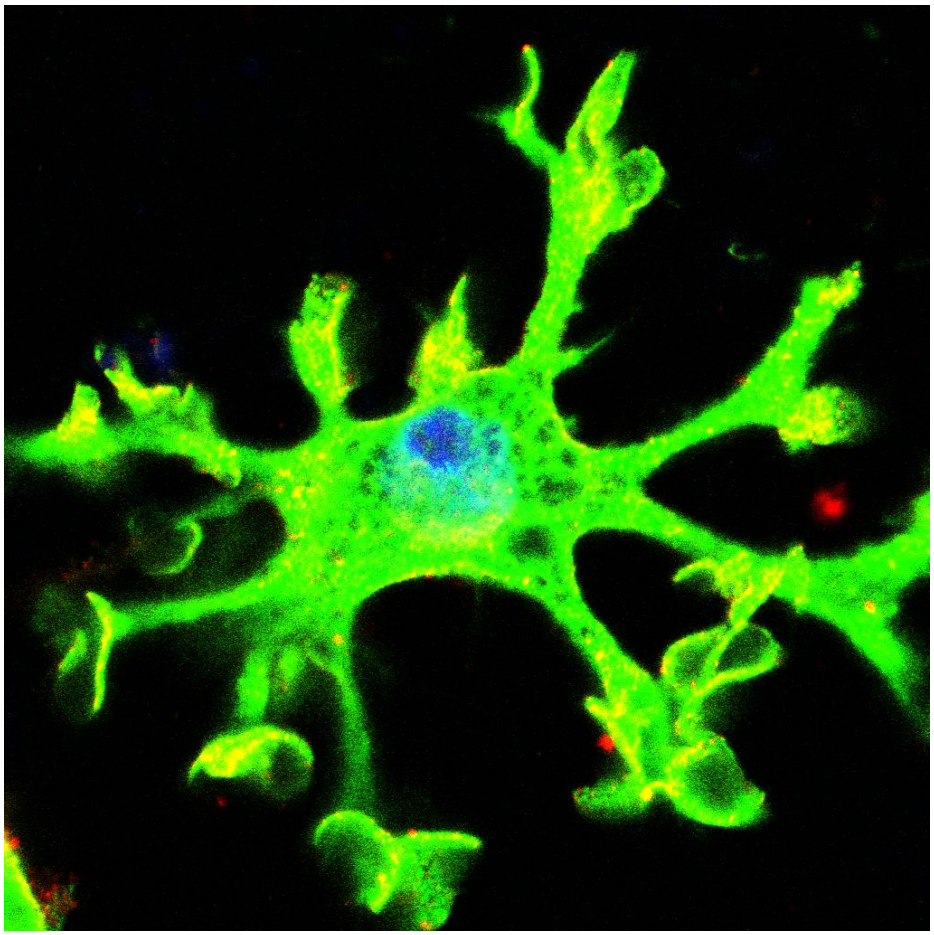

Maria Helena Bica Madeira

Role of adenosine A2A receptor in controlling neuroinflammation mediated by retinal microglial cells

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Investigação Biomédica. O trabalho foi realizado sob a orientação científica do Investigadora Doutora Ana Raquel Sarabando Santiago (Instituto Biomédico de Investigação da Luz e Imagem, Faculdade de Medicina da Universidade de Coimbra) e supervisão do Investigador Doutor António Francisco Rosa Gomes Ambrósio (Instituto Biomédico de Investigação da Luz e Imagem, Faculdade de Medicina da Universidade de Coimbra)

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UNIVERSIDADE DE COIMBRA

2011

Agradecimentos

Este espaço é dedicado a todos aqueles que me aconselharam, motivaram e orientaram. A todos eles expresso aqui o meu mais sincero agradecimento.

Agradeço à Doutora Raquel Santiago pela confiança em mim depositada na oportunidade de integrar o seu projecto de investigação. Obrigada pela orientação neste último ano, pelo incentivo, pela disponibilidade, pelas chamadas de atenção e pelas palavras amigas. Obrigada por todos os conselhos, por todos os momentos que passámos juntas este ano, que me ajudaram a crescer tanto como investigadora como pessoa.

Agradeço ao Doutor António Francisco Ambrósio, pela amabilidade e disponibilidade que demonstrou ao ser meu co-orientador neste trabalho. Obrigada pela confiança que depositou em mim na oportunidade de integrar o seu grupo de trabalho. Agradeço, ainda, como chefe de grupo, por me ter proporcionado as condições necessárias para a realização deste trabalho.

Agradeço profundamente aos meus colegas de laboratório. Obrigada pelo espectacular e descontraído ambiente de trabalho que me proporcionaram, pelo apoio que me deram em momentos menos bons, pela ajuda prestada e pelos conselhos que me deram. Sem vocês este trabalho teria sido mais difícil de concluir. À Joana, ao Filipe, ao Dan, à Áurea, ao Renato, à Camila, à Rosana e à Sandra, obrigada por me ajudarem a crescer mais um pouco, profissional e pessoalmente. Um agradecimento especial à Filipa, que tanto conselhos me deu, que tanto ajudou, e que tantas vezes nos cedeu o seu cantinho

no gabinete para uma conversa ou um momento de desabafo, e pelo seu sorriso, sempre presente para nos animar. Obrigada!

À Xana colega de mestrado e de laboratório, por se meter nesta aventura comigo, pelas ajudas, pelos desabafos, pelos nossos momentos de trabalho, pelos momentos de stress que partilhámos, pelos momentos bons que vivemos, por ser ela própria, e porque sem ela este ano não teria sido igual. Obrigada Xana!

Às minhas colegas de mestrado, em especial à Maria João e à Ana Soares pelos momentos que passámos nestes dois anos, pelas conversas desabafos, conselhos, e gargalhadas que partilhámos.

Agradeço ainda, à Carla Marques, à Joana Liberal e à Joana Gonçalves pela generosidade e pelos conselhos e sugestões que me deram entre conversas.

Sem amigos tudo se torna dificil, e há aqueles que mesmo longe nunca deixam de fazer parte das nossas vidas. Agradeço por isso a todos os meus amigos Alvaladenses, em especial à minha "afilhada" Rita, à Ângela, à Tânia, à Teresa e à Inês. Agradeço pela longa amizade, pelo apoio, incentivo e pelos bons momentos de distração e diversão que, sem dúvida, também ajudaram. Agradeço especialmente o facto de saber que aconteça o que acontecer, vocês estão sempre lá para me receber depois de uma das minhas longas ausências. Um enorme obrigado a todos eles!

Aos "Fantasmas", Mário, Curioso, Estica, Paco, Bacalhau, Luis, Samuel e Rui, por me acolherem em vossa casa, pelos bons momentos que me proporcionaram, por todas as pessoas que conheci através de vocês, pelas inúmeras amizades que fiz em vossa casa, pela vossa amizade e acima de tudo por serem a minha família em Coimbra. Um agradecimento especial ao meu "irmão" Milton que me integrou nesta família fantástica, pelo apoio que me deu, pela paciência, pelos jantares, pelos cafés, pelas conversas e pela amizade que temos desde sempre!

Ás minhas meninas, Jenny, Marta, e Mary, por me receberem, por me apoiarem em tantos momentos, pelo incentivo que me deram, pelas conversas e desabafos, pelos cafés, pelas noites que passámos juntas, por tantos momentos bons que nunca irei esquecer, e que sem dúvida foram essenciais. Agradeço acima de tudo a nossa amizade, que apesar de não ser de longa data será daquelas que me vão marcar para sempre!

Ao meu querido amigo Cyril, pela paciência que tem comigo, por me aturar, pela sua bondade e amizade que são tão grandes como ele!

Agradeço o meu Padrinho Zé, por ser uma espécie de ídolo desde infância, pelos abraços, pelo apoio, pela amizade, por ser um irmão que só não partilha o sangue.

À Marília, por ser a estrelinha mais brilhante no céu, pois sei que está a olhar por nós!

Ao meu irmão, pelo carinho, pelos fortes abraços, pelas suas palavras, e por ser desde sempre um exemplo e orgulho para mim! Obrigada Mano!

Aos meus sobrinhos, Miguel e João, que são o grande amor da minha vida, o meu maior orgulho e a minha maior motivação, pois são aqueles sorrisos que me iluminam a alma mesmo nos momentos mais complicados.

À minha irmã, que é também a melhor amiga, por todos os conselhos, pelo incentivo, pelo encoragamento, pelas chamadas de atenção, pelas nossa brincadeiras, pelas nossas zangas, pelas nossas conversas, por saber que está sempre lá para mim, por ser também ela um exemplo e um meus maiores orgulhos! Obrigada por seres parte de mim!

E finalmente aos meus Pais, agradeço por acreditarem em mim, por me apoiarem sempre e em qualquer situação, pela paciência, pelo carinho, amizade, pela compreensão nas minhas ausências. Obrigada por me educarem, por me formarem, por me ouvirem e por me proporcionarem sempre tudo o que precisei para chegar até aqui. Esta tese é dedicada especialmente a eles, os meus maiores mentores e exemplos na vida!

Contents

Abbreviations

A1R - Adenosine A_1 receptor

A2AR - Adenosine A_{2A} receptor

A2BR - Adenosine A_{2B} receptor

A3R - Adenosine A_3 receptor

ADP - Adenosine diphosphate

AMP - Adenosine monophosphate

AMPA - D,L -alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA - Analysis of variance

ARs - Adenosine receptors

ATP - Adenosine tri-phosphate

BSA - Bovine serum albumin

Ca^{2+} - Calcium

cAMP - Cyclic adenosine monophosphate

cDNA - Complementary deoxyribonucleic acid

CGS21680 - 4-[2-[[6-amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid

CNS - Central nervous system

CPA - N6-cyclopentyladenosine

CREB - cAMP responsive binding element

DAPI - 4',6-Diamidino-2-phenylindole

DMEM - Dulbecco's modified Eagle medium

eNOS - Endothelial nitric oxide synthase

EPAC - Exchange proteins directly activated by cAMP

ERK - Extracellular signal-regulated kinases

GABA - Gamma-aminobuytric acid

GCL - Ganglion cell layer

HBSS - Hank's balanced salt solution

IL-1β - Interleukin-1 beta

INL - Inner nuclear layer

iNOS - Inducible nitric oxide synthase

IOP - Intraocular pressure

IPL - Inner plexiform layer

KA - Kainate

K^+ - Potassium

KHR - Krebs-Henseleit Ringer

LPS - Lipopolysaccharide

MEM - Eagle's minimum essential medium

MHC - Major histocompatibility complex

mRNA - Messenger ribonucleic acid

NF-Kb - Nuclear factor kappa-light-chain-enhancer of activated B cell

NFL - Nerve fiber layer

nNOS - Neuronal nitric oxide synthase

NMDA - N-methyl-D-aspartate

NO - Nitric oxide

ONH - Optic nerve head

ONL - Outer nuclear layer

OPL - Outer plexiform layer

PBS - Phosphate buffer saline

PKA - Protein Kinase A

PKC - Protein Kinase C

PLC - Phospholipase C

RGC - Retinal ganglion cell

ROS - Reactive oxygen species

RPE - Retinal pigmented epithelium

SCH58261 - 2-(2-Furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine

SDS - Sodium dodecyl sulfate

SEM - Standard error of the mean

TEMED - N,N,N',N'-Tetramethylethylenediamine

TNF-α - Tumor necrosis factor-alpha

TUNEL - Terminal transferase dUTP nick end labeling

Resumo

A adenosina é um neuromodulador do sistema nervoso central (CNS) e as suas acções são mediadas via receptores purinérgicos do tipo P1 (receptores A_1 e A_3, inibitórios; e receptores A_{2A} e A_{2B}, facilitatórios). Várias evidências indicam que em situações nocivas para o cérebro o bloqueio dos receptores A_{2A} da adenosina (A2AR) confere potente neuroprotecção, principalmente através do controlo de neuroinflamação.

O glaucoma é uma doença degenerativa e progressiva da retina e a segunda causa de perda de visão em todo o mundo. No glaucoma, a neuroinflamação desempenha um papel importante. Nomeadamente, ocorre activação das células da microglia que libertam mediadores inflamatórios, promovendo a morte das células ganglionares da retina (RGC), uma característica do glaucoma. Na retina, as células da microglia estão localizadas na camada de RGC e expressam A2AR. Assim, o objectivo principal deste trabalho foi estudar se o bloqueio dos A2AR reduz a reactividade da microglia induzida por um estímulo pró-inflamatório, que desta forma pode contribuir para a protecção das RGC.

Culturas mistas de retina e culturas purificadas de microglia da retina foram tratadas com CGS21680 (agonista do A2AR) ou SCH58261 (antagonista do A2AR), antes da incubação com lipopolissacarídeo (LPS), o qual foi utilizado para mimetizar um estímulo inflamatório. Foram avaliados vários parâmetros, que são indicadores do estado de reactividade da microglia.

Os resultados indicam que o bloqueio dos A2AR pode modular a reactividade da microglia da retina. O bloqueio do A2AR previne os efeitos do LPS nas alterações morfológicas, na libertação de monóxido de azoto (NO) e de factor de necrose tumoral-alfa (TNF-α), e também na actividade fagocítica da microglia induzida por estímulos inflamatórios.

Em conclusão, estes resultados fornecem evidências da capacidade de bloqueio de A2AR para controlar a reactividade da microglia. Tendo em conta o papel das microglias na neuroinflamação, estes resultados abrem a possibilidade para o uso de antagonistas dos A2AR em doenças que envolvam neuroinflamação da retina, como é o caso do glaucoma. A diminuição da neuroinflamação da retina pode ter efeitos benéficos contra a morte das RGC, uma das características principais do glaucoma.

Abstract

Adenosine is a neuromodulator in central nervous system (CNS) and its actions are mediated via the type P1 purinergic receptors (inhibitory A_1 and A_3 receptors, and facilitatory A_{2A} and A_{2B} receptors). The blockade of adenosine A_{2A} receptors (A2AR) provides potent neuroprotection in several noxious brain conditions, mainly through the control of neuroinflammation.

Glaucoma is a progressive retinal degenerative disease and the second cause of vision loss worldwide. Neuroinflammation plays an important role in glaucoma. In particular, it occurs microglial activation, releasing inflammatory mediators that can promote retinal ganglion cell (RGC) death, a feature of glaucoma. In the retina, microglial cells are located in the ganglion cell layer and express A2AR. Therefore, the main aim of this work was to evaluate the effect of the blockade of A2AR in the control of microglial reactivity induced by an inflammatory stimulus, which can as a consequence contribute to the protection of RGC.

Primary retinal mixed cultures and purified retinal microglial cultures were pretreated either with CGS21680 (agonist of the A2AR) or SCH58261 (antagonist of the A2AR), and the cells were challenged with lipopolysaccharide (LPS) to mimic an inflammatory stimulus. Several parameters, indicators of the reactivity status of microglia were evaluated.

The results indicate that the blockade of the A2AR can modulate the microglial reactivity. A2AR blockade can prevent the LPS effects on morphological alterations, nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α) release, and on the phagocytic activity.

In conclusion, these results provide evidence of the ability of blockade of A2AR to control the microglial reactivity. Taking in account the role of microglial cells in

neuroinflammation, these data open the possibility for the use of A2AR antagonists in diseases involving retinal inflammation, as is the case of glaucoma. Decreasing retinal neuroinflammation may have beneficial effects against RGC death, the main characteristic in glaucoma.

Chapter 1 - Introduction

1.1 The Eye

The eye is a highly specialized and organized structure composed by an optical portion which focuses the visual image on the receptor cells, and by a neuronal component, which transforms the visual image into nerve signals that are transmitted to the brain (Seeley et al., 2003; Widmaier et al., 2007).

The ocular globe may be separated into three different layers. The outermost layer is composed by the sclera, a conjunctive tissue layer that helps maintaining the eye form and protects the internal structures, that becomes transparent in the front of the eye, forming the cornea, an avascular structure that allows the input of light into the eye causing reflection or refraction of light that enters (Kolb, 1995; Seeley et al., 2003).

