ladder (NZYDNA ladder V, Nzytech) as a DNA molecular weight marker. The results were observed in a translluminator (VersaDoc, Bio-Rad) (Figure 9).

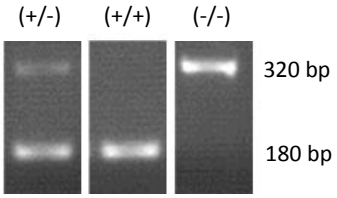

Figure 9: Representative PCR results from tail biopsy. The genotype of each sample is written above each lane, +/- genotype correspond to a heterozygote mice with $A_{2A}R$ present in one allele and absent on the other; +/+ genotype correspond to a homozygote mice with $A_{2A}R$ in both alleles and -/- genotype correspond to a homozygote mice without $A_{2A}R$ in both alleles, i.e., with a cassette of NEO in both alleles

2.3 Retinal organotypic cultures

Retinal organotypic cultures were prepared from retinas of wild type and $A_{2A}R$-knockout C57BL/6 mice. Briefly, mice were sacrificed by cervical dislocation, and after eye enucleation the retinas were dissected in a Ca^{2+} and Mg^{2+} free Hank's balanced salt solution

(HBSS, in mM: 137 NaCl, 5.4 KCl, 0.45 KH_2PO_4, 0.34 Na_2HPO_4, 10 HEPES, 5 glucose, 4 $NaHCO_3$, 1 $C_3H_3NaO_3$; pH 7.4). Retinas were cultured in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% of fetal bovine serum (FBS) and 0.1% of gentamicin for 4 days in culture inserts (12 mm, hydrophilic PTFE, 0.4 µm pore diameter, Milipore) at 37°C in a humidified atmosphere of 5% CO_2 (Figure 10A). Organotypic cultures were submitted to EHP (70 mmHg above atmospheric pressure) for 24 h, 48 h, 72 h or maintained in a standard incubator (at atmospheric pressure) (Figure 10B). Medium was changed at day 2 in culture.

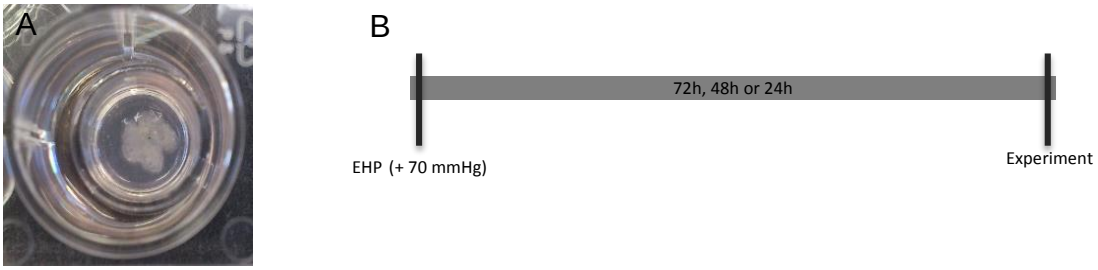

Figure 10: **(A)** Image from a retinal mice organotypic culture **(B)** Schematic representation of the experimental protocol in organotypic cultures

2.4 BV-2 cell line

Immortalized murine microglia cell line was cultured in 75 cm^2 flasks and maintained at 37°C in a humidified atmosphere of 5% CO_2, in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% of fetal bovine serum (FBS) and 1% of antibiotics (penincilin 100 U/ml, streptomycin 100 µg/ml). Culture medium was replaced every 2 days.

For experiments, BV-2 cells were maintained in RPMI supplemented with 2% FBS and 1% of antibiotics (penincilin 100 U/ml, streptomycin 100 µg/ml) and plated at a density of $6x10^4$ cells/cm^2 in 6-well-plates or at a density of $4x10^5$ cells/cm^2 in 12-well-plates.

2.5 Cell treatment

Cell cultures were incubated with $A_{2A}R$ selective antagonist (50 nM, SCH 58261) 45 min before the insult. The cells were submitted to elevated hydrostatic pressure (EHP) for 4 h or 24 h or incubated with lipopolysaccharide (LPS, 100 ng/mL), a classical inducer of

inflammation (Rebola et al., 2011) that triggers microglia activation (Santiago et al., 2014), for 4 h (Figure 11).

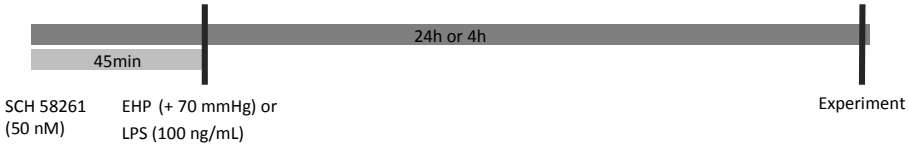

Figure 11: Schematic representation of the BV-2 cell line experimental procedure

The conditions for EHP were described previously (Sappington et al., 2006). Briefly, the pressure chamber has a water reservoir to keep humidity and is equipped with a regulator and a manometer and placed in a 37°C incubator (Figure 12). The EHP was raised to 70 mmHg above ambient pressure by filling the chamber with a mixture of 95% air and 5% CO_2. The CO_2 levels helped to maintain the pH between 7.3-7.4.

Control cell cultures were kept in a standard incubator at atmospheric pressure.

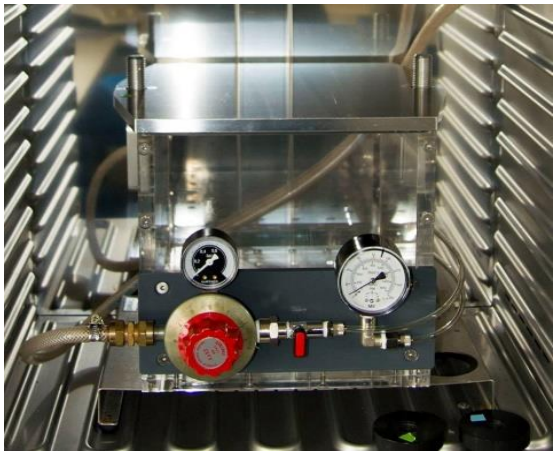

Figure 12: Pressure chamber used to expose cell cultures to elevated hydrostatic pressure (70 mmHg above atmospheric pressure)

2.6 Western Blot

Cells were washed twice with ice-cold phosphate-buffered saline (PBS, in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 1.8 KH_2PO_4, pH 7.4) at 4°C and whole cell extracts were lysed in RIPA buffer (50 mM Tris HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100; 0.5% DOC; 0.1% SDS; 1 mM DTT) supplemented with complete mini protease inhibitor cocktail tablets and phosphatase inhibitors (10 mM NaF and 1 mM Na_3VO_4), at 4°C. Then, the lysates were sonicated and centrifuged at 16100 x g for 10 min at 4°C. The supernatant was collected and stored at -80°C until protein quantification. The protein concentration was determined by bicinchoninic acid (BCA) method, following the instructions of the

manufacturer (Pierce Biotechnology). Samples were denatured by adding 6x concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M DTT, 0.012% bromophenol blue) and heating for 5 min at 95°C. To detect $A_{2A}R$, samples were denatured by adding 2x concentrated urea sample buffer (50 mM Tris-HCl, 1.6% SDS, 7% glycerol, 8 M urea, 4% β-mercaptoethanol, 0.016% bromophenol blue) and heating for 5min at 95°C. For each protein of interest, different amounts of protein (20-80 μg) were tested to select the amount of protein to use in the subsequent experiments. Then, equal amounts of protein (30 or 60 μg) (Table 2) were separated by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Then, proteins were transferred electrophoretically to polyvinyl difluoride (PVDF) membranes (Millipore). The membranes were blocked for 1h at room temperature in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) and 5% low-fat milk. Afterwards, the membranes were incubated overnight at 4°C and 1h at room temperature (the day after) with TBS-T supplement with 1% low-fat dry milk containing primary antibody against respective protein (Table 2). Membranes were washed 3 times in TBS-T and then incubated during 1h at room temperature with the respective secondary antibody (Table 2) prepared in TBS-T with 1% low-fat milk. The membranes were rinsing three times during 15 min in TBS-T and, then protein immunoreactive bands were visualized using Enhanced Chemi-Fluorescence system (ECF; GE Healthcare) on a Storm device (Molecular Dynamics, GE Healthcare) or with Enhance Chemiluminescence system (ECL; Bio-Rad) on a Versadoc (Table 2). Digital quantification of bands intensity was performed using Quantity One software (Bio-Rad). The membranes were re-incubated for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to control for similar amounts of protein loaded in the gels.

Table 2: List of antibodies used in western blot

Primary antibody	Denaturation buffer	Dilution	Protein (µg)	Molecular weight (kDa)	Supplier (cat no)	Secondary antibody	Dilution	Substrate
Anti-A_1	Sample buffer 6x	1:500	30	37	PA1-041A	Anti-rabbit	1:10000	ECF
Anti-A_3	Sample Buffer 6x	1:100	60	44/52/66	sc-13938	Anti-rabbit	1:10000	ECF
Anti-$A_{2A}R$	Urea Buffer 2x	1:200	30	45	sc-32261	Anti-mouse	1:10000	ECF
Anti-ADA	Sample Buffer 6x	1:100	60	41	sc-25747	Anti-rabbit	1:10000	ECF
Anti-ADK	Sample buffer 6x	1:100	60	48/38	sc-23360	Anti-goat	1:5000	ECL
Anti-CNT2	Sample buffer 6x	1:100	60	72	sc-134528	Anti-rabbit	1:10000	ECF
Anti-GAPDH	---	1:5000	---	37	Ab9485	Anti-rabbit	1:10000	ECF

2.7 ADA enzymatic assay

Cells were washed twice with ice-cold PBS and cells were extracted in 50 mM Tris HCl supplemented with complete mini protease inhibitor cocktail tablets, pH 7.2. To remove cell debris, the samples were centrifuged at 3000 x g for 10 min a 4°C. The supernatant was collected and kept at -20°C until use. ADA enzymatic activity was measured following the instructions provided by the manufacturer (Diazyme). Enzymatic ADA activity assay is based on the enzymatic deamination of adenosine to inosine which is converted to hypoxanthine by purine nucleoside phosphorylase (PNP). Hypoxanthine is then converted to uric acid and hydrogen peroxide (H_2O_2) by xanthine oxidase (XOD). Hydrogen peroxide further reacts with N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline (EHSPT) and 4-aminoantipyrine (4-AA) in the presence of peroxidase (POD) to generate quinone dye (Figure 13).

