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Uncovering the neuroprotective role of adenosine in diabetic retinopathy: Alterations in the adenosinergic system

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Professor Doutor Paulo Santos (Universidade de Coimbra)

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Table of contents

Abbreviations

5'-eN	5'-ectonucleotidase
APS	Ammonium persulfate
A_1AR	A_1 adenosine receptor
A_{2A}AR	A_{2A} adenosine receptor
A_{2B}AR	A_{2B} adenosine receptor
A_3AR	A_3 adenosine receptor
ADA	Adenosine deaminase
AK	Adenosine kinase
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
CLAP	Chymostatin, pepstatin, antipain and leupeptin
CMF	Ca^{2+} and Mg^{2+}-free Hank´s solution
CNS	Central nervous system
CREB	cAMP-response element-binding protein
DMSO	Dimethyl sulfoxide
DR	Diabetic retinopathy
DTT	Dithiothreitol
ECF	Enhanced chemifluorecense substrate
Ecto-NPP	Ecto-nucleotide pyrophosphatase/phosphodiesterase
EHNA	Erythro-9-(2-hydroxy-3-nonyl)adenine
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IP_3	Inositol 1,4,5-trisphosphate
LPS	Lipopolysaccharide

MAPK	Mitogen-activated protein kinase
MEM	Minimum Essential Medium
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
NFκB	Nuclear factor κB
NTPDase	Nucleoside triphosphate/diphosphohydrolase
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline with 0.1% Tween 20
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMBC	Peripheral mononuclear blood cells
PMSF	Phenylmethanesulphonyl fluoride
PVDF	Polyvinylidene difluoride
SAH	*S*-adenosylhomocysteine
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrilamide gel electrophoresis
STZ	Streptozotocin
TBS	Tris buffer saline
TBS-T	Tris buffer saline with 0.1% Tween 20
TEMED	Tetramethylethylenediamine
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α

Abstract

Diabetic retinopathy is the main cause of blindness in adults, and exhibits characteristics of chronic inflammation, such as microglia activation and release of inflammatory mediators. If microglia activation and pro-inflammatory mediator release could be inhibited, the effects of diabetes on vision loss could be reduced. Microglia, cells from the immune system, express all four types of adenosine receptors (A_1, A_{2A}, A_{2B}, A_3), a purine nucleoside with a marked effect on inflammatory situations. Adenosine is released under adverse conditions, and activation of its receptors decrease the expression and release of pro-inflammatory cytokines. Unlike other tissues, in the retina it is still unknown what effect diabetes/hyperglycemia exerts on the adenosinergic system. Therefore, in this work we have aimed to uncover the effects of diabetes on the adenosinergic system in the retina.

We focused our attention on the effect diabetes or hyperglycemia, considered the main cause of diabetes complications, has on the adenosine receptor total protein levels, the main conveyors of adenosine signaling and regulation, and on the protein levels and activity of adenosine deaminase (ADA), one of the enzymes responsible for adenosine removal from the extracellular space. We used as experimental models cultures of retinal cells isolated from 3-5 days newborn Wistar rats subjected to hyperglycemic conditions, and whole retinas isolated from control or diabetic rats. These diabetic animals were obtained after intraperitoneal injection of streptozotocin (STZ), which destroys pancreatic β-cells (type I *diabetes mellitus* model). We then analyzed the samples with Western Blot technique to assess the total protein levels. Our findings showed diabetes does affect the adenosinergic system in the retina: A_1AR, A_{2A}AR, A_3AR and ADA all had their protein levels altered by diabetic conditions; for A_3AR and ADA significant alterations occurred only on the retina of diabetic animals, while A_1AR and A_{2A}AR levels were altered in both cell cultures and animals.

We also investigated the enzymatic activity of ADA under hyperglycemic conditions in retinal cell cultures, and revealed a strong down-regulation of ADA's ability to deaminate adenosine in those conditions.

Finally, viability assays were performed with one A_{2A}AR agonist, CGS 21680, and one A_{2A}AR antagonist, SCH 58261, in retinal cell cultures subjected to a hyperglycemic environment to uncover the possible role of A_{2A}AR in the diabetic retina, and the outcome of A_{2A}AR altered levels in diabetic conditions. The treatment with CGS 21680 did prevent the retinal cell death occurring in high glucose conditions, while the treatment with SCH 58261 aggravated it.

Overall, this work shows that the retinal adenosinergic system is affected by diabetes/hyperglycemia. The effect on other adenosinergic system components will be necessary to finally understand the role of this system in diabetic retinopathy, and possibly open a new therapeutic window for the treatment of diabetic retinopathy in its early stages.

Keywords: *Diabetic retinopathy, adenosine, adenosinergic system, hyperglycemia, adenosine receptors, adenosine deaminase, inflammation.*

Resumo

A retinopatia diabética é a causa principal de cegueira em adultos, e exibe características típicas de inflamação crónica, como activação de microglias e libertação de mediadores inflamatórios. Se a activação de microglias e a libertação de factores pro-inflamatórios pudesse ser inibida, os efeitos da diabetes na visão poderiam ser reduzidos. As microglias, células do sistema imunitário, expressam todos os quatro tipos de receptores de adenosina (A_1, A_{2A}, A_{2B}, A_3), um nucleósido de purina com um efeito marcante em situações de inflamação. A adenosina é libertada em condições adversas, e a activação dos seus receptores diminui a expressão e libertação de citocinas pro-inflamatórias. Mas ao contrário de outros tecidos, na retina ainda não são conhecidos os efeitos que a diabetes/hiperglicémia causa no sistema adenosinérgico. Desta forma, neste trabalho o nosso objectivo foi o de investigar os efeitos provocados pela diabetes no sistema adenosinérgico da retina.

Focámos a nossa atenção no efeito que a diabetes ou hiperglicémia, considerada como a principal causa das complicações associadas à diabetes, tem nos níveis proteicos totais dos receptores de adenosina, os principais transmissores da sinalização e regulação comandadas por este nucleósido, e nos níveis proteicos e de actividade da adenosina deaminase (ADA), uma das enzimas responsáveis pela redução da adenosina do espaço extracelular. Usámos como modelos experimentais culturas de células da retina isoladas de ratos Wistar recém-nascidos, com 3-5 dias, sujeitos a condições de hiperglicémia, e retinas isoladas de ratos controlo ou diabéticos. Estes animais diabéticos foram obtidos após uma injecção intraperitonial de estreptozotocina (STZ), que destrói as células β-pancreáticas (modelo de *diabetes mellitus* tipo I). Analisámos então as amostras recorrendo à técnica Western Blot para determinar os níveis proteicos totais. Os nossos resultados mostram que a diabetes afecta de facto o sistema adenosinérgico na retina: os níveis proteicos de A_1AR, A_{2A}AR, A_3AR e ADA revelaram alterações provocadas pelas condições diabéticas; as

alterações significativas nos níveis de A_3AR e ADA ocorreram apenas na retina de animais diabéticos, enquanto que os níveis proteicos de A_1AR e A_{2A}AR foram alterados tanto em retinas de animais diabéticos, como nas culturas de células da retina sujeitas a condições de hiperglicémia.

Também investigámos os níveis de actividade enzimática de ADA em culturas de células da retina sujeitas a condições de hiperglicémia, e revelámos uma forte diminuição da capacidade de ADA para degradar adenosina nessas condições.

Por fim, ensaios de viabilidade celular foram efectuados com CGS 21680, um agonista de A_{2A}AR, e SCH 58261, um antagonista de A_{2A}AR, em culturas de células da retina sujeitas a condições de hiperglicémia, para desvendar um possível papel de A_{2A}AR na retina diabética, e as consequências dos níveis alterados de A_{2A}AR em condições diabéticas. O tratamento com CGS 21680 foi bem sucedido a prevenir a elevada morte de células da retina que ocorre em situações de hiperglicémia, enquanto o tratamento com SCH 58261 agravou a morte celular nessas mesmas condições.

Em resumo, este trabalho mostra que o sistema adenosinérgico na retina é afectado pela diabetes/hiperglicémia. Saber o efeito que a diabetes provoca em outros componentes do sistema adenosinérgico será necessário para verdadeiramente compreender o papel deste sistema na retinopatia diabética, e possivelmente expor uma nova vertente para o tratamento da retinopatia diabética nos seus estágios iniciais.

Palavras-chave: *Retinopatia diabética, adenosina, sistema adenosinérgico, hiperglicémia, receptores para adenosina, adenosina desaminase, inflamação.*

Chapter 1 Introduction

1.1 – Diabetic Retinopathy

Diabetic retinopathy (DR) has classically been regarded as a disease of the retinal microvasculature, and the progression of the disease has been divided into 4 stages: early or pre-retinopathy, non-proliferative (or background) stage, pre-proliferative stage and the later, proliferative stage. It is characterized by a progressive permeability of the blood-retinal barrier, blood flow alterations, capillary occlusion leading to tissue ischemia, migration of leukocytes, formation of macular edema and retinal detachment in the more severe cases. Recently, it is becoming apparent that cells of the neuroretina are affected in diabetes, causing subtle impairments in vision preceding the more detectable vascular lesions, an alteration that seems to happen before the blood-retinal barrier is significantly affected (Cunha-Vaz, 2007; Barber, 2003).

In fact, there are several degenerative changes occurring early on, usually associated with neurodegenerative and inflammatory conditions (Fig. 1) such as deregulation of glutamate metabolism, increased neural apoptosis, microglial cell activation and amplified production of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) (Santiago *et al.*, 2007; Santiago *et al.*, 2006; Krady *et al.*, 2005; Oshitari and Roy, 2005; Barber, 2003; Li and Puro, 2002). When all the retinal and systemic changes are considered together, a picture of chronic inflammation emerges and this perspective seems to be important in the pathogenesis of DR, since inhibition of the inflammatory cascade at any of multiple steps can inhibit the early stages of DR in animals, a result consistent with studies in the human eye (Kern, 2007). Preliminary results from our group showed that neutralization of TNF-α with anti-TNF-α antibodies prevented rat retinal cell death induced by exposure in culture to high concentrations of glucose (unpublished data). If microglia activation and the release of

pro-inflammatory mediators could be averted or reduced, this could limit the effect of diabetes on vision loss, and improve the quality of life for diabetic patients.

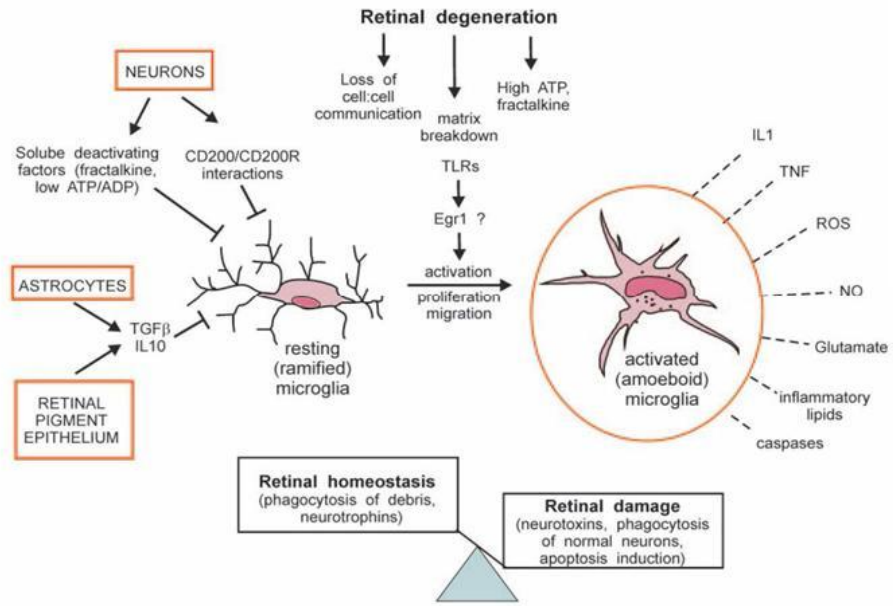

Figure 1: Representation of the inflammatory alterations occurring in the onset of diabetic retinopathy, triggered by the metabolic dysfunction caused by hyperglycemic conditions. (*in Langmann, 2007 Journal of Leucocyte Biology 81*)

1.2 – Retinal cells

The retina is composed of four major classes of cells (Fig. 2): blood vessel cells, macroglial cells, neuronal cells and microglial cells, and all of these cell types are affected by diabetes.

The blood vessels consist of both endothelial cells and pericytes that line them. Pericytes regulate retinal vascular flow while the endothelial cells regulate hemostatic functions and compose the blood–retinal barrier. Beyond the vascular cells we have macroglial cells, support cells that regulate retinal metabolism and modulate the function of neurons and blood vessels, and the main macroglia are Müller cells and astrocytes. Müller cells cross the whole thickness of the neural retina, constituting an

anatomical and functional connection between retinal neurons and retinal blood vessels, vitreous body and subretinal space and are key regulators of glutamate metabolism, extracellular ionic balance, and neuronal function. Astrocytes are limited to the nerve fiber layer where their processes wrap around blood vessels and ganglion cells. Together, Müller cells and astrocytes integrate vascular and neuronal activity in the retina.

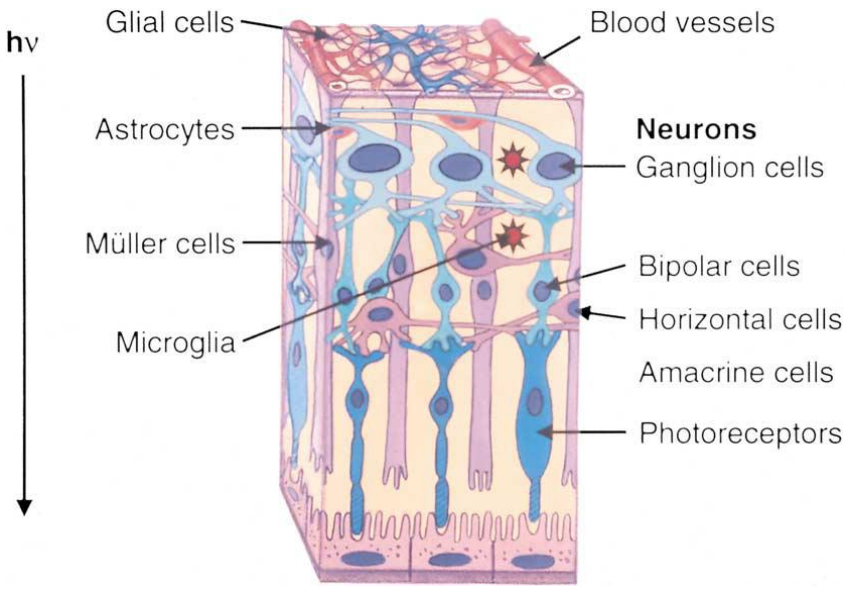

Figure 2: Representation of four major types of retinal cells: vascular (pericytes and endothelial cells); macroglial cells (Müller cells and astrocytes); neurons (photoreceptors, bipolar cells, amacrine and ganglion cells); and microglia. (*in Gardner et al., 2002)*

The third class of cells includes neurons: photoreceptors, bipolar cells, amacrine cells, and ganglion cells. These neurons mediate phototransduction, and modulate and communicate nerve impulses that are then transmitted to the brain through the axons of the ganglion cells that comprise the nerve fiber layer and optic nerve. Since neurons are the main players in vision, impairment of vision in DR and other disorders necessarily implies disturbance of their function.

The microglia constitutes the fourth class of cells. These cells are the immune cells characteristic of the nervous system and are present in the retina. Microglia are

normally quiescent, but their sensitivity to changes in the homeostatic state of the retina, means that when retinal homeostasis is disturbed or there is an inflammatory situation they rapidly become active, producing and releasing pro-inflammatory cytokines and gaining phagocytic activity, being at the core of inflammatory situations in the retinal tissue.

The interactions and functional integration of all of these cell types are required for normal vision so disruption of any of them may impair vision (Crooke *et al.*, 2008; Gardner *et al.*, 2002).

1.3 – Adenosine and Adenosine Receptors

1.3.1 – Adenosine as modulator and messenger

Adenosine is a purine nucleoside essential to all living cells that, beyond its role in energy metabolism and genetic transmission of information, is also a messenger that regulates numerous physiological processes in several tissues, particularly the heart and nervous system. Most of the effects of adenosine are related to energy expenditure, from reducing the activity of excitable tissues to promoting the delivery of metabolic substrates. But this simple view does not apply to other actions of adenosine, predominantly in the nervous system and inflammatory situations, where it is clear that adenosine has more than just one role as a messenger. Studies have shown the importance of adenosine in both normal and pathological situations, from regulation of sleep and linking energy demands to cerebral blood flow, to roles in inflammatory responses and in neuroprotection against ischemia and epilepsy. Adenosine also has a connection to several drugs of abuse, most notably to caffeine (Dunwiddie and Masino, 2001).