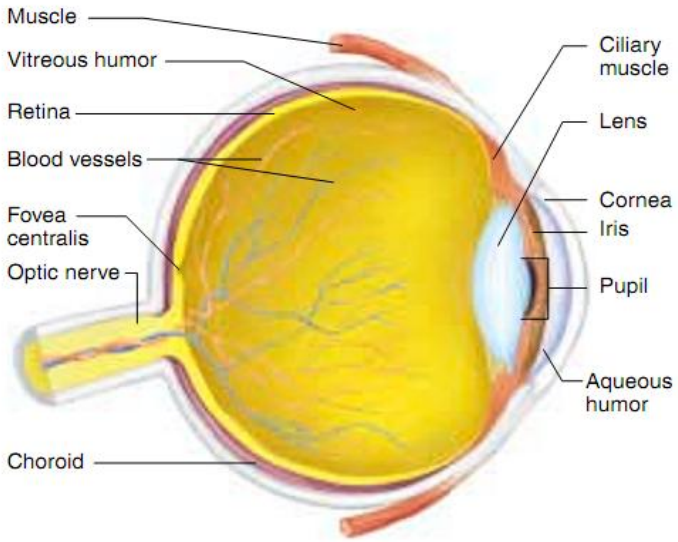

Figure 1 - Sagittal section of a human eye (Widmaier et al., 2007).

The middle layer of the eye is the uvea or uveal tract, which is divided into two parts, the anterior part containing the iris and ciliary bodies, and the posterior part formed by the choroid. The iris is a colored circular muscle that regulates the amount of light entering the eye by controlling the size of the pupil (the iris aperture). The ciliary bodies contain ciliary muscles that enable the lens to change shape during accommodation

(focusing near and distant objects). The choroid is the vascular layer containing connective tissue, lying between the retina and the sclera, which provides oxygen and nourishment to the outer layers of the retina (Seeley et al., 2003).

The internal layer of the eye is the retina, the part of the CNS responsible for transforming light rays in meaningful information to the brain.

The lens is a transparent, biconvex structure located behind the iris, which together with the cornea forms the optical system that focuses impinging light rays into an image upon the retina. The image is focused on a specialized area known as the fovea centralis (Kolb, 1995; Widmaier et al., 2007).

The eye has also three fluid chambers: the anterior chamber, between the cornea and the iris, the posterior chamber, located between the iris and the lens, both filled with aqueous humor; and the vitreous chamber, located between the lens and the retina, that is filled with vitreous humor, and which helps maintaining shape of the ocular globe and secure the lens and the retina in place (Kolb, 1995; Seeley et al., 2003). These two fluids are colorless and allow the transmission of light from the front of the eye to the retina (Widmaier et al., 2007).

1.1.1 Retina

The retina is a thin transparent layer of neural tissue lining the back of the eye, which is composed by three layers of cell bodies and two layers of synapses (Widmaier et al., 2007). The outermost layer of the retina is the retinal pigmented epithelium(RPE), which is followed by the outer nuclear layer (ONL) that contains cells bodies of photoreceptors (rods and cones). The inner nuclear layer (INL) contains cell bodies of the bipolar, horizontal and amacrine cells, and the ganglion cell layer (GCL) is composed by

the nuclei of retinal ganglion cells (RGCs) and of displaced amacrine cells (Fischbarg, 2006).

The retina is constituted by three main cell types: neurons (photoreceptors, horizontal cells, amacrine cells and RGC), glial cells (Müller cells, astrocytes and microglial cells) and cells that constitute the retinal vessels (endothelial cells and pericytes). The communication between the different retinal cells types is crucial to a normal vision.

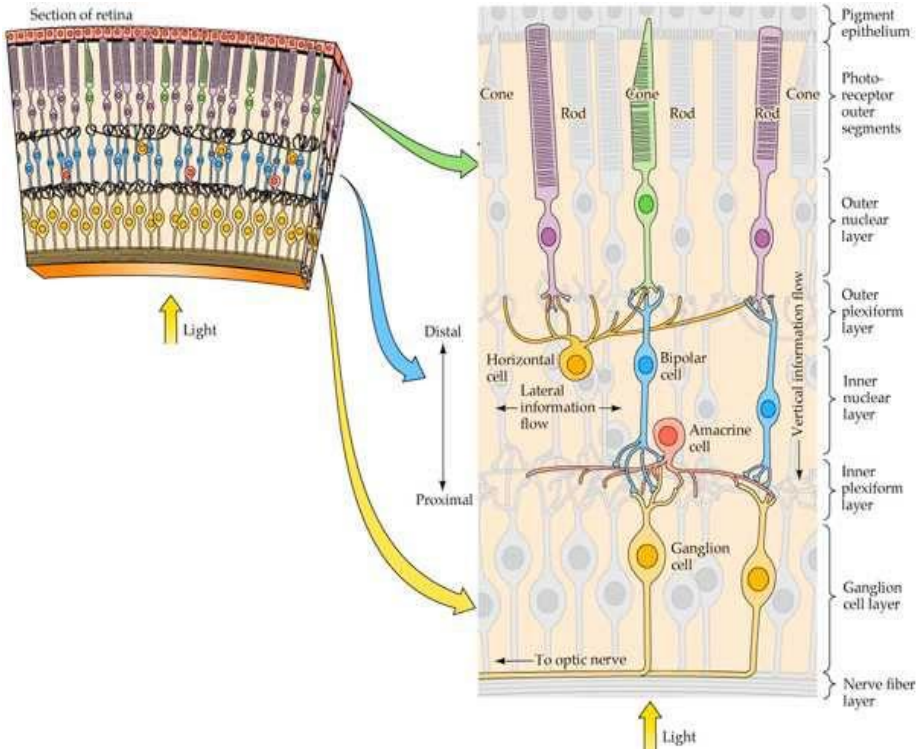

Figure 2 - The anatomy of the retina. **(A)** Schematic representation of the retina. **(B)** Diagram of the basic circuitry of the retina. Photoreceptors, bipolar cells, and ganglion cells provide the most direct route for transmitting visual information to the brain. Horizontal cells and amacrine cells mediate lateral interactions in the outer and inner plexiform layers, respectively (Adapted from Purves, 2004).

In the retina, neurons mediate phototransduction and transmit the visual impulses to the brain through the axons of RGCs. The transduction of light into electrical activity occurs in the photoreceptors. Photoreceptors synapse with bipolar cells in the OPL and bipolar cell to RGC neurotransmission occurs in the synaptic zones of the IPL (vertical

neurotransmission). There are cells mediating horizontal neurotransmission in both the outer and inner plexiform layers, and these are vital in shaping the temporal and spatial qualities of scotopic and photopic vision. Horizontal cells synapse in the OPL, affecting photoreceptor/bipolar cell interactions, while amacrine cells perform a similar role in the INL for bipolar to RGC transmission. The RGCs axons, that are located in brain visual centers, conduct the retinal output to the brain (Fischbarg, 2006; Kevany and Palczewski, 2010; Kolb, 1995).

The retina contains three types of glial cells: Müller cells, astrocytes and microglial cells, which contribute to the maintenance of homeostasis, support and protection of neighbor cells (Fischbarg, 2006; Kolb, 1995).

Müller cells are the principal glial cells of the retina, spanning radially across the retina (Kolb, 1995). Mülller cells are the major regulators of glutamate metabolism, extracellular ionic balance, and neuronal function, among other functions (Gardner et al., 2002). Together, Müller cells and astrocytes integrate vascular and neuronal activity in the retina (Gardner et al., 2002; Kolb, 1995).

Astrocytes are characteristic star-shaped glial cells, with flattened cell body and fibrous series of radiating processes. Astrocytes enter the developing retina from the brain along the optic nerve. The presence and distribution of retinal astrocytes correlate with the presence and distribution of retinal blood vessels. Both the cell bodies and processes of astrocytes are almost entirely restricted to the nerve fiber layer (NFL) of the retina and wrap around blood vessels and RGCs.

The third glial cell type is the microglial cell, which is ubiquitous in the human retina, being found in every layer in the retina. Microglial cells may be of two types, and both can be stimulated into a macrophagic function in traumatic conditions to the retina,

reacting to the inflammatory stimuli and engaging in phagocytosis of degenerating retinal neurons.

Retinal endothelial cells and pericytes constitute the retinal vessels. Endothelial cells are the primary physical barrier between blood and the retinal tissue (Fischbarg, 2006). The tight junctions between these cells constitute the inner blood-retinal barrier that limits the fluid flow from the circulation to the retina, to regulate its extracellular chemical composition, particularly ions important for neuronal activity. The blood-retinal barrier protects the retina from circulating inflammatory cells and their cytotoxic products (Fischbarg, 2006; Gardner et al., 2002). Pericytes are smooth muscle-like cells that envelope capillaries and have contractile functions (Shepro and Morel, 1993).

1.2 Microglial Cells

Microglial cells are the major immunocompetent cells in the CNS, being ubiquitous in the human retina, once they are found in all retinal layers (Gyoneva et al., 2009; Kolb, 1995). Microglial cell are capable of phagocytosis, antigen presentation and expression of numerous immune-related factors (Gyoneva et al., 2009).

Microglial cells are considered to derive from cells of the monocyte lineage that invade the brain in early development. Indeed, they express many features of monocytes, including signaling cascades well established in the immune system, involving chemokines and cytokines and other receptor systems. Microglial cells also respond to specific signaling substances, namely neurotransmitters (Kettenmann, 2005).

In the healthy brain, microglial cells are characterized by a ramified morphology, exhibiting small cell bodies with extremely branched processes, highly motile, being thought to be the most dynamic cells in the CNS. Microglial cells act as patrolling cells constantly surveying their microenvironment, being characterized by low expression of

major histocompatability complex (MHC) proteins and other antigen-presenting surface receptors (Block, 2010; Gyoneva et al., 2009; Karlstetter et al., 2010; Kettenmann, 2005).

Microglial cells communicate with other glial cells and neurons, which regulate their activation status and their capacity for phagocytosis of cellular debris (Dare et al., 2007). In response to stress conditions, microglial cells become into an active state, adopting a rounded, non ramified morphology, which may favor phagocytosis, similar to that of the initial infiltrating precursors (Karlstetter et al., 2010; Kettenmann, 2005). These morphological alterations are accompanied by changes in signaling and gene expression, release of pro- or anti-inflammatory factors, such as the cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), nitric oxide (NO) and reactive oxygen species (ROS), and recruitment of molecules, that affect the inflammatory response (Block, 2010; Gyoneva et al., 2009; Karlstetter et al., 2010).

Depending on the pathological stimulus, microglial activity can have a neurotoxic or a neuroprotective role. It was shown that neurotoxic microglial response was caused by lipopolysaccharide (LPS), whereas IL-1β induced a neuroprotective phenotype (Biber et al., 2007). Numerous neuroprotective effects of activated microglia have been established, like its benefic effect in a model of NO-dependent excitotoxicity and in animal models for some neurodegenerative diseases. Contrarily, microglial neurotoxicity can occur after excessive and uncontrolled stimulation of microglia or when microglia function is impaired (Biber et al., 2007).

Microglial cells can provide trophic support to neurons through the release of nerve growth factors, neurotrophins and other neurothropic factors. These cells are also able to assist in synaptic plasticity and have also been shown to surround damaged neurons and participate in an anti-inflammatory manner, in synaptic stripping, a process of removing branches from damaged neurons to promote repair and regrowth (Block,

2010). In fact, the mainstream of microglial functions is beneficial and necessary to the CNS, as activated microglia are vital for CNS damage repair. Evidence supports that microglia become neurotoxic due to both the loss of the beneficial functions and/or shift to a pro-inflammatory phenotype (Block, 2010).

Although the precise mechanisms are not completely understood, the release of pro-inflammatory or cytotoxic factors have an impact on neurons inducing neurodegeneration (Block, 2010; Liu et al., 2001). TNF-α and IL-1β are known cytotoxic factors for some neurons and can exacerbate neuroinflammation in the brain. Injuries in surrounding cells can be a consequence of NO release, a reactive free radical that reacts with superoxide anion to form the highly toxic radical peroxynitrite. (Liu et al., 2001).

1.2.1 Retinal Microglial Cells

In contrast to the vast amount of research data from the brain, relatively little is known about microglial homeostasis in the retina. However, over the last few years, this situation has changed considerably as more retinal disorders have come into focus, such as age-related macular degeneration and many rare monogenic disorders. Therefore, new genetic and experimental mouse models have been developed to mimic various forms of retinal degeneration and novel macrophage/microglia reporter mice were established, allowing the monitoring of retinal microglial *in situ* and *in vivo* (Karlstetter et al., 2010).

A large number of morphological and functional studies have revealed similarities between retinal and brain microglia. Retinal microglia also presents the immunological role that brain microglia, which was previously thought to be maintained by blood-retinal barriers, the absence of a lymphatic drainage and the inability of producing immunological responses (Chen et al., 2002).

Several studies suggest a hematopoietic origin of retinal microglia. Microglial precursors invade the developing retina from two main sources, the retinal margin and the optic disc, most likely via blood vessels of the ciliary body and iris, and the retinal vasculature, respectively. After microglial precursors enter the retina they migrate to the axon fascicles of the NFL and are subsequently spread through the retinal parenchyma to reach their final destination. These cells have ameboid morphology, being round and with short and broad branches. They are reactive microglia, playing active roles during development. Finally, they differentiate in so-called ramified microglia and become resting and mature (Chen et al., 2002).

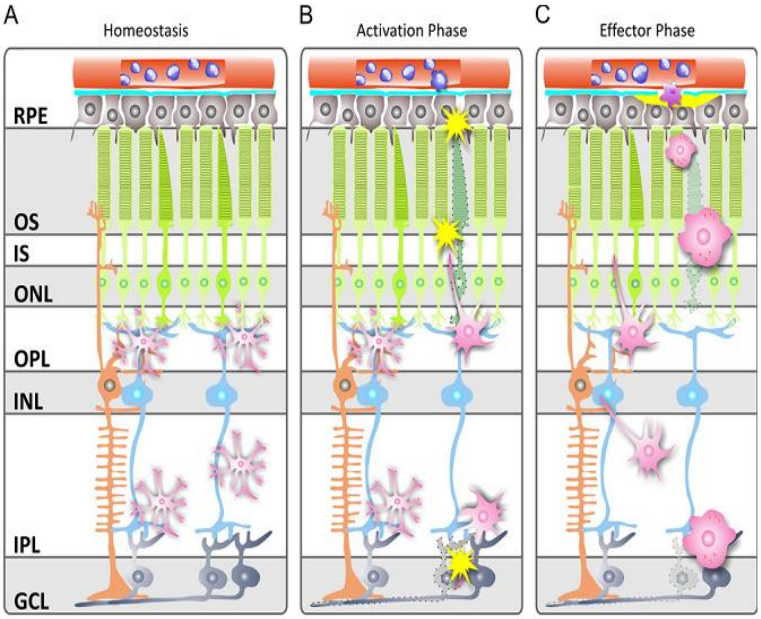

Figure 3 - Schematic representation of three common phases of microglial cells in the retina. **(A)** In normal retina, resting microglial cells (pink) mainly populate the plexiform layers. **(B)** When the retina is subjected to abnormal stimuli (yellow) microglial cells become active. **(C)** Resident microglia migrate to the lesion sites where they transform into ameboid phagocytes (Karlstetter et al., 2010).

In the mouse retina, immunofluorescence assays have demonstrated that resting microglial cells have a pluristratified distribution and they reside in the inner and plexiform layers (Hume et al., 1983; Karlstetter et al., 2010; Sasmono, 2003). Ramified

microglia situated in the plexiform layers, GCL and NFL survey the different retinal regions with their highly motile protrusions, without actively penetrating the nuclear layers (Figure 3A). Resting microglial cells are considered critical in host defense against invading microorganisms, immunoregulation, and tissue repair. Retinal microglial cells become activated by various stimuli, including nerve degeneration, inflammation and traumatic nerve lesions, suffering a rapid morphological transition of ramified microglia into non ramified phagocytes with only a few branched processes (Figure 3B). In the effector phase, microglial cells accumulate in the nuclear layers and the subretinal space where they participate in the phagocytosis of debris and facilitate regenerative processes (Figure 3C) (Chen et al., 2002; Karlstetter et al., 2010; Lee et al., 2008).