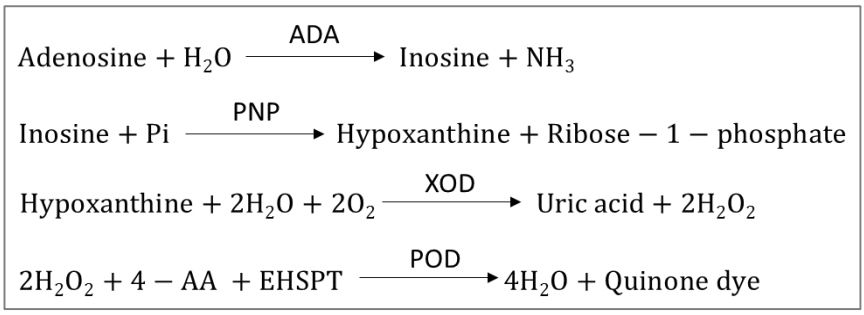

Figure 13: Scheme of the reactions that occur for the deamination of ADA enzymatic activity.

Briefly, 5 µL of sample were added to 180 µL of Reagent solution 1 (50 mM Tris HCl, pH=8; 0.2 mM 4-AA; 0.1 U/mL PNP; 0.2 U/mL XOD; 0.6 U/mL Peroxidase and stabilizers). After 3 min, 90 µL of Reagent solution 2 (50 mM Tris HCl, pH=4.0; 10 mM adenosine; 2 mM EHSPT) were added to the mixture. The amount of quinone dye formed was determined by spectrophotometry (550/620 nm), during 3 min. All the procedure was performed at ±37°C. The ADA activity was determined using a calibration curve obtain by successively dilutions of calibrator included in the kit (ADA= 48 U/L) and divided for 3 min in order to obtained ADA activity = value U/L/min.

2.8 Quantification of pro-inflammatory cytokines

IL-1β and TNF levels were measured by enzyme-linked immunosorbent assay (ELISA), following the instructions provided by the manufacturer (R&D System). Briefly, culture medium was collected and kept at -80°C until further analysis. Before using, the lysates were centrifuged at 16100 x g for 10 min at 4°C. Then, both standard and samples were placed in a 96-well plate, pre coated with monoclonal antibody specific for IL-1β or TNF, during 2h at room temperature. The plate was washed 5 times and incubated with IL-1β or TNF conjugate, respectively. After 2h, the plate was washed and incubated with substrate solution during 30 min, protected from light. After that, stop solution was added and the optical density was measured at 450 nm with correction reading for 540 nm or 570 nm, using a microplate reader.

2.9 Immunocytochemistry

2.9.1 Cell cultures

The BV-2 cells were washed twice with ice-cold PBS and fixed using 4% of paraformaldehyde (PFA) with 4% sucrose for 10 min at room temperature. After fixation, the cells were washed again with PBS and permeabilized during 5 min with 1% Triton X-100 in PBS. Next, the cells were blocked with 3% of bovine serum albumin (BSA) and 0.2% of Tween-20 in PBS for 1h to prevent the non-specific bindings. Then, cells were incubated with primary antibody diluted in the block solution for 90 min (Table 3). After washing with blocking solution, the cells were incubated with secondary antibody in the same solution (Table 3) for 1h at room temperature in the dark. Then, cells were rinsed three times with PBS. Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) (1:2000) and F-actin was stained with phalloidin conjugated to Tetramethylrhodamine B isothiocyanate, (1:500). Upon rising three times with PBS, the coverslips were mounted in glycergel mounting medium and observed with a fluorescence microscope (Axio observer. Z1), using a LD Plan-Neofluar 40x/ 0.6 Korr Ph2 M27 objective.

Table 3: List of primary and secondary antibodies

Primary antibody	Dilution	Source (cat no)	Secondary antibody	Dilution
IL-1β	1:50	AF-501-NA	Rabbit anti-goat 488	1:200
iNOS	1:1000	SC-650	Goat anti-rabbit 488	1:200
$A_{2A}R$	1:50	SC-7504	Rabbit anti-goat 488	1:200
CD73	1:50	SC-14684	Rabbit anti-goat 488	1:200

Quantitative analysis of total cells fluorescence was performed from images of BV-2 cells immunostained to IL-1β, iNOS and CD73, using ImageJ software (http://rsb.info.nih.gov/ij/). Cell immunoreactivity was calculated using the correct total cell fluorescence (CTCF) formula, as previously described (Gavet and Pines, 2010):

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

Representative images were acquired with a confocal microscope (LSM 710, Zeiss) on an Axio Observer Z1 microscope using Plan-Apochromat 63x/ 1.40 Oil Dic M27 objective.

2.9.2 Organotypic retinal cultures

Organotypic cultures were washed twice with PBS and fixed in 100% ice-cold ethanol during 10 min at 4°C. After washing with PBS, the cultures were blocked with 0.1% of Triton X-100, 3% of BSA and 10% of goat serum in PBS for 1h at room temperature and then were incubated for 72 h at 4°C with goat anti-Brn3a (santa cruz). After extensive washing, the samples were incubated with donkey anti-goat 568 overnight at 4°C in the dark and then washed again and incubated with DAPI (1:1000) during 15 min at room temperature. Upon rising with PBS, the organotypic cultures were mounted in Glycergel mounting medium and observed with confocal microscope (LSM 710, Zeiss) on an Axio Observer Z1 microscope using Plan ApoChromat 20x/0.8 objective.

Representative images were acquired also with a confocal microscope (LSM 710, Zeiss), using Plan-Apochromat 63x/ 1.40 Oil Dic M27 objective.

2.10 Real-time PCR

2.10.1 RNA extraction

Cells were washed with ice-cold autoclaved PBS and then lysed using TRIZOL reagent (Life Technologies). The cells were disrupted using a p1000 pipet tip and kept at -80°C overnight. Samples were defrosted and chloroform was added and the tubes were gently shaken to homogenize the mixture and kept 5 min in ice. Then, the samples were centrifuged at 12000 x g for 15 min at 4°C to assure phase distribution. Upper aqueous phase was collected to a new tube and RNA was precipitated with isopropanol. Glycogen (20 μg) (Invitrogen, Life Technologies) was added as carrier, to facilitate precipitation. Samples were kept overnight at -20°C and centrifuged at 12000 x g for 30 min at 4°C. The pellets were washed twice with ethanol, and RNA was resuspended in RNase-free water. The purity and concentration of RNA samples were determined using NanoDrop. Then, the RNA samples were treated with Deoxyribonuclease I (DNase I, Life technologies) to eliminate possible DNA contamination.

2.10.2 Reverse transcription

RNA samples were reversed transcribed to cDNA using NZY M-MuLV First-Strand cDNA synthesis kit according to manufacturer's instructions (NZYtech). Briefly, RNA was incubated for 10 min at 25°C with NZYRT 2x Master Mix and NZYRT Enzyme Mix, followed by a 30 min step at 50°C and a final step at 85°C to stop the reaction. To eliminate remaining RNA-DNA hybrids, the obtained cDNA was treated with RNAse H at 37°C during 20 min. Then, to confirm the absence of genomic DNA a standard endpoint PCR for β-actin using, intron spanning primers (Table 4) was performed and 2x MyTaq Red Mix (Bioline). The cDNA (1 µL) was incubated with 2x MyTaq Red Mix under the following conditions: 1 min initial denaturation at 95°C, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, elongation at 72°C during 10 seconds. The cDNA resulting samples were separated by electrophoresis in a 1.5% agarose gel with 5% ethidium bromide during 35 min at 150 V. The results were observed in a translluminator (VersaDoc, Bio-rad) and the samples were diluted 1:2 and kept at -20°C until qPCR analysis.

Table 4: List of primers sequences used in PCR

Target gene	Primers sequences	Melting temperature
ywhaz	F: CAGCAAGCATACCAAGAAG	60°C
	R: TCGTAATAGAACACAGAGAAGT	
gadph	F: CGACTTCAACAGCAACTC	60°C
	R: TGTAGCCGTATTCATTGTCATA	
hprt	F: TCCATTCCTATGACTGTA	55°C
	R: CATCTCCACCAATAACTT	
inos	F: CCACCATCAAGGACTCAA	60°C
	R: GGCAACCTGACCACTCTC	
Ada	F: AATCAGAAGACCGTGGTGGC	60°C
	R: TTTACTGCGCCCTCATAGGC	
adora2A	F: TCCTGCTAATACACTACTCTC	56°C
	R: TCCTCACATTGTTATCTTCTTG	
β-actin	F: GCTCCTCCTGAGCGCAAG	60°C
	R: CATCTGCTGGAAGGTGGACA	

2.10.3 Real-time quantitative PCR

The BV-2 cells mRNA expression was quantified by qPCR in a 7500 Real-time PCR System (Appied Byosystems). The qRT-PCR was performed using iTaqTMSYBR® Green Supermix (Bio-Rad) using pre-optimized and custom-designed primers (Sigma-Aldrich). Samples were loaded in duplicates into a 96-well plate and tested according to the following protocol (Figure 14):

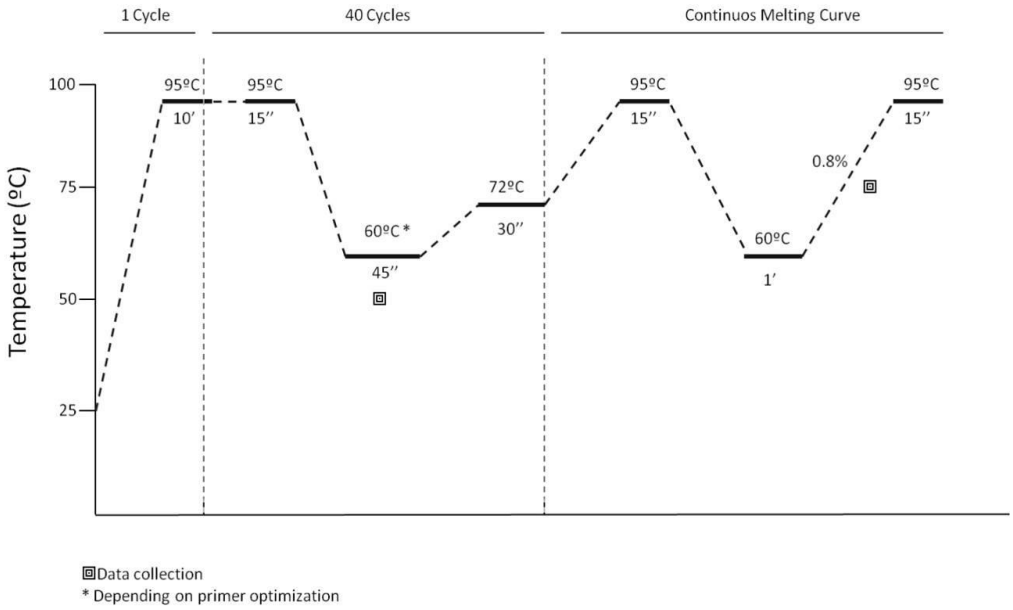

Figure 14: Schematic representation of the qPCR temperature cycles profile.