1.3.2 – Adenosine receptors

The actions of adenosine are mediated by specific receptors in the cell membrane. Based on differences in molecular structure and pharmacological profile, four different receptors have been cloned and characterized: A_1, A_{2A}, A_{2B}, and A_3. All these receptors are G protein coupled receptors, depicting seven transmembrane domains of hydrophobic amino acids, connected by three extracellular and three cytoplasmatic hydrophilic loops of unequal size. The N-terminal of the protein is on the extracellular side, and the C-terminal is on the cytoplasmatic side of the membrane (Ralevic and Burnstock, 1998). Although adenosine receptors govern cell function mainly by coupling to G proteins, some G-protein-independent effects have also been reported (Haskó *et al.*, 2008; Ralevic and Burnstock, 1998).

1.3.2.1 – A_1 receptor

A_1 adenosine receptor (A_1AR) is a very widely distributed receptor, and the most abundant adenosine receptor in the brain. It is present in glial cells and neurons, both pre and post-synaptically. It is also the receptor with the highest affinity for adenosine, with K_m = ±70 nM (Dunwiddie and Masino, 2001; Wardas, 2002). The receptor couples with G_i and G_o proteins and the most common signaling pathway is the inhibition of adenylate cyclase, which leads to a decline in the levels of cAMP, an important second messenger (Dunwiddie and Masino, 2001). This regulates the activity of cAMP-dependent protein kinase A (PKA), inhibiting the phosphorylation of various target proteins. Beyond the direct effect on adenylate cyclase, this inhibition also counters the effect of other agents that stimulate adenylate cyclase (Wardas, 2002).

Another important signaling mechanism mediated by A_1AR is the activation of phospholipase C (PLC), which increases the production of diacylglycerol and inositol

1,4,5-triphosphate (IP_3). IP_3 stimulates the release of stored Ca^{2+} by interacting with specific receptors on the sarcoplasmic reticulum. This increase in Ca^{2+} levels can then stimulate several other proteins and pathways, including protein kinase C (PKC), nitric oxide synthase and Ca^{2+}-dependent K^+ channels. In some tissues A_1AR interacts directly, through G proteins, with other types of K^+ channels. A_1AR also disrupt Ca^{2+} currents, by inhibiting membrane Ca^{2+} channels (Santos *et al.*, 2000; Ralevic and Burnstock, 1998).

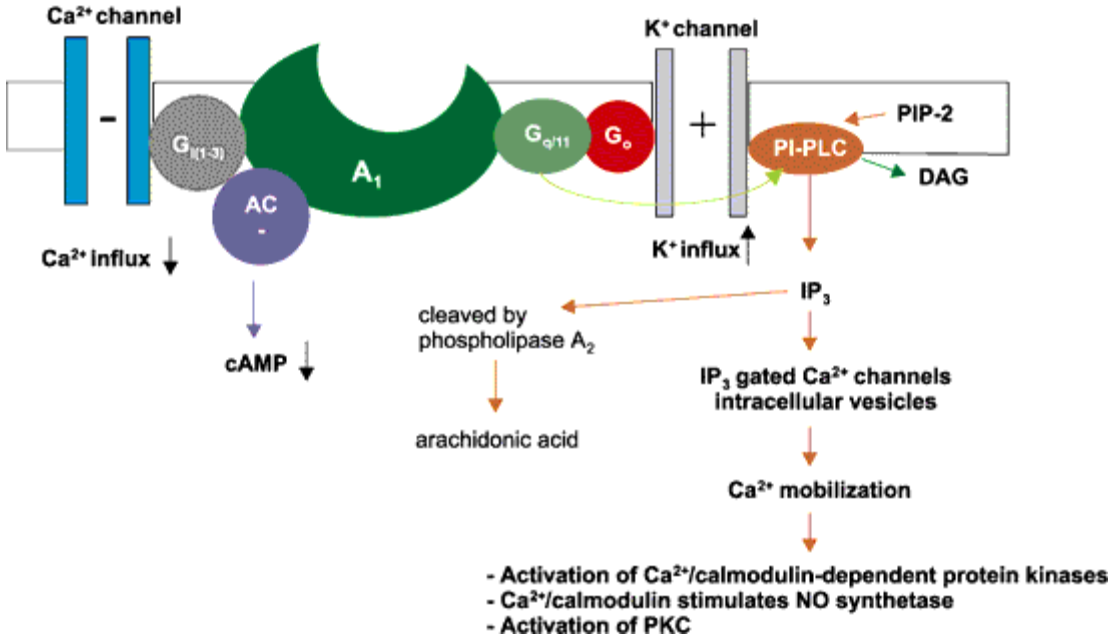

Figure 3: Representation of possible signaling pathways activated by a ligand binding to A_1 adenosine receptor. (*in Marx et al., 2001*)

The overall effect of A_1AR leads to a state of reduced activity, particularly in neurons where there is a decrease in neuronal excitability, firing rate and neurotransmitter release. It is largely due to these actions that adenosine plays a neuroprotective role during hypoxia and ischemic conditions (Ralevic and Burnstock, 1998).

1.3.2.2 – A_{2A} receptor

A_{2A} receptors ($A_{2A}AR$) are present in many tissues, but with a more focused distribution, including immune tissues and the central and peripheral nervous system. $A_{2A}AR$ are the adenosine receptor with the second highest affinity rate, K_m = ±150 nM (Dunwiddie and Masino, 2001). The receptor couples with G_s protein mainly, though other G proteins may be involved (Ralevic and Burnstock, 1998). The most common signal pathway for $A_{2A}AR$ is the activation of adenylate cyclase, increasing the levels of cAMP, and consequently the activity of PKA. This pathway may be responsible for the potentiation of synaptic transmitter release observed with $A_{2A}AR$ activation. Triggering the cascade downstream from PKA can result in phosphorylation of the transcription factor CREB (cAMP-response element-binding protein), on serine residue 133, activating it. Once active, CREB can mediate gene expression directly by interacting with gene promoters or indirectly by competing with nuclear factor κB (NFκB) or other factors for the CREB-binding protein (CBP) (Haskó *et al.*, 2008).

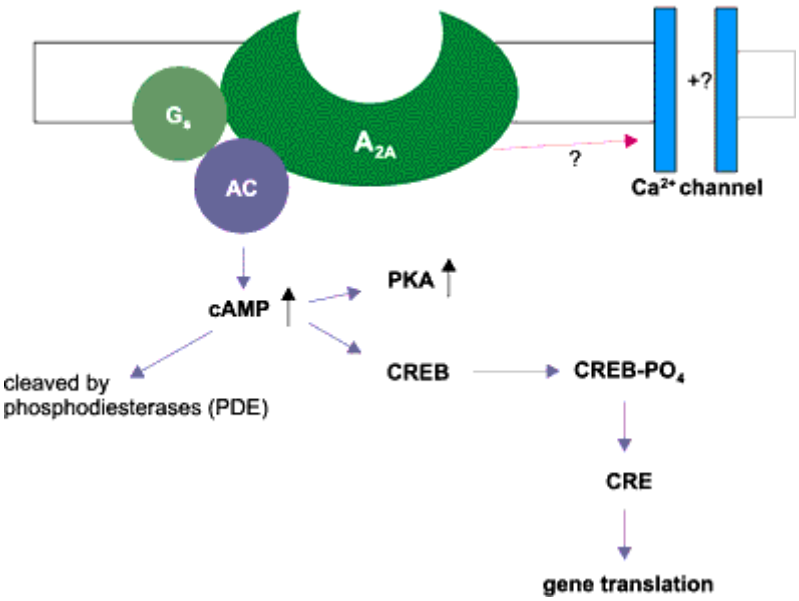

Figure 4: Representation of possible signaling pathways activated by a ligand binding to A_{2A} adenosine receptor. (*in Marx et al., 2001*)

Several cAMP-independent signal pathways have been proposed for $A_{2A}AR$, including modulation of P-type and N-type Ca^{2+} channels, activation of a serine/threonine protein phosphatase and the activation of mitogen-activated protein kinase (MAPK) (Kreckler *et al.*, 2006; Ralevic and Burnstock, 1998).

A_{2A} receptor influences several processes, from locomotion, behavior, promoting relaxation on blood vessels, to a very active role on the strong anti-inflammatory effects of adenosine (Ralevic and Burnstock, 1998).

1.3.2.3 – A_{2B} receptor

A_{2B} receptors ($A_{2B}AR$) are found in almost every cell, but in small number and require higher concentrations of adenosine to elicit a response, having an affinity rate of 5.1 µM. These receptors couple with G_s and G_q proteins and activate adenylate cyclase, being also able to activate PLC and promote a IP_3-dependent increase of intracellular Ca^{2+} (Dunwiddie and Masino, 2001; Ralevic and Burnstock, 1998).

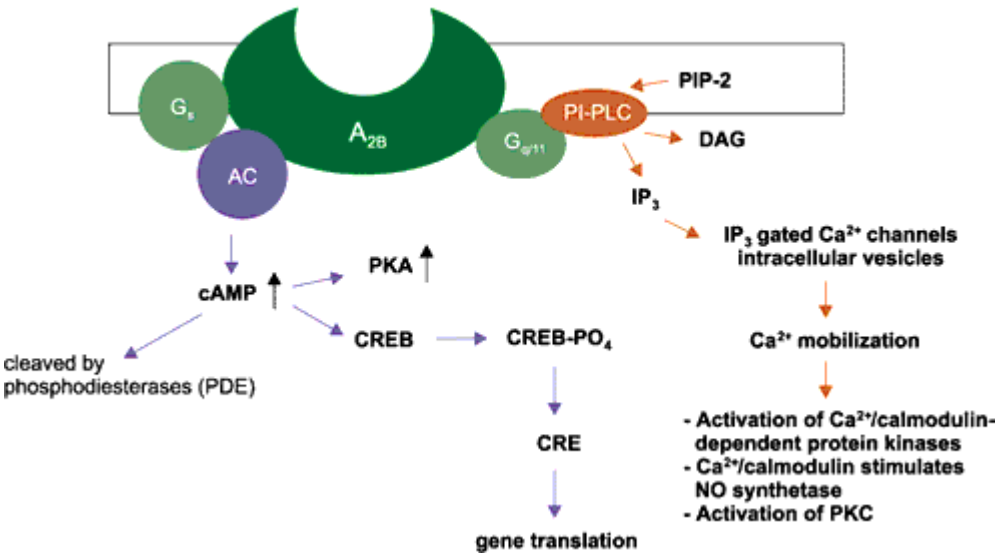

Figure 5: Representation of possible signaling pathways activated by a ligand binding to A_{2B} adenosine receptor. (*in Marx et al., 2001*)

For a long time there was a lack of selective A_{2B}AR agonists and antagonists, which made the study of its physiological role challenging. In blood vessels, A_{2B}AR are coupled to vasodilatation in both smooth muscle and endothelium and in the central nervous system they have been linked to increased neurotransmission. A_{2B}AR role in the immune system is somewhat contradictory: Earlier studies revealed that A_{2B}AR activation increased the release of several pro-inflammatory messengers, such as IL-8 and IL-6, in different cell types. Later it was shown that A_{2B}AR activation in macrophages suppressed TNF-α expression and could have, with selective agonists and in certain pathological situations, an effective anti-inflammatory role (Kreckler, 2006; Dunwiddie and Masino, 2001; Ralevic and Burnstock, 1998).

1.3.2.4 – A_3 receptor

The A_3 receptor (A_3AR) is widely distributed in the organism and is the receptor with the lowest affinity for adenosine (K_m = ±6.5 µM) (Dunwiddie and Masino, 2001). The A_3AR is probably the adenosine receptor that is less known and most intriguing. Although several studies have been performed in the years to clarify its physiological function, it still presents in several cases a double nature in different pathophysiological conditions (Gessi *et al.*, 2008).

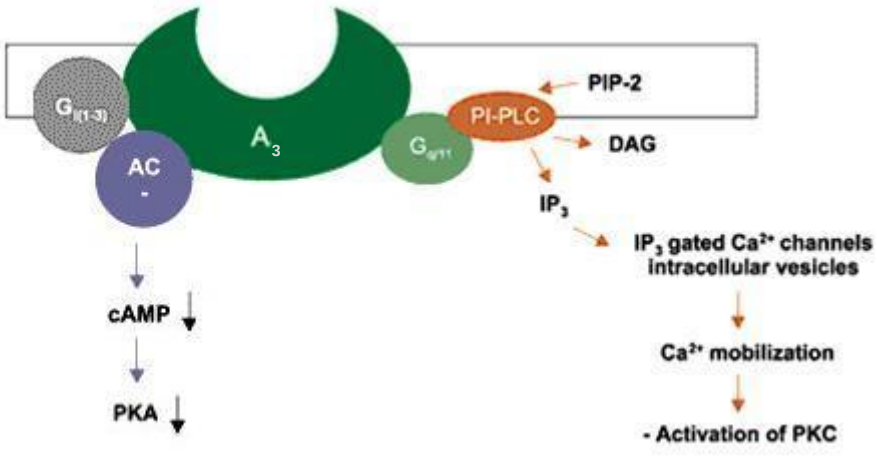

Figure 6: Representation of possible signaling pathways activated by a ligand binding to A_3 adenosine receptor. (*adapted from Marx et al., 2001*)

A_3AR couples with G_i and G_q proteins and triggers PLC, promoting a IP_3-dependent increase of intracellular Ca^{2+} (Dunwiddie and Masino, 2001). This receptor also inhibits adenylate cyclase and, via a PKC-dependent mechanism, is able to uncouple metabotropic glutamate receptors, suggesting a role in the modulation of receptor activity (Dunwiddie and Masino, 2001). A_3AR activation also mediates phosphorylation of protein kinase B and is able to decrease the levels of PKA (Fishman *et al.*, 2002). A study on microglial cells revealed an A_3AR mediated phosphorylation of extracellular signal-regulated kinase (ERK) 1/2, of the mitogen-activated protein kinase (MAPK) family, a signaling pathway involved in the modulation of microglia cells (Hammarberg *et al.*, 2003). A_3AR also plays a protective role in retinal ganglion cells, along with A_1 receptors (Zhang *et al.*, 2006).

1.3.2.5 – Interaction between adenosine receptors

The different adenosine receptors have distinct but frequently overlapping distributions. Due to this, the extracellular adenosine concentration and receptor affinity are responsible for the differential activation of one or more co-expressed subtypes. The co-expression of adenosine receptors allows adenosine to activate multiple signaling pathways and offers a self-modulation in several cases, with A_2 receptors activating adenylate cyclase and A_1AR and A_3AR inhibiting it. On the case of A_{2A}AR and A_{2B}AR, the interactions may be synergic, with the lower affinity A_{2B}AR supporting the higher affinity A_{2A}AR, which may suffer desensitization faster (Ralevic and Burnstock, 1998).

There is also evidence of situations where one adenosine receptor may act as a modulator of other adenosine receptors: A_3AR is able to uncouple A_1AR, via a PKC-dependent mechanism (Dunwiddie and Masino, 2001), and a study performed in avian retinal cells showed a connection between high levels of A_{2A}AR activation and an up-regulation of A_1AR, via a cAMP/PKA dependent pathway (Pereira *et al.*, 2010).

1.4 – Adenosine Cycle

In many systems, the basal concentrations of extracellular adenosine (25-250 nM in the brain) are able to activate a considerable number of high-affinity adenosine receptors, A_1AR and A_{2A}AR, suggesting that the interactions between these two receptors set a basal purinergic balance that shows an equilibrium between the release and uptake of extracellular adenosine, a signal for homeostasis in the system (Fredholm, 2007; Dunwiddie and Masino, 2001).

There are two main mechanisms for increasing the levels of extracellular adenosine: 1) extracellular conversion of adenine nucleotides to adenosine and 2) the release of adenosine from the cell, mediated by transporters.

1.4.1 – Extracellular conversion of adenine nucleotides

Several extracellular enzymes handle the conversion of adenine nucleotides. Through several enzymes, nucleoside triphosphate diphosphohydrolases (NTPDase), ecto-nucleotide pyrophosphatase/phosphodiesterases (ecto-NPP), apyrases, virtually all adenine nucleotides can be dephosphorylated to 5'-AMP, which is then dephosphorylated to adenosine by 5'-ectonucleotidase (5'-eN). These enzymes have a rather broad specificity and are generally rapid in their action (Dunwiddie and Masino, 2001). 5'-eN is a homodimer linked to the plasma membrane through a glycosyl-phosphatidylinositol lipid anchor, and is inhibited by ATP and ADP. Due to this inhibition, the conversion of 5'-AMP to adenosine becomes the rate-limiting step in the catabolism of ATP and ADP (Latini and Pedata, 2001). It has been observed an up-regulation of this enzyme on ischemic situations, in glial cells, speeding up the removal of extracellular ATP, decreasing its citotoxic effects, and increasing the neuroprotective actions of adenosine (Latini and Pedata, 2001; Braun *et al.*, 1998).