1.3 Adenosine

Adenosine is a ubiquitous purine nucleoside with a neuromodulatory role in the CNS. It is mainly produced by the degradation of adenosine tri-phosphate (ATP) which is involved in key pathways of primary metabolism, such as nucleotide and nucleoside metabolism, amino acid metabolism, trans-methylation reaction and handling of ammonia (Cunha, 2005). It has been reported that adenosine is able to prevent or decrease neuronal damage under different noxious conditions, such as hypoxia/ischemia, excitotoxicity, chemotoxicity or trauma.

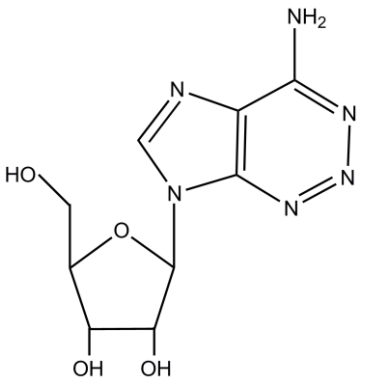

Figure 4 - Molecular structure of adenosine.

Purines are released upon depolarization of presynaptic terminals during physiological neurotransmission, and modulate nerve cell activity via both pre and postsynaptic specific receptors (Abbracchio et al., 1988). Adenosine is released upon conditions of metabolic stress and it is able to decrease the release of excitatory aminoacids, hyperpolarize neurons, restrain the activation of *N*-methyl-D-aspartate (NMDA) receptors, limit calcium (Ca^{2+}) influx, inhibit free radical formation and exert modulatory effects in astrocytes and microglia (Rebola et al., 2005).

There are two sources of extracellular adenosine: release of adenosine from intracellular space; and extracellular conversion of released adenine nucleotides (such as ATP, ADP and AMP) by a cascade of ectonucleotidases that are expressed in the extracellular surface of several cell types, including microglial cells, thus enabling a rapid increase in local adenosine (Dare et al., 2007; Haskó et al., 2005).

The actions of adenosine are mediated by four types of receptors (A1R, A2AR, A2BR and A3R), that couple to heterotrimeric G proteins. Adenosine receptors are pleiotropic receptors, once they have potential to couple to different G proteins and different transducing systems, according to their degree of activation and cellular and sub-cellular localization (Cunha, 2005; Haskó et al., 2005). These receptors have been linked to both inhibition (A1R and A3R) and activation (A2AR and A2BR) of adenylate cyclase activity, stimulation of phosphoinositide metabolism and modulation of K^+ and Ca^{2+} conductance (Abbracchio et al., 1988).

1.3.1 Adenosine A_1 Receptors

The adenosine A_1 receptor (A1R) was the first subtype identified, and is widely distributed in the CNS and peripheral tissues. In the CNS, this receptor exerts a global inhibitory modulation of synaptic transmission (Schenone et al., 2010). This receptor

couples to G_i-protein leading to the inhibition of adenylate cyclase and activation of K^+ channels. As consequence, it renders the postsynaptic cells less excitable, leads to inhibition of Ca^{2+} channels, decreasing the release of excitatory neurotransmitters such as glutamate, acethylcholine and dopamine and to activation of phospholipase C (PLC) (Cunha, 2005; Schenone et al., 2010).

The A1R is expressed in high density in the cerebral cortex, hippocampus, cerebellum, thalamus, brain stem, spinal cord and in the retina (Cunha, 2005; Trincavelli et al., 2010). The A1Rs are mostly found in neurons with a particular density at synapses, where they can act presynaptically refraining the evoked release of excitatory neurotransmitters and postsynaptically controlling Ca^{2+} entry through inhibition of voltage-sensitive Ca^{2+} channels (Cunha et al., 2007).

Several biological studies suggested that, even though the A1R does not play an essential role in the normal physiology of nervous tissue, this receptor has important effects in the pathophysiologic conditions such as noxious stimulation and hypoxia (Schenone et al., 2010). An increase in adenosine levels is associated with several sorts of stress or brain injury, and the activation of A1R appears as an endogenous neuroprotective agent, aimed at limiting the release and damaging effects of excitatory neurotransmistters, such as glutamate (Schenone et al., 2010).

Numerous CNS diseases including Huntington's disease and multiple sclerosis are known to have the involvement of the A1R, and the administration of A1R agonists reveals positive effects. However, chronic administration of such compounds is ineffective probably because of functional desensitization of the receptors. The involvement of A1Rs in Alzheimer disease has been also investigated, but, at the moment, it is not yet clearly understood (Schenone et al., 2010).

1.3.2 Adenosine A_{2A} Receptor

The A2AR couples to G_s-protein, increasing cyclic adenosine monophosphate (cAMP) levels through stimulation of adenylate cyclase, leading to downstream activation of protein kinase A (PKA), cyclic nucleotide-gated ion channels and exchange proteins directly activated by cyclic cAMP (EPACs) (Moreau and Huber, 1999). One key target for PKA is the cAMP responsive binding element (CREB) an important mediator of activity-dependent transcription which is critical for many forms of neuronal plasticity as well as other neuronal functions (Greer and Greenberg, 2008; Trincavelli et al., 2010). Stimulation of these receptors also results in activation of the extracellular signal-regulated kinase (ERK) signaling cascade through a number of different mechanisms that vary between cell types (Palmer, 2011).

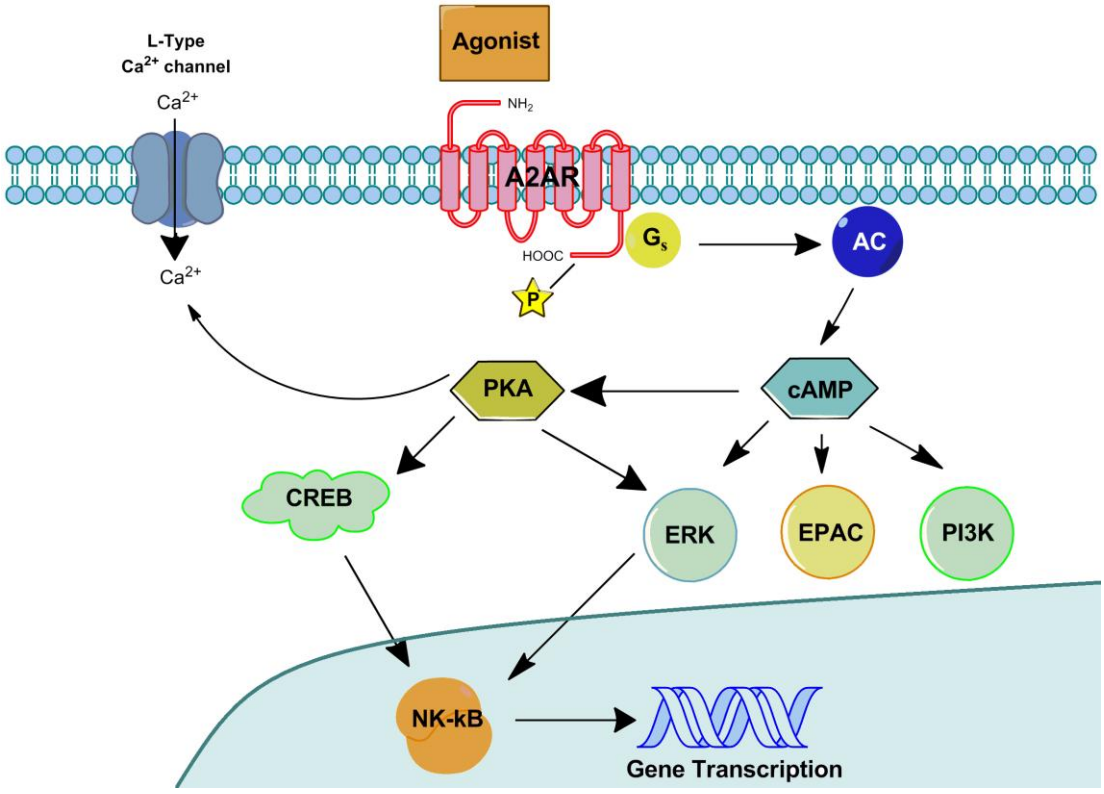

Figure 5 - Simplified overview of the A2AR induced signal transduction. The activation of Gs coupled receptors results in activation of several transcription factors via ERK1/2 activation, and/or CREB (modified from Fredholm, 2007; Schulte and Fredholm 2003). Abbreviations: AC, adenylate cyclase NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PI3K, phosphatidylinositol 3-kinase.

In the CNS, the A2AR can be detected in all brain regions being highly expressed in the striatum, nucleus accumbens and olfactory tubercles especially in GABAergic stratopallidal projection neurons and cholinergic interneurons. A2AR is also expressed in neurons and microglia in most other brain regions (Moreau and Huber, 1999). In the periphery, A2AR is highly expressed in the spleen, thymus, leukocytes and blood platelets, and intermediate levels are found in the heart, lung and blood vessels (Trincavelli et al., 2010).

Numerous investigations in cellular and animal model systems have provided evidence that A2AR signaling pathways are active in limiting inflammation and tissue injury. In different cell types, the expression of A2AR increases after exposure to proinflammatory stimuli, such as TNF-α and IL-1β (Blackburn et al., 2009).

The mechanism by which A2AR impact on neurodegeneration remains to be defined (Cunha et al., 2007). At this moment, there is no consensus about the mechanism by which A2AR blockade confer a robust neuroprotection in noxious conditions. Two leading hypothesis are currently being explored to explain the neuroprotection afforded by A2AR blockade: control of glutamate excitotocity and control of neuroinflammation (Canas, 2009; Cunha, 2005).

An aspect that is important to the understanding of the role of A2AR in the control of inflammation and neuroinflammation is the paradoxical modulation that this receptor has (Sitkovsky, 2003). Activation of A2AR prevents peripheral inflammation, however, in the CNS, it is the blockade of the A2AR that prevents neuroinflammation (Cunha et al., 2007). This contradictory modulation by A2AR can reflect the complexity of A2AR actions on neuronal, glial and vascular components, which may have distinct effects in brain injury (Canas, 2009; Chen et al., 2007; Cunha et al., 2007).

1.3.3 Adenosine A_{2B} Receptor

The adenosine A_{2B} receptor (A2BR) positively couples to adenylate cyclase through G_s proteins, and it can couple to PLC, representing the most important pathway responsible for A2BR mediated effects. It is a low affinity receptor able to activate second messenger systems during limited oxygen availability and it could be mainly activated under pathological conditions (Martinelli and Ortore, 2010; Stone et al., 2009).

The A2BR has been found in several organs, including spleen, lung, colon, and kidney, being the vasculature the primary site of expression in all of these tissues. Smooth muscle cells, endothelial cells and macrophages exhibit a high level of expression (Trincavelli et al., 2010).

The A2BRs are the only subtype that so far has been shown to activate ERK1/2, JNK and p38 pathways, activating the mitogen-activated protein kinase (MAPK) through a mechanism involving G_q proteins, and this effect appears to be relevant for IL-8 secretion and consequent mast cell activation, enhancing the release of inflammatory mediators in addition to pro-inflammatory effects (Martinelli and Ortore, 2010).

Some authors reported a prospective function of the A2BR antagonism due to the stimulation of proliferation, differentiation and migration of retinal endothelial cells, which could inhibit retinal angiogenesis and provide a novel therapeutic approach to the treatment of diseases associated with alterations in the neovascularization, such as diabetic retinopathy (Martinelli and Ortore, 2010). However, in neurodegenerative disorders, such as Alzheimer's disease, activation of these receptors might have neuroprotective implications, since IL-6 released from astrocytes upon A2BR activation is protective against hypoxia and glutamate neurotoxicity (Martinelli and Ortore, 2010).

1.3.4 Adenosine A3 Receptor

The adenosine A3 receptor (A3R) is the less well studied receptor. It was originally cloned in 1991 as an orphan receptor from rat testis, and subsequently cloned from a variety of species (Stone et al., 2009; Taliani et al., 2010). The A3R is expressed in the hypothalamus and thalamus, but at relatively low levels. The highest levels of these receptors have been found in the lung and liver, but they are believed to occur on neuronal and glial cells membranes in most species, including human (Stone et al., 2009; Trincavelli et al., 2010).

Classically, A3R couples to the G_i protein, which inhibits adenylate cyclase, and to the G_q protein that stimulates PLC, inositol triphosphate and the uptake of Ca^{2+}. The A3R has been implicated in mediating allergic responses, airway inflammation and apoptotic events. Furthermore, it is involved in the control of the cell cycle and inhibition of tumor growth. In fact, it has been demonstrated to be more expressed in tumoral cells then in healthy cells, suggesting a role as a tumor marker (Taliani et al., 2010; Trincavelli et al., 2010).

1.3.5 Adenosine and Neuroprotection

Neuroprotection can be defined as the use of therapeutic agents to prevent, hinder, and in some instances reverse neuronal cell death whatever the primary injury. Several neuroprotective treatments have been established in CNS diseases, such as for Alzheimer's, Parkinson's and Huntington's diseases (Cheung et al., 2008).

Adenosine has neuroprotective properties, since it is able to decrease excitatory amino acid release, hyperpolarize the neuronal membrane, restrain the activation of NMDA receptors, limit Ca^{2+} influx, inhibit free radical formation and exert modulatory

effects at astrocytic and microglial cells (de Mendonça et al., 2000; Gomes et al., 2011; Liu et al., 2009; Stone et al., 2009).

The adenosine neuromodulatory role relies on a balanced activation of inhibitory A1R and facilitatory A2AR, mostly controlling excitatory synapses: A1R imposes a tonic brake on excitatory transmission, whereas A2ARs are selectively engaged to promote synaptic plasticity phenomena (Gomes et al., 2011). Activation of A1R only effectively controls neurodegeneration if activated in the temporal vicinity of CNS insults; in contrast, the blockade of A2AR alleviates the long-term burden of CNS disorders in different neurodegenerative conditions (Gomes et al., 2011).

The acute administration of A1R agonist or the use of strategies aimed to enhance the extracellular levels of adenosine afford neuroprotection against different types of insults both *in vivo* and *in vitro* models (Cunha, 2005).

Evidence has accumulated supporting the hypothesis that released adenosine activates A1Rs, and plays a neuroprotective role during hypoxia (Leshem-Lev et al., 2010). Activation of A1R protects against ischemic brain injury in adult animals in global or transient ischemia (Rudolphi et al., 1992) and against other brain noxious stimulus such as excitotoxicity induced by kainate (KA) and quinolinic acid (MacGregor et al., 1997) or against dopaminergic neurotoxicity (Delle Donne and Sonsalla, 1994). On the other hand, the acute administration of A1R antagonists aggravates brain damage (Cunha et al., 2007; de Mendonça et al., 2000). However, the A1R system is prone to a rapid desensitization and the neuroprotection that it affords is time-limited, once the effects operated by these receptors undergo desensitization upon chronic noxious brain conditions (Cunha, 2005; Cunha et al., 2007).

Neuroprotection by an antagonist of A2AR was first described in the gerbil brain against ischemic damage (Gao and Phillis, 1994; Stone et al., 2009). Other studies

indicated that activation of A2AR can produce protection of the CNS against several insults, including excitotoxins such as KA (Jones et al., 1998), glutamate (Pintor et al., 2004) and quinolinic acid (Scattoni et al., 2007; Stone et al., 2009). The potential neuroprotective effect of A2AR blockade in Parkinson's disease is further substantiated by caffeine or specific A2AR antagonists (Ross and Petrovitch, 2001) in the attenuation of dopaminergic neurotoxicity and neurodegeneration in animal models of Parkinson's disease. Moreover, A2AR antagonism-mediated neuroprotection can be extended to a variety of other brain injuries induced by stroke, excitotoxicity and mitochondrial toxins (Kalda et al., 2006).

The activation of A2AR promotes responses that are benefic to the cell, such as trophic and anti-inflammatory effects (Milne and Palmer, 2011). However, pharmacological, neurochemical, molecular and/or genetic approaches to the complex actions of A2AR in different cellular elements suggest that A2AR activation can be detrimental or protective after brain insults, depending on the nature of brain injury and associated pathological conditions (Chen et al., 2007).