Three housekeeping candidate genes were tested (YWAHAZ, HPRT and GAPDH) and the most stable gene among all samples and conditions was used as housekeeping gene. All samples were analyzed using NormFinder Software (Andersen et al., 2004). In our conditions, YWHAZ was the most stable gene. Relative quantification of mRNA expression was calculated by the comparative cycle threshold (Ct) method after the target genes levels were normalized to the expression of YWHAZ for each sample. The fold difference in gene expression between treated groups was calculated as follows:

Fold difference: $2^{-\triangle\triangle Ct}$

Where $\triangle\triangle Ct$:

$\triangle\triangle Ct = (Ct\ target - Ct\ YWHAZ)treated\ sample - (Ct\ target - Ct\ YWHAZ)control\ sample$

2.11 Statistical analyses

Results are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism Version 6 (GraphPad Software). Data was analyzed using the non-parametric Kruskall-Wallis test, followed by Dunn's multiple comparison test or to non-parametric Mann-Whitney test. Differences were considered significant for $p<0.05$.

CHAPTER 3

Results

3. RESULTS

Since IOP is considered one of the main risk factors of glaucoma, we used an *in vitro* model of elevated pressure to mimic ocular hypertension. Previous results obtained by our group showed that both ATP and adenosine levels increase in BV-2 microglial cells exposed to EHP for 4 h and 24 h. Additionally, BV-2 microglial cells become reactive when subjected to EHP (data not published).

In order to better understand the effects of EHP in the adenosinergic system of microglial cells, we aimed to investigate whether EHP alters the transporters, enzymes and receptors of the adenosinergic system. Furthermore, we also aimed to investigate whether the modulation of $A_{2A}R$ controls EHP-induced alterations in microglial cells.

3.1 Elevated hydrostatic pressure significantly increases the CNT2 protein levels in BV-2 microglial cells

Since our previous results showed increased levels of adenosine in the culture media of BV-2 cells challenged with EHP, we first evaluated the effect of EHP in the levels of the adenosine transmembrane transporters ENT and CNT. BV-2 cells were challenged with EHP for 4 h and 24 h and CNT2 protein levels were evaluated by Western blot (Figure 15).

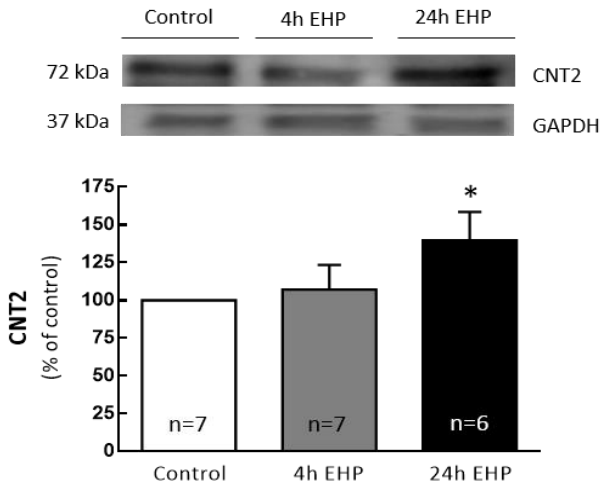

Figure 15: Elevated hydrostatic pressure increased the protein levels of CNT2 in BV-2 microglial cells. BV-2 cells were exposed to EHP for 4 h or 24 h. The protein levels of CNT2 were analyzed by Western blot in total cellular extracts. Representative images for CNT2 and GAPDH (loading control) were presented above the graph. Results were expressed as percentage of control, from the number of experiments indicated within each graph bar. *p<0.05, different from control, Kruskal-Wallis test, followed by Dunn's multiple comparison test.

When cells were exposed to EHP for 4 h, the CNT2 protein levels were not significantly different from the control (107.2±16.1% of the control). However, a significant increase of the CNT2 protein levels were found when microglia were exposed to EHP for 24 h (139.6±18.9% of the control, $p<0.05$). Despite the different attempts, detection of ENT1 and ENT2 by Western blot was not obtained.

3.2 Elevated hydrostatic pressure increases the CD73 immunoreactivity in microglial cells

ATP can be converted to ADP or AMP and adenosine by CD39 and CD73 (Zimmermann, 2000, Melani et al., 2012), so we analyzed the effect of EHP in the levels of CD73.

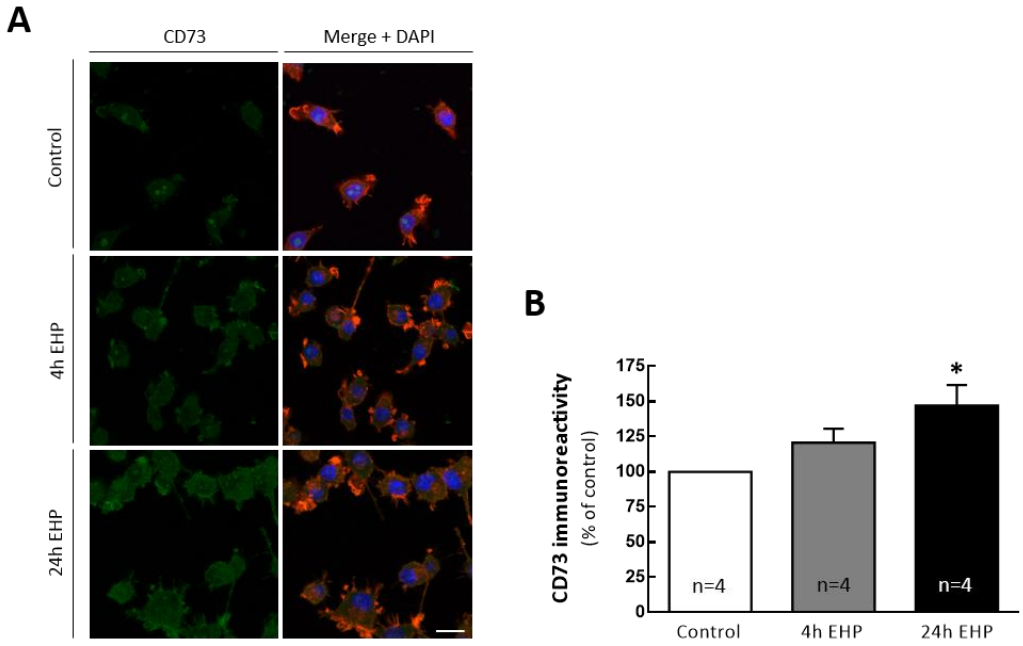

Figure 16: Elevated hydrostatic pressure increases CD73 immunoreactivity in microglial cells. CD73 immunoreactivity was evaluated in BV-2 cells after exposure to EHP for 4 h or 24 h. **(A)** Immunocytochemistry was performed using anti-CD73 (green) antibody. Phalloidin (red) was used to stain F-actin, allowing observation of cell limits. Nuclei were counterstained with DAPI (blue). Scale bar: 20 µm. **(B)** CD73-immunoreactivity was quantified using ImageJ in 10 random fields. Results were expressed as percentage of control, from the number of experiments indicated within each graph bar. *$p<0.05$, different from control, Kruskal-Wallis test, followed by Dunn's multiple comparison test.

BV-2 cells were exposed to EHP for 4 h and 24 h and processed for immunocytochemistry. Cells were labeled with an antibody that specifically recognizes

CD73 (green), and phalloidin (red) staining to identify cell limits (Figure 16A). We observed that the exposure of BV-2 cells to EHP for 4 h increased CD73 immunoreactivity (Figure 16B), as compared with the control (120.7±9.9% of control). Further exposure to 24 h significantly increased CD73-immunoreactivity (137.1±14.3% of the control; $p<0.05$).

3.3 Effect of elevated hydrostatic pressure in ADA mRNA levels, protein levels and enzymatic activity

We also evaluated the effects of EHP in ADA mRNA, protein levels and activity, the enzyme responsible for the conversion of adenosine into inosine.