There are several mechanisms for adenine nucleotide release, and they vary according to the regions of the central nervous system (CNS) and methods of stimulation used (Latini and Pedata, 2001). ATP is colocalized with neurotransmitters such as acetylcholine, dopamine, 5-HT and norepinephrine, and is coreleased upon electrical stimulation. Extracellular ATP activates P2 receptors and is quickly converted to adenosine, serving a dual purpose: removal of ATP and activation of more receptors (Dunwiddie and Masino, 2001). cAMP can also be released into the extracellular space in a number of systems, through an non-specific energy-dependent transporter, providing another source for adenosine production. This was observed with stimulation of adenylate cyclase, by activating receptors or by using forskolin, and suggests that the receptors that transmit the signal through adenylate cyclase may have a role in regulating the basal extracellular adenosine levels. cAMP can also be converted to 5'-AMP inside the cells, with the latter being the released source of adenosine, a possibility raised upon observation of a NMDA-evoked adenosine release in rat cortical slices (Latini and Pedata, 2001).

1.4.2 – Adenosine transport and release

Another mechanism that regulates extracellular adenosine levels is the release of intracellular adenosine by bi-directional nucleoside transporters, although a neuronal Ca^{2+}-dependent release may be possible (Latini and Pedata, 2001).

There are two functionally distinct groups of these transporters: equilibrative nucleoside transporters, carrying nucleosides through the cell membrane according to their concentration gradient (more abundant in the CNS); concentrative nucleoside transporters that use the Na^{+} gradient to provide energy for the transport (Latini and Pedata, 2001). The equilibrative transporters are classified according to their sensitivity to the selective inhibitor nitrobenzylthioinosine, with the insensitive transporters

presenting a lower affinity for substrates than the sensitive transporters. The concentrative transporters have also been divided into five subclasses.

In homeostatic situations, the intracellular adenosine concentration is low due to the high activity of adenosine kinase (AK), making the net flux across the equilibrative transporters inwardly directed. But in conditions where the concentration of adenosine inside the cell rises, the flux shifts and these transporters release adenosine to the extracellular space. Similarly, the concentrative transporters could invert the flux when intracellular adenosine concentration is high and the Na^+ gradient is diminished, such as hypoxia, ischemia and seizures, making these transporters another possible mechanism of adenosine release (Dunwiddie and Masino, 2001).

1.4.3 – Removal of extracellular adenosine

To ensure the proper function of the adenosine signaling pathways, extracellular adenosine must be cleared or inactivated afterwards, preventing unwanted receptor activation.

The primary mechanism is the uptake across the cell membrane by neurons and neighboring cells. Once inside, adenosine can be either phosphorylated to AMP by AK or deaminated to inosine by adenosine deaminase (ADA). Both enzymes are involved in the regulation of intracellular adenosine levels, and the different effects of ADA and AK inhibitors in several experiments can be caused by their different affinity values. Since AK presents a much lower K_m (2 µM for AK while ADA features a K_m of 17-45 µM, in rat whole brain), it becomes easily saturated with basal concentrations of adenosine, which are in the nanomolar range. Due to this, it is probable that phosphorylation by AK is the main pathway of adenosine metabolism in normal physiological conditions, while deamination by ADA gains a more important role when adenosine concentrations rise, such as in ischemic situations (Dunwiddie and Masino, 2001; Latini and Pedata, 2001).

Another mechanism for the inactivation of extracellular adenosine is the deamination to inosine by the extracellular form of ADA, although most of the extracellular adenosine is cleared by reuptake in normal conditions. This extracellular form does not have its own hydrophobic/transmembrane domain, being present on the surface of many cell types by alternative anchoring mechanisms, such as association with CD26 (Dong *et al.*, 1996) and interactions with A_1AR and A_{2B}AR (Franco *et al.*, 1997). During hypoxia and ischemia ecto-ADA may gain a more prominent role in regulating extracellular adenosine concentrations, since these situations raise the intracellular levels of adenosine, affecting the gradient concentration and disrupting the inward flow of nucleotides through the transporters (Dunwiddie and Masino, 2001).

1.4.4 – Regulation of intracellular adenosine

Due to the presence of equilibrative transporters, the concentration of intracellular adenosine directly affects the extracellular levels of this nucleoside, making the regulation of the enzymes involved in the adenosine cycle critical.

There are two soluble 5'-nucleotisases found in the cytosol: one IMP-selective and another AMP-selective, with an affinity 15-20 times higher for AMP than IMP. Since the K_m values for both enzymes (1-14 mM) are much higher than the intracellular concentration of AMP under physiological conditions (0.1-0.5 mM), it is probable that a large increase in AMP concentrations is required to activate these enzymes, which occurs during high metabolic activity. Since the intracellular concentration of ATP exceeds that of AMP roughly 50x, small variations in ATP catabolism are enough to significantly alter the concentration of AMP, which means this interaction can function as a sensitive signal of increased metabolic rates or metabolic stress (Latini and Pedata, 2001).

Another possible cytosolic source of adenosine stems from the hydrolysis of *S*-adenosylhomocysteine (SAH) by SAH hydrolase, in a pathway not closely linked to the

energy cycle of the cell. Despite providing one third of the adenosine production in homeostatic conditions in organs such as the heart, studies with selective inhibitors indicate that this pathway is not particularly relevant in the brain. The reversible reaction catalysed by SAH hydrolase can also function as a possible pathway for adenosine inactivation, using it and $_L$-homocysteine to form SAH. $_L$-homocysteine availability limits the use of this pathway and, since its levels are very low in the brain, SAH synthesis is not a major player in adenosine metabolism in the central nervous system under physiological conditions (Latini and Pedata, 2001).

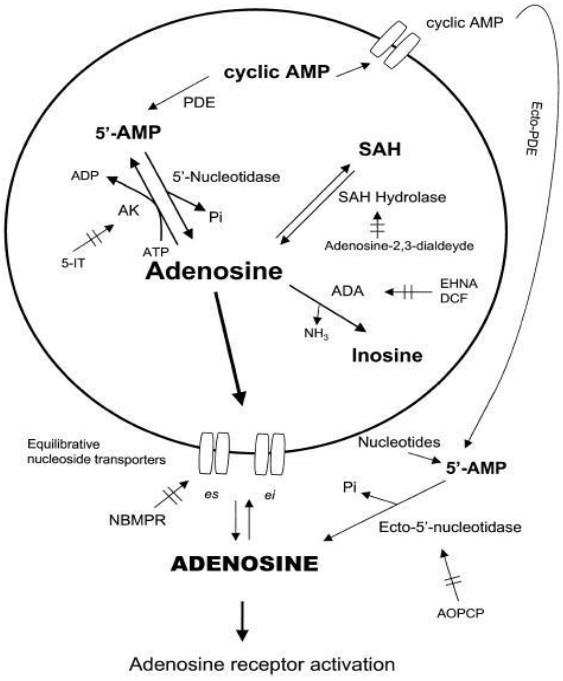

Figure 7: Intra and extracellular pathways of adenosine production, metabolism and transport, with indication of several inhibitors and their target enzymes. Abbreviations not on text: es, equilibrative-sensitive nucleoside transporter; ei, equilibrative-insensitive nucleoside transporter; PDE, phosphodiesterase; AOPCP, α,β-metylene ADP; DCF, deoxycoformycin; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenosine; NBMPR, nitrobenzyl-thioinosine. (*in Latini and Pedata, 2001*)

1.4.5 – Stimuli that can induce adenosine release

There are numerous physiological manipulations that can increase extracellular adenosine, although several pathways used are not well understood. Adenosine can be released in response to such stimuli as high concentrations of K^+, electrical stimulation, glutamate receptor agonists, hypoxia, ischemia, hypoglycemia and seizures. Many of them are related to the energy pathways in the brain, showing that manipulations causing energy requirements too high for the cell's ATP production rates (by a higher energy demand or impairment of ATP synthesis) increase the levels of extracellular adenosine, showing once again the probable role of adenosine levels as an indicator of the metabolic state (Dunwiddie and Masino, 2001; Latini and Pedata, 2001).

1.5 – Adenosine in inflammation

Recent studies exposed the role of adenosine as a key regulatory molecule in the pathophysiology of inflammatory diseases, exerting a protective effect on the majority of situations. The four adenosine receptors are expressed on virtually all cell types involved in orchestrating an inflammatory/immune response, including microglial cells (Haskó and Pacher, 2008; Liou *et al.*, 2008). The precise roles of the different receptors appear to differ in a time- and tissue-dependent manner. What is beneficial in one tissue at one time may be detrimental at another time or place.

1.5.1 – A_{2A} receptor in inflammation

Of the four adenosine receptors, $A_{2A}AR$ are considered the primary mediators of the immunologic effect of extracellular adenosine, down-regulating inflammation and immunity, for the greater part (Haskó and Pacher, 2008; Liou *et al.*, 2008). The $A_{2A}AR$

gene expression is driven by at least four independent promoters, and their regulation is under strong investigation, already becoming clear that A_{2A}AR gene expression is very sensitive to alterations in the extracellular environment, such as concentrations of exogenous and endogenous inflammatory factors (Haskó and Pacher, 2008). Exposure of human and murine macrophages to lipopolysaccharide (LPS) induced a dramatic increase in the expression of A_{2A}AR mRNA and protein, through activation of NFκB (Murphee *et al.*, 2005). This same factor was also activated on human monocytes, by

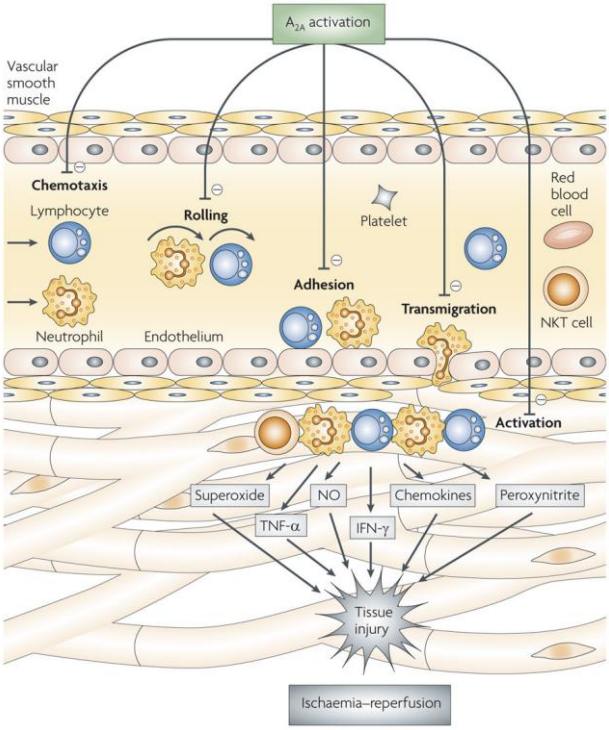

Figure 6: A_{2A}AR activation protects organs from ischemia–reperfusion injury by inactivating the ischemia–reperfusion-induced inflammatory response. A_{2A}AR activation reduces rolling, adhesion and transmigration of several inflammatory cells, including natural killer T (NKT) cells, lymphocytes and neutrophils. A_{2A}AR stimulation also limits inflammatory cytokine and chemokine production, superoxide release and interferon-γ (IFN-γ) secretion. NO, nitric oxide; TNF-α, tumor-necrosis factor-α. (*in Haskó et al., 2008*)

endogenous TNF-α and IL-1α (Khoa *et al.*, 2001), and on human lymphocytes and neutrophils by endogenous TNF-α (Varani *et al.*, 2009). Beyond the effects on A_{2A}AR

expression, TNF-α can also forestall A_{2A}AR desensitization by preventing translocation of G protein-coupled receptor kinase 2 and β-arrestin to the plasma membrane, leading to an increased receptor sensibility to its agonist (Khoa *et al.*, 2006).

The effects of A_{2A}AR activation on immune cells are considerable. On monocytes and macrophages, A_{2A}AR activation reduces migration, Toll-like receptors (TLR)-induced activation of NFκB and the production of several pro-inflammatory cytokines, such as TNF-α (Haskó *et al.*, 2008;). A_{2A}AR exerts similar effects on other immune cells, such as lymphocytes, neutrophils, peripheral blood mononuclear cells (PBMC), natural killer T cells and dendritic cells (Varani *et* al, 2009; Hamano *et al.*, 2008; Fredholm, 2007). On microglia, A_{2A}AR show an anti-inflammatory action, by preventing pro-inflammatory cytokine expression and release (Liou *et al.*, 2008).

1.5.2 – Other adenosine receptors in inflammation

Beyond the dominant action of A_{2A}AR, numerous studies highlighted the role of other adenosine receptors in the anti-inflammatory effects of adenosine, but the research is sometimes conflicting, since it is difficult to study the important tissue resident cells in isolation in a defined activation state, and many of the earlier works used agonists and antagonists with low specificity (Haskó *et al.*, 2008). The main contributor seems to be A_3AR, with several studies showing that its activation suppresses pro-inflammatory cytokine expression in several immune cells, including inhibition of TNF-α production in microglial cells (Varani *et al.*, 2009; Lee *et al.*, 2006; Haskó and Cronstein, 2004).

A study using a combined approach of using A_{2A}AR knockout mice and the A_{2B}AR antagonist MRS 1754 supports a role for A_{2B}AR in the inhibition of TNF-α release (Kreckler *et al.*, 2006). However, it appears that A_{2B}AR become operational only when their effect is not masked by A_{2A}AR, because both MRS 1754 and genetic deletion of A_{2B}AR in the presence of functional A_{2A}AR fails to affect the suppression of

TNF-α production (Haskó* *et al.*, 2008). A_{2B}AR have opposing effects from tissue to tissue, and are being increasingly recognized as important mediators of inflammation. A_{2B}AR promote the inflammatory response of mast cells, epithelial cells, smooth muscle cells and fibroblasts, contributing to the pathophysiology of asthma and colitis, but prevent tissue injury after hypoxia and ischemia by limiting endothelial cell inflammatory responses and suppressing macrophage activation (Haskó* *et al.*, 2008).

Most of the signaling pathways of A_1AR were uncovered in non-immune cells, making the A_1AR signaling mechanisms in cells of the immune system largely unknown. There are, however, several examples of A_1AR activity in inflammatory situations: There are studies revealing that A_1AR regulates innate inflammatory responses in macrophages and neutrophils (Linden, 2001), and one work showing that reduced A_1AR expression in microglia/macrophages leads to enhanced pro-inflammatory responses and the release of cytotoxic factors, while up-regulation of A_1AR through use of caffeine attenuates the inflammatory response (Tsutsui *et al.*, 2004). A_1AR also appears to enhance adhesion on neutrophils, but the effect may be due to A_1AR activation on endothelial cells and not the neutrophils themselves (Fredholm, 2007). They also regulate, along with A_3AR, the activation and recruitment of immature dendritic cells (Haskó *et al.*, 2008).

1.6 – The adenosinergic system in diabetes

The adenosinergic system is the target of several alterations caused by various forms of stress and pathological conditions. Ischemia and/or hypoxia can directly increase the expression of A_{2B}AR via hypoxia-inducible factor 1α and causes a down-regulation of A_1AR (Fredholm, 2007). Hypoxia also stimulates adenosine receptor trafficking in some types of cells, and can increase the expression of 5'-eN and ecto-NTPDase as well as decrease the expression of nucleoside transporters, that in this condition tend mainly to reduce adenosine levels (Fredholm, 2007). The induction of

epilepsy, either through kainate injection, electrical kindling or sub-chronic restraint stress led to a down-regulation of inhibitory A_1AR and to an up-regulation of A_{2A}AR in cortical regions, independently of neuronal loss (Duarte *et al.*, 2006). Thus, there seems to be a general occurrence of adaptative changes of the density of adenosine receptors upon prolonged noxious conditions. Diabetes, being a prolonged condition that causes metabolic alterations to the cellular environment, was likely to influence the adenosinergic system as well. Four recent studies investigated the effect of diabetes and hyperglycemia on adenosine receptors and several of the enzymes responsible for the regulation of extracellular adenosine levels.

In the hippocampus of streptozotocin (STZ)-induced diabetic rats, A_1AR density was reduced 7 days after induction of diabetes and this down-regulation was maintained after 30 and 90 days. In contrast, the density of A_{2A}AR was increased 7 days after induction of diabetes and this up-regulation also persisted after 30 and 90 days. These changes are similar to modifications occurring upon aging, where there is also an up-regulation of A_{2A}AR and down-regulation of A_1AR in the hippocampus, and may be related to the cognitive deterioration associated with diabetes (Duarte *et al.*, 2006).