The mechanism for the neuroprotection afforded by A2AR blockade has not yet been elucidated (Stone et al., 2009). One currently open hypothesis is the ability of the blockade of the A2AR to control the excitotoxicity mediated by glutamate release (Cunha et al., 2007). Glutamate excitotoxicity has been implicated in acute injury to the CNS and in chronic neurodegenerative disorders, such as Alzheimer's, Parkinson's and Huntington's diseases (Choi, 1988; Lee et al., 1999; Lipton and Rosenberg, 1994). Several studies identified A2AR as being responsible for the release of glutamate in noxious situations (Melani et al., 2003; Popoli et al., 2007). Therefore, blockade of A2AR can be used as a neuroprotective strategy, by preventing glutamate-induced excitotoxicity that is a major feature in several neurodegenerative disorders.

The other raising possibility is the neuroprotection afforded by the blockade of A2AR, which may result from the control of glia cell-mediated neuroinflammation (Cunha et al., 2007).

1.3.6 Adenosine and neuroinflammation

A burst of interest in the role of neuroinflammation in the demise of CNS degeneration has followed the increased recognition that inflammatory features seem to be present in most conditions of CNS damage, either acute traumatic conditions or neurodegenerative disorders (Cunha et al., 2007). Evidences demonstrate that adenosine is a key signaling molecule and ARs are important molecular targets in the pathophysiology of inflammation. The consequences of AR activation have been investigated and have identified numerous approaches for adenosine-based therapeutic intervention (Blackburn et al., 2009).

Although neuroinflammation is present in different conditions of brain damage, it should be made clear that it may play a dual role, possibly contributing for brain damage, but also for the repair and regeneration of brain tissue (Canas, 2009).Some actions evoked by the A2AR activation, such as potentiation of glutamate release, NO release and microglial activation may be related with inflammatory processes and this can indicate an important role of the inflammatory control in the neuroprotective processes mediated by the A2AR. (Cunha, 2005; Cunha et al., 2007).

There is a paradoxical modulation by A2AR of peripheral inflammation and neuroinflammation in chronic brain noxious conditions. In the control of peripheral inflammation (where the activation of A2AR affords protection) A2AR might have opposite effects compared to the control of neuroinflammation-associated with chronic

damage to CNS (where the evidences indicate that it is the blockade of A2AR that affords protection) (Cunha et al., 2007).

At a peripheral level, findings in disease-relevant animal models suggest that A2AR activation on immune cells is beneficial in environments associated with acute inflammation and hypoxia (Sitkovsky et al., 2004). Agonists of A2AR have remarkable anti-inflammatory and tissue protective effects in models of ischemic liver damage to periphery organs (Blackburn et al., 2009; Day et al., 2005).

In contrast to this clear effect of the A2AR as main "OFF" signal of the peripheral inflammatory system, the role of A2AR in the control of immune responses in the CNS is considerably less explored and certainly less clear (Cunha et al., 2007). An obvious difference between central and peripheral inflammation resides in the type of cells involved in these two processes and the existence of cell types in the brain (microglia and astrocytes) that do not participate in peripheral inflammation (Cunha et al., 2007). This contradictory modulation by A2AR can reflect the complexity of A2AR actions on neuronal, glial and vascular components, which may have distinct effects in brain injury (Chen et al., 2007).

In conditions of acute damage to the CNS, it appears that activation of A2AR may attenuate neuronal damage, possibly through the control of inflammatory processes in brain. However, if A2ARs are activated long after injury the receptor leads to a deleterious effect and it is the A2AR antagonist that is now able to afford tissue protection and functional recovery (Cunha et al., 2007). However, in a chronic noxious stimulus, the blockade of A2AR mediates neuroprotection, by controlling neuroinflammation, for example in Parkinson's disease models (Kalda et al., 2006) and in ischemia models (Yu et al., 2004).

The lack of A2AR in transgenic animals or the administration of antagonists is neuroprotective in animal models of Parkinson's disease, Huntington's disease, ischemic stroke or excitotoxic neuronal death (Rebola et al, 2011; Saura et al., 2005). Even though, the mechanisms by which A2AR blockade controls brain damage and affords neuroprotection have not yet been elucidated. An A2AR antagonist is currently in phase IIb of a clinical trial as anti-Parkinsonian, based on the simultaneous ability to normalize motor function and afford marked neuroprotection (Rebola et al, 2011). The main hypothesis for the control of CNS damage mediated by A2AR modulation would be that A2AR stimulation modulates astroglial and/or microglial cell function, resulting in deleterious effects for surrounding neurons, due to the increased release of inflammatory mediators by activated microglia (Rebola et al, 2011; Saura et al., 2005).

Several evidences, in *in vitro* models and in neurodegenerative disease models, point toward the A2AR signaling as an endogenous protective anti-inflammatory and immunosuppressive system in the treatment of many diseases where inflammation is a detrimental component, while A2AR antagonists may be beneficial in the treatment of neurological disorders.

Control of neuroinflammation mediated by A2AR blockade might be the strongest candidate mechanism to explain the neuroprotection against brain injury afforded by A2AR blockade in adult animals. However, it seems that this conclusion might only be valid for particular conditions of brain injury where neuroinflammation acquires a chronic profile (Blackburn et al., 2009; Cunha et al., 2007).

1.3.7 Adenosine in the retina

Adenosine is present in all cells and body fluids and is known to function as a modulator of a variety of biological processes (Kvanta et al., 1997).

In the cat and rabbit retinas, it was demonstrated, by autoradiographic techniques, that adenosine is present in the GCL and INL (Blazynski et al., 1989). The same work demonstrates that adenosine has complex modulatory effects, involving RPE, neuronal structures, blood vessels and glial cells (Blazynski et al., 1989). It has also been demonstrated that endogenous adenosine is present in the GCL in rodents (Braas et al., 1987). This work also revealed that the distribution of AlR sites closely parallels that of retinal neurons and fibers, suggesting a role for endogenous adenosine as a co-neurotransmitter in ganglion cells and their fibers in the optic nerve (Braas et al., 1987).

In ocular tissue, adenosine has been suggested to regulate intraocular pressure (IOP) (Crosson, 1995), corneal endothelial ion transport (Riley et al., 1996) retinal and choroidal blood flow (Gidday and Park, 1993), the hypoxic induction of vascular growth factor and its receptors on retinal capillary endothelial cells and pericytes (Takagi et al., 1996).

Purine release experiments have demonstrated that adenosine and ATP are released from rabbit and chick retina and that adenosine deaminase is active in the retina (Blazynski and Perez, 1991; Kvanta et al., 1997). Biological, pharmacological and anatomical studies have provided convincing evidences for the presence of ARs in the retina of several species (Blazynski and Perez, 1991). Using in situ hybridization, it was demonstrated the expression of mRNA for A1R, A2AR and A2BR in the rat eye (Kvanta et al., 1997). The expression of A1R mRNA was mainly detected in GCL, although some cells within the INL also express A1R. The A2AR mRNA in the retina was mainly found in the INL and GCL and to a lesser extent in the ONL. The A2AR transcripts appeared to be expressed in most cells in the GCL, suggesting the expression of A2AR also in endothelial cells (Kvanta et al., 1997).

It has been suggested that the activated A2AR in retinal microglial cells plays a major anti-inflammatory role in the retina, being the cannabinoids anti-inflammatory effects linked to the inhibition of adenosine uptake in the retina (Liou et al., 2008). A more recent study has demonstrated, for the first time, that RGCs express the A3R (Zhang et al., 2006).

Several *in vitro* studies have shown that adenosine can protect against different insults. Adenosine blocked the glutamate or KA-induced cell death in cultures of chick retinal neurons (Ferreira and Paes-de-Carvalho, 2001). Furthermore, adenosine regulates the survival of developing retinal neurons by a long-term activation of A2AR and the increase of cAMP levels (Paes-de-Carvalho et al., 2003).

Adenosine is a major component of the retina's endogenous reaction to ischemia (Li et al., 1999). In the rat retina, the increases in the concentration of adenosine and its metabolites depend upon the duration of ischemia, and the concentrations remained elevated during the subsequent reperfusion period (Roth et al., 1996). In the ischemia-reperfusion injury adenosine plays an important protective role possibly through activation of A1R and/or blockade A2AR, which have a well established neuroprotective effect in several CNS areas where the nucleoside is released during hypoxia and ischemia (Paes-de-Carvalho, 2002).

Ischemic preconditioning protects the rat retina against the injury that ordinarily follows severe ischemia (Roth et al., 1998). The involvement of A1R and A2AR is required for ischemic preconditioning protection, increasing the expression of protective proteins and decreasing the expression of pro-apoptotic proteins (Roth, 2004).

The A3R is responsible for attenuating intracellular calcium increase after insult of NMDA (Zhang, 2010). The A3R also protects RGC after stimulation of receptors associated with cell death (Zhang et al., 2006).

1.4 Glaucoma

Glaucoma is the second cause of irreversible blindness, affecting approximately 70 million people, and approximately 2% of the population over the age of 40 (Cheung et al., 2008; Fuse, 2010). It is defined as a group of chronic degenerative optic neuropathies characterized by their irreversible and progressive loss of RGCs and their axons accompanied by excavation and degeneration of the optic nerve head (ONH) which leads to visual field loss (Chiu et al., 2010).

Glaucoma is a multifactorial disease where family history, systemic hypertension, diabetes and cigarette smoking are known risk factors associated with the disease development, but the main risk factor is elevated intraocular pressure (IOP) (over 21.5 mmHg) is the major risk factor (Qu et al., 2010). Elevated IOP has long been thought to increase the risk of glaucoma by causing abnormalities of the ONH at the level of the lamina cribrosa, affecting the intracellular transport within the RGCs axons or by causing vascular abnormalities that lead to ischemic damage (Caprioli and Coleman, 2010).

Increased IOP is frequently a consequence from alterations in aqueous humor dynamics due to changes in the trabecular meshwork, which leads to impaired drainage of aqueous humor. Some studies report a relationship between changes in IOP and RCG death in glaucomatous rats, and a positive association has also been observed between the level and duration of elevated IOP and RCG loss, since the loss of half of the RGCs occurs during the initial two to three months of elevated IOP (Kaushik S. et al., 2003; Qu et al., 2010). However, the central role of raised IOP is being questioned. Among glaucoma patients, only one-third to half have elevated IOP at the initial stages and, on average, 30 to 40% of the patients with glaucomatous visual loss are being diagnosed as having normal tension glaucoma (Agarwal et al., 2008; Kaushik S. et al., 2003). It has become clear that in addition to pressure control, neuroprotective measures are relevant in

the treatment of glaucoma, such as neuroprotection of RGCs and the central visual pathway neurons (Cheung et al., 2008; Naskar, 2002).

Progressive degeneration of retinal RGCs and their axons is the primary cause of glaucomatous visual loss. Even though, growing evidence now supports that not only the events intrinsic to RGCs, but also environmental signals from other cells are critical to overcome cell death stimuli, and RGC-glia interactions are critically important for different aspects of glaucomatous neurodegeneration (Tezel, 2009). The process of RGC death is thought to be biphasic: a primary injury responsible for the initiation of damage that is followed by a slower secondary degeneration related to noxious environment surrounding the degenerating cells, which may include excitotoxicity damage caused by glutamate release, and oxidative damage caused by over-production of NO and other ROS (Agarwal et al., 2008; Kaushik S. et al., 2003). Despite these evidences, the pathophysiology of glaucomatous optic neuropathy is still not well understood, and it remains uncertain whether the primary damage is in the RGCs body or in their axons. Nevertheless, no matter the initial site of neuronal injury and the mechanism involved, the terminal outcome is the death of RGCs and their axons, which leads to an irreversible visual loss (Agarwal et al., 2008).

1.4.1 Neuroinflammation in Glaucoma

During glaucomatous neurodegeneration, signals arisen from the surrounding environment are critically important for the RGCs fate. Macroglial cells, including astrocytes and Müller cells, constitute the major cell type exhibiting important homeostatic interactions with RGCs. Another glial cell type, also having important impacts in glaucomatous neurodegeneration, is microglia. Studies have reported the progressive degeneration of optic nerve axons and RGCs in human glaucoma

accompanied by chronic alterations in structural and functional characteristics of glial cells in the ONH and retina (Baltmr et al., 2010; Tezel, 2009).

Several studies about the role of glial cells in glaucoma have shown that the high level of plasticity of glial cells allows them to rapidly respond to any homeostatic imbalance by exhibiting a phenotype commonly referred to as activated, suffering dramatic alterations in cell morphology, gene expression involved in signal transduction, cell proliferation, cell interaction, cell adhesion, extracellular matrix synthesis, and immune response (Tezel, 2009). In human glaucoma there is an abnormal microglia reactivity and redistribution within the ONH, where the optic nerve pathology is first detectable (Quigley, 1983). However, the mechanism controlling microglial recruitment and activation in human glaucoma or animal models are not established, and it is unclear when during disease microglia undergo these changes (Bosco et al., 2011) A recent study have demonstrated that microglial activation is an early event in experimental glaucoma that coincides with the onset of RGC death and suggests that microglia may play a role in the initiation of RGC loss (Taylor et al., 2011).

Although the relationship of glial reactivity to neurodegeneration in glaucoma has not been established, increased production of some inflammatory mediators like NO (Liu and Neufeld, 2000; Neufeld et al., 1997) and TNF-α (Yuan and Neufeld, 2000) may contribute to an environment that is directly or indirectly neurotoxic and also inhibitory for axonal regeneration in the RGCs in glaucoma (Baltmr et al., 2010; Tezel, 2000).

TNF-α is a potent immunomediator and proinflammatory cytokine that is rapidly upregulated in CNS after injury (Liu et al., 1994). TNF-α is synthesized and released from astrocytes and microglia in the CNS, where it plays a crucial role in several diseases. TNF-α can trigger a caspase-dependent mitochondrial cell death promoting

pathway that activate caspase 8, which in turn activates caspase 3, promoting apoptotic cell death (Baltmr et al., 2010; Tezel, 2000).

In the eye tissues, increased production of TNF-α has been detected in several diseases, including glaucoma (Yan et al., 2000; Yuan and Neufeld, 2000). In a mouse model of glaucoma, TNF-α mRNA is upregulated in the retina, with RGC and oligodendrocyte cell loss and consecutive optic atrophy (Balaiya et al., 2011). Moreover, TNF-α through binding to its receptor TNF-R1, and in association with JNK signaling pathway, has also been proposed to be a mediator of RGC death in glaucomatous optic nerve degeneration (Tezel, 2004; Tezel et al., 2001).

Nitric oxide is an important physiological and pathological inflammatory response mediator, which is synthesized by several isoforms of NOS: neuronal NOS (nNOS) and endothelial NOS (eNOS) which are constitutively present in a variety of cells, and iNOS, which can synthesize excessive amounts of NO that might be cytotoxic to neighboring cells (Yuan and Neufeld, 2000). Increased production of NO in retinal glial cells that have been exposed to different stress conditions induce RGC death (Tezel, 2000). In glaucomatous ONH, iNOS is expressed in reactive astrocytes but not in normal ONH, where it may be neurodestructive, locally, to the axons of RGCs (Liu and Neufeld, 2000; Neufeld et al., 1997).

The sustained neuronal damage in glaucoma can trigger immune responses. Among diverse roles of glial cells during glaucomatous neurodegeneration as neurosupportive or neurodestructive, one is linked to their immunoregulatory functions. Given their roles in phagocytosis, glial cells, mainly including microglia, are important components of immune surveillance involved in protection of the injured tissue (Tezel, 2010). Similarly to CNS glia, retinal and optic nerve glial cells express MHC molecules and function as resident antigen presenting cells, which can be extreme upon

glaucomatous neurodegenerative conditions, thereby enhancing the inflammatory cascade leading to tissue damage (Baltmr et al., 2010; Tezel, 2009; Tezel, 2010).

A critical dynamic balance of cellular interaction and intracellular pathways determines neuronal fate in response to stressful conditions. Immune response to a stressful insult may initially be beneficial in limiting neurodegenerative consequences. However, growing evidences support that failure to properly control immune activity may subsequently convert protective immunity into an auto-immune neurodegenerative process in glaucoma, resulting in much more extensive neuronal injury and glial dysfunction (Tezel, 2009).