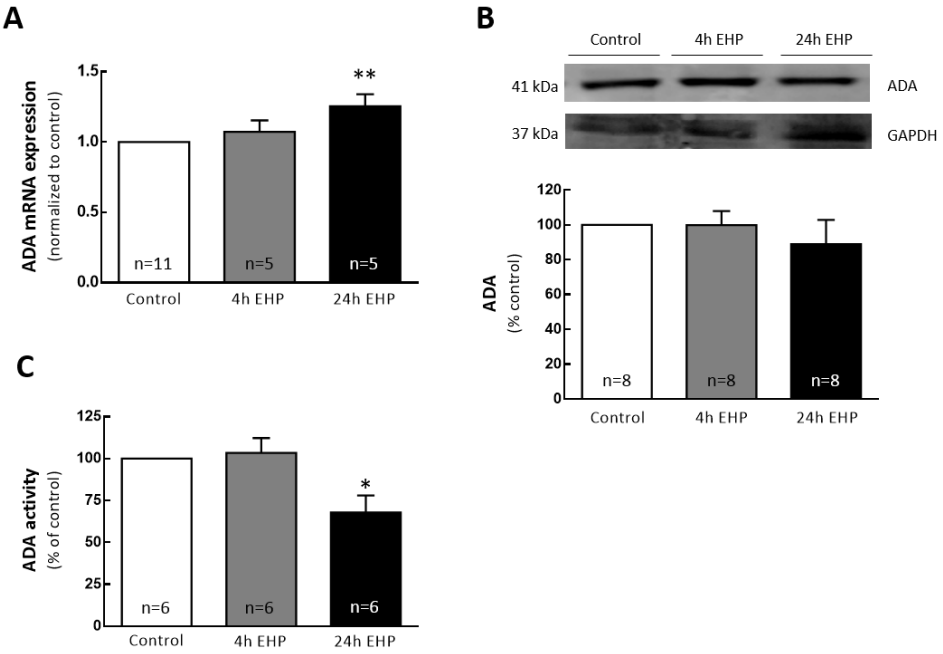

Figure 17: Elevated hydrostatic pressure decreases ADA enzymatic activity, increases the ADA mRNA levels but not ADA protein levels. BV-2 microglial cells were challenged with EHP for 4 h or 24 h. **(A)** ADA mRNA levels were quantified by qPCR and the results were expressed as fold change of control, from the number of experiments indicated within each graph bar. **(B)** The protein levels of ADA were analyzed by Western blot in total cellular extracts. Representative images for ADA and GAPDH (loading control) were presented above the graph. Results were expressed as percentage of control, from the number of experiments indicated within each graph bar. **(C)** ADA enzymatic activity was quantified in cellular extracts and the results were presented as percentage of control, from the number of experiments indicated within each graph bar. *$p<0.05$, **$p<0.01$, different from control, Kruskal-Wallis test, followed by Dunn's multiple comparison test.

We first analyzed ADA mRNA expression by qPCR (Figure 17A). The exposure of BV-2 cells for 4 h to EHP did not alters the mRNA levels of ADA (1.1±0.1 fold-change) when

compared with control conditions. However, further exposure for 24 h significantly increased ADA expression when compared with control (1.3±0.1 fold-change; $p<0.01$).

The protein levels of ADA were evaluated by Western blot (Figure 17B). No alterations were observed when BV-2 cells were exposed to EHP for 4 or 24 h when comparing with the control (99.9±7.99% and 88.94±14.0% of control, respectively).

The enzymatic activity of ADA (Figure 17C) was assessed in cellular extracts upon exposure to EHP for 4 h and 24 h. We found that ADA activity was not altered when cells were exposed to EHP for 4 h (103.4±8.8% of control). Nevertheless, 24 h of exposure to EHP decreased the enzymatic activity of ADA (67.9±10.2% of control; $p<0.05$).

3.4 Effect of elevated hydrostatic pressure in the ADK protein levels in BV-2 cells

We have also analyzed the levels of ADK, the enzyme responsible for the conversion of adenosine in AMP.

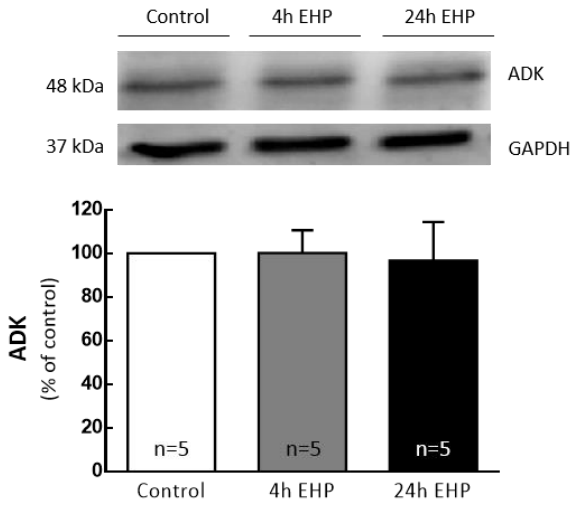

Figure 18: Effect of elevated hydrostatic pressure in the ADK protein levels in BV-2 microglial cells. BV-2 cells were exposed to EHP for 4 h or 24 h. ADK protein levels were evaluated in total cellular extracts, by Western blot. Representative images for ADA and GAPDH (loading control) were presented above the graph. Results were expressed as percentage of control, from the number of experiments indicated within each graph bar.

BV-2 cells were exposed to EHP for 4 h or 24 h and ADK protein levels were evaluated by Western blot (Figure 18). No significant alterations in the protein levels of ADK were found after EHP for 4 h and 24 h (100.1±5.3% or 96.7±8.9% of control, respectively).

3.5 Effect of elevated hydrostatic pressure in protein levels of A_1, A_{2A} and A_3 adenosine receptors in BV-2 microglial cells

The effect of EHP on the levels of adenosine receptors (A_1, A_{2A}, A_{2B} and A_3) was also studied.

BV-2 cells were challenged with EHP for 4 h and 24 h and the protein levels of A_1R (Figure 19A), $A_{2A}R$ (Figure 19B) and A_3R (Figure 19C) were evaluated by Western blot.

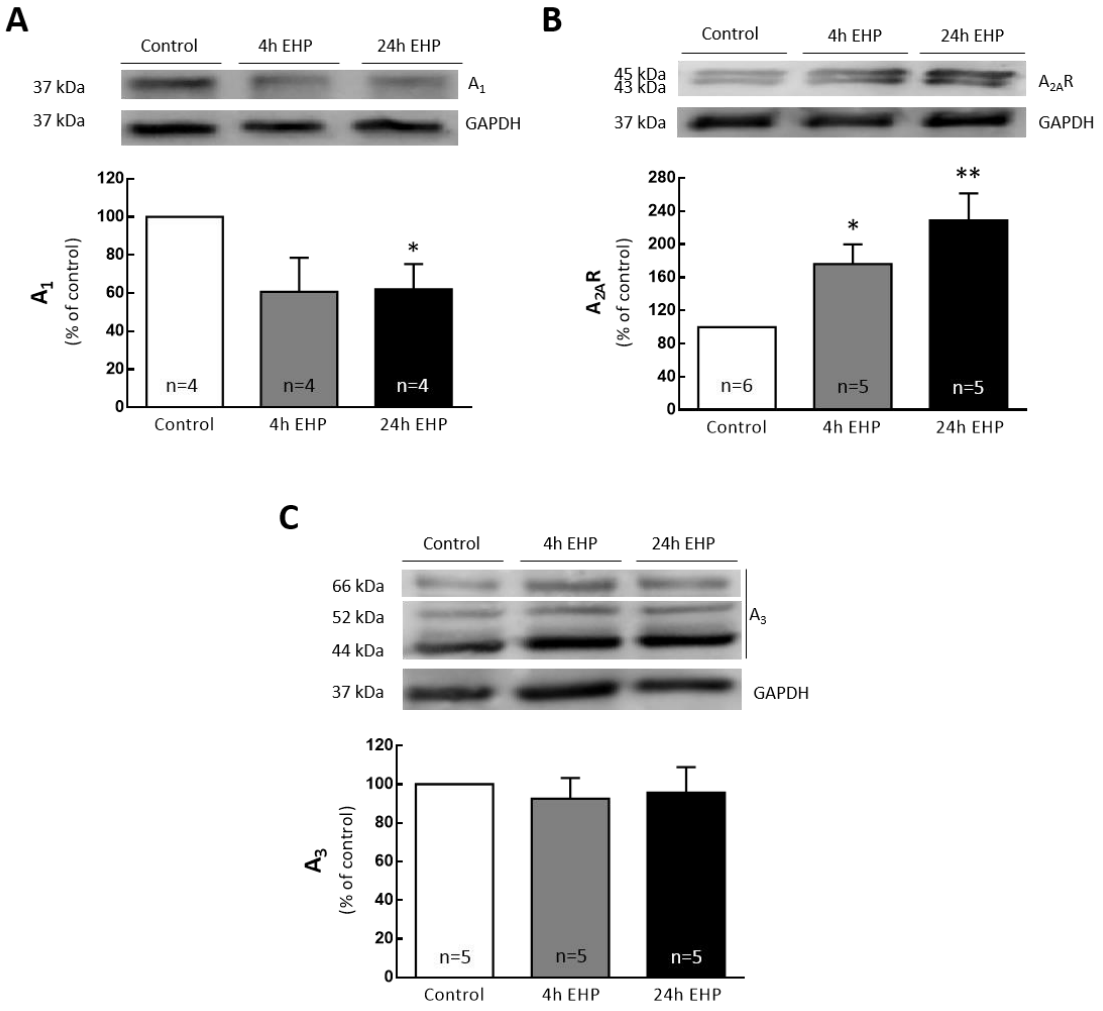

Figure 19: Effect of elevated hydrostatic pressure in protein levels of A_1, $A_{2A}R$ and A_3 adenosine receptors. BV-2 cells were exposed to EHP for 4 h or 24 h. A_1 **(A)**, $A_{2A}R$ **(B)** and A_3 **(B)** protein levels were evaluated in total cellular extracts by Western blot. Representative images for A_1, $A_{2A}R$ and A_3 and GAPDH (loading control) were presented above the graph. Results were expressed as percentage of control, from the number of experiments indicated within each graph bar. *p<0.05, **p<0.01 different from control, Kruskal-Wallis test, followed by Dunn's multiple comparison test.

A_1R protein levels decreased after exposure to 4 h of EHP (60.6±8.9% of control). When cells were exposed to 24 h of EHP, the A_1R density significantly decreased to

62.1±6.6% of control ($p<0.05$). $A_{2A}R$ density was significantly increased 175.97±24.0% of control ($p<0.05$) (4 h of EHP) and to 228.85±32.49% of control ($p<0.01$) (24 h of EHP). Nevertheless, after both 4 h and 24 h of EHP, the A_3R protein levels were not changed (92.54±4.78% or 95.7±5.86% of control, respectively).

3.6 Elevated hydrostatic pressure increases $A_{2A}R$ mRNA and immunoreactivity in microglial BV-2 cells

The effects of EHP on $A_{2A}R$ mRNA (Figure 20A) and immunoreactivity (Figure 20B) were also evaluated in BV-2 cells. We found that the exposure of BV-2 cells to EHP for 4 h and 24 h significantly increased mRNA $A_{2A}R$ expression (1.50±0.19 and 1.49±0.12 fold change, respectively; $p<0.01$), in agreement with the results obtained with Western blot (Figure 19B). Furthermore, by immunocytochemistry, we observed that $A_{2A}R$ (green) immunoreactivity increased upon exposure to EHP, in accordance with the results obtained by Western blotting and qPCR.