On human blood platelets, a comparative study between *Diabetes Mellitus* Type II patients and a healthy control group of similar age and sex was carried to evaluate the activity of NTPDases and 5'-nucleotidase, two enzymes involved in the regulation of extracellular adenosine levels. The activity of both enzymes was increased in patients with diabetes and associated pathologies, demonstrating that the hydrolysis of adenine nucleotides is affected by hyperglycemic conditions (Lunkes *et al.*, 2008).Another study performed in platelets of STZ-treated rats, demonstrated that NTPDase, ecto-NPP, 5′-nucleotidase and ADA activities were increased in diabetic rats, indicating an up-regulation of the enzymes connected to adenosine extracellular levels. This increased activity could be related to a compensatory organic response (Schmatz *et al.*, 2009).

Finally, a study performed in the retina of STZ-treated rats showed that adenosine is heavily involved in the inhibition of osmotic glial swelling during diabetes, and that diabetic conditions prompt a differential expression of the nucleotide degrading enzyme NTPDase 1, which hydrolyzes ATP and ADP about equally well, from being restricted to blood vessels in control animals to being present in the retina, in glial cells in diabetic animals (Wurm *et al.*, 2008).

1.7 - Objectives

There is compelling evidence that diabetes can modulate the adenosinergic system in several tissues. However, little is known about the effect diabetes or hyperglycemia may have in this same system in the retina. With the onset of diabetic retinopathy triggered by inflammatory reactions in response to the alterations caused by hyperglycemic conditions, and the marked anti-inflammatory role of adenosine, it is imperative we understand if diabetes or hyperglycemia can alter the adenosinergic system in the retinal tissue. Thus, this project aimed at studying whether the adenosinergic system in the retina was altered when the shift from normal physiological conditions to diabetic/hyperglycemic pathophysiological conditions occurred. We examined if diabetic conditions affected the protein levels of adenosine receptors and ADA in the retina, and also focused on the activity levels of ADA under hyperglycemic conditions. Finally, viability assays were performed to begin understanding the outcome of these alterations in DR.

Chapter 2 Materials and Methods

2.1 – Materials

Minimum Essential Medium (MEM), penicillin and streptomycin, protease inhibitors chymostatin, pepstatin, antipain and leupeptin (CLAP), and streptozotocin (STZ) were acquired from Sigma-Aldrich Química S.A. Trypsin UPS grade was obtained from GIBCO GRL. Life Fetal Bovine Serum (FBS) was obtained from Biochrom. BCA Protein Assay Kit was acquired from Thermo Scientific. Sodium dodecyl sulfate (SDS) solution 10%, bromophenol blue, bis-acrylamide solution 30% were acquired from Bio-Rad Laboratories. Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Madrid, Spain). Tween-20 was purchased from Sigma-Aldrich Química S.A. and low-fat dry milk used was from the Nestlé brand. Enhanced chemifluorecense substrate (ECF) was purchased from GE Healthcare. Triton X-100 and fatty acid-free bovine serum albumin (BSA) were purchased from Merck. All other reagents were obtained from Fisher Scientific, Sigma-Aldrich and Merck. Antibodies used are described in Table I. All solutions were aqueous if not described, expect for phenylmethanesulphonylfluoride (PMSF) which was prepared in dimethyl sulfoxide (DMSO).

Table I: Protocol details of antibodies used in Western Blot and Immunocytochemistry

	Host	Type	Dilution Western Blot	Dilution Immuno cytochemistry	Origin
Primary antibodies					
Anti-A_1	Rabbit	Polyclonal	1:1000	1:100	Calbiochem
Anti-A_1	Rabbit	Polyclonal	1:300	-	Alomone Labs
Anti-A_{2A}	Rabbit	Polyclonal	-	1:200	Alomone Labs
Anti-A_{2A}	Rabbit	Polyclonal	1:500	-	Santa Cruz Biotechnology
Anti-A_{2B}	Rabbit	Polyclonal	1:1000	1:200	Alomone Labs
Anti-A_3	Rabbit	Polyclonal	1:500	1:200	Alomone Labs
Anti-ADA	Rabbit	Polyclonal	1:500	1:200	Santa Cruz Biotechonology

Anti-CD11b	Mouse	Polyclonal	-	1:200	Chemicon
Anti-β tub	Mouse	Monoclonal	-	1:400	Lab Vision
Anti- GFAP	Mouse	Polyclonal	-	1:400	Invitrogen
Anti-Vimentin	Mouse	Monoclonal	-	1:400	Lab Vision
Anti-actin	Mouse	Polyclonal	1:20000	-	Invitrogen
Secondary antibodies					
Alkalyne phosphatase-conjugated Anti-rabbit	Goat	Polyclonal	1:20000	-	GE Healthcare
Alkalyne phosphatase-conjugated Anti-mouse	Rabbit	Polyclonal	1:20000	-	GE Healthcare
Alexa Fluor 594 Anti-rabbit	Goat	Polyclonal	-	1:200	Invitrogen
Alexa Fluor 488 Anti-mouse	Donkey	Polyclonal	-	1:200	Invitrogen

2.2 – Cellular Model

Retinal cell cultures were obtained from new-born (3-4 days old) Wistar rats. Each culture was made from a single litter of 8 to 12 animals. The rats were decapitated and retinas were dissected under sterile conditions in a Ca^{2+} and Mg^{2+}-free Hank´s solution (CMF, in mM: NaCl 32, KCl 5.4, KH_2PO_4 0.45, Na_2HPO_4 0.45, $NaHCO_3$ 4, glucose 2.8, HEPES 5, pH 7.1). The retinas were then subjected to digestion in CMF supplemented with trypsin (2 mg/ml), at 37 ºC for 12-15 minutes. After this incubation the tissue was mechanically dissociated using a glass pipette and the cellular suspension was gently centrifuged (59 g/ min). The resulting supernatant was discarded and the pellet resuspended in Modified Eagle's medium (MEM), supplemented with fetal bovine serum (FBS) 10% (v/v), penicillin 100 U/ml, streptomycin 100 µg/ml, HEPES 25 mM and $NaHCO_3$ 26 mM. Cellular viability and density were measured by a viable cell count using Trypan Blue staining. Cells were

diluted 1:50 in CMF with Trypan Blue 0.4% and placed in a hemocytometer under a optical microscope for counting.

The cells were plated at a density of 2.0 x 10^6 cells/cm^2 on plastic multi-well plates coated with poly-D-lysine (0.1 mg/ml) and multi-wells containing glass coverslips with 1.6 cm diameter. Cells were then maintained at 37 °C, in a humidified atmosphere of 95% air and 5% CO_2. After two days, the culture medium was supplemented with D-glucose 25 mM, reaching a final concentration of glucose 30 mM to simulate high glucose conditions in diabetes, or with D-mannitol 25 mM (plus glucose 5 mM), which was used as an osmotic control. The concentration of glucose in control conditions was 5 mM. The cells were used for experimentation seven days after the incubation.

2.2.1 – Whole cell extracts

After nine days in culture, the multi-wells were removed from the incubator and rapidly place on ice. The culture medium was removed and cells were washed three times with ice-cold Krebs buffer solution (in mM: NaCl 111, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 24.8, glucose 11.1, HEPES 15, pH 7.4), and whole cell extracts were prepared by scrapping the plates in 80 µl of lysis buffer (NaCl 137 mM, Tris 20 mM, Nonidet P-40 1% (v/v), glycerol 10%) supplemented with protease inhibitors (PMSF 0.1 mM, CLAP 1 µg/ml, DTT 1mM, orthovanadate 1mg/ml, NaF 1 M), on ice. The suspension obtained was then sonicated five times with five second pulses and cleared of their insoluble fraction by centrifugation (15700 g, 10 min, 4 ºC). The protein concentration was determined by bicinchoninic acid (BCA) method using the BCA protein kit and its recommended protocol. 6x concentrated sample buffer was added (4x Tris-HCl, glycerol 30%, SDS 10%, DTT 600 mM, bromophenol blue 0.012%) and the extracts were denatured with heat (95 °C for 10 minutes).

2.3 – Animal model

In this work we have used a model of type 1 diabetes. Eight week old male Wistar rats, purchased from Charles River Laboratories (Spain), were used for whole retina extracts. The induction of diabetes was performed by intraperitoneal injection of STZ, 65 mg/kg (animal weight) in sodium citrate 10 mM. Weight and blood sugar levels were recorded for each animal on the day of injection and two days afterwards to confirm the effects of the drug. The animals were considered diabetic when presenting blood sugar levels above 250 mg/dl. The animals were maintained on a regular chow diet, *ad libitun,* for three different time periods: 7 days, 30 days and 60 days, after which the animals were sacrificed.

2.3.1 – Whole retina extracts

The animals were anesthetized and decapitated. The eyes were removed and the retinas extracted and placed in 200 µl of lysis buffer, supplemented with protease inhibitors (PMSF 0.1 mM, CLAP 1 µg/ml, DTT 1mM, orthovanadate 1mM, NaF 1 M) and homogenized with a small piston on ice. The extracts were sonicated five times with 10 second pulses and then frozen and thawed 3 times, using dry ice and ethanol 70%. Afterwards the extracts were cleared of their insoluble fraction by centrifugation (15700 g, 10 min, 4 ºC). The protein concentration was determined by BCA method using the BCA protein kit and its recommended protocol. 6x concentrated sample buffer was added and the samples were denatured with heat (95 °C for 10 minutes).

2.4 – SDS-PAGE and Western Blot

Samples were electrophoresed in polyacrilamide gels using MiniPROTEAN® 3 systems (Bio-Rad Laboratories). Electrophoresis Resolving gel composition was: bis-acrilamide concentration was either 10% (v/v) or 7.5% (v/v) according to the targeted protein, Tris-HCl 1.5M 25%, SDS 0.1% (v/v), TEMED 0.05%, APS 0.05% (v/v) in ultra-pure water, 10 ml per gel. For the stacking gel: bis-acrilamide 4% (v/v), Tris-HCl 0.5M 25%, SDS 0.1% (v/v), TEMED 0.05%, APS 0.05% (v/v) in ultra-pure water, 5 ml per gel.

Loaded volumes of each input were determined so that the applied protein mass was the same in each lane for the targeted protein (40-60 µg); gels were submerged in running buffer (Tris 25 mM, glycine 190 mM, SDS 0.1%, pH 8.3) and electrophoresis was carried at 60 V for 10 minutes and then 130 V until proper molecular weight separation was reached (30-40 min); the proteins were then electrotransferred (750 mA, for 150 min) to PVDF membranes, submerged in electrophoresis buffer (CAPS 10mM, methanol 12.5%, pH 11 adjusted with NaOH), using Trans-Blot Cell apparatus (Bio-Rad Laboratories). After this procedure, membranes were immersed in Tris-buffered saline (Tris 2.4 g/L, NaCl 8 g/L, pH 7.6 adjusted with HCl) containing 0.1% (v/v) Tween 20 (TBS-T) and 5% (w/v) low-fat dry milk for 1 hour. Primary antibodies were diluted in TBS-T containing 1% (w/v) low-fat dry milk, and the membranes were incubated overnight, at 4 °C. Afterwards membranes were washed with TBS-T (3x 10 min each) and then incubated with a suitable alkaline phosphatase-conjugated secondary antibody diluted in the above solution for 1 hour at room temperature. Membranes were again washed, and then dried before being resolved using enhanced chemifluorecense substrate (ECF). The scanning was performed with the VersaDoc Imaging System (Bio Rad Laboratories), and the data analysed with Quantity One (Bio Rad Laboratories). The exception to this protocol was the anti-A_{2B} antibody: membranes to be incubated with this antibody were

blocked overnight, at 4 °C, using phosphate buffer saline solution (PBS, in mM: KH_2PO_4 1.4, Na_2HPO_4 8, NaCl 140, KCl 2.7, pH 7.3) with 0.1% (v/v) Tween 20 (PBS-T) instead of TBS-T, along with 0.025% azide (v/v) and the washing was done using PBS-T as well.

For the loading control, membranes were stripped using 0.1 M glycine, pH 2.3 and then reprobed with anti-actin antibody diluted in TBS-T containing 1% (w/v) low-fat dry milk, for 1 hour at room temperature. The described protocol was then followed for the remainder of the technique.

2.5 – Enzymatic assay

For the (ADA) activity assay, cell lysates were prepared in Tris-HCl 50 mM, PMSF 0.2 mM, CLAP 1 µg/ml, pH 7.2 and cleared of their insoluble fraction by centrifugation (3000 g, 10 min, 4 ºC). The protein concentration was determined by BCA method using the BCA protein kit and its recommended protocol. The protein content in the samples used was between 0.7 and 0.9 mg/ml.

The activity of ADA was determined according to Guisti and Galanti (1984) based on the Bertholet reaction. This method is based on the direct production of ammonia when ADA acts in excess of adenosine, where ADA activity is then measured by the formation of colored indophenol complex from the ammonia produced and quantified spectrophotometrically. 100 µl of the samples were added to 500 µl of a solution of 21 mM of adenosine in 50 mM phosphate buffer [$NaH_2PO_4(H_2O)$ 4.73 g/L, $Na_2HPO_4(H_2O)_{12}$ 5.62 g/L], pH 6.5 and were incubated at 37 °C for 60 min. For a standard, a solution of ammonium sulphate [$(NH_4)_2SO_4$ 75 mM in phosphate buffer] was used and for a reagent control, phosphate buffer was used. No samples were added to these two conditions nor to the sample control, which contained only adenosine solution. Afterwards, 1.5 ml of a phenol solution (phenol 106 mM, sodium nitroprusside 170 µM) and a sodium hypochlorite solution (NaOCl 11 mM, NaOH 125

mM) were added and the samples incubated at 37 °C for 30 min. The final products were quantified spectrophotometrically at a wavelength of 620 nm. Results were calculated according to the formula: [(sample - sample control)/ (standard - reagent control)] x50; results were expressed in units per liter (U/L). One unit (1 U) of ADA is defined as the amount of enzyme required to release 1 mmol of ammonia per minute from adenosine at standard assay conditions (Schmatz *et al.*, 2009).

2.6 – Immunocytochemistry

Glass coverslips containing the retinal cells were removed from the culture wells and washed three times with ice-cold PBS. Cells were fixed by addition of a solution of 4% paraformaldehyde, 4% sacarose in PBS. After 20 minutes, at room temperature, cells were washed as previously described and then permeabilized, using a solution of 1% Triton X-100 in PBS for 5 minutes. Blockade was performed with PBS with 3% fatty acid-free bovine serum albumin (BSA) and 0.2% Tween-20. Another set of washing was done between each step. After blocking, cells were incubated for 1 hour at room temperature with primary antibodies diluted in the blocking solution according to manufacturer specifications. Then the cover slips were washed and incubated with the appropriate secondary antibodies coupled to Alexa Fluor 594 or 488 for 1 h at room temperature, after which cells were incubated with Hoechst 33342, 1 µg/ml, for 10 minutes. After washing four times with PBS, the excess liquid was gently absorbed with tissue paper and the cover slips were mounted using Dako fluorescence mounting medium (Dako) onto microscope slides. The slides were left to dry overnight in a dark container, and afterwards the coverslips were contoured with clear nail polish. The slides obtained were visualized using the fluorescence microscope Zeiss Axioshop 2 Plus coupled to a digital camera, Axiocam HRc.

For preparations with the anti-A_{2A} and ADA antibodies, the incubation was performed overnight, at 4 °C, and followed by the secondary antibody. Any additional marking was performed separately, following the described protocol.

2.7 – Viability assay

To attest the viability of cells in the presence of the A_{2A}AR antagonist, we used the MTT assay, a colorimetric method based on the enzymatic reduction of MTT (methylthiazolyldiphenyl-tetrazolium bromide) (Mosmann, 1983). MTT, when taken up by living cells, is converted from a yellow to a water-insoluble purple colored precipitate (formazan) by cellular dehydrogenases. For this, cells cultured in 24-well plates were incubated with 100 nM SCH 58261 or 30 nM CGS 21680 (Tocris Bioscience), according to the experiment's requirements, 4 days after plating and the assay performed 5 days later. Cells were gently washed with Krebs solution, 300 µl of MTT solution was added to each well and the plate covered and incubated for 1 hour at 37 °C, 5% CO_2. MTT solution was then removed and 300 µl of a solution of HCl 40 mM in isopropanol was added to each well, to dissolve the formazan crystals, and the covered plate was lightly shaken to help the process. The final product was quantified spectrophotometrically at the wavelengths of 570 and 620 nm for normalization, with the final result achieved by subtracting the results of 620 nm reading from the 570 nm reading results.

Chapter 3 Results

3.1 – Effect of adenosine A_{2A} receptors modulation on cell death induced by hyperglycemia

Diabetic retinopathy exhibits the classical findings of chronic inflammation. It is known that in diabetes there is an activation of microglial cells and a concomitant release of inflammatory mediators that cause death of retinal neurons, even before the detectable vascular lesions (Barber, 2003). It is known that activation of A_{2A}AR reduces the levels of inflammatory molecules released by microglia upon stimulation (Lee et al., 2006; Liou et al., 2008). Therefore, we investigated if A_{2A}AR can modulate the neuronal cell death observed in retinal cell cultured in high glucose conditions.