Modulation of the neuroinflammatory response in glaucomatous neurodegeneration would probably be an interesting approach to control the RGCs injury and death during glaucoma. The control of the TNF-α signaling pathway or the modulation of microglial reactivity are two important possible therapeutic targets to take into account during the development of new biological studies in the glaucomatous degeneration field.

1.5 Objectives of the study

Glaucoma is the second leading cause of blindness and is characterized by RGCs death.

Adenosine is a neuromodulator that operates via G-protein-coupled receptors (A1R, A2AR, A2BR and A3R). Several evidences have shown that the blockade of A2AR confers potent neuroprotection in several CNS noxious conditions. The mechanism by which the blockade of A2AR is neuroprotective in neurodegenerative models is currently unclear. However, several hypothetic mechanisms have been postulated. It can involve control of glutamate excitotoxicity, the control of apoptosis or the control of neuroinflammation. In glaucoma, neuroinflammation plays an important role, with microglia releasing pro-inflammatory cytokines, which may contribute to RGC death.

Therefore, the main aim of this project was to investigate the ability of A2AR to control retinal microglial reactivity induced by a pro-inflammatory stimulus (exposure to LPS). In order to achieve this goal, two different culture preparations were used: primary retinal mixed cultures and purified cultures of retinal microglial cells. Microglial activation status upon a pro-inflammatory stimulus was evaluated in the presence or absence of selective A2AR agonist or antagonist.

Chapter 2 - Materials and Methods

2.1 Materials

LPS, 4',6-diamidino-2-phenylindole (DAPI), latex beads (amine-modified polystyrene, fluorescent yellow-green), and Sulfanilamide, N(1-naphtyl) ethylenodiamine were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Gentamicine, fetal bovine serum and Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) with GlutMAX were acquired from Invitrogen (Life Technologies, Carlsbad, CA, USA) Macrophage colony stimulating Factor (M-CSF) and the antibody rabbit ant-TNF-α were bought from Peprotech (London, UK).

4-[2-[[6-amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid (CGS21680) and 2-(2-Furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH58216), were obtained from Tocris Bioscience (Cambridge, UK). In situ cell death detection kit, fluorescein (TUNEL) was purchased from Roche (Basel, Switzerland).

Primary antibody mouse anti-Cd11b was purchased from AbdSerotec (Oxford, UK). Primary antibody goat anti-A2A was acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody rabbit anti-iNOS was obtained from BDBiosciences (Franklin Lakes, NJ, USA) and the mouse anti-iNOS from Abcam (Cambridge, UK). Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 488 mouse anti-goat, Alexa Fluor 568 goat anti-mouse secondary antibodies and 4-amino-5-methylamino-2′,7′-difluorescein diacetate (DAF-FM diacetate) were obtained from Molecular Probes (Invitrogen, Life Technologies, Carlsbad, CA, USA).

All other reagents were acquired from Sigma-Aldrich Corporation (St. Louis, MO, USA).

2.2 Primary retinal cell cultures

Primary cell cultures were prepared from the retinas of 3-4 days old Wistar rats as described previously (Santiago et al., 2006). Briefly, rats were euthanized by decapitation, and after enucleation the retinas were dissected in a Ca^{2+} and Mg^{2+} free Hanks balanced salt solution (HBSS in mM: 137 NaCl, 5.4 KCl, 0.45 KH_2PO4, 0.34 Na_2HPO_4, 4 $NaHCO_3$, 5 glucose; pH 7.4). The retinas were digested with 0.1% trypsin (w/v) for 12 minutes at 37ºC. After dissociation, cell suspension was centrifuged and the cells were ressuspended in Eagle's minimum essential medium (MEM) supplemented with 26 mM $NaHCO_3$, 25 mM HEPES, 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml).

The cells were plated at a density of $2x10^6$ cells/cm^2, on 12-well plates with glass coverslips, for immunocytochemistry pre-coated with poly-D-lysine (0.1 mg/ml) and maintained at 37ºC humidified atmosphere of 5%CO_2, for seven days.

The mixed primary cultures contain microglial cells, astrocytes and Müller cells, and retinal neurons.

2.3 Microglial cell cultures

Microglial cell cultures were prepared as described previously (Liou et al., 2008), with some minor modifications. The mixed retinal cell culture was obtained from the retinas of 7-9 days old Long Evans rats, as described above. The cells were plated at a density of $1.5x10^6$ cells/cm^2 in T75-culture flasks, coated with poly-D-lysine (0.1 mg/ml) and maintained at 37ºC under humidified atmosphere of 5% CO_2, for three weeks, in DMEM-F12 with GlutaMAX I, supplemented with 10% heat-inactivated FBS, 0.1% gentamicin and 2 ng/ml M-CSF.

Microglial cells were obtained from the mixed primary culture by shaking. The culture flasks were placed in an orbital shaker, at 200 rpm for 120 minutes, at 37ºC

under humidified atmosphere of 5% CO_2. The cells were collected by centrifugation, and plated at a density of $1.3x10^6$ cells/cm^2 on 12-well plates with glass coverslips, for immunocytochemistry, DAF-FM DA fluorescence, coated with poly-D-lysine (0.1 mg/ml) and maintained at 37ºC under humidified atmosphere of 5%CO_2, for three days.

2.4 Cell treatment

Cell cultures were incubated with LPS (1 ng/ml), for 24 h. Cells were pre-treated with adenosine A2AR agonist (30 nM CGS21680) or antagonist (50 nM SCH58261) for 45 minutes before LPS incubation.

2.5 Immunocytochemistry

The cells were washed with phosphate-buffered saline (PBS; in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, and 1.8 KH_2PO_4; pH 7.4) and fixed with 4% paraformaldehyde with 4% sucrose for 10 minutes. After washing in PBS, cells were permeabilized in 1% Triton X-100 in PBS for 5 minutes. Cells were blocked with 3% BSA and 0,2% Tween, in PBS, for 60 minutes, and then incubated with the primary antibody (mouse anti-Cd11b at a dilution of 1:100, rabbit anti-iNOS at a dilution of 1:100, rabbit anti-TNF-α at a dilution of 1:100 or goat anti-A_{2A}R at a dilution of 1:50, in blocking solution), for 90 minutes. Following washing in blocking solution, cells were incubated with the secondary antibody (Alexa Fluor-568 goat anti-mouse IgG at a dilution of 1:200, Alexa-Fluor-488 goat anti-rabbit IgG at a dilution of 1:200 or Alexa-Fluor-488 rabbit anti-goat IgG at a dilution of 1:200, in blocking solution), for 60 minutes. The cells were then washed in PBS and incubated with DAPI (1:2000) for 10 minutes, to stain nuclei. After washing the cells, the coverslips were mounted with Glycergel mouting medium.

All coverslips were observed with a confocal microscope (LSM 710, Zeiss) and the settings and exposure times were kept identical for all the control and stimulated conditions, to obtain an accurate representation of the differences in immunoreactivity intensities of iNOS and A2AR. Densitometric analysis for the different antibodies was performed using the public domain ImageJ program (http://rsb.info.nih.gov/ij/) and analyzed using the Graphpad Prism 5 Software.

2.6 Nitrite Quantification assay

NO production was assessed by Griess reaction, a colorimetric assay that involves a diazotization reaction to spectrophotometrically detect nitrite formed by the spontaneous oxidation of NO under physiological conditions.

The culture medium was collected and centrifuged to remove cell debris, and incubated (1:1) with Griess reagent mixture (1% sulfanilamide, in 5% phosphoric acid with 0.1% N-1-naphtylenediamine) for 30 minutes, in dark conditions. The optical density was measured at 550 nm using a microplate reader (Synergy HT; Biotek, Winooski, USA).

The nitrite concentration was determined from a sodium nitrite standard curve.

2.7 Terminal transferase dUTP nick end labeling (TUNEL) staining

The TUNEL assay is a method used to detect and quantify cell death (apoptosis) by detection and labeling of DNA strand breaks in individual cells by fluorescence microscopy.

The cells were processed for immunocytochemistry against Cd11b, as described previously. After the incubation with the secondary antibody, the cells were washed in PBS and incubated with the TUNEL reaction mixture containing the enzyme and

fluorescein dUTP, for 60 minutes, in a humidified chamber. The cells were then washed in PBS and incubated with DAPI (1:2000) for 10 minutes, to stain nuclei. After washing the cells, the coverslips were mounted with Glycergel mouting medium.

All coverslips were observed with an inverted fluorescence miscroscope (Leica DM IRE2) and the number of Cd11b-positive and TUNEL-positive cells was counted. The results were analyzed using the Graphpad Prism 5 software.

2.8 Nitric Oxide Quantification by DAF-FM diacetate

The NO indicator DAF-FM diacetate was employed to detect NO production. Briefly, the cell medium was collected and stored, and the cells were incubated with 8 µM DAF-FM in Krebs-Henseleit Ringer solution (KHR; in mM: 140 NaCl, 1 EDTA, 10 HEPES, 3 KCl, 5 glucose; pH 7.4) for 60 minutes at 37ºC in a humidified atmosphere of 5% CO_2. Then, the DAF-FM solution was replaced by previously collected medium, and the cells placedin cell incubator for 24 h. Next, the cells were processed for immunocytochemistry, as described above.

Cells were observed with a confocal microscope (LSM 510, Zeiss), and from each condition, at least seven random fields were acquired. Densitometric analysis for the DAF-FM fluorescence was performed using the public domain ImageJ program (http://rsb.info.nih.gov/ij/) and analyzed using the Graphpad Prism 5 software.

2.9 Phagocytosis assay

Cells were incubated with 0.0025% fluorescent 1 µm diameter latex beads for 75 minutes at 37ºC, in the cell incubator. After the incubation, cells were fixed, permeabilized and blocked as described above. The cells were then incubated with the primary antibody mouse anti-cd11b (1:100) for 90 minutes at room temperature,

followed by incubation with the secondary antibody, AlexaFluor 568 goat anti-mouse (1:200) for 60 minutes. Nuclei were counterstained with DAPI, as described previously, and mounted in Glycerogel mounting medium.

Cells were observed with an inverted fluorescence microscope (Leica DM IRE2), and from each condition, at least seven random fields were acquired. The number of Cd11b-positive cells accumulating beads and the number of beads per Cd11b-positive cell were counted and analyzed using the Graphpad Prism 5 Software.

2.10 Statistical analysis

The results are presented as mean ± standard error of the mean (SEM). The data were analyzed using one-way analysis of variance (ANOVA), followed by Bonferroni's or Dunnett's post-hoc test. The statistical analysis was performed in Prism 5.0 Software (GraphPad Software). p values less than 0,05 were taken as significant.

Chapter 3 - Results

3. Results

In this work, we aimed to study whether the modulation of A2AR controls retinal microglial cell reactivity, using primary retinal mixed neuronal cultures and purified retinal microglial cultures.

3.1 Blockade of A2AR prevents microglial cell activation induced by LPS in primary retinal mixed cultures

Modification of microglial cell morphology is one of the hallmarks of its activation profile and has been widely used to categorize different activation states. LPS is a part of Gram negative bacteria wall and is a classical activator of the immune system, including microglial cells. Therefore, cells were exposed to LPS (1 ng/ml for 24 hours) to mimic an inflammatory condition. The morphology of microglial cells was assessed by immunocytochemistry using an antibody anti-Cd11b, a marker of microglia (Jensen et al., 1997). In control conditions, most of microglial cells (Cd11b-positive cells) were ramified, as assessed by the ratio non-ramified/ramified Cd11b-positive cells (Figure 6), indicating that microglial cells are in a non reactive state. Treatment of cells with LPS changed the morphology of microglia to a large and non-ramified shape, a phenotype that is characteristic of activated or reactive microglia. Pre-treatment with A2AR agonist (CGS21680, 30 nM) significantly increased the number of cells with a round morphology induced by LPS (which corresponds to a fold increase of 1.6 as compared to LPS). When cells were pretreated with 50 nM SCH58261 (A2AR antagonist), the number of cells with ramified morphology was similar to the control.

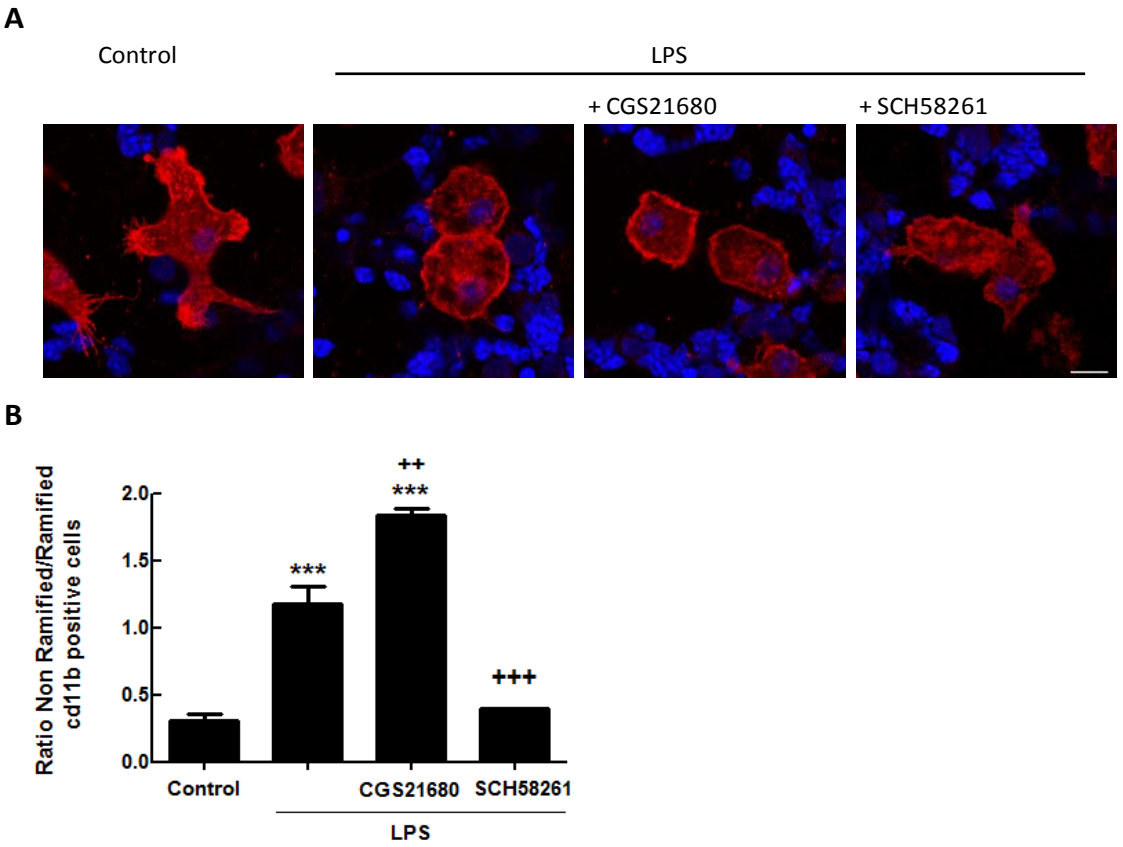

Figure 6 - Blockade of adenosine A2AR prevents microglial cell activation induced by LPS in primary retinal mixed cultures. Cells were challenged with 1 ng/mL LPS in the absence or in the presence of 30 nM CGS21680 or 50 nM SCH58261. The morphology of retinal microglial cells immunoreactive to Cd11b (marker of microglial cells) was evaluated 24 hours later. **(A)** Representative images for each condition. Cells were stained with an antibody anti-Cd11b (red). Nuclei were counterstained with DAPI (blue). **(B)** The number of round and ramified microglia (active microglia) and ramified microglia (resting microglia) was determined by counting more than 30 random fields with Zeiss LSM 710 confocal microscope. Results represent the non-ramified /ramified cells ratio [and are expressed as mean ± SEM, from at least three independent experiments. ***$p<0.001$, significantly different from control; one-way ANOVA followed by Dunnett's post-hoc test; +++$p<0.001$, significantly different from LPS condition; one-way ANOVA followed by Bonferroni's post-hoc test. Bar: 20 µm.