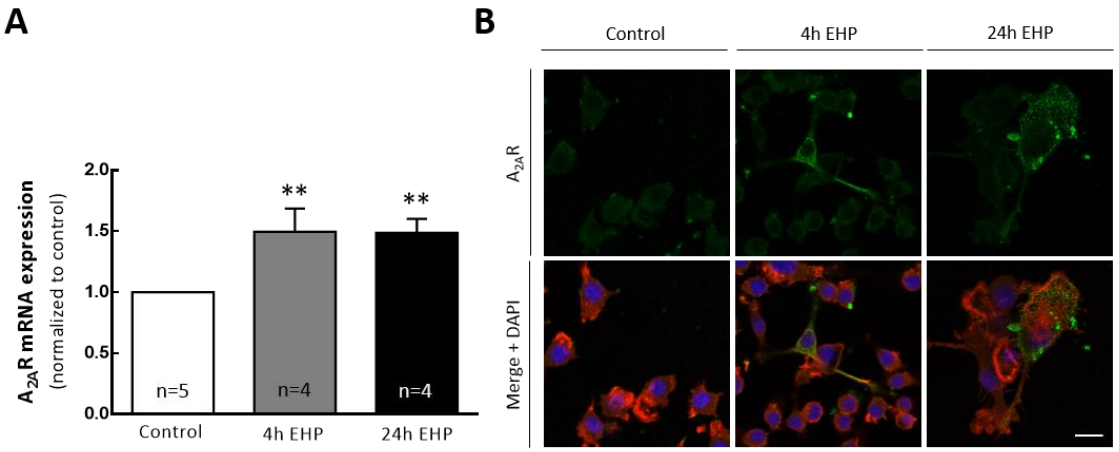

Figure 20: Elevated hydrostatic pressure increases mRNA levels, protein levels and immunoreactivity of the $A_{2A}R$ in microglial BV-2 cells. BV-2 cells were challenged with EHP for 4 h or 24 h. **(A)** $A_{2A}R$ mRNA levels were quantified by qPCR and the results were expressed as fold change of control, from the number of experiments indicated within each graph bar. **(B)** Cells were stained with an anti-$A_{2A}R$ (green) antibody. Phalloidin (red) was used to stain F-actin, allowing observation of cell limits. Nuclei were counterstained with DAPI (blue). Scale bar: 20 µm. **p<0.01, different from control, Kruskal-Wallis test, followed by Dunn's multiple comparison test.

3.7 Effect of the blockade of $A_{2A}R$ in iNOS mRNA levels and immunoreactivity in microglial cells after exposure to elevated hydrostatic pressure

Previous studies have demonstrated that blockade of $A_{2A}R$ prevents microglial reactivity and production of inflammatory mediators (Saura et al., 2005, Rebola et al., 2011, Gomes et al., 2013, Santiago et al., 2014). Since EHP increased the levels of $A_{2A}R$ in BV-2 microglial cells, we analyzed the effects of $A_{2A}R$ blockade in the inflammatory response induced by EHP.

BV-2 microglial cells were challenged with EHP for 4 h or 24 h in the presence or absence of a selective antagonist of $A_{2A}R$ antagonist (50 nM SCH 58261). iNOS mRNA (Figure 21A) and immunoreactivity (Figure 21B,C) were evaluated.

The iNOS mRNA levels were significantly increased to 1.71±0.29 over control ($p<0.01$) upon 4 h of EHP. The $A_{2A}R$ blockade did not change the EHP-induced iNOS mRNA increase (1.37±0.20 fold-change of the control; $p<0.05$). Exposure of BV-2 microglial cells to EHP for 24 h did not alter the iNOS transcript levels, both in the presence or absence of the $A_{2A}R$ antagonist (1.02±0.11 and 1.14±0.14 fold-change of the control, respectively).

The immunoreactivity of iNOS was assessed by immunocytochemistry (Figure 21C). BV-2 cells show similar iNOS-staining expression in the presence or absence of SCH 58261 upon exposure to EHP for 4 h, comparing with control. Moreover, iNOS staining is more intense when cells were challenged with EHP for 24 h. The blockade of $A_{2A}R$ reduced iNOS immunoreactivity, when comparing with EHP for 24 h.

The exposure of BV-2 cells to EHP for 4 h did not significantly alter iNOS immunoreactivity, in the presence or absence of SCH 58261 (97.55±6.16% and 92.63±8.83% of control, respectively). However, when the cells were challenged with EHP for 24 h, iNOS immunoreactivity was significantly increased when compared with control (154.85±12.48% of control; $p<0.01$). Furthermore, the blockade of $A_{2A}R$ seems to have a tendency to prevent the EHP-induced effects (113.85±5.35% of control).

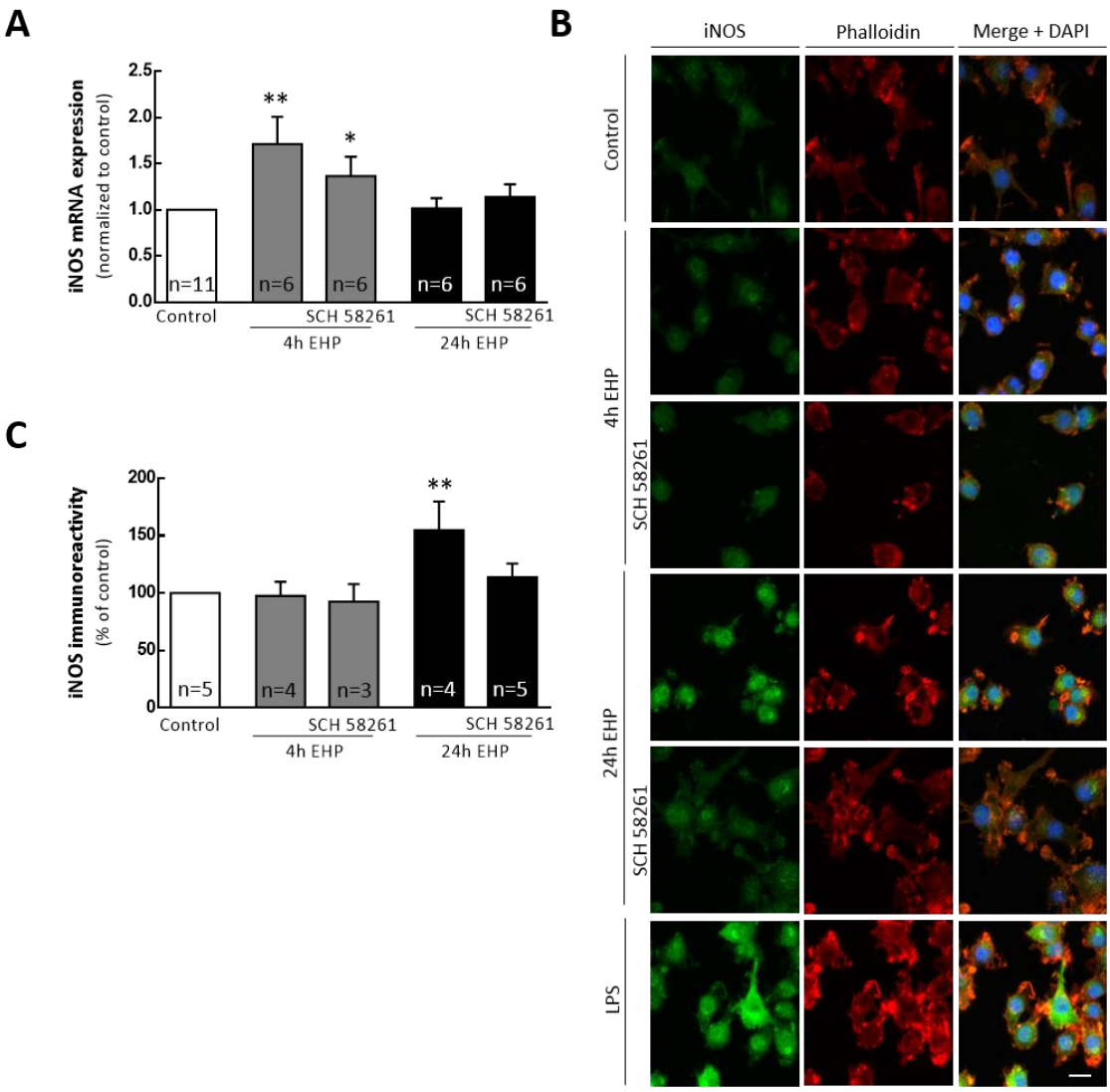

Figure 21: Effect of $A_{2A}R$ blockade in iNOS from BV-2 cells after exposure to elevated hydrostatic pressure. BV-2 microglial cells were pre-treated with 50 nM SCH 58261 followed by EHP exposure for 4 h and 24 h. **(A)** iNOS mRNA levels were assessed by qPCR and the results were expressed as fold change of control, from the number of experiments indicated within each graph bar. **(B)** iNOS-immunoreactivity was evaluated by immunocytochemistry using anti-iNOS (green) antibody. Phalloidin (red) was used to stain F-actin, allowing observation of cell limits. Nuclei were counterstained with DAPI (blue). Scale bar: 20 µm. **(C)** iNOS-immunoreactivity was quantified using ImageJ in 10 random fields from the number of experiments indicated within each graph bar, and the results are expressed as percentage of control; *p<0.05, **p<0.01, different from control, Kruskal-Wallis test, followed by Dunn's multiple comparison test.

3.8 Effect of the blockade of $A_{2A}R$ in IL-1β immunoreactivity in microglial BV-2 cells

IL-1β expression was assessed by immunocytochemistry, by labeling the cells with an antibody that specifically recognizes IL-1β (green). Phalloidin (red) staining was used to identify cell limits (Figure 22A). Cells show a similar staining for IL-1β (green) in the same condition. BV-2 cells with or without pre-treatment with SCH 58261, upon EHP for 4 h, show IL-1β staining similar with the control. Nevertheless, the exposure of EHP for 24 h increases IL-1β-staining in BV-2 cell. Moreover, BV-2 cells treated with SCH 58261 show an IL-1β-staining similar with cells exposed to EHP 24 h, demonstrating that the blockade of $A_{2A}R$ is not able to prevent these effect.