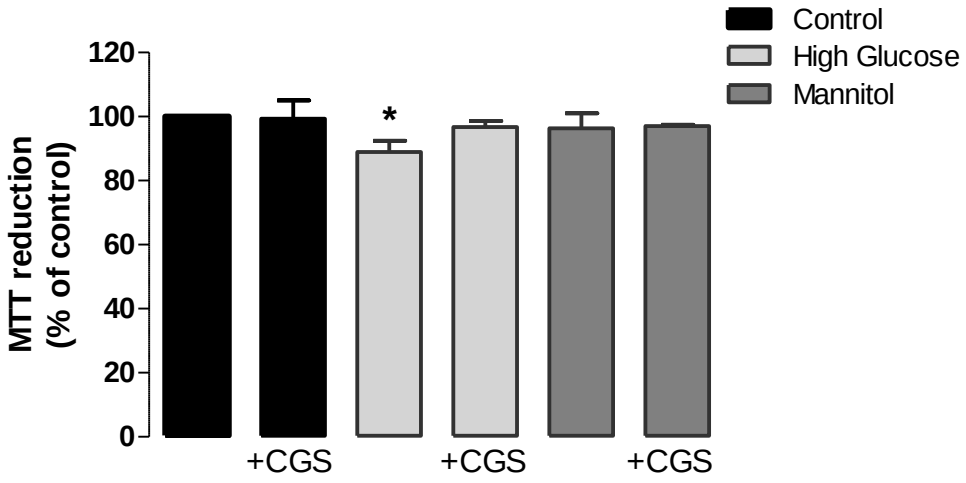

Figure 9: Activation of A_{2A}AR prevented retinal cell death induced by high-glucose. High Glucose group was obtained by incubating retinal cells with 30 mM glucose to mimic hyperglycemic conditions for a period of 7 days. Osmotic control was performed by incubating cells with 25 mM mannitol. Cells were cultured in the presence of 30 nM CGS 21680 (+CGS). The cellular viability was assessed, as previously described, by MTT assay. MTT, when taken up by living cells, is converted from a yellow to a water-insoluble blue colored precipitate by cellular dehydrogenases. The mean±SEM of 3-8 independent experiments was analyzed with one-way ANOVA test followed by the Dunnett post hoc test. Each experiment had 3-4 replicas. * p <0.05

Activation of A_{2A}AR with a specific agonist CGS 21680 (30 nM; Fig. 9), prevented the decrease in cell viability, measured by the MTT method, a method widely used for the determination of cell viability (Santiago *et al.*, 2007; Mosmann, 1983). The decrease in cell viability to 90.0±3.3% of control, observed in cells cultured in high glucose conditions, was reduced to only 96.7±1.9% of control in cells that were cultured in both high glucose conditions and in the presence of 30 nM CGS 21680.

On the other hand, inhibition of A_{2A}AR with a specific antagonist, SCH 58261 (100 nM; Fig. 10), produced opposing and therefore concurrent results.

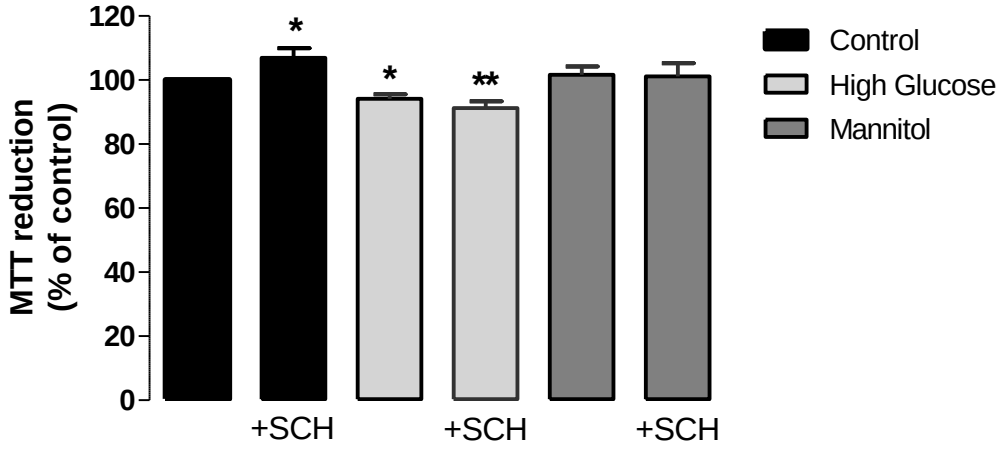

Figure 10: Blockade of A_{2A}AR aggravated retinal cell death induced by high-glucose. Retinal cells cultured in control conditions (5 mM glucose; Ctrl) or in the presence of 30 mM glucose (High Glucose) or 25 mM mannitol plus 5 mM glucose (Mannitol), were cultured in the presence of 100 nM SCH 58261 (+SCH). The cellular viability was assessed, as previously described by the MTT assay. MTT, when taken up by living cells, is converted from a yellow to a water-insoluble blue colored precipitate by cellular dehydrogenases. The mean±SEM of 6-12 independent experiments was analyzed with one-way ANOVA test followed by the Dunnett post hoc test. Each experiment had 3-4 replicas. ** p <0.01; * p <0.05

A blockade of A_{2A}AR by SCH 58261 in cells subjected to high glucose conditions worsened their already lower viability when compared to control: cell viability in high glucose conditions was 94.2±1.3% of control, but when treatment with SCH 58261 was added to the high glucose environment, viability dropped to 91.2±2.1% of control.

Worth noticing also was the significant increase in cell viability observed in control conditions treated with SCH 58261 (106.9±3.0% of control).

3.2 – Effects of diabetes/hyperglycemia on adenosine receptors

It has been shown that diabetes/hyperglycemia alters the protein levels of some adenosinergic system components, such as adenosine receptors and enzymes involved in the adenosine cycle. Therefore, we set out to determine the effect of diabetes on the protein levels of adenosine receptors in retinas isolated from Wistar rats and also in retinal cultured cells exposed to high glucose concentrations.

3.2.1 – Total protein levels of A_1 adenosine receptor in retinal tissue

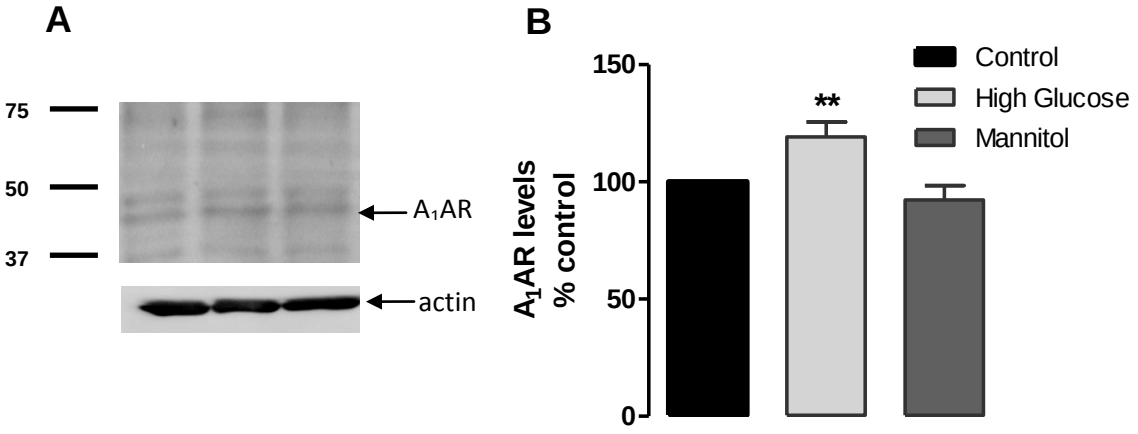

Figure 11: Adenosine A_1 receptor levels in cultured retinal cell. (A) Cells were incubated with 30 mM glucose to mimic hyperglycemic conditions for a period of 7 days. Osmotic control was performed by incubating cells with 25 mM mannitol. 60 µg of protein content from each sample was loaded into a 7,5% gel, electrophoresed and probed for the presence of A_1AR. Molecular weight markers are indicated in kDa. Membranes were later reprobed for actin as a loading control. **(B)** Total protein levels were normalized by the loading control, and expressed as percentage of the control group. The mean±SEM of 5-7 independent experiments was analyzed with one-way ANOVA test and Tukey's multiple comparison test. ** $p < 0.01$

As illustrated in Fig 11, A_1AR total protein levels showed a significant increase in retinal cell cultures subjected to high glucose conditions, rising to 119.1±6.4% of control whereas the osmotic control (mannitol cultured cells) remained close to control levels (92.3±6.2% of control).

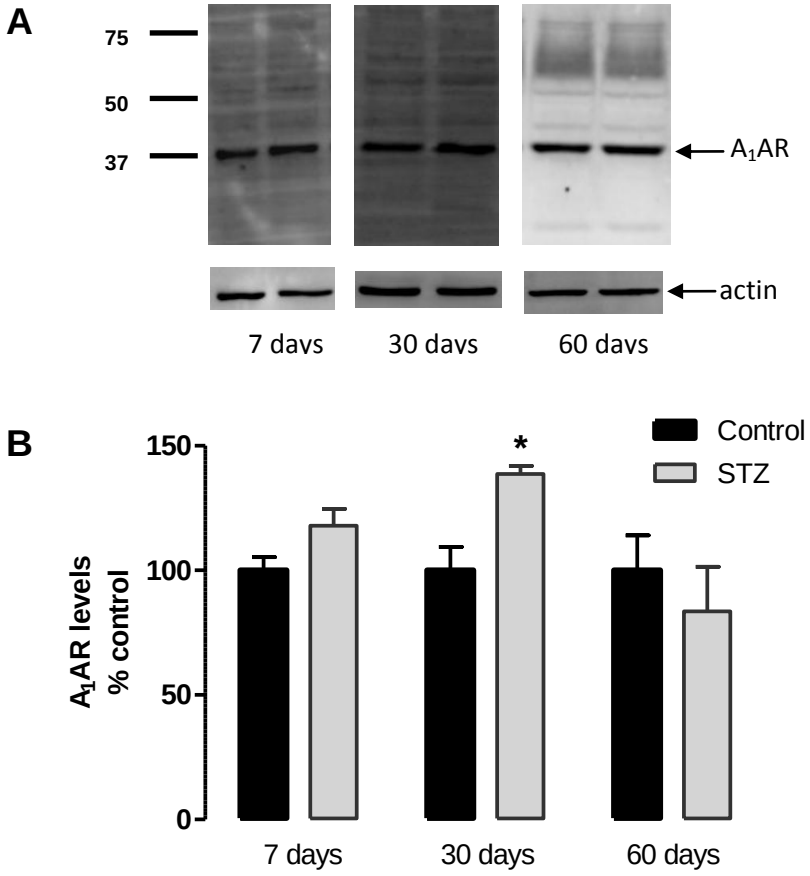

Figure 12: Effect of streptozotocin-induced diabetes on rat retinal adenosine A_1 receptors protein levels. (A) Rats were subjected to an intraperitoneal injection of STZ and maintained for a period of 7 days, 30 days or 60 days. 50 µg of retinal protein content from each sample was loaded into a 7,5% gel, electrophoresed and probed for the presence of A_1AR. Molecular weight markers are indicated in kDa. Membranes were later reprobed for actin as a loading control. **(B)** Total protein levels were normalized by the loading control, and expressed as percentage of the control group. The mean±SEM of 3-5 samples for each condition was analyzed with T test and F test. * $p< 0.05$

In the animal models of diabetes, seven days after induction of diabetes the retinal protein levels of A_1AR were augmented when compared to control animals (113.2±7.0% of control). However, this potentiation only attained statistical significance after 30 days of diabetes. In these animals, the A_1AR levels increased up to 126.7±12.2% of control. After 60 days of diabetes the protein levels decreased to levels observed in control animals (83.6±17.6% of control) (Fig 12).

3.2.2 – Total protein levels of A_{2A} adenosine receptor in retinal tissue

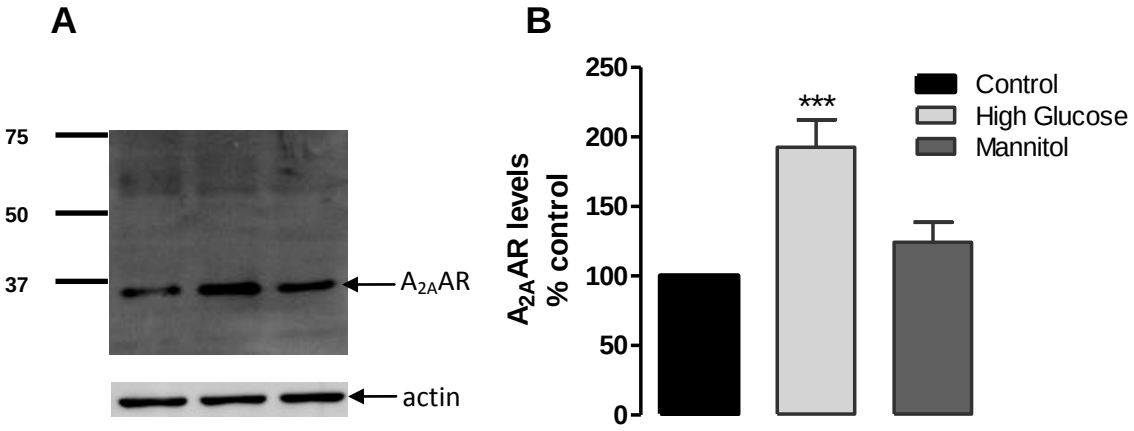

Figure13: Adenosine A_{2A} receptor levels are highly increased in retinal cells cultured in high glucose conditions. (A) Cells were incubated with 30 mM glucose to mimic hyperglycemic conditions for a period of 7 days. Osmotic control was performed by incubating cells with 25 mM mannitol. 50 µg of protein content from each sample was loaded into a 7.5% gel, electrophoresed and probed for the presence of A_{2A}AR. Molecular weight markers are indicated in kDa. Membranes were later reprobed for actin as a loading control. **(B)** Total protein levels were normalized by the loading control, and expressed as percentage of the control group. The mean±SEM of 5-6 independent experiments was analyzed with one-way ANOVA test and Tukey's multiple comparison test. *** $p<0.001$

As shown in Fig 13, the total protein levels of A_{2A}AR increased dramatically in retinal cell cultures subjected to high glucose conditions during 7 days (192.5±19.9% of

control), while the osmotic control levels remained at 124.3±14.5% of control, removing the possibility of an osmotic disturbance.

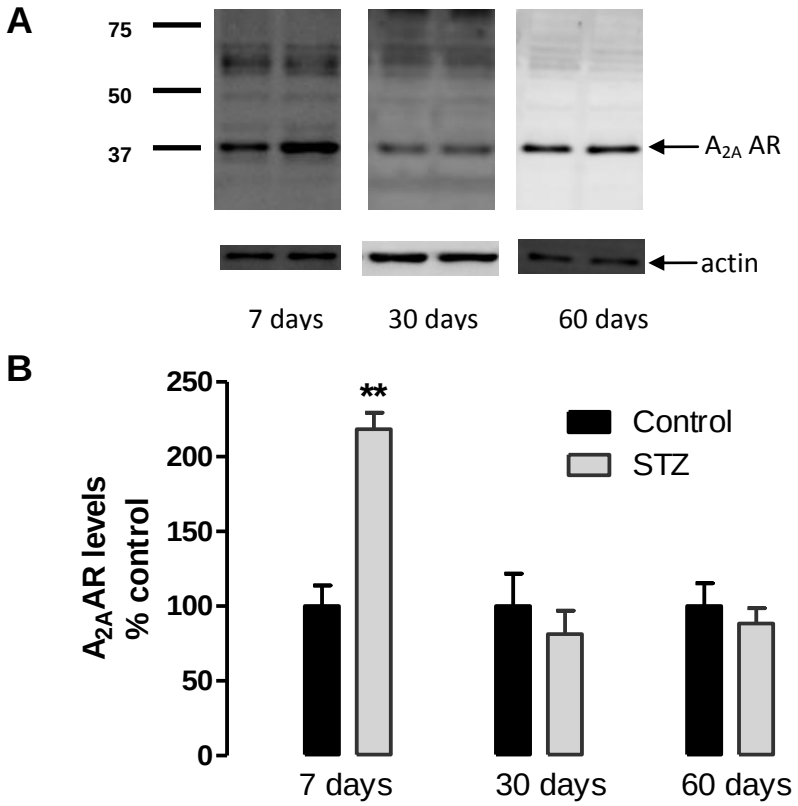

Figure 14: Effect of streptozotocin-induced diabetes on rat retinal adenosine A_{2A} receptor protein levels. (A) Rats were subjected to an intraperitoneal injection of STZ and maintained for a period of 7 days, 30 days and 60 days. 50 µg of protein content from each sample was loaded into a 7,5% gel, electrophoresed and probed for the presence of A_{2A}AR. Molecular weight markers are indicated in kDa. Membranes were later reprobed for actin as a loading control. **(B)** Total protein levels were normalized by the loading control, and expressed as percentage of the control group. The mean±SEM of 2-4 samples for each condition was analyzed with T test and F test. ** $p< 0.01$

This steep increase was mirrored in the results from STZ treated rats maintained for 7 days (218.4±11.1% of control), but the levels normalized after 30 days (81.3±15.7% of control), and stabilized at 88.5±10.1% of control after 60 days (Fig 14).