3.2 Effect of A2AR blockade in LPS-induced NO production in primary mixed retinal cultures.

Previous data have reported that exposure of microglial cells to LPS leads to the production of inflammatory and cytotoxic factors like NO, both *in vivo* and *in vitro* (Block, 2010; Chao et al., 1992).

The effect of the modulation of A2AR on the production of NO was assessed indirectly by evaluating the iNOS immunoreactivity (Figure 7A) and by nitrites quantification using the Griess reaction (Figure 7B). In control conditions, iNOS immunoreactivity was almost undetectable, indicating that the expression of this enzyme in basal conditions was very low, and it was not restricted to microglial cells. After LPS incubation, the immunoreactivity of iNOS increased, particularly in microglial cells (Cd11b-positive cells). When cells were pre-treated with the A2AR agonist CGS21680 the LPS-induced iNOS immunoreactivity showed a tendency to increase, while the A2AR antagonist SCH58261 decreased iNOS immunoreactivity induced by LPS. By Griess reaction (Figure 7B) nitrites concentration was determined, and in the control it was 1.0±0.19 µM. The nitrites concentration significantly increased to 7.7±0.46 in LPS-treated cells. Pre-treatment with A2AR agonist or antagonist did not significantly change nitrites concentration, when compared with LPS.

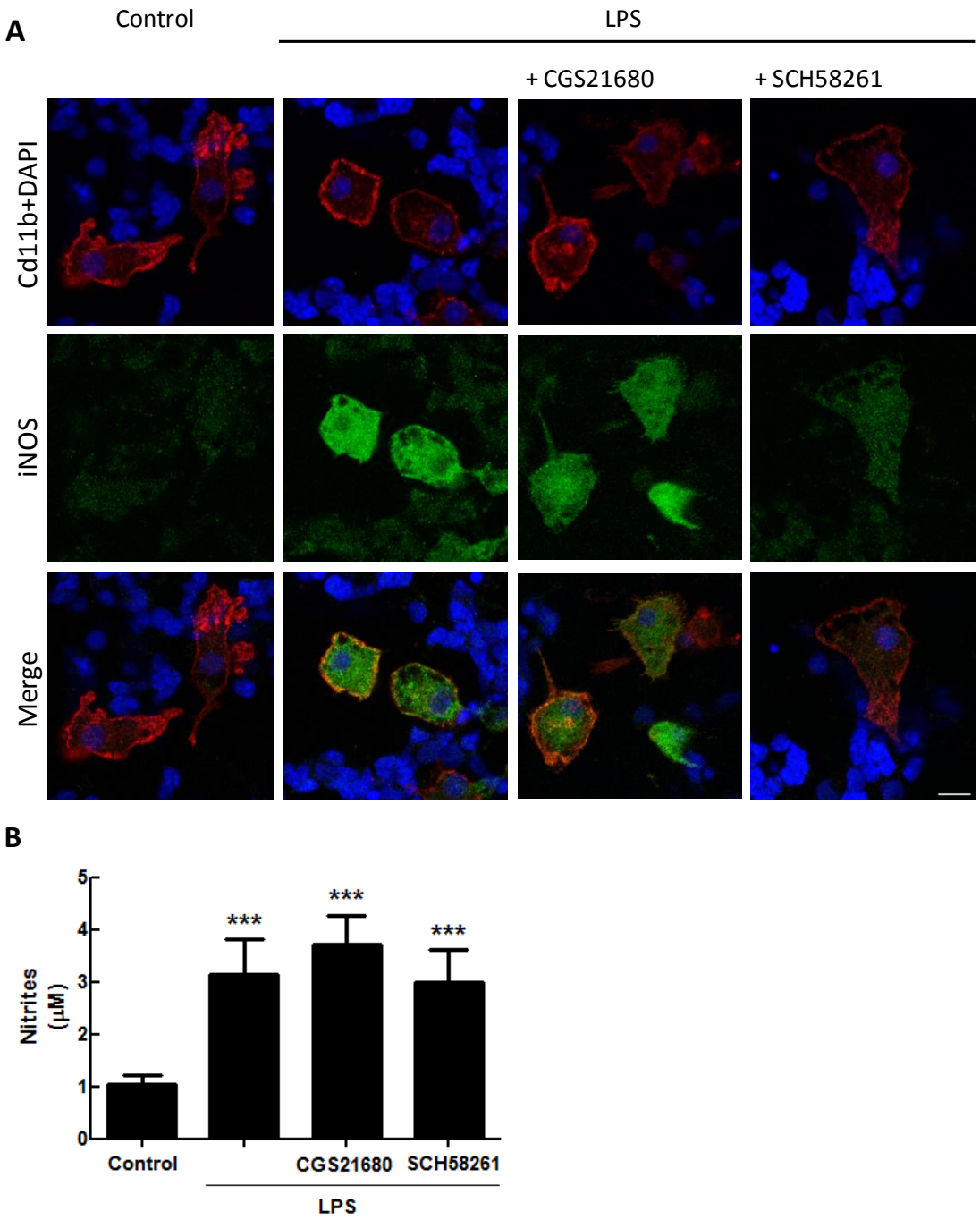

Figure 7 - The number of microglial cells with increased iNOS immunoreactivity induced by LPS is decreased by A2AR blockade, without changes in nitrites production. Cells were challenged with 1 ng/mL LPS in the presence of 30 nM CGS21680 or 50 nM SCH58261. **(A)** Representative images of Cd11b (red) and iNOS immunoreactivity (green). Nuclei were counterstained with DAPI (blue). **(B)** Nitrite levels were determined in the culture medium supernatant by the Griess reaction. Results represent nitrite concentration (µM) and are expressed as mean ± SEM, from at least five independent experiments, performed in duplicate. ***p<0.001, significantly different from control; one-way ANOVA followed by Dunnett's post-hoc test. Bar: 20 µm

3.3 Microglial cells express A2AR in primary retinal mixed cultures

The primary mixed retinal neural culture contains neurons, astrocytes, Müller cells and microglia (Santiago et al., 2006). It was already documented that retinal microglial cells express A2AR mRNA (Liou et al., 2008). In addition, it was also reported that A2AR expression increases after LPS incubation in brain microglial cells (Canas et al., 2004; Saura et al., 2005). Therefore, mixed retinal cultures were challenged with LPS for 24 hours and immunocytochemistry was performed to study whether retinal microglial cells express A2AR. Cells were labeled with an antibody that specifically recognizes A2AR and microglial cells were labeled with an antibody anti-Cd11b (Figure 8). As it can be observed, both in control and LPS-challenged cells, A2AR immunoreactivity is mainly found in Cd11b-positive cells, indicating that in primary mixed retinal cultures microglial cells express A2AR. Following an inflammatory stimulus, A2AR immunoreactivity tends to increase in microglia, although not significantly.

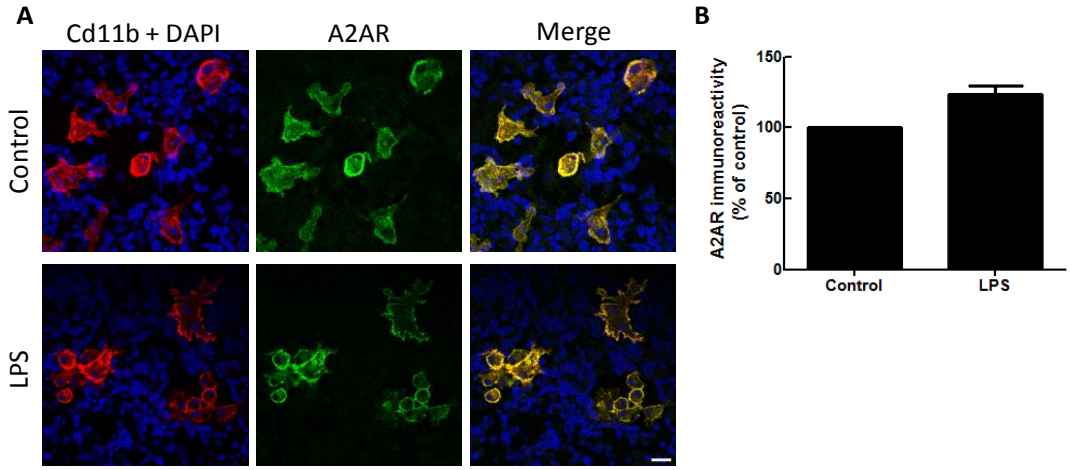

Figure 8 - Microglial cells express A2AR in primary retinal mixed cultures. Cells were incubated with 1 ng/ml LPS for 24 hours. **(A)** Immunocytochemistry was performed using antibodies against Cd11b (red) and A2AR (green). Nuclei were counterstained with DAPI (blue). Preparations were visualized in the Zeiss LSM 710 confocal microscope. **(B)** The mean grey value of A2AR in Cd11b-immunoreactive cells was analyzed with ImageJ image analysis software using the 'mean grey value' built-in function. Data represent the mean ± SEM and are expressed as percentage of control of three independent experiments. Bar: 20 µm.

3.4 Retinal microglial cells express A2A receptor in purified cultures

Previous results using mixed cultures showed that blockade of A2AR may prevent microglia reactivity and that A2AR is mainly found in microglial cells. Therefore, purified cultures of retinal microglial cells were prepared and the role played by A2AR in the control of microglial reactivity was evaluated.

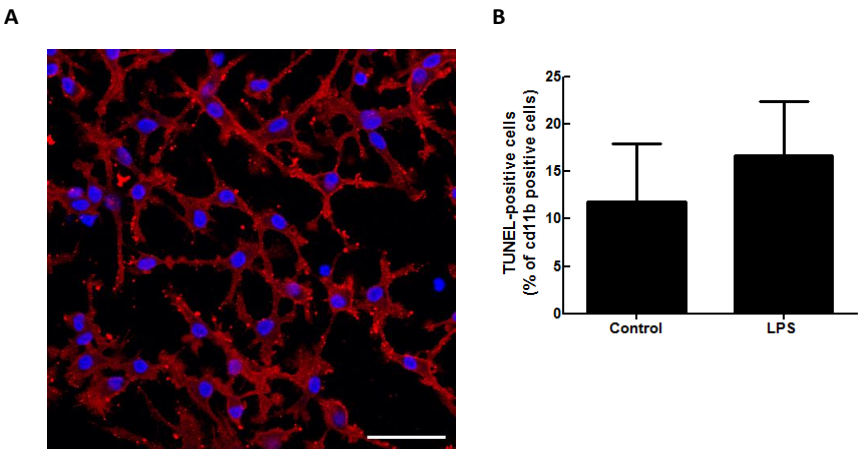

Figure 9 - Microglial cells in the purified culture (A) Microglial cell cultures were stained with the antibody anti-Cd11b (red) and the nuclei were counterstained with DAPI (blue). The purity of the culture was assessed by counting the number of Cd11b-positive cells in relation to the total number of cells, from six independent cultures in at least 10 assigned fields. A representative image is shown. **(B)** TUNEL assay was performed to evaluate cell death. Cells were incubated with 1 ng/ml LPS for 24 hours. Data represent the number of TUNEL-positive microglial cells and are expressed as percentage of Cd11b-positive cells, from four independent experiments. Preparations were visualized in the Zeiss LSM 710 confocal microscope. Bar: 20 µm.

The purified culture of microglial cells was obtained from mixed cell cultures. The purity of the culture was assessed by immunocytochemistry using a microglial cell marker (Cd11b) and counting the total number of cells (nuclei were stained with DAPI). In control conditions, the percentage of cells that were Cd11b-positive was 93.0±2.3% (Figure 9A), and LPS incubation (1 ng/ml for 24 hours) did not significantly change the purity of cell culture (data not shown). In addition, cell death was assayed evaluated using the TUNEL assay (Figure 9B). In the control conditions, the percentage of microglial

cells (Cd11b-positive) that were TUNEL-positive was 11.9±6.1%. When cells were challenged with LPS, the percentage of microglial cells that were also TUNEL-positive was not significantly different as compared to the control (16.7±5.6%).

The protein expression of A2AR in the purified culture of microglial cells was assessed by immunocytochemistry (Figure 10), and it was observed that microglial cells express the A2AR (Figure 10A), and that LPS challenge significantly increased A2AR immunoreactivity, suggesting an increase in A2AR expression after LPS incubation (Figure 10B).

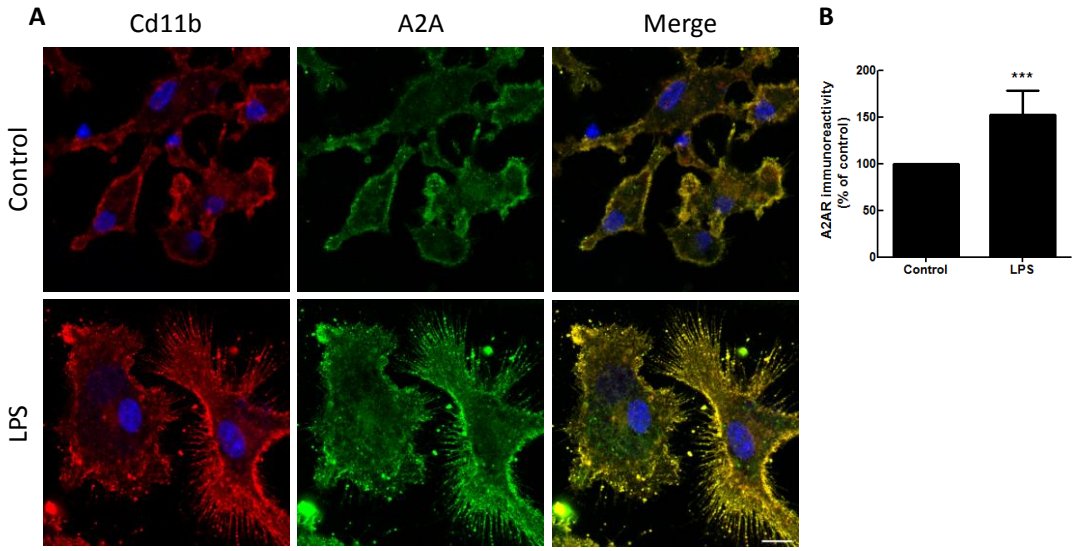

Figure 10 - Microglial cells express A2AR in purified cultures. Cells were incubated with 1 ng/ml LPS for 24 hours. **(A)** Immunocytochemistry was performed using antibodies against Cd11b (red) and A2AR (green). Nuclei were counterstained with DAPI (blue). Preparations were visualized in the Zeiss LSM 710 confocal microscope. **(B)** From each condition, at least five random fields were acquired and the mean grey value was quantified using ImageJ software. The results represent the mean ± SEM, obtained from four independent experiments, and are expressed as percentage of control. ***p<0.001, significantly different from control; two-tailed Student's t-test. Bar: 20 µm.

3.5 Blockade of A2AR decreases LPS-induced NO production

Nitrite production was quantified in purified microglial cell cultures by the Griess reaction method, as an indirect measurement of NO production (Figure 11). In control conditions, nitrite concentration was 0.7±0.06 µM, which was significantly increased after LPS incubation to 7.77±0.46 µM (which corresponds to a fold increase of 11.0, as compared to control). Activation of A2AR with CGS21680 (30 nM) prior to LPS treatment did not change nitrites production, as compared to LPS. The pre-treatment with the A2AR antagonist (SCH58261, 50 nM) significantly decreased nitrite concentration, as compared to LPS condition. Either A2AR agonist or antagonist, when incubated alone, did not significantly change nitrite concentration as compared to control conditions.