Analysis of immunoreactivity (Figure 22B) revealed that exposure to EHP for 4 h did not significantly alter the expression of IL-1β (114.45±2.89% of control), as well as blockade of $A_{2A}R$ (107.67±2.08% of control). Nevertheless, exposure to 24 h of EHP significantly

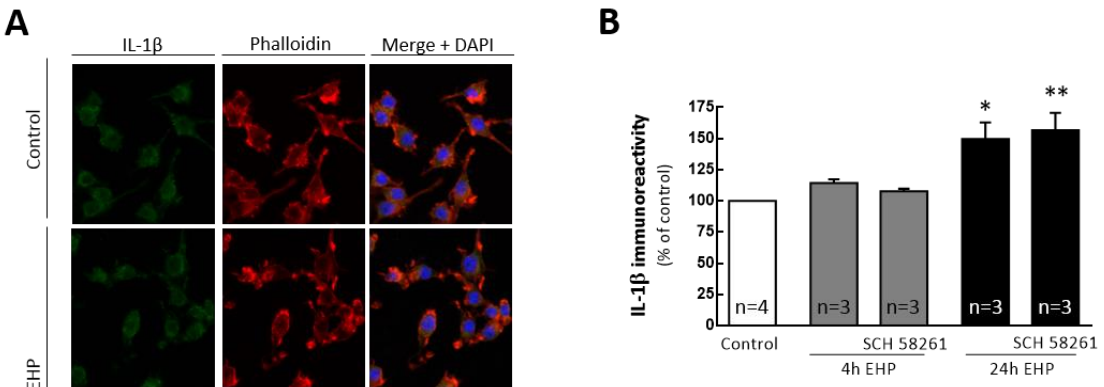

Figure 22: Effect of $A_{2A}R$ blockade in IL-1β immunoreactivity in microglial BV-2 cells. BV-2 cells were pre-treated with 50 nM SCH 58261 followed by EHP exposure for 4 h and 24 h. **(A)** IL-1β immunoreactivity was evaluated by immunocytochemistry using an anti-IL-1β antibody (green). Phalloidin (red) was used to stain F-actin, allowing observation of cell limits. Nuclei were counterstained with DAPI (blue). Scale bar: 20 µm. **(C)** IL-1β-immunoreactivity were quantified using ImageJ in 10 random fields from the number of experiments indicated within each graph bar. Results were expressed as percentage of control; *p<0.05, **p<0.01, different from control, Kruskal-Wallis test, followed by Dunn's multiple comparison test.

increased the IL-1β immunoreactivity to 149.56±13.23% of control ($p<0.05$). Blockade of $A_{2A}R$ did not alter the EHP-induced increased in IL-1β immunoreactivity (156.73±13.8% of control, $p<0.01$).

3.9 $A_{2A}R$ blockade prevent the TNF release from BV-2 microglial cells after exposure to elevated hydrostatic pressure

We then assessed the effects of EHP, and potential effects of $A_{2A}R$ blockade, in the release of TNF to the culture medium.

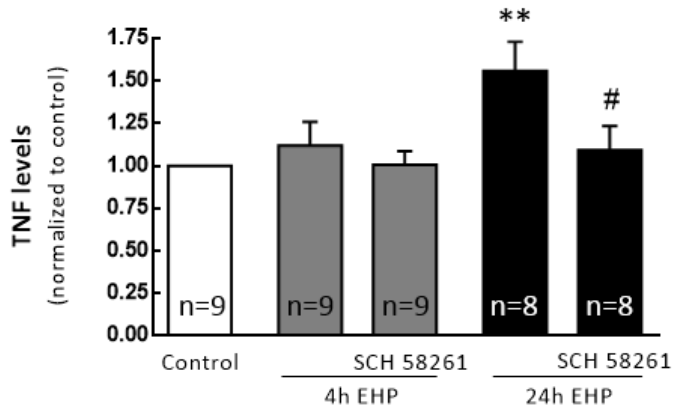

Figure 23: $A_{2A}R$ blockade prevents TNF release from BV-2 microglial cells triggered by elevated hydrostatic pressure. BV-2 cells were pre-treated with 50 nM SCH 58261 before the exposure to EHP for 4 h and 24 h. Extracellular levels of TNF were quantified by ELISA in cell culture medium supernatants. Results were expressed as fold change of control, from the number of experiments indicated within each graph bar. **p<0.01, different from control, #p<0.05, different from 24 h of EHP, Kruskal-Wallis test, followed by Dunn's multiple comparison test.

TNF protein levels were quantified by ELISA (Figure 23), in culture medium of BV-2 microglial cells challenged with EHP for 4 h or 24 h. The exposure to EHP for 4 h did not alter the TNF levels (1.12±0.14 fold), as well as the treatment with SCH 58261 prior EHP (4 h) (1.00±0.01 fold), comparing with the control. Still, when the cells were challenge with EHP for 24 h, TNF levels were significantly increased (1.56±0.17 fold-increase of the control, $p<0.01$). Treatment with $A_{2A}R$ antagonist prior exposure of EHP (for 24 h) significantly prevented the EHP-induced increase of TNF protein levels (1.25±0.20 fold to control, $p<0.05$).

3.10 Effect of elevated hydrostatic pressure on the levels of IL-1β and TNF in organotypic cell cultures

To investigate the effect of EHP in a more complex system and the ability of $A_{2A}R$ genetically blockade to modulate these alterations, organotypic cultures were obtained from $A_{2A}R$-KO mice and wild type.

To assess the effect of EHP in the production of inflammatory mediators, retinal organotypic cultures were challenge with EHP for 24, 48, 72 h and obtained from wild-type or $A_{2A}R$-KO mice (to evaluate the effect of $A_{2A}R$ absence).

IL-1β (Figure 24A) and TNF (Figure 24B) extracellular levels were measured from the culture medium using ELISA.

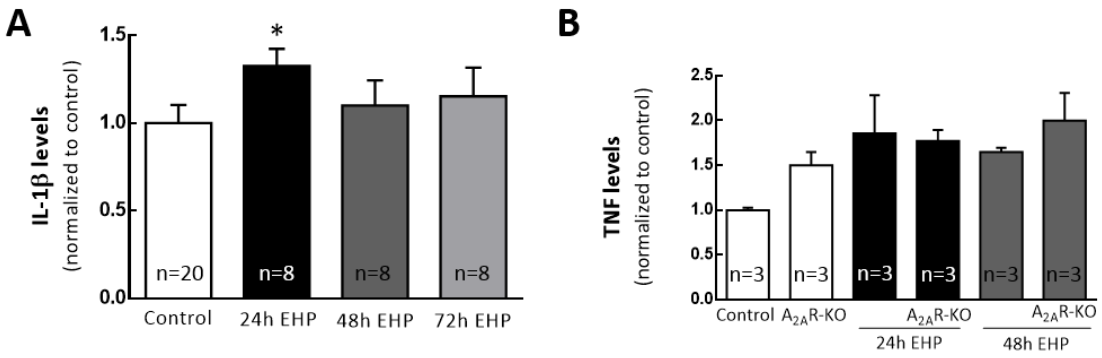

Figure 24: Effect of elevated hydrostatic pressure on the levels of IL-1β and TNF in retinal organotypic cultures. Organotypic cultures obtained from wild-type and $A_{2A}R$-KO mice were cultured for 4 days and exposed to EHP for 24 h, 48 h and 72 h. Extracellular levels of IL-1β **(A)** and TNF **(B)** were quantified by ELISA in culture medium supernatants. Results were expressed as fold change of control, from the number of experiments indicated within each graph bar. *p<0.05, different from control, Mann-Whitney test.

Exposure to 24 h of EHP significantly increases the protein levels of IL-1β present in the culture to the medium (1.33±0.1 fold of control, p<0.05), whereas when organotypic cultures were challenged with 48 h and 72 h of EHP, the IL-1β levels were 1.15±0.16 and 1.10±0.14 fold-increase of the control.

The protein levels of TNF in the culture medium of retinal organotypic cultures exposed to EHP for 24 h or 48 h were 1.86±0.43 and 1.65±0.05% of control, respectively. Still, the genetically inactivation of $A_{2A}R$ did not prevent the effects caused by EHP ($A_{2A}R$-KO 24 h of EHP: 1.77±0.12 and $A_{2A}R$-KO 48 h of EHP: 2.00±0.31% of control). Moreover, is important to notice that is an n=3, small for the variability of the technique and statistical analysis were not performed.

3.11 Elevated hydrostatic pressure decreases Brn3a positive-cells in organotypic cell cultures

In order to understand whether EHP affects the RGCs, we exposed retinal organotypic cultures for 24 h or 48 h to EHP. Immunohistochemistry was performed in organotypic cultures with an antibody that specifically recognizes Brn3a (red), which is a transcript expressed in RGCs (Nadal-Nicolas et al., 2009) (Figure 25A). Brn3a immunoreactive cells were counted (Figure 25B) showing that exposure to EHP for 24 h decreased the number of Brn3a-positive cells decrease to 82.27±6.61% of control. Challenge with 48 h of EHP significantly decrease the number of Brn3a-positive cells to 60.25±4.63% of control ($p<0.05$).