3.2.3 – Total protein levels of A_{2B} adenosine receptor in retinal tissue

Unlike the effect observed in $A_{2A}AR$, the results we obtained showed that the total protein levels of $A_{2B}AR$ were unaltered by high glucose conditions in cell culture (Fig 15).

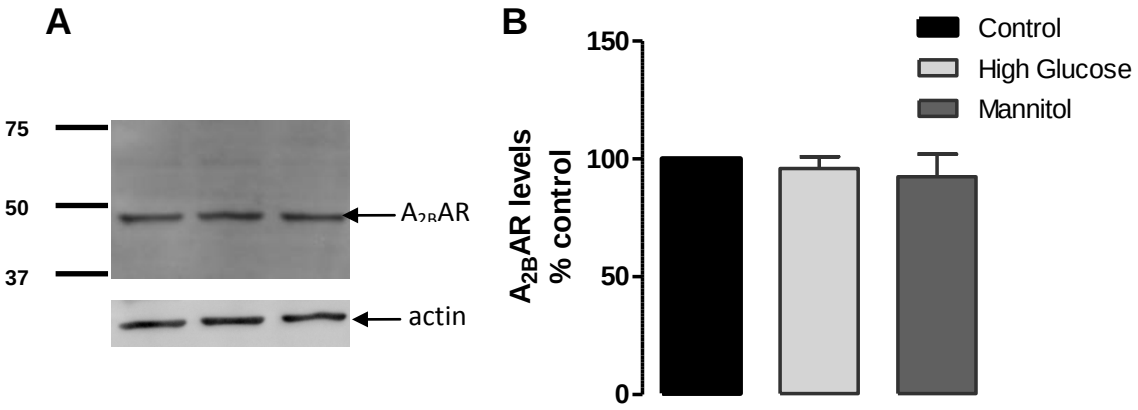

Figure 15: A_{2B} adenosine receptor levels in cultured retinal cells is not affected by high glucose conditions. (A) Cells were incubated with 30 mM glucose to mimic hyperglycemic conditions for a period of 7 days. Osmotic control was performed by incubating cells with 25 mM mannitol. 40 µg of protein content from each sample was loaded into a 7,5% gel, electrophoresed and probed for the presence of $A_{2B}AR$. Molecular weight markers are indicated in kDa. Membranes were later reprobed for actin as a loading control. **(B)** Total protein levels were normalized by the loading control, and expressed as percentage of the control group. The mean±SEM of 3-4 independent experiments was analyzed with one-way ANOVA test and Tukey's multiple comparison test.

The immunoblot showed no significant differences between the control, osmotic control cultures (92.4±9.6% of control) and cells maintained in high glucose conditions (96.0±5% of control).

The lack of alterations of $A_{2B}AR$ matched the results obtained in the diabetic animal models. In fact, in all three periods of diabetes, 7, 30 or 60 days, the protein levels of $A_{2B}AR$ were not significantly different from control conditions, and attained

111.1±12.3%, 93.4±5.2% or 97.5±13.2% of control for 7, 30 and 60 days after STZ injection, respectively, (Fig 16).

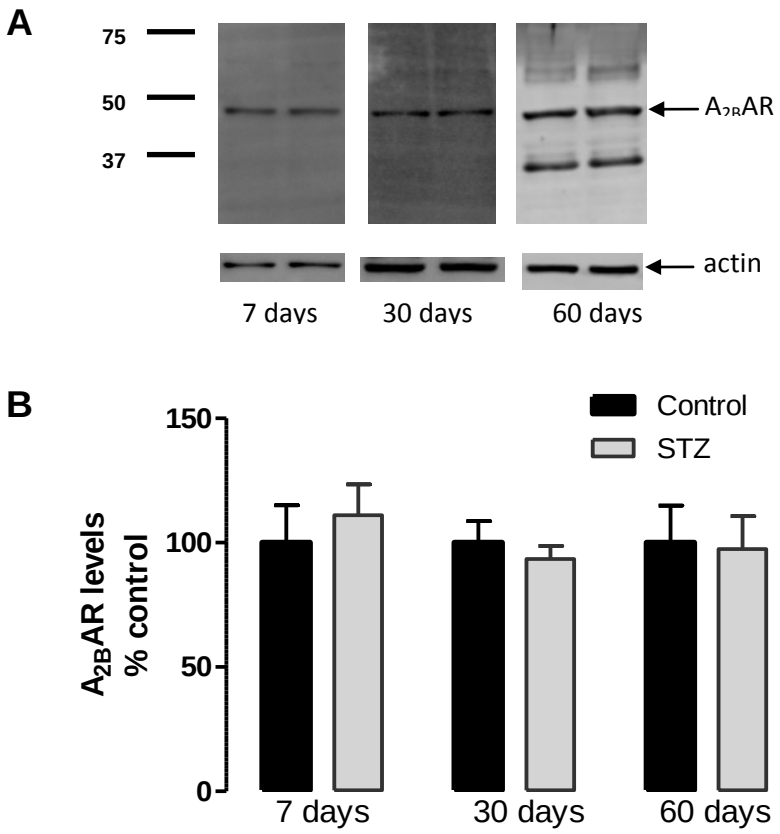

Figure 16: Effect of streptozotocin-induced diabetes on rat retinal adenosine A_{2B} receptor protein levels. (A) Rats were subjected to an intraperitoneal injection of STZ and maintained for a period of 7 days, 30 days and 60 days. 40 µg of protein content from each sample was loaded into a 7,5% gel, electrophoresed and probed for the presence of A_{2B}AR. Molecular weight markers are indicated in kDa. Membranes were later reprobed for actin as a loading control. **(B)** Total protein levels were normalized by the loading control, and expressed as percentage of the control group. The mean±SEM of 3-5 samples for each condition was analyzed with T test and F test.

3.2.4 – Total protein levels of A_3 adenosine receptor in retinal tissue

In retinal cell cultures, western blot analysis of A_3AR total protein levels revealed no significant alterations between control, osmotic control (91.7±11.2% of control) and high glucose (81.3±9.0% of control) despite a small decrease (Fig 17), leading us to presume that A_3AR levels seemed unaffected by diabetic conditions.

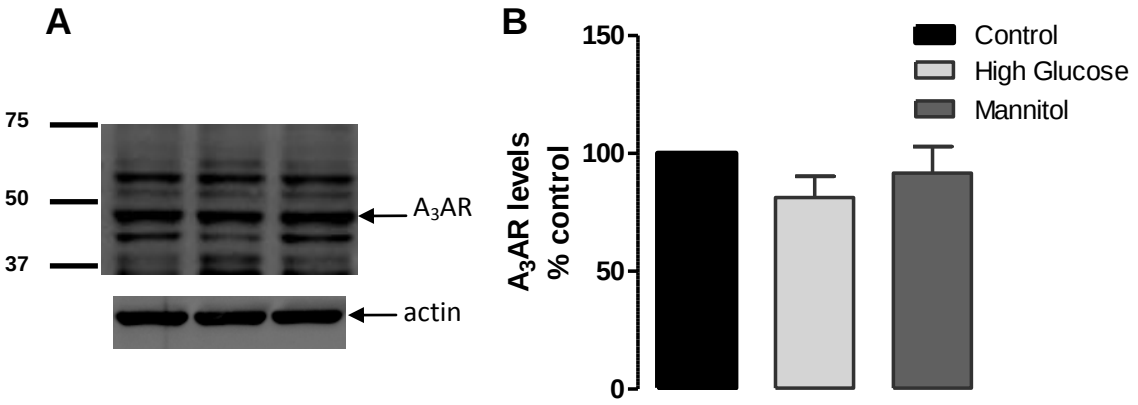

Figure 17: A_3 adenosine receptor levels in cultured retinal cells are not affected by high glucose conditions. (A) Cells were incubated with 30 mM glucose to mimic hyperglycemic conditions for a period of 7 days. Osmotic control was performed by incubating cells with 25 mM mannitol. 50 µg of protein content from each sample was loaded into a 10% gel, electrophoresed and probed for the presence of A_3AR. Molecular weight markers are indicated in kDa. Membranes were later reprobed for actin as a loading control. **(B)** Total protein levels were normalized by the loading control, and expressed as percentage of the control group. The mean±SEM of 5 independent experiments was analyzed with one-way ANOVA test and Tukey's multiple comparison test.

Contrary to that observed in cell cultures, the results obtained with STZ injected rats maintained for the same period of 7 days showed a significant raise (150.6±11.7% of control) in A_3AR total protein levels (Fig 18), seemingly in contradiction of the results obtained in cultures. However, this increase was of short duration, being followed by a

steep decrease to 77.7±3.6% of control at 30 days, and after 60 days the protein levels of the diabetic animals remained at 107.0±21.0% of control.

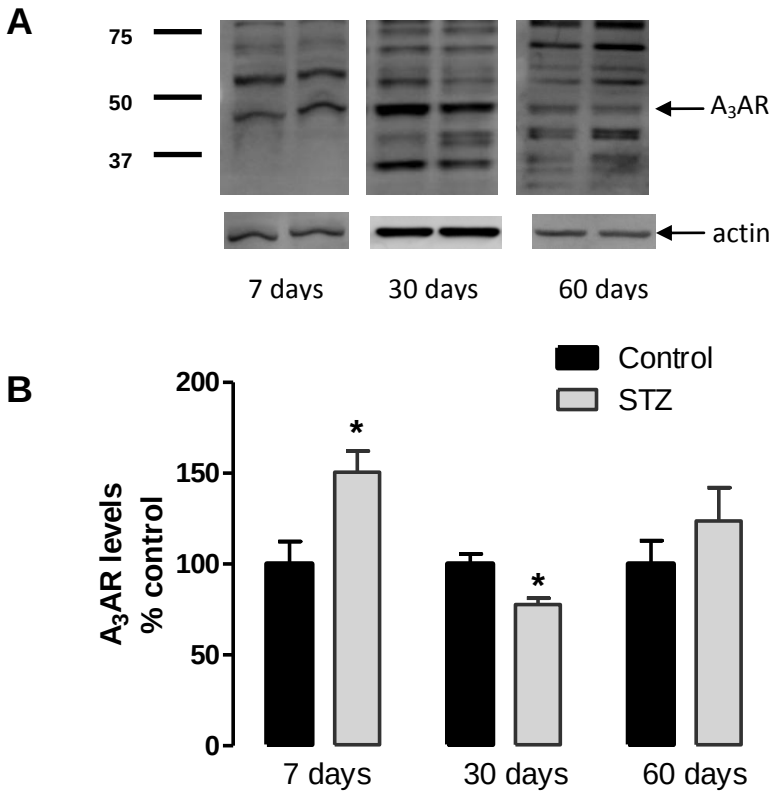

Figure 18: Effect of streptozotocin-induced diabetes on rat retinal adenosine A_3 receptor protein levels. (A) Rats were subjected to an intraperitoneal injection of STZ and maintained for a period of 7 days, 30 days and 60 days. 50 µg of protein content from each sample was loaded into a 10% gel, electrophoresed and probed for the presence of A_3AR. Molecular weight markers are indicated in kDa. Membranes were later reprobed for actin as a loading control. **(B)** Total protein levels were normalized by the loading control, and expressed as percentage of the control group. The mean±SEM of 4-5 samples for each condition was analyzed with T test and F test. * p<0.05

3.3 – Adenosine Deaminase

Adenosine deaminase is one of the enzymes that catabolizes adenosine, and is present both intra and extracellularly. Its role in adenosine removal is highlighted in situations where adenosine levels increase dramatically, such as hypoxia and ischemia

(Dunwiddie and Masino, 2001), and diabetes has been shown to affect the activity of ADA, in human platelets (Schmatz *et al.*, 2009). With this in mind, we set out to discover if diabetic conditions in rat retina have any effect on ADA.

3.3.1 – ADA activity is reduced under diabetic conditions

To assess the activity levels of ADA in diabetic conditions, we used mixed retinal cells cultured in 6-well plates, in control, osmotic control and high glucose conditions, and obtained cell extracts to perform the enzymatic activity assay based on the Bertholet reaction, all according to the protocol described in Chapter 2.

As shown in Fig 19, ADA activity was severely compromised in retinal cell cultures after 7 days in high glucose conditions.

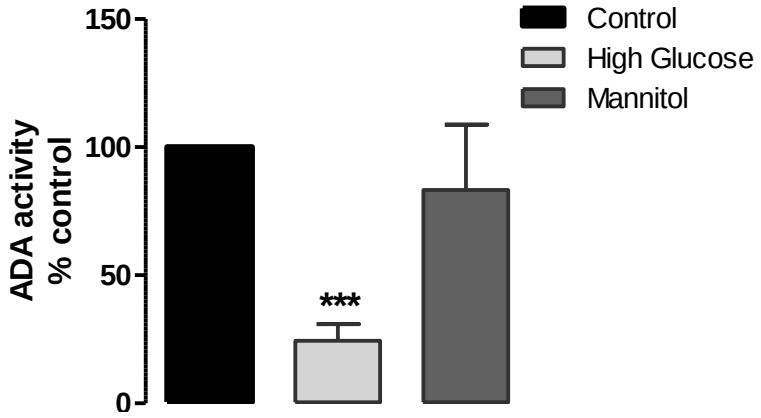

Figure 19: The adenosine deaminase activity is significantly decreased in retinal cells cultured in high glucose conditions. Cells were incubated with 30 mM glucose to mimic hyperglycemic conditions for a period of 7 days. Osmotic control was performed by incubating cells with 25 mM mannitol. 100 µl of the samples were added to 500 µl of a solution of 21 mM of adenosine in 50 mM phosphate buffer and were incubated at 37 °C for 60 min. Afterwards, 1.5 ml of a phenol solution and a sodium hypochlorite solution were added and the samples incubated at 37 °C for 30 min. The final products were quantified spectrophotometrically at a wavelength of 620 nm. Protein content in the samples was between 0.7 and 0.9 mg/ml. Results are expressed as percentage of the control group. The mean±SEM of 4-5 independent experiments was analyzed with one-way ANOVA test and Tukey's multiple comparison test. *** $p<0.001$

Values for activity were reduced to 24.4±6.5% in relation to the control group while the osmotic control numbers remained relatively unchanged (83.3±25.4% of control), foreshadowing a decline in the adenosine removal levels during diabetic conditions. This could signify a physiological reaction to the adverse high glucose environment or a consequence of those conditions.

This alteration could, however, also be associated to a decrease in ADA total protein levels in these settings, so the next step was to examine the total protein levels of the enzyme.

3.3.2 – ADA total protein levels in retinal tissue

Although cells cultured in high glucose conditions presents a drastic decrease in ADA activity, the protein levels of this enzyme were not affected by the culture conditions (Fig 20).

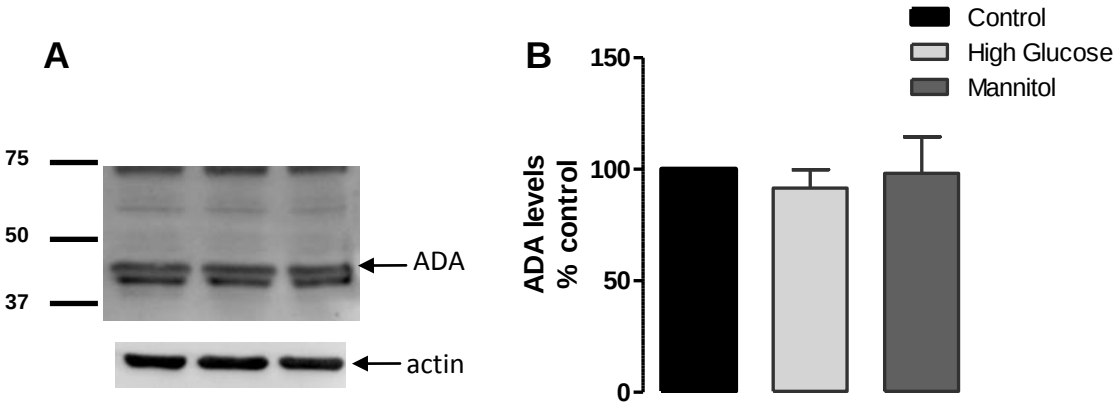

Figure 20: Adenosine deaminase protein levels were not altered in retinal cells cultured in high glucose conditions. (A) 50 µg of protein content from each sample was loaded into a 7.5% gel, electrophoresed and probed for the presence of ADA. Molecular weight markers are indicated in kDa. Membranes were later reprobed for actin as a loading control. **(B)** Total protein levels were normalized by the loading control, and expressed as percentage of the control group. The mean±SEM of 4-6 independent experiments was analyzed with one-way ANOVA test and Tukey's multiple comparison test.