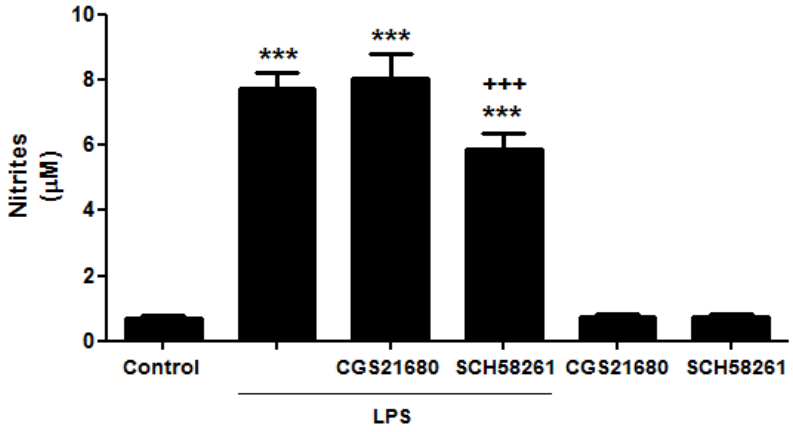

Figure 11 - Blockade of A2AR partially inhibits LPS-induced nitrites production. Cells were challenged with 1 ng/mL LPS in the absence or in the presence of 30 nM CGS21680 or 50 nM SCH58261. Nitrite levels were determined in the culture medium supernantant by the Griess reaction. Results represent nitrite concentration (µM) and are expressed as mean ± SEM, from at least five independent experiments, performed in duplicate. ***$p<0.001$, significantly different from control, one-way ANOVA followed by Dunnett's post-hoc test; +++$p<0.001$, significantly different from LPS, one-way ANOVA followed by Bonferroni's post-hoc test.

In order to accurately quantify NO production in the purified culture of microglia, cells were loaded with DAF-FM DA, which has been reported as the most successful indicator for NO (Sheng, 2005). The culture medium was collected and the cells were incubated with DAF-FM DA, which passively diffuses across cellular membranes. Once inside the cell, DAF-FM DA is deacetylated by intracellular esterases to DAF-FM, which increases fluorescence after reacting with NO. After 1 hour the medium was replaced and the cells pre-treated with CGS21680 30 nM or with SCH58261 50 nM, and then challenged with LPS for 24 hours. Cells were fixed and labeled with an antibody anti-Cd11b to identify microglial cells. Incubation with LPS for 24 hours significantly increased DAF-FM staining in microglia as compared to control (Figure 12), indicating that LPS increased NO production.

Activation of the A2AR with CGS21680 significantly potentiated the effect of LPS, while the blockade of the A2AR with SCH58261 significantly inhibited the effect of LPS on the DAF-FM fluorescence.

To determine how A2AR blockade was involved in the inhibition of NO production, we tested whether SCH58261 was affecting the synthesis of the converting enzyme iNOS, the isoform present in microglia. By immunocytochemistry, it was found that iNOS immunoreactivity significantly increased after LPS challenge as compared to control (Figure 13). Pre-treatment with the A2AR agonist or antagonist did not significantly change iNOS immunoreactivity induced by LPS.

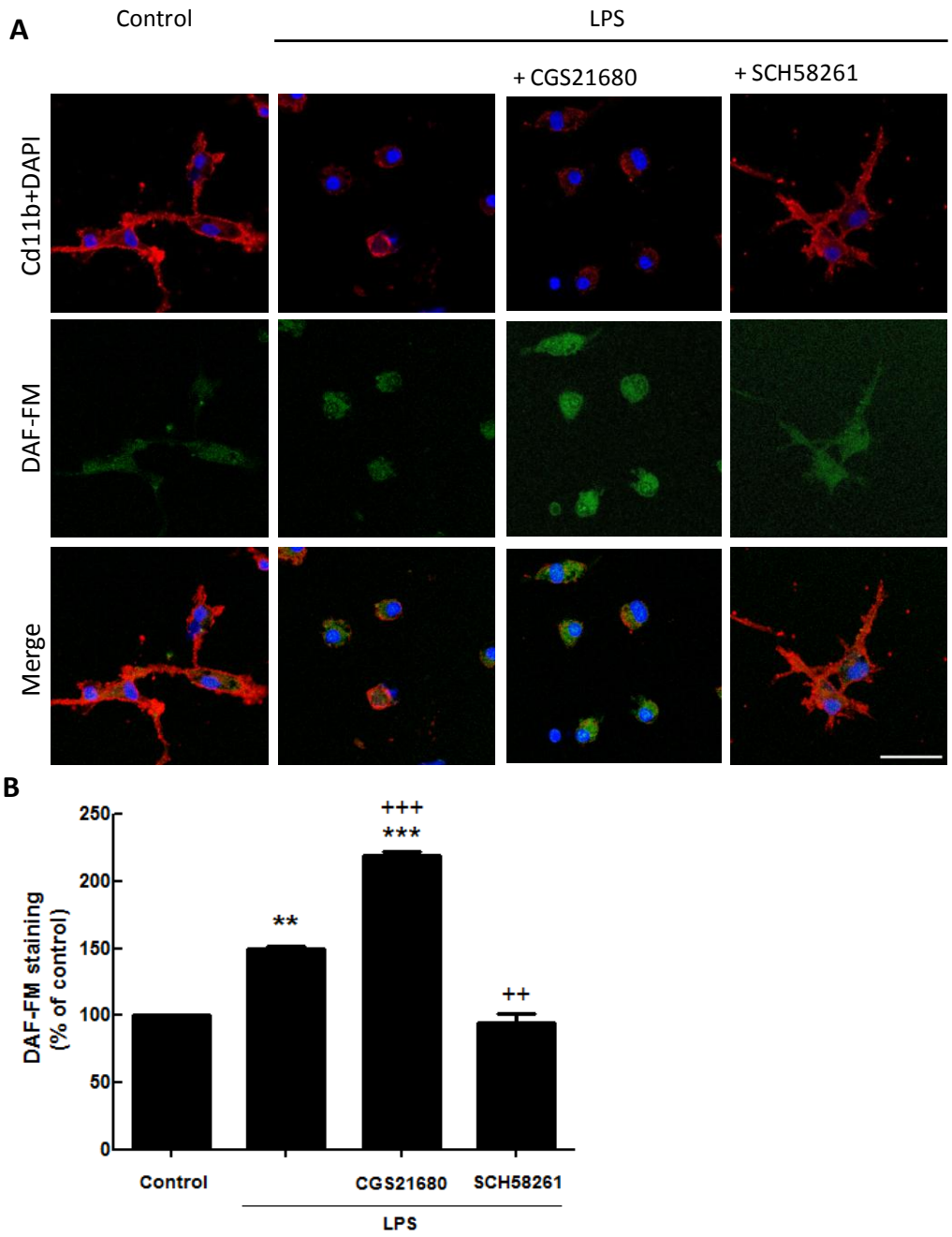

Figure 12 - Blockade of A2AR inhibits LPS-induced NO production. Cells were loaded with the NO-sensitive probe DAF-FM DA for 1 hour and then challenged with 1 ng/ml LPS in the absence or in the presence of 30 nM CGS21680 or 50 nM SCH58261 for 24 hours. **(A)** Representative images of DAF-FM fluorescence (green) in microglial cells (red). Nuclei were counterstained with DAPI (blue). Preparations were visualized using a Zeiss LSM 710 confocal microscope. **(B)** DAF-FM intensity was quantified in the digitized pictures using ImageJ software in at least five random fields. The results represent the mean ± SEM and are expressed as percentage of control, from two independent experiments. **p<0.01, ***p<0.001, significantly different from control, one-way ANOVA followed by Dunnett's post-hoc test; ++p<0.01, +++p<0.001, significantly different from LPS, one-way ANOVA followed by Bonferroni's post-hoc test. Bar: 20 µm.

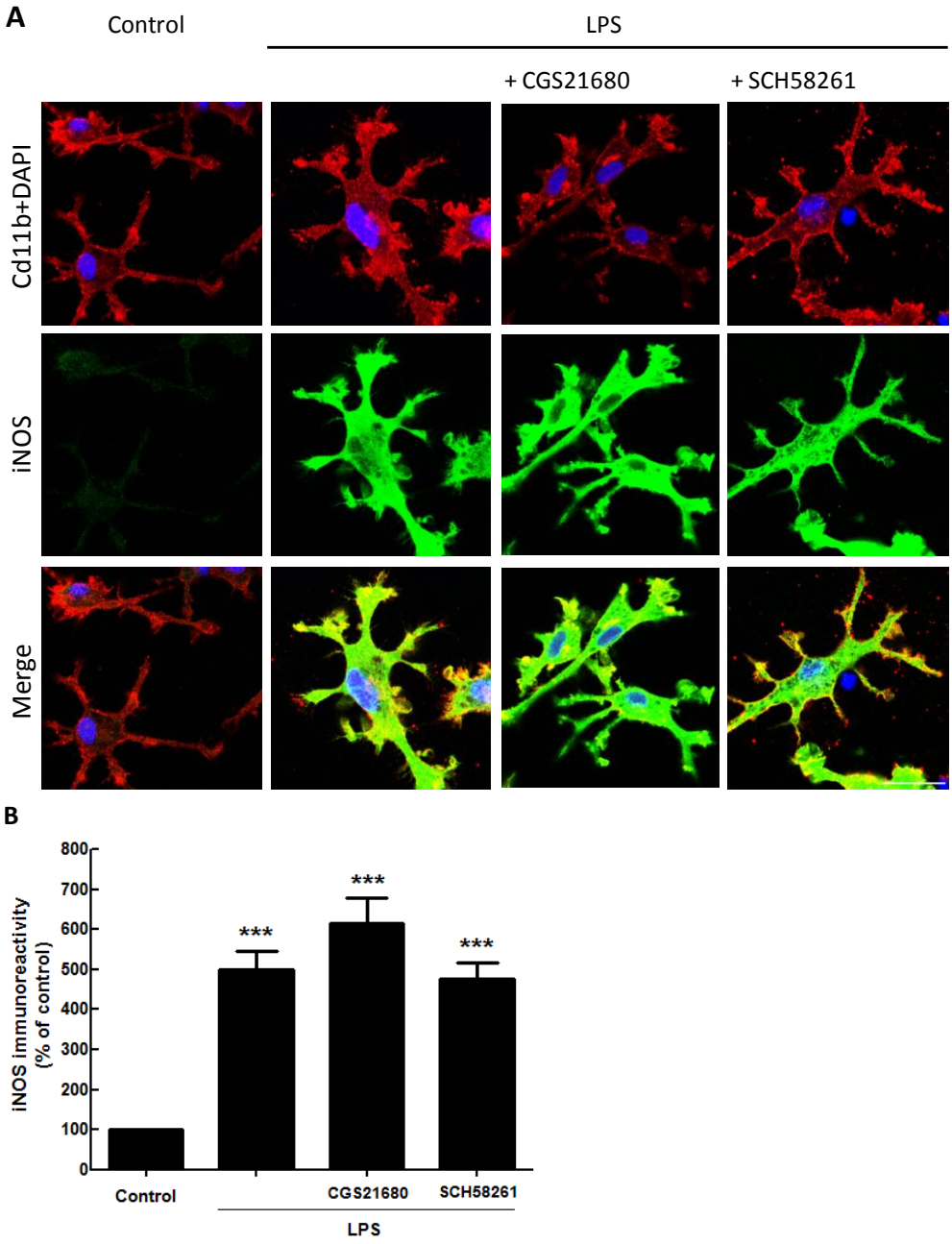

Figure 13 - LPS challenge increases iNOS immunoreactivity in purified microglial cell cultures. Cells were challenged with 1 ng/mL LPS in the absence or in the presence of 30 nM CGS21680 or 50 nM SCH58261. **(A)** Cells were stained with the primary antibodies mouse anti-Cd11b (red) and rabbit anti-iNOS (green), and the nuclei were counterstained with DAPI (blue). **(B)** Densitometric analysis of the iNOS immunoreactivity was performed using the public domain ImageJ software in at least 8 assigned fields per coverslip. Results represent the percentage of control of the iNOS immunoreactivity, and are expressed as mean ± SEM, from at least three independent experiments. Preparations were visualized in a Zeiss LSM 710 confocal microscope. ***p<0.001, significantly different from control; one-way ANOVA followed by Dunnett's post-hoc test. Bar: 20µm.

3.6 Blockade of A2AR inhibits LPS-induced TNF-α expression in purified microglial cell cultures

Microglia detect and respond to pro-inflammatory triggers by changing to an activated phenotype, resulting in a shift of cellular function to release cytotoxic factors such as TNF-α (Block, 2010). The release of this pro-inflammatory cytokine has been used as a parameter to measure microglial cell activation (Saura et al., 2005).

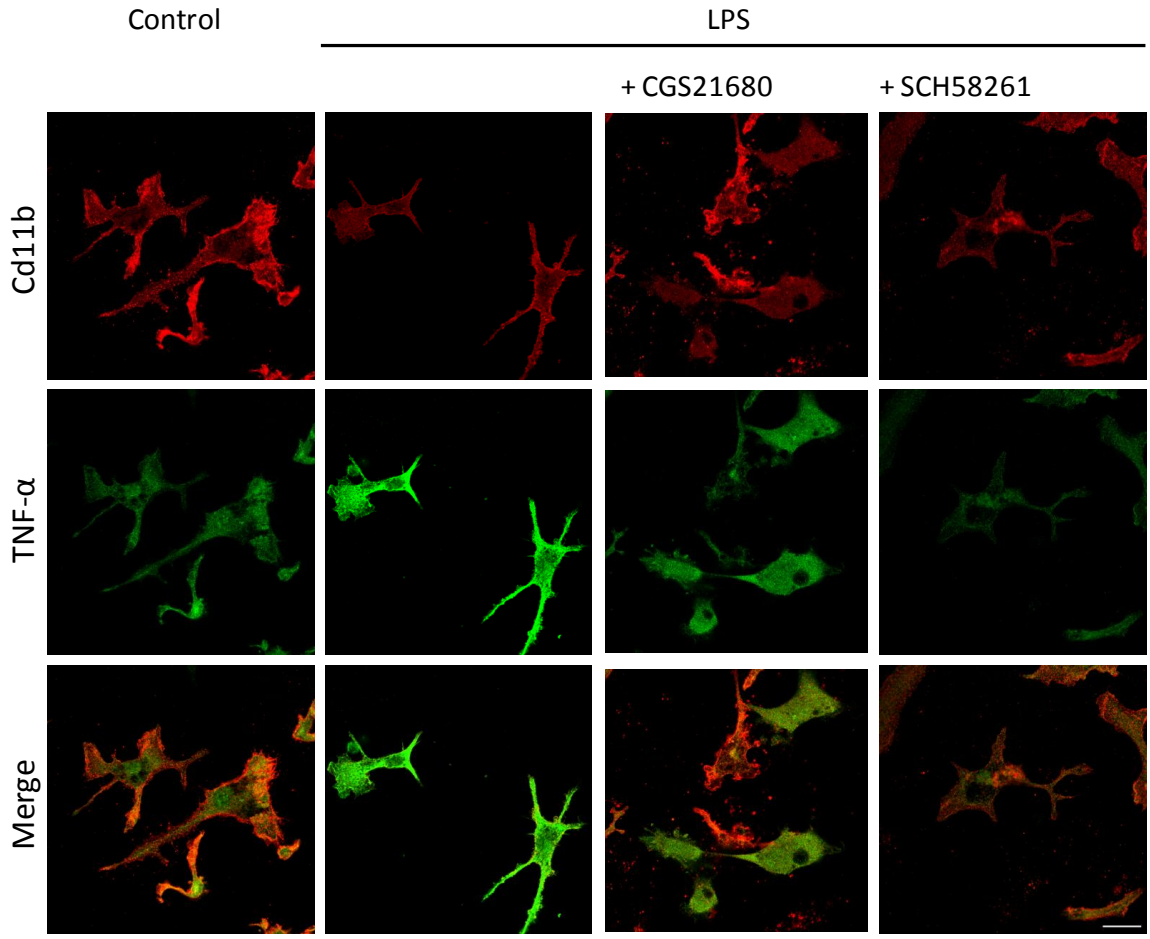

Figure 14 - Blockade of A2AR inhibits LPS-induced TNF-α expression. Cells were challenged with 1 ng/mL LPS in the absence or in the presence of 30 nM CGS21680 or 50 nM SCH58261. Representative images of Cd11b (red) and TNF-α immunoreactivity were obtained in a Zeiss LSM 710 confocal microscope. Bar: 20µm.