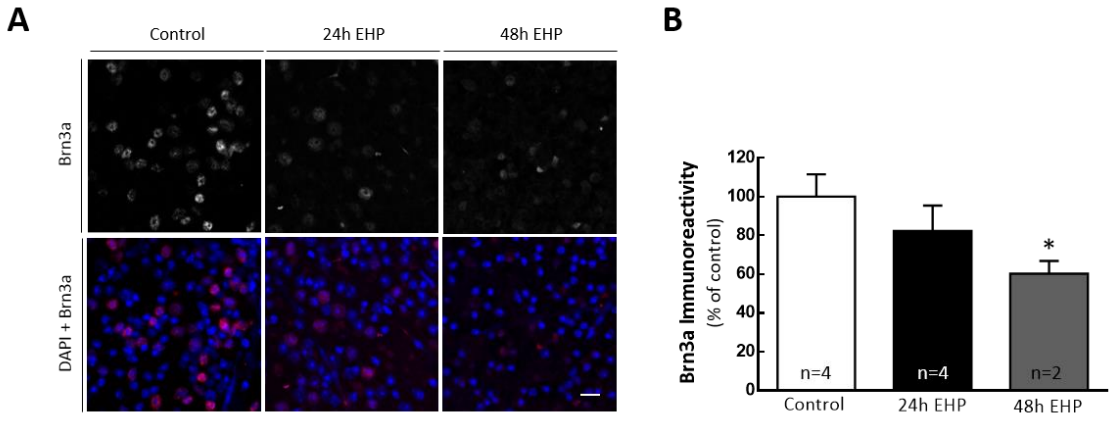

Figure 25: Elevated hydrostatic pressure decreases triggers loss of retinal ganglion cells. Organotypic cultures were challenged with EHP for 24 h and 48 h. **(A)** Retinal ganglion cells were labeled by immunohistochemistry using an antibody anti-Brn3a (RGC marker; grey/red) and nuclei were counterstained with DAPI (blue). Scale bar: 20 µm. **(B)** Brn3a-immunoreactive cells were counted in 12 random fields (3 fields per retinal quadrant). Results were expressed as percentage of control, from the number of experiments indicated within each graph bar. *$p<0.05$ different from control, Kruskal-Wallis test, followed by Dunn's multiple comparison test.

CHAPTER 4

Discussion

4. DISCUSSION

Glaucoma is characterized by progressive loss of RGCs and optic nerve fibers, which leads to vision loss. Elevated IOP is the major risk factor for the development of glaucoma (Song et al., 2015), and the current treatments are based on lowering the IOP. Nevertheless, despite successful IOP control, some patients still progress to blindness (Nucci et al., 2013), supporting the concept that IOP-independent mechanisms may also play a role in the pathogenesis of this disease (Zhong et al., 2013). It is well established that neuroinflammation plays a role in glaucoma (Madeira et al., 2015a). Indeed, several evidences show an increase in the production of pro-inflammatory mediators in human glaucoma (Borkenstein et al., 2013) and in several experimental models (Tezel and Wax, 2000, Yoneda et al., 2001, Roh et al., 2012). Moreover, exacerbated microglia reactivity has been reported before the loss of RGCs (Bosco et al., 2011, Bosco et al., 2015, Wang et al., 2015), which highlights the importance of modulating microglia reactivity to attenuate disease progression.

Adenosine is an important neuromodulator in the CNS that acts through the activation of four ARs. The A_1R, A_2AR and A_3R were already described in microglial cells (Hasko et al., 2005). Moreover, we have recently reported that in organotypic rat retinal cultures, $A_{2A}R$ blockade prevents microglia reactivity triggered by EHP (Madeira et al., 2015b). In addition, previous results obtained by our group have shown that the exposure of BV-2 cells to EHP increases the extracellular levels of both ATP and adenosine (data not published). Therefore, in this work we aimed to investigate the impact of EHP in the adenosinergic system of microglia cells, namely on the expression of adenosine receptors, transporters and enzymes involved in the adenosine metabolism. In addition, we also aimed to study whether EHP triggers the release of inflammatory mediators from BV-2 and retinal organotypic cultures and whether $A_{2A}R$ blockade prevents these effects.

CD73, also known as ecto-5'-nucleotidase, is a membrane-bound glycoprotein with enzymatic capacity to catalyze the dephosphorylation of extracellular AMP into adenosine (Regateiro et al., 2013). We found that the exposure of BV-2 cells to EHP increased the protein levels of CD73, which could explain the increase in extracellular adenosine levels upon exposure to EHP. Indeed, several physiological (Cunha et al., 1996, Cunha et al., 2001) and pathological conditions (Kobayashi et al., 2000, Napieralski et al., 2003) cause a parallel

increase in ecto-5'-nucleotidase activity and $A_{2A}R$ expression, suggesting that ecto-5'-nucleotidase and $A_{2A}R$ interact. In fact, in the striatum, CD73 is responsible for the formation of adenosine, thus providing the source for $A_{2A}R$ activation (Augusto et al., 2013), suggesting CD73 modulation as an alternative target for the treatment of neurodegenerative diseases (Augusto et al., 2013).

ADK is the main pathway for adenosine removal in normal physiological conditions, becoming easily saturated with basal concentrations of adenosine (Dunwiddie and Masino, 2001, Latini and Pedata, 2001). The increase of hydrostatic pressure did not change ADK protein levels in BV-2 cells, indicating that intracellular adenosine is not being converted to AMP by ADK, which may lead to increased adenosine levels. Previously, it was reported that in the retina of diabetic Wistar rats, both mRNA and protein levels of ADK decrease, without changes in retinal cell cultures incubated with elevated glucose concentration (Vindeirinho et al., 2013). This emphasizes the potential difference between *in vitro* and animal models, and therefore we can not extrapolate that ADK is not altered in retinal microglia from glaucomatous animals. Nevertheless, it was also reported that both mRNA and protein levels of ADK increase in a traumatic optic neuropathy mouse model (Ahmad et al., 2014). Moreover, the authors demonstrated that the inhibition of ADK attenuates retinal inflammation due to increased levels of adenosine that act through $A_{2A}R$ (Ahmad et al., 2014). This hypothesis is not in agreement with the several evidences demonstrating neuroprotection with the blockade of $A_{2A}R$ in several neurodegenerative diseases (Cunha, 2005, Santiago et al., 2014). This controversial idea of the activation/ blockade of $A_{2A}R$ as neuroprotective approach can by explained. Ahmad et al. (2014) administered the ADK inhibitor through intraperitoneal injection, having a high contribution of the peripheral system, which corroborate with some studies that report protection mediated by $A_{2A}R$ activation upon peripheral inflammation (Hasko et al., 2005). On the other hand, results obtained by our group demonstrated that the administration of the $A_{2A}R$ antagonist intravitreally, reduced the pro-inflammatory response and RGCs loss, being more directed to the CNS and, consequently, reducing the peripheral system contribution.

ADA is mainly a cytosolic enzyme, but it also operates at the cell surface (ecto-ADA). Ecto-ADA irreversibly deaminates extracellular adenosine to inosine (Regateiro et al., 2013). Under pathological conditions, in which adenosine levels increase, ADA gains a more relevant role (Dunwiddie and Masino, 2001, Latini and Pedata, 2001). In our work, we

showed that the exposure to EHP decreased ADA activity in BV-2 cells. This reduced ADA activity may indicate a lower rate of adenosine removal in the extracellular space, which can be correlated with our previous observations, showing increased levels of adenosine under EHP. Particularly, EHP leads to an increase of ADA mRNA levels, which is not paralleled by an increment in the protein. In fact, EHP did not alter ADA protein levels, although the enzymatic activity was shown to be decreased. This increase in ADA expression might be a mechanism to compensate for the reduced ADA enzymatic activity, trying to reestablish the basal levels of adenosine. In the retina, others also demonstrated that ADA activity decreases in cultured retinal cells incubated with elevated glucose concentration, to mimic hyperglycemic conditions (Vindeirinho et al., 2013). Other study, performed with rats in hyperoxic condition (0.79 bar of O_2 above to the control), demonstrated a parallel increase in CD73 expression and in ADA enzymatic activity, suggesting that the low extracellular adenosine levels did not result from a decreased synthesis of adenosine by CD73 but from an exacerbated ADA activity (Bruzzese et al., 2015). However, hyperbaric conditions induced by an increase of 1.0 bar of N_2 relative to the control did not affect the extracellular levels of adenosine neither the CD73 nor the ADA expression (Bruzzese et al., 2015). The potential differences between these results and ours may be explained by the conditions used to increase the pressure (while we injected mixture of 95% air and 5% CO_2, the group of Bruzzese used N_2) or by the experimental model (BV-2 cell line vs. animal model).

CNT2 mediates the transport of adenosine using the sodium gradient. We found that CNT2 levels were increased upon EHP, suggesting that BV-2 cells are trying to compensate for the increased levels of extracellular adenosine. Others have also demonstrated that mRNA and protein for CNT2 are increased in PC12 cells, a neuronal cell line, differentiated with nerve growth factor, further indicating the fundamental role of CNT2 in the regulation of adenosine homeostatic (Medina-Pulido et al., 2013). Moreover, under hypoxia, CNT2 is downregulated in PC12 cells and in a rat model of transient focal ischemia (Medina-Pulido et al., 2013). Nevertheless, we should take into account that the different results obtained may be related with the different insults (EHP vs. hypoxia) and/or different cell types (microglia vs. neurons). In addition, although we observed an upregulation of CNT2 protein levels, the function of the transporter was not assessed,

which would provide us a better understanding about the impact of EHP on CNT2 in BV-2 cells.

The actions of extracellular adenosine are mediated through the activation of four different ARs. We found that EHP induces a decrease A_1R expression and significantly increase of $A_{2A}R$ expression in BV-2 cells. Moreover, EHP did not affect the expression of A_3R in BV-2 cells. The role of A_3R in inflammation is still controversial and poorly understood. Activation of A_3R was shown to confer neuroprotection after brain ischemia, by reducing the migration/infiltration of microglia/macrophages and inflammation (Choi et al., 2011). The activation of A_3R suppresses TNF production in LPS-treated microglial cells (Lee et al., 2006). Moreover, the extension of microglial processes towards the local of the injury is triggered by the $P2Y_{12}$ receptors. An agonist of ADP (2MeSADP), that can activate the $P2Y_{12}$ receptors, failed to induce the process extension of microglia. Furthermore, A_3R agonist promoted the 2MeSADP-stimulated migration. Contrarily, the A_3R antagonist inhibited ADP-induced microglial migration, demonstrating that the A_3R activation is extremely essential for microglia migration and process extension, which contributes for the correct surveillance of the environment (Ohsawa et al., 2012). In the retina, A_3R protein levels increase in diabetic Wistar rats, without alterations in retinal cell cultures exposed to high glucose, to mimic hyperglycemia, (Vindeirinho et al., 2013), emphasizing the potential difference between *in vitro* and animal models.