In fact, the results obtained in high glucose conditions were not significantly different from the control conditions (91.6±8.2% of control) and also similar to those registered for the osmotic control (98.2±16.3% of control).

After 7 days of diabetes induction by STZ injection, the retinal ADA protein levels were not significantly different from the levels observed in control animals (97.3±7.8% of control). However, after 30 days of diabetes induction it was observed a marked decrease in ADA protein levels, down to 71.8±6.4% of control, and that reduction was sustained after 60 days, despite a small upsurge (86.0±1.0% of control) (Fig 21).

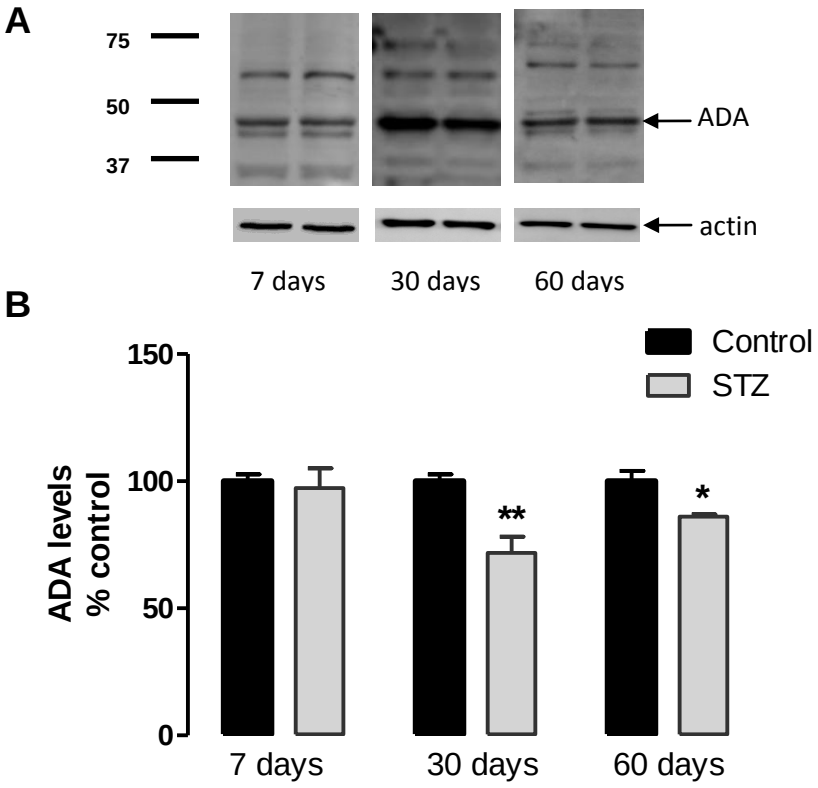

Figure 21: Effect of streptozotocin-induced diabetes on rat retinal adenosine deaminase protein levels. (A) STZ treated rats were maintained for a period of 7 days, 30 days and 60 days. 50 µg of protein content from each sample was loaded into a 7.5% gel, electrophoresed and probed for the presence of ADA. Molecular weight markers are indicated in kDa. Membranes were later reprobed for actin as a loading control. **(B)** Total protein levels were normalized by the loading control, and expressed as percentage of the control group. The mean±SEM of 2-5 samples for each condition was analyzed with T test and F test. ** $p<0.01$; * $p<0.05$

These results suggest that ADA protein levels were affected by diabetic conditions in STZ treated rats, though not immediately at the onset of the environmental alterations.

Chapter 4 Discussion

The inflammatory conditions characteristic of the early onset of DR are emerging as a potential area for preventive treatment of the disease. Adenosine is a ubiquitous messenger and modulator and the adenosinergic system is not only heavily active in inflammatory situations, it is also very sensitive to signs of metabolic stress. These traits, coupled with a known adaptability to prolonged adverse conditions, make the adenosinergic system a prime target for research in the early stages of DR. In this study, we aimed to better understand the effects diabetic conditions cause in several key components of the adenosinergic system in the retina, revealing the role of adenosine in the cellular response to diabetes/hyperglycemic conditions.

4.1 – Effect of diabetes/hyperglycemia on adenosine A_1 receptors

In this study we have observed that in diabetes/hyperglycemic conditions there is an increase in the protein levels of this inhibitory receptor.

The chronic inflammation environment created by hyperglycemic conditions in the retina may be the reason for this increase. It has been shown in several reports that different agents and conditions can stimulate A_1AR expression in an NFκB-dependent manner (Jhaveri *et al.*, 2007; Hammond *et al.*, 2004; Nie *et al.*, 1998). The activity of NFκB, a transcription factor with putative binding sites found in all adenosine receptor genes (St. Hilaire *et al.*, 2009), was shown to be regulated by TNF-α in peripheral blood monocytes (Dichamp *et al.*, 2007), revealing a possible connection between the high levels of TNF-α in hyperglycemic conditions and the increased protein levels of A_1AR detected under the same conditions. Furthermore, a study performed in avian retinal cells showed a connection between high levels of A_{2A}AR receptor activation and an up-regulation of A_1AR receptor, via a cAMP/PKA dependent pathway (Pereira *et al.*, 2010). This up-regulation was blocked when NFκB inhibitors were used, indicating another link between NFκB and A_1AR regulation. The exact mechanism for this alteration remains to be known, but the above possibilities are intriguing. A study on

A_1AR levels using A_{2A}AR antagonists could answer the question of a possible connection between the levels of these two adenosine receptors.

Although A_1AR role in inflammation is less known than other adenosine receptors, there are some studies that suggest that these receptors may play anti-inflammatory roles in certain situations: a study in murine dendritic cells showed that suppression of dendritic cell activation upon an inflammatory stimulus was mediated by A_1AR (Desrosiers *et al.*, 2007); a study in a model of multiple sclerosis showed that up-regulation and activation of A_1AR in microglia suppressed pro-inflammatory and increased anti-inflammatory responses (Tsutsui *et al.*, 2004).

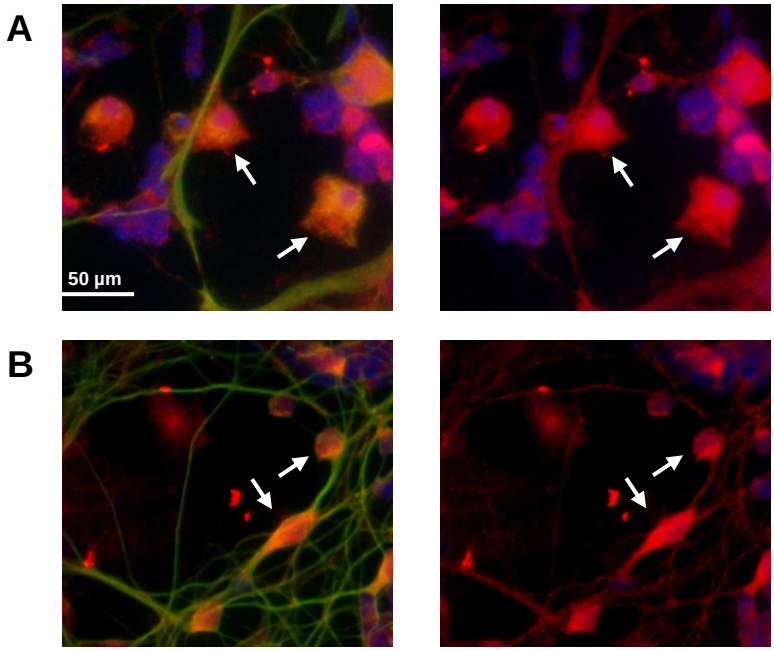

Figure 22: Immunolocalization of A_1AR in cultured rat retinal cells. Procedure performed in 7 days old primary cultures of mixed retinal cells. Cell nucleus stained in blue with Hoechst 33342. Magnification 400x. **(A)** Immunoreactivities for A_1AR (red) and Vimentin (green). Arrows indicate microglial cells. Co-localization of A_1AR was further confirmed with microglia marker CD11b (not shown) **(B)** Immunoreactivities for A_1AR (red) and neuron marker β-tubulin (green). Arrows indicate the central body of neurons.

The presence of A_1AR in our cultures was confirmed by immunocytochemistry studies (Fig 22). Although immunoreactivity to A_1AR was observed to some degree in macroglial cells (data not shown), A_1AR showed a more intense staining in microglia and neurons (Fig 22). The strong presence of A_1AR in retinal neurons may reflect the neuroprotective nature of these receptors and the possible role they play in the context of DR. High glucose conditions in the retina alter the levels of glutamate receptors (Santiago *et al.*, 2009) and trigger a disturbance in glutamate metabolism, causing its concentration to increase (Lieth *et al.*, 1998; Kowluru *et al.*, 2001). Furthermore, the same high glucose conditions create an increase in intracellular Ca^{2+} responses (Pereira *et al.*, 2010), which can lead to retinal ganglion cell death (McKernan *et al.*, 2007) and excessive release of glutamate. This glutamate release can in turn enhance Ca^{2+} influx and also induce retinal cell death (Duarte *et al.*, 1998; Hartwick *et al*, 2008). The ability of A_1AR to inhibit the release of retinal neurotransmitters, and decrease the influx of Ca^{2+} (Santos *et al.*, 2000) gains a new importance in these circumstances. The increase in A_1AR protein levels in hyperglycemic conditions may have a neuro-protective effect, by down-regulating excessive excitatory neurotransmission and decreasing high Ca^{2+} influx levels caused by the diabetic environment.

In light of these alterations, is important to understand the effects caused by an increase in the protein levels of A_1AR in the context of the diabetic retina, and how that increase is related to the other modifications occurring at the same time. The first step to take should be to determine the nature of the alterations to A_1AR protein levels: RT-PCR should be performed to analyze the expression levels of A_1AR, allowing us to understand how the high levels of A_1AR occur, if by an increase in gene expression, in m-RNA translation or in both; Another experiment to carry out is binding studies using radio-labeled A_1AR antagonist ^{3}H-DPCPX to measure alterations to receptor density in the cell membrane, letting us look at how hyperglycemia affects the distribution of A_1AR. Finally, viability assays should be performed to determine if the potential

protective effect of A_1AR is observed in retinal cells subjected to high glucose conditions.

4.2 – Effect of diabetes/hyperglycemia on adenosine A_3 receptors

A_3AR has a known role in several inflammatory conditions (Varani *et al.*, 2009; Haskó and Cronstein, 2004), playing a part in the modulation of microglia activity (Lee *et al.*, 2006) and protection of retinal ganglion cells (Zhang *et al.*, 2006), along with A_1AR. It is no surprise, then, that the chronic inflammation imposed by diabetic conditions in the retina caused alterations to the protein levels of this receptor. Although not visible in retinal cell cultures, A_3AR showed a marked increase in protein levels after 7 days of STZ induced diabetes.

One hypothesis for this alteration is to trace it back to the inflammatory environment provided by hyperglycemic conditions. Studies made in rheumathoid arthritis models revealed an up-regulation of A_3AR in human peripheral blood mononuclear cells caused by inflammatory cytokines, such as TNF-α and IL-2 (Madi *et al.*, 2007). Additionally, these alterations were directly correlated to an increase in NFκB. In fact, a blockade of IL-2 and TNF-α prevented the increase in both A_3AR and NFκB. A recent study performed in murine microglia and astrocytes revealed a LPS-induced NFκB activation pathway in both cells, where the production of TNF-α was stimulated (Lu *et al.*, 2010). Another study in BV2 microglial cells showed an A_3AR-mediated inhibition of LPS-induced TNF-α expression that was associated with the inhibition of LPS-induced activation of PI_3K/Akt and NFκB pathway (Lee *et al.*, 2006). These findings shows a strong likelihood of NFκB being involved in the alterations witnessed in our results, and hint at the possible existence of a negative feedback mechanism in the shape of a regulation circle: TNF-α may be involved in regulating A_3AR levels, while A_3AR itself can regulate TNF-α production. It would be important to explore this hypothesis in several ways: RT-PCR studies would allow us to determine if

the rise in A_3AR levels is due to an increase in expression or if it's due to a amplified translation process, and measuring A_3AR protein levels on samples subjected to a blockade to TNF-α receptors would give us an answer to the hypothesis of TNF-α involvement in the alterations to A_3AR protein levels.

Despite the increase in protein levels of A_3AR in the first 7 days of diabetes, after 30 days those levels dropped and there was actually a decrease observed, before control levels were recovered after 60 days. The actual decline in A_3AR levels after 30 days can perhaps be a secondary effect of high activity of A_{2A}AR and A_3AR itself. A_{2A}AR levels are greatly inflated after 7 days of diabetes, and the consequence of their activation to TNF-α levels and possibly NFκB activation levels may have the secondary effect of down-regulating A_3AR.

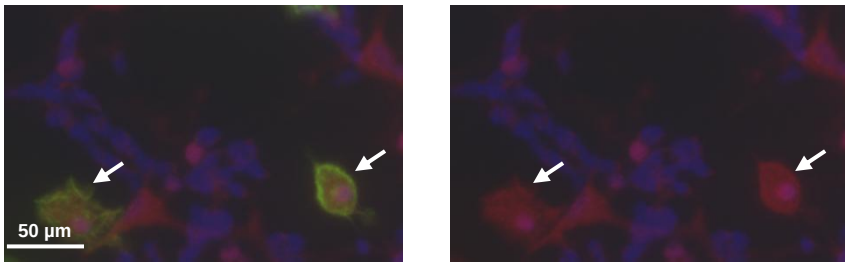

Figure 23: Immunolocalization of A_3AR in cultured rat retina cell cultures Procedure performed in 7 days old primary cultures of mixed retinal cells. Cell nucleus stained in blue with Hoechst 33342. Magnification 400x. Immunoreactivities for A_3AR (red) and microglia cell marker CD11b (green). Arrows indicate microglial cells.

There is a strong possibility that the transient increase in A_3AR receptor levels is part of a negative feedback mechanism triggered by the increased levels of pro-inflammatory cytokines resulting from hyperglycemic conditions. In an immunocytochemistry procedure, A_3AR showed a very significant staining in microglia (Fig 23). One of the more relevant effects of A_3AR activation in microglia is that it can down-regulate the production of TNF-α, in an anti-inflammatory response (Gessi *et al.*,

2008). Beyond microglia, A_3AR can also, in astrocytes, stimulate the production of CCL2, a neuroprotective chemokine (Gessi *et al.*, 2008).

Although we can see the most likely outcomes of the A_3AR alterations observed through past studies in other cells and tissues, it will be necessary to confirm our hypothesis in the diabetic retina, and to map out the key players, and how they relate to the hyperglycemic environment, and the other modifications occurring simultaneously.

4.3 – Effect of diabetes/hyperglycemia on adenosine A_{2A} receptors

Widely regarded as a main player in inflammatory conditions, A_{2A}AR is a receptor with potential to represent a key role not only in the modifications occurring in the retina in diabetic conditions, but also in the response to those alterations. The viability assay we performed showed that engaging the A_{2A}AR with a specific agonist, CGS 21680, prevented the increase in cell death caused by hyperglycemic conditions in retinal cells. Another viability assay performed, this time with A_{2A}AR specific antagonist, SCH 58261, further confirmed the protective role of A_{2A}AR in diabetic conditions: blockade of A_{2A}AR with SCH 58261 aggravated the cell death observed in high glucose situations.

This may be due, in large part, to the ability of A_{2A}AR to regulate inflammatory cytokines in many immune cell types, down-regulating the levels of several pro-inflammatory cytokines, such as IL-6, IL-8, IL-12 and TNF-α (Varani *et* al, 2009; Hamano *et al.*, 2008; Haskó *et al.*, 2008; Liou *et al.*, 2008; Fredholm, 2007), with the latter being directly linked to the rise in cell death witnessed in diabetic conditions (unpublished data). One way to confirm this connection would be to measure the levels of produced and released TNF-α in the presence of CGS 21680 and SCH 58261, and compare the results with a control and osmotic control group.

Beyond affecting the survival of retinal cells in diabetic conditions, A_{2A}AR itself was affected, revealing a large increase in total protein levels in cell cultures and

retinas subjected to hyperglycemic conditions or diabetes, respectively. If this increase is due to an up-regulation of expression or an increase in translation is a question that has to be answered in the future, and could be cleared by RT-PCR assays to measure m-RNA levels. Another important issue to address in the future is the levels of receptor density in the membrane. Binding studies using radio-labeled A_{2A} antagonist ^{3}H-SCH 58261 can tell us if the rise in A_{2A}AR protein levels is reflected in membrane density.