Cells were incubated either with the A2AR agonist or antagonist and then challenged with LPS (1 ng/ml) for 24 hours. Expression of TNF-α was assessed by

immunocytochemistry with an antibody anti-TNF-α (Figure 14). Microglial cells were labeled with an antibody anti-Cd11b. TNF-α immunoreactivity is found in all cells in control conditions. After cell challenge with LPS, TNF-α immunoreactivity increased, when compared with the control condition. Pre-treatment with CGS21680 did not change TNF-α immunoreactivity when compared with LPS. Though, pre-treatment with the A2AR antagonist SCH58261 decreased TNF-α immunoreactivity, as compared with the LPS condition, suggesting that the blockade of A2AR decreases TNF-α expression.

3.7 The increase in phagocytic activity induced by LPS in microglial cells is inhibited by A2AR blockade

Phagocytosis is one of the main features of microglial activation dumping cell debris prior to cell regeneration, and can also be involved in the pathogenesis of several CNS dysfunctions (Silva et al., 2010). We have evaluated the microglial phagocytic activity by incubating cells with fluorescent latex beads, and examining the incorporated beads. Microglial cells were stained by immunocytochemistry using an antibody anti-Cd11b. As shown in Figure 15A, in control cells it is possible to observe some beads incorporated into microglial cells (2.6±0.24 beads per cell). With LPS, the number of beads per cell significantly increased to 5.1±0.78 (which corresponds to a fold increase of 1.96). Pre-treatment with CGS21680 or with SCH58261 did not change significantly the number of beads per cell (4.3±0.78 and 3.7±0.34, respectively), although in the case of A2AR activation there was a trend to increase comparing to the control.

The number of cells that incorporated beads was also analyzed (Figure 15C). In control conditions, 58.50±6.08% of cells incorporated beads. When cells were exposed to LPS, the number of microglial cells incorporating beads significantly increased to 90.10±3.23%. Pre-treatment with CGS21680 significantly increased the number of

microglia with beads (86,00±2.44%), as compared to the control. Also, the blockade of A2AR significantly decreased the percentage of microglial cells with beads to 73.70±4.41% of the control. These results demonstrated that although the blockade of A2AR did not significantly decrease the number of beads that were phagocytosed, it decreased the number of cells that were phagocytosing.

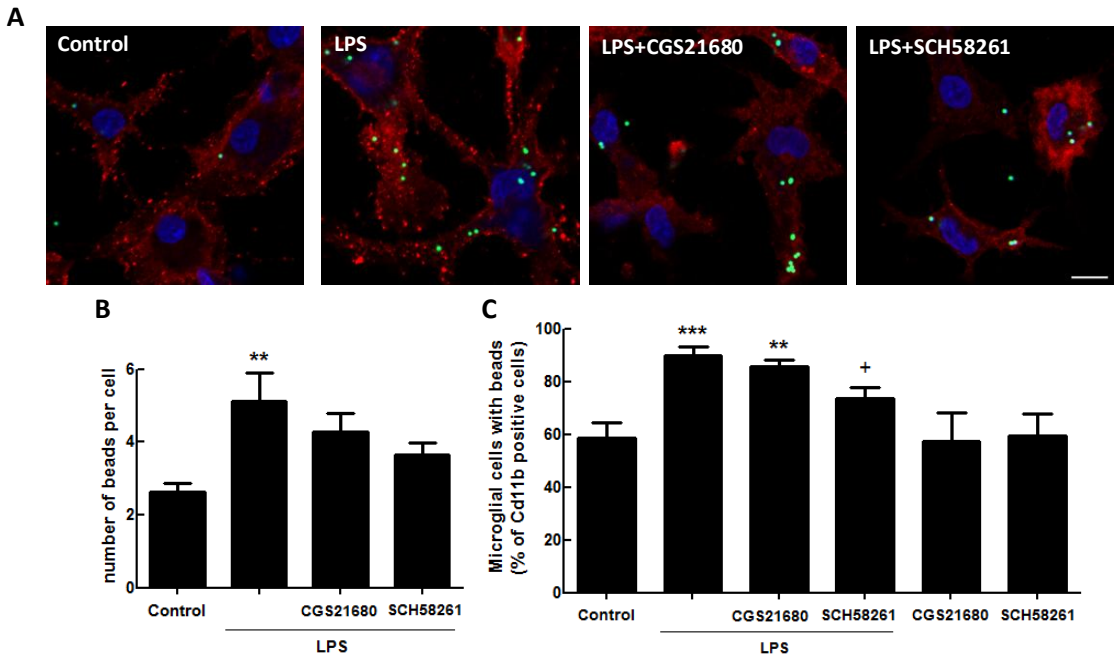

Figure 15 - The increase in phagocytic activity induced by LPS in microglial cells is inhibited by A2AR blockade. Cells were challenged with 1 ng/mL LPS in the absence or in the presence of 30 nM CGS21680 or 50 nM SCH58261. Cells were incubated with fluorescent beads (green) and immunocytochemistry was performed using an antibody against Cd11b (red). Nuclei were counterstained with DAPI (blue). Preparations were visualized in a Zeiss LSM 710 confocal microscope. **(A)** Representative images for each condition. From each condition, the number of beads per cell **(B)** and the number of cells with beads **(C)** was counted in five random fields. The results represent the mean ± SEM, obtained from five independent experiments. **p<0.01, ***p<0.001, significantly different from control, one-way ANOVA followed by Dunnett's post-hoc test; +p<0.05, significantly different from LPS, one-way ANOVA followed by Bonferroni's post-hoc test. Bar: 10 µm.

Chapter 4 - Discussion

4. Discussion

Glaucoma is a progressive and non-curable retinal degenerative disease and the second cause of vision loss in the world. It is characterized by an irreversible and progressive loss of RGCs and their axons, accompanied by excavation and degeneration of the ONH, which leads to visual field loss (Chiu et al., 2010; Resnikoff et al., 2004).

As the resident immune surveillance cells in the CNS, microglia are exquisitely sensitive to tissue stress and injury, and are linked to several neurodegenerative diseases, including glaucoma (Neufeld, 1999). Neuroinflammation plays an important role in glaucoma and microglial cells become activated, occurring clustering and redistribution of microglia specifically in the region of the ONH (Neufeld, 1999). Microglial activation is associated with microglial proliferation and upregulation of various inflammatory molecules including NO and TNF-α (Yuan and Neufeld, 2000), which can promote cell death.

Adenosine is a neuromodulator in the CNS that acts by coupling with inhibitory (A1 and A3) and facilitatory (A2A and A2B) receptors. It has been claimed that adenosine is one of the most promising neuroprotective systems in the CNS (Cunha, 2005). Much attention is being given to A2AR activity modulation as potential therapeutic target. The antagonists of A2AR have been shown to protect against a broad spectrum of brain insults such as ischemia, excitotoxicity, and mitochondrial toxicity (Cunha, 2005).

It is still unknown the role of A2AR in RGC neuroprotection in glaucoma. Since microglial cells and RGCs are in the GCL, and microglia express A2AR, this study aimed to evaluate the effect of A2AR modulation in the control of retinal neuroinflammation through the reduction of microglia reactivity. This work found that the blockade of A2AR may reduce microglial reactivity induced by a pro-inflammatory stimulus.

In healthy conditions, microglial cells are characterized by ramified morphology, being highly motile patrolling cells, constantly surveying their microenvironment. Through numerous types of disturbances in the CNS, microglial cells rapidly become in active state, adopting a rounded (ameboid), non-ramified morphology, which may favor phagocytosis (Chew et al., 2006; Silva et al., 2010). In primary retinal mixed cultures, a pro-inflammatory stimulus like LPS induced microglia activation, as assessed by cell morphology. The activation of A2AR potentiated the effect of LPS, which was completely blocked with the A2AR antagonist, suggesting a role for A2AR modulation in the control of microglial cell reactivity. When we assessed which cell types express A2AR, we found that A2AR was preferentially expressed by microglial cells, in accordance with a previous report (Liou et al., 2008). Moreover, when cells were challenged with LPS, there was a trend to an upregulation of A2AR in microglial cells. In fact, it was reported that LPS challenge increases A2AR mRNA (Wittendorp et al., 2004) and protein (Canas et al., 2004) in brain microglial cells in culture. Previous data have observed that microglial cells express A2AR in cortical samples from Alzheimer's disease patients but not in controls (Angulo et al., 2003), further demonstrating that A2AR increases upon microglia activation

Activated microglial cells release a number of potentially neurotoxic factors. Especially convincing is the evidence showing the neurotoxic effects of NO produced by iNOS in microglia, which can induce damage to neighboring glia or neurons (Pang et al., 2010; Saura et al., 2005). We found that, in mixed cultures, the blockade of A2AR decreased iNOS expression in microglial cells. The enzyme iNOS produces high amounts of NO (Liversidge et al., 1994). Therefore, we hypothesized that A2AR blockade could decrease LPS-induced NO production. However, we found no changes in nitrite quantification, through the Griess reaction method. One possibility for this discrepancy would be that NO is also produced by the other cells in culture. Astrocytes

have the capacity to secrete or respond to a variety of cytokines including IL-1, IL-6, IL-3, and TNF-α (Chung and Benveniste, 1990). In addition, astrocytes are endowed with NOS (Kozuka et al., 2007; Murphy, 2000) and they can produce NO in response to activation by LPS (Kozuka et al., 2007) It was already reported that the treatment of brain astrocytes with the A2AR agonist CGS21680 inhibited NO production induced by stimulation with either LPS/IFN-γ or TNF-α/IL-1β (Brodie et al., 1998). NO production may also result from nNOS and eNOS.

In purified microglial cell cultures, the blockade of A2AR partially decreased nitrites concentration induced by LPS, when assessed by Griess reaction. However, when NO was assessed directly with DAF-FM, considered to be one the most accurate probes to detect NO (Kojima et al., 1999), the activation of A2AR potentiated the effect of LPS, while the blockade of A2AR significantly decreased NO production induced by LPS. Griess diazotization reaction detects nitrite formed by the spontaneous oxidation of NO. Nitrates are not taken into account unless converted to nitrites by enzymatic using nitrate reductase. The probe DAF-FM directly detects NO intracellularly. Also, since the microglial culture is not a pure culture, we cannot rule out the possibility that astrocytes present in the culture are also being stimulated by LPS, and therefore producing NO that it will be taken into account in Griess reaction. When NO production was assessed by DAF-FM staining only microglial cells (Cd11b-positive cells) were evaluated.

In contrast to previous studies reporting activation of microglial cells by CGS21680 (Gebicke-Haerter et al. 1996; Fiebich et al. 1996; Heese et al. 1997; Kust et al. 1999), and despite the fact that, in our conditions, A2ARs are expressed in non-activated microglial cells, we did not observe any NO release induced by CGS21680 alone. However, a marked potentiation of NO production was observed if cells were also treated with LPS. Activated microglia therefore appears to be especially sensitive

to A2AR stimulation. The up-regulation of the receptor in LPS-activated cells, which is in agreement with previous results (Canas et al., 2004; Wittendorp et al., 2004), may also account for the effect. Another possible explanation would be that these findings indicate that A2AR agonists regulate NO production in microglia by a signaling pathway that is operative in activated but not in resting cells. This explanation is compatible with a previous report, showing that activation of A2AR potentiates the effect of LPS stimulus on NO release by mixed glial cultures (Saura et al., 2005). In microglia cultures, the protein expression of iNOS was significantly increased after LPS incubation, confirming that this enzyme has an important role in NO production induced by LPS. However, iNOS expression was not altered when A2AR agonist or antagonist were present, implying that this receptor does not control *de novo* synthesis of this enzyme. Nevertheless, LPS-induced NO production decreased with A2AR blockade, thus suggesting that A2AR can control iNOS activity.

In the retina, microglial cells express iNOS and TNF-α in response to inflammatory stimuli and tissue hypoxia, where they are involved in mediating neuronal cell death in retinal inflammatory and degenerative disease (Stevenson et al., 2010). Furthermore, in a mouse model of glaucoma with RGC loss and consecutive optic atrophy, TNF-α mRNA is upregulated in the retina (Balaiya et al., 2011). In microglial cell cultures, TNF-α was upregulated after an inflammatory stimulus which was inhibited by A2AR blockade. The TNF-α expression was not potentiated by A2AR agonist, as NO production was, suggesting that not every effect of LPS on microglial cells is enhanced by A2AR activation. This finding is in accordance with a previous study that demonstrated that CGS21680 potentiated the effect of LPS on NO release without effects on TNF-α release (Saura et al., 2005).

Microglial cells are the CNS-resident innate immune cells endowed with sensor and effector functions as well as with phagocytic capacity during physiological and

pathological conditions. Clearance of tissue debris by microglia is essential for tissue homeostasis and may have a neuroprotective outcome (Kettenmann et al., 2011; Silva et al., 2010). Activated microglial cells can migrate to the site of injury, proliferate, and phagocyte cells and cellular compartments (Kettenmann et al., 2011). In microglial cultures, LPS induced an increase in the microglial phagocytic activity, typical of an activated state. The A2AR agonist had no effect in the microglial phagocytic capacity alone or together with LPS. The blockade of A2AR decreased the number of cells with beads, without effects on the number of beads per cell. This data suggests that blockade of A2AR inhibited the phagocytic activity of activated microglial cells. During neurodegeneration, activated microglial cells participate in the phagocytosis of debris and facilitate regenerative processes (Chen et al., 2002). In the retina, the main functions of microglia are phagocytosis and elimination of cellular debris from apoptotic neurons in the GCL and INL (Langmann, 2007). Activation of microglial cells in a rat model of glaucoma indicate that these cells are involved in glaucomatous pathophysiology (Naskar, 2002). However, abnormal accumulation and reactivity of microglial cells could result in excessive phagocytosis that can be deleterious to the RGC, as previously observed in retinal dystrophy (Thanos, 1992).

Taken all results together, we have demonstrated that A2AR blockade prevents microglia reactivity induced by a pro-inflammatory stimulus. The blockade of A2AR decreased the effect of LPS on the release of NO and TNF-α and on the phagocytic activity. Interestingly was the fact that A2AR does not appear to control iNOS *de novo* synthesis, but it may regulate the activity of the enzyme.

Previous studies have demonstrated that microglial cell activation is an early alteration in glaucoma models (Bosco et al., 2011). In glaucoma, the increased levels of NO and TNF-α (Balaiya et al., 2011) may contribute to the disease process. The increased presence of iNOS in the lamina cribosa of glaucoma patients suggest that the

glaucomatous optic nerve head is exposed to excessive levels of nitric oxide, which may be neurodestructive, locally, to the axons of the RGC (Neufeld et al., 1997). TNF-α contributes to the progression of optic nerve degeneration in glaucoma by both a direct effect on the axons of the RCG (Yuan and Neufeld, 2000) and via microglial activation and oligodendrocyte death (Nakazawa et al., 2006). Moreover, TNF-α blockade inhibit the deleterious effects of IOP in a glaucoma model (Nakazawa et al., 2006).

Our findings propose a potential role of the modulation of the A2AR, particularly the blockade of these receptors, in the control of the retinal microglial reactivity. Our main hypothesis is that the blockade of the A2AR can control retinal neuroinflammation by controlling microglial reactivity. These findings may have implication in RGC neuroprotection, opening new perspectives for pharmacological intervention in glaucoma.

Chapter 5 - Conclusions and Future Directions

5. Conclusions and Future Directions

With this work we have demonstrated that the modulation of the A2AR can, at least partially, control the microglial cell reactivity in the retina.

Regarding the effects of the agonist CGS21680, it potentiated LPS-induced morphological alterations and NO production in retinal microglial cells.

The antagonist SCH58261 appears to have a stronger modulatory effect in retinal microglial cells. The blockade of the A2AR prevented the morphological alteration induced by LPS typical of microglial cell activation. This antagonist partially blocked the production of NO by microglial cells, having no direct effect on the iNOS expression. Furthermore, the blockade of A2AR inhibits TNF-α expression by microglial cells.

Taking into account the results obtained, it is clear that more studies must be performed to a better understanding of the modulation of the retinal microglial cell reactivity by A2AR. The levels of important inflammatory mediators, such as NO, TNF-α, IL-1β, and also the levels of A2AR, should be assessed by real-time quantitative RT-PCR in the different conditions. Moreover, the cross-talk between microglial cells and other retinal cells, such as RGCs, should be evaluated to explore possible ways of controlling microglial cell reactivity through A2AR receptor modulation to prevent RGC death, which might help developing a potential new therapy for the treatment of glaucoma.

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