Our results suggest that under EHP, the effects of adenosine are mediated through the activation of $A_{2A}R$. It is well documented that under noxious conditions, A_1Rs are downregulated with a concomitant increase in $A_{2A}R$ density (reviewed in Cunha (2005). In fact, A_1R expression is downregulated in neurodegenerative conditions, such in Alzheimer's disease (Kalaria et al., 1990, Ulas et al., 1993, Deckert et al., 1998) and in several brain regions follow ischemia (Lee et al., 1986, Nagasawa et al., 1994). Contrarily, the density and expression of $A_{2A}R$ increase in several models of neurodegenerative diseases such Parkinson's, Alzheimer's and Huntington's diseases (Calon et al., 2004, Tomiyama et al., 2004, Tarditi et al., 2006, Boison, 2008). Specifically, different brain insults that trigger neuroinflammation also lead to an upregulation of $A_{2A}R$ in microglial cells(Gomes et al., 2013). Accordingly, retinal microglial cells also increase $A_{2A}R$ expression upon inflammatory conditions, such LPS (Liou et al., 2008, Madeira et al., 2012, Madeira et al., 2015b).

Moreover, the exposure of retinal organotypic cultures to EHP for 24 h also increase the expression of $A_{2A}R$ in microglia (Madeira et al., 2015b).

Taking into account our results demonstrating upregulation of $A_{2A}R$ under EHP conditions, and the beneficial properties of $A_{2A}R$ blockade against microglia-mediated neuroinflammation, we also aimed to investigate the effects of $A_{2A}R$ blockade in microglia challenged with EHP, namely on the iNOS, IL-1β and TNF levels.

Several reports show the involvement of microglia-mediated neuroinflammatory markers in animal models of glaucoma and in patients (reviewed in Madeira et al. (2015a). Indeed, numerous evidences support the idea that these inflammatory mediators released by microglia can be deleterious to RGCs (reviewed in Soto and Howell (2014) Karlstetter et al. (2010). In order to understand the effect of EHP in inflammatory response, we took advantage of two distinct experimental models: 1) the BV-2 cells, to specifically study the response of microglial cells to EHP; 2) retinal organotypic cultures obtained from mice, a more complex system were the structural integrity of the retina is maintained. Although in BV-2 cells (microglial cells line) we dissect only the microglia contribution, in the retinal organotypic cultures, where retina structure is maintained, we have to take into consideration the contribution of other glial cells (astrocytes and Muller cells). In fact, we can not isolate the contribution of microglial cells *per se* from the other cells. However, this system allows us to use the genetic $A_{2A}R$ blockade approach. Since it is more realistic to take into account the contribution of all glial cells in the inflammatory response, the use of a complex system, were the structural integrity of the retina is maintained, might be seen as a more accurate approach.

Previous results from our group demonstrated that EHP increases microglia iNOS and NO production in retinal neural cell cultures (data not published) and in cultured rat retinal organotypic cultures (Madeira et al., 2015b). Accordingly, we found that EHP exposure increased iNOS in BV-2 cells. These results are also in agreement with previous reports showing increased iNOS and NO in glaucoma animal models (Neufeld, 1999b, Cho et al., 2011) and patients (Neufeld, 1999b, Schneemann et al., 2003, Rokicki et al., 2015).

Pro-inflammatory cytokines TNF and IL-1β are also increased in glaucoma models (Tezel and Wax, 2000, Yoneda et al., 2001, Roh et al., 2012). In this work, we found that the exposure to EHP increased IL-1β in BV-2 cells and in retinal organotypic cultures. Moreover,

we have also detected an increase in TNF in BV-2 cells and in retinal organotypic cultures, demonstrating that EHP triggers an inflammatory response in microglia. In fact, we have previously demonstrated that IL-1β and TNF levels increase in retinal neuronal cell cultures upon EHP (data not published). Moreover, EHP leads to an increase of both transcript levels and extracellular levels of IL-1β and TNF in retinal rat organotypic cultures (Madeira et al., 2015b). Nonetheless, several studies demonstrated that after LPS, both IL-1β and TNF protein levels were increased in BV-2 cells (Dang et al., 2014, Li et al., 2014, Wu et al., 2015).

It is becoming widely accepted that inflammation contributes to retinal neural loss (Karlstetter et al., 2010). The demise of RGCs is one feature of glaucoma, and it was already reported that microglia reactivity contributes to RGC loss (Bosco et al., 2011, Bosco et al., 2015, Madeira et al., 2015b). Herein, we demonstrated that EHP elicits loss of RGCs in mice retinal organotypic cultures, as observed by a decrease in the number of cells immunoreactive to Brn3a, a RGC marker (Nadal-Nicolas et al., 2009). These results are similar to previous results in organotypic cultures obtained from rats, where blockade of $A_{2A}R$ was able to prevent the loss of RGCs induced by EHP (Madeira et al., 2015b). The current work, performed in mice, opens the possibility for investigating the effect of the lack of $A_{2A}R$ responses using $A_{2A}R$ KO mice. The loss of RGCs triggered by EHP is consistent with previous results demonstrating an increase in cell death in retinal neural cell cultures (data not published).

Since microglial cells have a strongly contribution in inflammatory response follow EHP, the control the microglia reactivity seems to be an important approach to prevent the neuronal loss.

The blockade of $A_{2A}R$ has been shown to afford robust neuroprotection in several neurodegenerative diseases (Rebola et al., 2011, Li et al., 2015). Moreover, several evidences have been demonstrated that blockade of $A_{2A}R$ controls microglia reactivity (Santiago et al., 2014). In the retina, we have been describing that microglia reactivity elicited by LPS can be prevented by the blockade of $A_{2A}R$ (Madeira et al., 2012). Accordingly, we observed that $A_{2A}R$ blockade reduced iNOS immunoreactivity in BV-2 cells upon EHP, without changing iNOS mRNA. These results suggested us that the modulation of $A_{2A}R$ does not control *de novo* synthesis of iNOS. In addition, blockade of $A_{2A}R$ was not able to prevent the increase in IL-1β immunoreactivity in BV-2 cells triggered by EHP. Contrary, previous

results from our group demonstrated that the blockade of $A_{2A}R$ prevents the increase of IL-1β mRNA and extracellular levels in purified retinal microglial cell cultures upon EHP. However, in the BV-2 cell line we did not find an increase in extracellular levels of IL-1β, leading us to hypothesize that this can be a limitation of the cell line. However, further studies are necessary to address this result. Moreover, we can not establish a parallelism between the results in microglia purified cultures and BV-2 cells because the IL-1β was evaluated through different techniques. The pharmacological blockade of $A_{2A}R$ prevented the increase in the extracellular levels of TNF in BV-2 cells, but the genetic inactivation of $A_{2A}R$ ($A_{2A}R$ KO animals) did not alter TNF levels in culture supernatants. This discrepancy of the results can be justified by the use of two different models. In BV-2 cells, only microglial cells contribute for the TNF release and the pharmacological blockade has an effect directly in microglia. In retinal organotypic cultures, the TNF can be released from other glial cells, astrocytes and Müller cells. Moreover, since the genetic blockade of $A_{2A}R$ is not conditional but total, i.e., occurs in all cells that expresses this receptor, the absence of this receptor *per se* can lead to an inflammatory response. Nevertheless, more experiments are necessary to elucidate whether the genetic blockade of $A_{2A}R$ can be neuroprotective. In addition, evidences indicate that the silencing of $A_{2A}R$ in newborn animals has deleterious effects (Aden et al., 2003) while $A_{2A}R$ removal in adulthood affords protection against noxious conditions (Cunha, 2005). Previous results from our group demonstrated that the intravitreal injection of $A_{2A}R$ antagonist (SCH 58261) in ischemia-reperfusion (I-R) animal model reduces both IL-1β and TNF levels and prevents RGCs loss. Moreover, intravitreal injection of antibodies against of IL-1β and TNF, in the same model, showed a reduction in this cytokines and in RGCs loss, suggesting that the blockade of $A_{2A}R$ can confer neuroprotection through the control of the neuroinflammation. However, it is important notice that I-R model is a different model compared with the retinal organotypic cultures.

In conclusion, with this work we have demonstrated that EHP impaired the adenosinergic system by the alteration of CNT2 adenosine transporter, ADA and CD73 enzymes and the density of A_1R and $A_{2A}R$. In addition, taking in to account our results on the upregulation of $A_{2A}R$ in BV-2 cells upon EHP and increased adenosine levels, we hypothesize that the signaling mechanisms operated through $A_{2A}R$ are overactivated. Furthermore, EHP led to a proinflammatory environment, potentially deleterious to cells.

Since we found that blockade of $A_{2A}R$ is able to attenuate some microglia inflammatory responses, it could be envisaged as a potential therapeutic strategy to afford neuroprotection to RGCs by the control of microglial overeactivity. Still, we can not exclude the contribution of cells to the inflammatory milieu, like astrocytes or Müller cells.

CHAPTER 5

Concluding remarks and future perspectives

5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The results presented here allow us to conclude that EHP model can mimic some of the features of glaucoma, and that microglial cells might play a pivotal role in the development of this disease. Moreover, blockade of $A_{2A}R$ seems to control some of the inflammatory alterations mediated by microglia, although more experiments are needed. In fact, we demonstrated that EHP:

1) Impacts the adenosinergic system in microglia, promoting the increase in adenosine in both intra- and extracellular spaces;
2) Increases $A_{2A}R$ and decreases A_1R but does not alter A_3R expression;
3) Increases the inflammatory response from microglia, namely, iNOS, TNF and IL-1β levels both in BV-2 cells and retinal organotypic cultures;
4) Induces loss of RGCs in retinal organotypic cultures.

Likewise, blockade of $A_{2A}R$ decreased the levels of some pro-inflammatory mediators that contribute to the toxic milieu triggered by EHP.

Taking into consideration our findings, it will be interesting to evaluate the alterations in adenosinergic system triggered by EHP in a more complex system, like retinal organotypic cultures and in an animal model of glaucoma.

Considering that the activation of microglial cells and the loss of RCGs that occurs in glaucoma, future studies might be directed to explore the crosstalk between these two cell types, using co-culture system that may also allow evaluation of the role of $A_{2A}R$ for the protection of RGCs under EHP.

CHAPTER 6

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