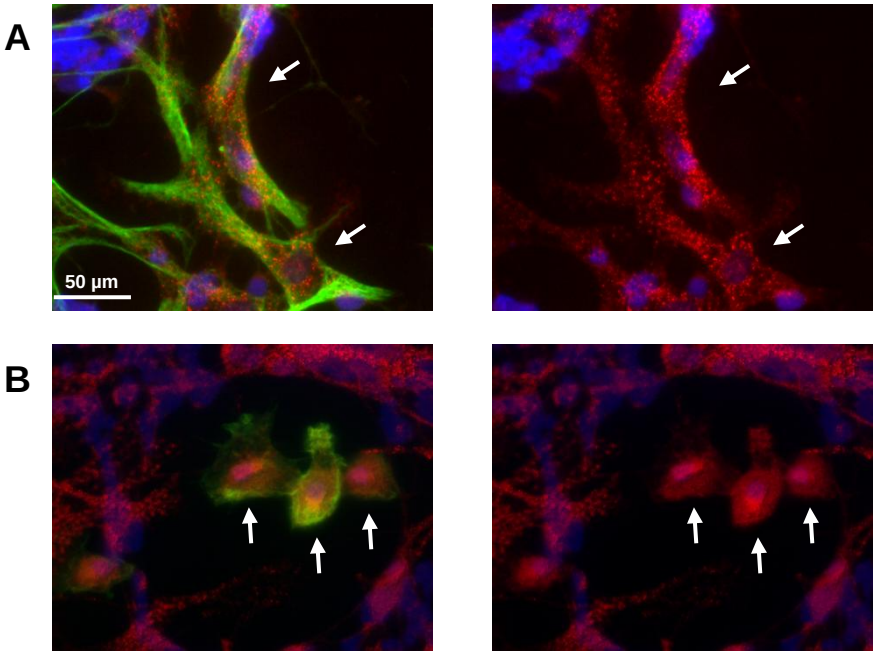

Figure 24: Immunolocalization of A_{2A}AR in rat retina cell cultures. Procedure performed in 7 days old primary cultures of mixed retinal cells. Cell nucleus stained in blue with Hoechst 33342. Magnification 400x. **(A)** Immunoreactivities for A_{2A}AR (red) and Müller cell marker Vimentin (green). Arrows indicate Müller cells. **(B)** Immunoreactivities for A_{2A}AR (red) and microglia marker CD11B (green). Arrows indicate microglial cells.

A_{2A}AR regulation is very sensitive to alterations in the extracellular environment, such as concentrations of exogenous and endogenous inflammatory factors (Haskó *et al.*, 2008). In fact, A_{2A}AR can be regulated by the transcription factor NFκB (Varani *et al.*, 2009; Murphree *et al.*, 2005), a factor that was shown to be regulated by endogenous TNF-α on several occasions (Lu *et al.*, 2010; Varani *et al.*, 2009; Dichamp

et al., 2007). It would be interesting to observe how the levels of A_{2A}AR are altered by a neutralization of endogenous TNF-α, or a blockade of TNF-α receptors TNFR1 and TNFR2 in order to establish the link between A_{2A}AR increase and TNF-α.

The distribution of A_{2A}AR in retinal cells observed with immunocytochemistry showed A_{2A}AR presence in all cell types present in our rat retinal cultures. However, the more intense staining occurred in microglia and Müller cells, as shown in Fig 24. Microglial cells, as the immune cells in retinal tissue and very sensitive to shifts in homeostasis, are the key players in the inflammatory conditions caused by hyperglycemia, and a marked A_{2A}AR presence there further supports the anti-inflammatory role of this receptor. Müller cells, spanning the thickness of the retina, are major regulators of glutamate metabolism, extracellular ionic balance and neuronal function (Gardner *et al.*, 2002); high A_{2A}AR levels there correlate to A_{2A}AR role in synaptic transmission and modulation of P-type and N-type Ca^{2+} channels, beyond other regulatory roles through the activation of PKA-dependent and MAPK-dependent pathways (Kreckler *et al.*, 2006; Ralevic and Burnstock, 1998).

The observed increase in A_{2A}AR protein levels, ,along with the A_3AR and A_1AR alterations, all with a possible link to NFκB and increased levels of TNF-α, reveals a possible negative feedback mechanism working in a circular pattern: When TNF-α levels are increased, TNF-α itself activates the signaling pathways that work to recover the homeostatic state. Another player in this possible scenario may be A_{2A}AR itself, beyond its role in down-regulating TNF-α levels, for A_{2A}AR is known to be able to regulate A_1AR levels through a cAMP/PKA dependent pathway (Pereira *et al.*, 2010), a pathway blocked by NFκB inhibition. In fact A_{2A}AR was shown to be able to block NF-κB activation, most likely through competition with CREB, for the CREB-binding protein (Haskó *et al.*, 2008). On this context it would be interesting to try linking the adenosine receptors, by assessing A_1AR and A_3AR levels in an animal model knock-out for A_{2A}AR subjected to diabetic conditions.

In the possible mechanisms for the alterations occurring in $A_{2A}AR$ protein levels, we see again NFκB as a potential mediator of the regulation of an adenosine receptor in the inflammatory environment caused by diabetic conditions in the retina (Fig 25). This suggests there might be a common link in the mechanisms responsible for the different alterations in adenosine receptor levels in the form of NFκB. Since NFκB regulation pathways are very complex, and differ in action according to different cell stimulus, disease states and cell types, the first step would be to determine the actual role of NFκB in this context of inflammatory conditions caused by hyperglycemia in the retina, by measuring expression and total protein levels, activation levels and the effects caused by a blockade of this transcription factor.

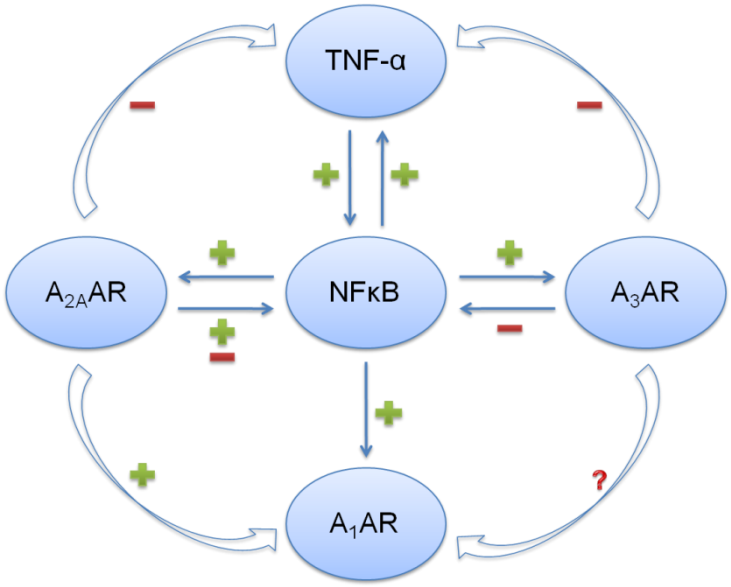

Figure 25: Representation of possible signaling mechanisms that can exist in retinal cells under diabetic conditions. NFκB regulation pathways are very complex, and differ in action according to different cell stimulus, disease states and cell types.

In fact it seems that, beyond linked in the regulation, the adenosine receptors are also linked when it comes to possible outcomes of their respective altered protein levels, in a synchronized effort to reestablish homeostasis. This is quite an enticing

possibility, and the several experiments needed to confirm or deny it, and to explain it should be a priority in the near future of diabetic retinopathy studies.

4.4 – Adenosine A_{2B} receptors are unaffected by diabetes/hyperglycemia

As a receptor with low affinity for adenosine, A_{2B}AR is most active when pathological situations occur, due to the high levels of adenosine released into the extracellular space when homeostasis is disrupted (Fredholm, 2007). They can have opposing effects from tissue to tissue, and are being increasingly recognized as important mediators of inflammation (Haskó *et al.*, 2008; Kreckler, 2006; Dunwiddie and Masino, 2001). In other circumstances their role is more supportive of A_{2A}AR, becoming active in situations where A_{2A}AR can become desensitized (Ralevic and Burnstock, 1998).

It has been shown that inflammatory situations and even a potential regulation by TNF-α may increase A_{2B}AR levels (St Hilaire *et al.*, 2009; Haskó* *et al.*, 2008). However, we did not observed such increase in our experimental models, leading us to question if A_{2B}AR is regulated by other means beyond an increased gene expression or protein translation, or if it is simply unaffected by the inflammatory environment surrounding the diabetic retina. There is one study, in human astrocytes, that shows a possible mechanism of A_{2B}AR regulation: in it TNF-α was found to increase selectively the receptor activity of A_{2B}AR without increasing mRNA or protein levels, possibly by inhibiting receptor desensitization (Trincavelli *et al*., 2004).

To answer the A_{2B}AR riddle, we would need to determine the mRNA levels of this receptor, and perform binding studies, not only to observe possible variations in A_{2B}AR membrane density, but to test the inhibition of desensitization in the presence of TNF-α.

4.5 – Effect of diabetes/hyperglycemia on ADA

Adenosine deaminase is one of the key elements responsible for the removal of intracellular and extracellular adenosine, degrading the nucleoside to inosine. The role of ADA in normal physiologic conditions is secondary, with the larger share of adenosine removal from the extracellular space being done by reuptake with nucleoside transporters. However, during disruptive situations, such as ischemia and hypoxia, where adenosine levels rise, ADA gains a more predominant role (Dunwiddie and Masino, 2001; Latini and Pedata, 2001). Beyond its primarily role in adenosine removal, one study performed in murine dendritic cells revealed a regulatory role of ADA, directly tied to its enzymatic activity. The study showed that ADA activity was increased in dendritic cells upon inflammatory stimulus, blocking the inhibitory effect of adenosine and allowing dendritic cells to sustain their activation (Desrosiers *et al.*, 2007).

Our results on ADA enzymatic activity in retinal cell cultures show an opposite effect: ADA activity levels plummet down in response to high glucose conditions. As the study described above showed, ADA activity can be used to regulate the activity of dendritic cells in inflammatory situations, and we can suggest the same happened in our cultures. A low activity rate for ADA means a lower rate of adenosine removal from the extracellular space, keeping higher concentrations of adenosine in that space for longer periods of time, which can result in higher levels of adenosine receptor activation. Such an increase would affect the intensity of signaling transmitted by the adenosine receptors, particularly A_{2B}AR and A_3AR, the receptors with lower affinity for adenosine, creating a more powerful immunosuppressive response.

A strong presence of ADA in microglial cells, as shown in Fig 26, further supports the regulatory role proposed for ADA. Although present in all cell types found in our retinal cultures, the immunocytochemistry showed an intense staining in microglia, in both control and high glucose situations.

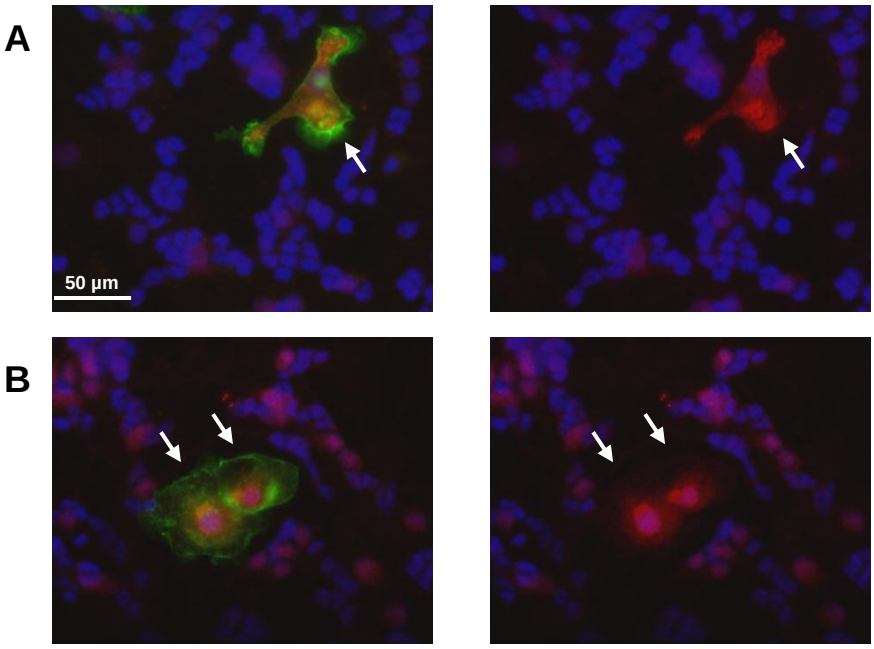

Figure 26: Immunolocalization of ADA in rat retinal cell cultures. Procedure performed in 7 days old primary cultures of mixed retinal cells. Cell nucleus stained in blue with Hoechst 33342. Magnification 400x. Arrows indicate microglial cells **(A)** Control retinal cell culture: immunoreactivities for ADA (red) and microglia marker CD11B (green).**(B)** High glucose conditioned retinal cell culture: immunoreactivities for A_{2A}AR (red) and microglia marker CD11B (green).

To better understand the effect of this decrease in enzymatic activity, and further grasp the role of ADA in the diabetic retina, we could measure the levels of extracellular adenosine in control and high glucose conditions, and measure them again in the presence of ADA inhibitor EHNA. The use of EHNA could be extended to other experiments, to determine if the blockade of ADA might have an effect on the alterations occurring in adenosine receptor protein levels. Of particular interest is the possibility of ADA inhibition affecting the activation levels of NFκB, the transcription factor that can regulate not only the adenosine receptors, but also TNF-α and other cytokines. On the study previously mentioned EHNA and high concentrations of adenosine (100 µM) blocked NFκB activation while a blockade to nucleoside transporters failed to affect NFκB activity, further stressing the importance of ADA

activity in that scenario. It is an experiment worth trying in the context of the diabetic retina.

Highlighting the decrease in activity of ADA is the fact that ADA protein levels were unaltered in retinal cell cultures during high glucose conditions, and in the retinas of rats diabetic for 7 days. Only later did ADA protein levels begin to decrease, after 30 days, and remaining low after 60 days, which coincides with the decrease in the levels of A_{2A}AR and A_3AR and the increase in the levels of A_1AR. It would be interesting to analyze if there is any connection between these alterations.

Chapter 5 Closing Remarks

5.1 – Conclusion

The main purpose of this work was to investigate how diabetes affects the performance of the adenosinergic system in the retina and how those modulations affect the survival of retinal cells in those conditions. The early onset of DR presents the characteristics of a chronic inflammatory state, and adenosine is widely regarded as a significant modulator of the inflammatory response, revealing the adenosinergic system as a potential player in the events unfolding in early DR. In this work, we had particular focus on some of the key elements of the adenosinergic system: the adenosine receptors A_1, A_{2A}, A_{2B} and A_3, and the adenosine degrading enzyme, adenosine deaminase.

Using Western Blot analysis we were able to identify alterations to the protein levels of A_1AR, A_{2A}AR, A_3AR and ADA in diabetes, revealing a clear modulation of the adenosinergic system by diabetes in the retina. The enzymatic assay to determine ADA activity showed a steep decrease in ADA activity in diabetic conditions, further confirming this modulation. MTT viability assays performed began to unveil the consequences of the alterations observed in the adenosinergic system components, hinting at an anti-inflammatory and possibly neuroprotective feedback response to the high levels of pro-inflammatory cytokines present in the diabetic retina.

5.2 – Future perspectives

Beyond the future work proposed in Chapter 4, needed to explore and clarify the role of the adenosinergic system components we studied, our work showed the importance of investigating the other elements responsible for the adenosine role as a modulator and messenger.

The nucleoside transporters have a very important role in adenosine signaling, removing adenosine from extracellular space and, in specific conditions, they may also contribute to the release of the nucleoside to the extracellular space. As revealed in one study, the nucleoside transporters took a backseat to ADA activity in the removal of extracellular adenosine, in a case of inflammation in dendritic cells (Desrosiers *et al.*, 2007), meaning it will be important to explore their impact on adenosine release and removal in DR.

The enzymes that convert several adenine nucleotides into 5'-AMP, NTPDase, ecto-NPP, apyrase and the enzyme responsible for converting 5'-AMP to adenosine in the extracellular space, 5'-eN, are prime targets for analysis in the future. If there is a down-regulation of adenosine degradation rate due to diabetic conditions, we need to determine if those conditions can affect the rate of nucleotide conversion to adenosine. A previous study from our group showed that ATP extracellular concentrations increase in diabetic conditions, partly due to a lower rate of extracellular ATP catabolism (Costa *et al.*, 2009). This most likely means alterations to the levels and/or activity of one or more of the nucleotide degrading enzymes and will have consequences for the extracellular adenosine levels.

Once we expose the modifications occurring in the adenosinergic system in DR, it is of vital importance that we understand their roles and mechanisms. It will be essential to understand how each change affects the survival of the retinal cells, which are the concerted efforts and the individual players, and how do these changes affect the later progression of the disease. It will open the door to possible manipulations of this system, in order to help prevent or forestall the development of diabetic retinopathy.

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